Full title: Gram-scale production of recombinant microbial enzymes in shake flasks

Short title: Gram-scale production of recombinant microbial enzymes

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Abstract

Heterologous production of recombinant proteins is a cornerstone of microbiological and biochemical research as well as various biotechnological processes. Yields and quality of produced proteins have a tremendous impact on structural and enzymology studies, development of new biopharmaceuticals and establishing new biocatalytic processes. Majority of current protocols for recombinant protein expression in *Escherichia coli* exploit batch cultures with complex media, often providing low yields of the target protein due to oxygen transfer limitation, rapid depletion of carbon sources, and pH changes during the cultivation. Recently introduced EnBase technology enables fed-
batch-like cultivations in shake flasks with continuous glucose release from a soluble starch. In this study, we critically compare the yields of fourteen model enzymes in *E. coli* cultured in a novel semi-defined medium and in a complex medium. Significant improvements of the volumetric yields 2-31 times were observed for all tested enzymes expressed in enzymatic fed-batch-like cultures with no adverse impact on enzyme structure, stability, or activity. Exceptional yields, higher than one gram of protein per litre of culture, were obtained with six enzymes. We conclude that the novel semi-defined medium tested in this study provides a robust improvement of protein yields in shake flasks without investment into costly bioreactors.

**Keywords**

Bioprocess engineering, *Escherichia coli*, Heterologous expression, Microbial enzymes, Recombinant protein, Shake flask
Introduction

Heterologous expression is extensively used to produce large amounts of recombinant proteins of interest for biochemical and structural analyses as well as in various biotechnological processes. Although there are numerous host organisms and expression systems available, production of the recombinant proteins in *E. coli* is still the most frequently used technique (Terpe 2006; Li et al. 2014). The main advantages of this bacterial host are the relatively small genome, well-known metabolism, short life cycle, easy cultivation conditions, well-known genetics, and the variety of commercially available plasmid vectors (Terpe 2006; hoi et al. 2006; Gopal et al. 2013). When expressing a protein in *E. coli*, the limitations of this organism can sometimes be problematic, including the low expression of a foreign gene, formation of inclusion bodies, and proteolytic degradation of the target protein (Gopal et al. 2013; Gnoth et al. 2008). Several strategies can be applied to overcome or avoid these limitations, including altering the gene sequence, changing the vector, changing the host strain, selecting the proper inducing agent, and optimizing the cultivation conditions (Gopal et al. 2013; Sorensen and Mortensen 2005; Papanoeophytou and Kontopidis 2014).

The most common procedure for recombinant protein production in a laboratory is batch cultivation in shake flasks. Complex media such as Luria-Bertani or Lysogeny Broth (LB; Bertani 1951) and Terrific Broth (TB; Tartoff and Hobbs 1987) are used for routine protein expressions in shake flasks. Although such cultivations are easy to perform, the utilization of complex media in shake flasks has several obstacles. Complex broths usually contain peptones that are metabolized as carbon sources, leading to the alkalization of the medium up to pH 9, which is toxic for the cells due to the excretion of ammonium (Sezonov et al. 2007; Hortsch and Weuster-Botz 2011; Small et al. 1994). This physiologically unfavorable pH and the depletion of usable carbon sources in the LB medium, caused by insufficient concentration of fermentable sugars, results in the unstable growth of the culture, with a maximum reachable optical density (OD$_{600}$) of 7 (Sezonov et al. 2007). At the same time, shake flasks can cause oxygen transfer limitation in the cultures, leading to a decrease in protein yield due
to anoxic conditions. Complex media in shake flasks are therefore not optimal for the efficient production of recombinant proteins. Besides complex media, defined or minimal media can be used to express recombinant proteins. Due to the slow growth of cultures and low protein yields, defined media are not usually the first choice for routine shake flask scale expression of unlabeled proteins. They are more often used to produce labeled proteins for NMR studies or neutron and X-ray crystallography (Tyler et al. 2005). Several defined, semi-defined, and complex autoinduction media have been described for high-level recombinant protein production (Studier 2005; Blommel et al. 2007; Li et al. 2011). Such media take advantage of expression controlled by the lac operon, which is repressed by glucose in the initial phase of the culture. This limits the choice of expression vector as well as of strain. Preparation of such media is laborious and most are not commercially available.

While typical laboratory-scale protein production in shake flasks is operated as a batch culture, the usual mode of operation in pilot to industrial-scale bioreactors is the fed-batch. The most commonly applied principle is glucose-limited fed-batch, in which feeding of a highly concentrated glucose solution is started after an initial batch phase (Sanden et al. 2003; Lee 1996). Proper feeding strategy and composition of the medium enable prolonged controlled growth of the culture and better yield of target protein. This strategy allows E. coli to grow to cell densities over 150 g/l of dry cell biomass (Markl et al. 1993; Lee et al. 1994). Defined or semi-defined media are considered to be the most suitable for fed-batch high cell density cultures. Reasonable feeding of a carbon source prevents the formation of acetate, which occurs mainly in broths containing glucose (Salehmin et al. 2013). Enhanced aeration and stirring of the culture enables sufficient oxygen supply and prevents oxygen limitations in the dense cultures (Salehmin et al. 2013; Hewitt et al. 2007).

Innovative EnBase technology combines the advantages of cultivation in shake flasks and fed-batch cultures. It is easy to perform, and high cell densities can be achieved due to the constant glucose release throughout the cultivation. The method of enzymatic glucose release from polymer in shake flasks was introduced by Panula-Perala et al (2008). The gel layer containing starch was later replaced...
by soluble starch, which is easily accessible for glucoamylase (Krause et al. 2010). Cells are grown in a defined medium with glucose before induction and in a semi-defined medium after induction. The mineral medium is composed of Na$_2$SO$_4$, (NH$_4$)$_2$SO$_4$, NH$_4$Cl, K$_2$HPO$_4$, NaH$_2$PO$_4$·H$_2$O, (NH$_4$)$_2$-H-citrate, MgSO$_4$, thiamine hydrochloride and trace elements as described by Krause et al. (2010). Besides the mineral salts, boosting tablets contain also complex additives such as yeast extract. In addition to the expression of recombinant proteins in shake flasks, EnBase technology can be used for large-scale protein production in bioreactors (Glazyrina et al. 2010, 2012; Siurkus et al. 2010) or high-throughput screening of large libraries (Tegel et al. 2011). The most important advantages of EnBase technology are the high cell densities of the cultures, enhanced yields of recombinant proteins, and pH maintenance throughout the whole cultivation process. Moreover, the buffering capacity of the medium prevents pH changes during cultivation (Hortsch and Weuster-Botz 2011, Krause et al. 2010). The use of baffled Ultra Yield Flasks (UYFs) introduced by Ukkonen et al. (2011) attempted to address the problem of oxygen limitation in the shaken flask cultures. Higher oxygen transfer rates are achieved in UYFs than in the routinely used Erlenmeyer flasks.

In this study, we investigate the production of a set of selected recombinant enzymes in novel EnPresso B medium and conventional LB medium in shake flasks with the goal of critically comparing volumetric yields of the soluble and active proteins in these media. The difference between herein presented EnPresso B and classical EnPresso is a new polysaccharide, which allows better control of substrate consumption, lower amount of yeast extract and addition of three additional trace elements. Biotechnologically relevant haloalkane dehalogenases (HLDs) were selected as the model enzymes for this study. HLDs are predominantly microbial enzymes which catalyze hydrolysis of carbon-halogen bonds in halogenated aliphatic hydrocarbons, releasing halide ions and corresponding alcohols as the reaction products. HLDs find broad usage in biodegradation, biosensing, biocatalysis, and cell imaging (Koudelakova et al. 2013). A set of fourteen biochemically characterized HLDs including DadB (Li and Zhao 2014), DatA (Hasan et al. 2011), DbeA (Chalopkova et al. 2014), DbjA (Sato et al. 2005), DhaA (Yokota et al. 1987), DhlA (Keuning et al. 1985), DmbA...
(Jesenska et al. 2005), DmlA (Sato et al. 2005), DmmA (Gehret et al. 2012), DmsA and DmxA (unpublished data), DpcA (Drienovska et al 2012), DppA (Hesseler et al. 2011), and LinB (Nagata et al. 1999) was selected for testing.

Materials and methods

Bacterial strains and chemicals

_E. coli_ BL21, _E. coli_ BL21(DE3) (New England Biolabs, USA) and _E. coli_ ArcticExpress (DE3) cells (Stratagene, USA) were used in this study. All chemicals used in the study were purchased from Sigma-Aldrich, unless stated otherwise.

Gene isolation, synthesis and cloning

The genes encoding DbeA, DbjA, DmlA, DmbA, LinB, DhaA, and DhlA were isolated from original hosts, as described previously (Chaloupkova et al. 2014; Yokota et al. 1987; Jesenska et al. 2005; Nagata et al. 1999; Kulakova et al. 1997; Janssen et al. 1989). The remaining genes encoding HLDs used in this study were synthesized artificially (Mr. Gene, Germany). Gene constructs containing C-terminal hexahistidyl tag enabling purification by the metallo-affinity chromatography were subcloned to pAQN (Nagata et al. 1999, Terada et al. 1990), pET21b, pET24a (Novagen, USA) or pET28a (Invitrogen, USA) expression vectors (Table 1) carrying ampicillin (pAQN and pET21b) or kanamycin (pET24a and pET28a) resistance. The plasmids containing subcloned genes encoding particular HLDs were transformed to _E. coli_ BL21(DE3) strain, with the exception of the pAQN::dmsA construct, which was transformed to _E. coli_ BL21, and the pET21b::dpcA construct, which was transformed to _E. coli_ ArcticExpress (DE3).
Cultivation in semi-defined and complex medium

The cultivations in EnPresso B medium were performed according to manufacturer’s instructions (BioSilta, Finland). Novel EnPresso B medium contains a polysaccharide serving for a better control of substrate consumption, lower amount of yeast extract and three additional trace elements supporting bacterial cell growth. Inoculum was prepared by picking 1 colony of transformed E. coli carrying the gene coding a target enzyme to 1 mL of LB medium with 2 g/L glucose and shaken 6 hrs at 37°C and 200 rpm. Prior to the cultivation, white-bag tablets were dissolved in 50 mL of sterile water in UYF (Thomson Instrument Company, USA). The cultivation was initialized by the addition of respective antibiotics (ampicillin to a final concentration of 100 µg/mL; kanamycin to a final concentration of 30 µg/mL; gentamycin and tetracycline to a final concentration of 20 µg/mL), 1 mL of pre-culture, 25 µL of Reagent A (glucoamylase) and 100 µL/L antifoam agent Struktol SB2020. The flask mouth was covered with the AirOtop cover (Thomson Instrument Company, USA). The culture was grown for 18 hrs before induction at 30°C and 250 rpm. At the induction time, a black-bag booster tablet, 75 µL of Reagent A, and 0.5 mM final Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the shake flasks. The glucose release rate was 0.048 g.L⁻¹.h⁻¹ at the beginning of cultivation and 0.18 g.L⁻¹.h⁻¹ after the boosting step with a second dose of Reagent A. The induced cultures were incubated for 24 hrs in 30°C (15°C for DpcA) with constant shaking at 250 rpm. OD₆₀₀ was measured at the time of induction and at harvesting. Cultured cells were harvested by centrifugation at 6,000 g and 4°C, stored at -70°C, and defrosted before purification.

For comparative purposes, expression of selected HLDs was performed also in LB medium (Sigma-Aldrich, USA). The medium was composed of 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride (Lennox 1955). Precultures were prepared by picking one colony of transformed E. coli carrying the gene coding a target enzyme to 10 mL of LB medium with respective antibiotics and incubated overnight at 37°C and 200 rpm. One L of LB medium with the respective antibiotic was inoculated with the overnight culture and incubated at 37°C and 120 rpm. When the culture reached
OD$_{600}$ 0.5, the expression was initiated by the addition of IPTG to a final concentration of 0.5 mM. *E. coli* BL21(DE3) and BL21 cells were further incubated overnight at 25°C, *E. coli* ArcticExpress (DE3) cells were grown overnight at 15°C. Cells were harvested by centrifugation at 6,000 g and 4°C, stored at -70°C and defrosted before purification.

Protein purification

Harvested cells were disrupted by sonication using a Soniprep 150 (Sanyo Gallenkamp PLC, UK). The supernatants were collected after centrifugation at 21,000 g for 1 h. The crude extracts were further purified on Ni-NTA Superflow Cartridge (Qiagen, Germany) as described previously (e.g. Drienovska et al. 2012; Hasan et al. 2011; Chaloupkova et al. 2014). The purified proteins were dialyzed against 50 mM phosphate buffer (pH 7.5) overnight. All enzymes were stored at 4°C in a 50 mM potassium phosphate buffer prior to analysis. Expression profiles, solubility, and purity of the enzymes were checked by SDS-PAGE; the amount of target enzyme in the fractions on SDS gel was determined by a GS-800 Calibrated Densitometer (Bio-Rad, USA). The concentration of purified enzyme was determined with the Bradford method (Bradford 1976), using bovine serum albumin as a standard.

Determination of specific activity

Enzymatic activity was assayed using the colorimetric method developed by Iwasaki et al. (1952). The release of halide ions was measured spectrophotometrically at 460 nm using a SUNRISE microplate reader (Tecan, Switzerland) after reaction with mercuric thiocyanate and ferric ammonium sulfate. Dehalogenation reactions were performed at 37°C (25°C for DpcA) in 25-mL Reacti-flasks closed by Mininert valves. The reaction mixture contained 10 mL of glycine buffer (100 mM, pH 8.6) and 10 μL of halogenated substrate. 1,2-dibromoethane was selected as a substrate for the activity measurement of most tested HLDs, except the activity of DmsA which was measured with 1-
bromobutane. The reactions were initiated by the addition of appropriate amounts of the enzymes depending on their activities. The reactions were monitored by withdrawing 1 mL samples at periodical intervals from the reaction mixture and immediately mixing the samples with 0.1 mL of 35% nitric acid to terminate the reaction. Dehalogenation activities were quantified by a slope of product formation with time.

Analysis of structure and thermostability

Circular dichroism (CD) spectra were recorded at room temperature using a Chirascan spectrometer (Applied Photophysics, UK) equipped with a Peltier thermostat. Data were collected from 185 to 260 nm, at a scan rate of 100 nm/min, 1 s response and 1 nm bandwidth using a 0.1 cm quartz cuvette containing 0.2 mg/mL enzyme in a 50 mM potassium phosphate buffer (pH 7.5). Each collected spectrum represents an average of five individual scans and has been corrected for absorbance caused by the buffer. CD data were expressed in terms of the mean residue ellipticity ($\Theta_{\text{MRE}}$) using the following equation:

$$\Theta_{\text{MRE}} = \left(\Theta_{\text{obs}} \cdot M_w \cdot 100\right) / \left(n \cdot c \cdot l\right)$$

where $\Theta_{\text{obs}}$ is the observed ellipticity in degrees, $M_w$ is the protein molecular weight, $n$ is number of residues, $l$ is the cell path length, $c$ is the protein concentration, and the factor of 100 originates from the conversion of the molecular weight to mg/dmol. Thermal unfolding of studied HLDs was followed by monitoring the ellipticity at 222 nm over a temperature range of 20 to 80°C, using a resolution of 0.1°C and heating rate of 1°C/min. The resulting thermal denaturation was roughly normalized to
represent a signal change between approximately 1 and 0 and fitted to sigmoidal curves using Origin
6.1 software (OriginLab, USA). The melting temperature ($T_m$) was evaluated as a midpoint of the
normalized thermal transition.

Results and discussion

The EnBase platform has been developed as a fed-batch-like cultivation to be performed in shake
flasks, providing high cell densities and enhanced volumetric yields of recombinant proteins (Krause
et al. 2010, 2016). Cultivations are easy to perform under laboratory conditions with minimized input
of an operator (Krause et al. 2010; Mahboudi et al. 2013; Ukkonen et al. 2013; Peck et al. 2014; Li et
al. 2014; Zarschler et al. 2013). Here we critically compare the expression of a set of fourteen model
enzymes in semi-defined EnPresso B medium and complex LB medium. The objective is to
systematically analyze yield, stability, and activity of proteins obtained from enzymatic fed-batch-like
cultures with those obtained from the traditional complex media. The majority of cultures grown in
EnPresso B medium reached OD$_{600}$ between 23.4 to 58.7 at harvesting time, 24 hours after induction
at 30°C (Table 1). The only exception was DpcA, originating from a psychrophilic organism, expressed
in *E. coli* ArcticExpress (DE3) cells at 15°C, reaching approximately half of the cell density observed
with proteins from mesophilic organisms. The OD$_{600}$ reached at harvesting time in LB medium ranged
between 2.5 and 6.4 (Table 1), which is typical of this medium. It is described in the literature that
OD$_{600}$ in LB medium should not exceed 7 (Sezonov et al. 2007).

Most of the HLDs cultured in semi-defined EnPresso B medium exhibited significant improvement in
the volumetric yield of soluble protein. Yields of DadB, DmA, DhA, and DmA exceeded 2 grams of
protein per liter of culture, which to the best of our knowledge has never been reported for the
EnBase technology (Table 1). Yields achieved in LB shown in Table 1 correspond well with previously
published results (Koudelakova et al. 2013; Hasan et al. 2011; Chaloupkova et al. 2014; Drienovska et
al. 2012). The most beneficial effect of EnPresso B was observed for DbjA, with the volumetric yield
increased 31 times, from 50 mg/L to 1572 mg/L. No correlation was observed between the yields in both tested media, suggesting that the improvement of yield is protein-specific. While improvement of purified enzyme yield in eight out of fourteen tested cultures was achieved by higher cell density, the relative yield increase observed with six cultures also shows positive impact of EnPresso B on per cell basis yield (Table 1). It is clear that significant increase of cell density brings benefits in terms of volumetric yield in the shake flask.

SDS-PAGE confirmed that all enzymes were purified to homogeneity by metallo-affinity chromatography with purity ≥ 95% (data not shown). A comparison of soluble and insoluble protein fractions revealed that EnPresso B medium does not fully prevent formation of inclusion bodies during the induction phase. Nevertheless, the ratio of soluble and insoluble recombinant enzymes was better in EnPresso B than in LB (Table 2, Figures S1-S2), with the exception of DmlA, DmmA and LinB. This disadvantage was overcome by high cell density in EnPresso B. No difference was observed between the CD spectra of HLDs expressed in EnPresso B and LB, considering the preserved structure of HLDs from both tested media (Figures S3-S4). All produced HLDs exhibited CD spectra with one positive maximum at 195 nm and two negative features at 208 and 222 nm characteristic of α-helical content (Fasman 1996). Thermally-induced denaturation experiments revealed comparable melting temperatures ($T_m$) for the proteins produced in both media (Table 3). Observed differences in protein stability were within the error of the method.

The catalytic activities of most of the purified enzymes were similar regardless of the cultivation medium (Table 3). Small differences in specific activity values can be explained by varying concentrations of substrate in the reaction mixture, mainly corresponding to the limited solubility and volatility of the used halogenated compounds. Observed activity levels of tested enzymes are in good agreement with values from the biochemical characterization of individual enzymes. Therefore, we conclude that cultivation in EnPresso B medium did not change the functional properties of the tested enzymes.
**Conclusions**

Heterologous expression of proteins in *E. coli* cells is the most widely used approach to obtain recombinant proteins in laboratory as well as industrial scale. In this study, we demonstrate a positive effect of enzymatic fed-batch-like cultures employing novel semi-defined EnPresso B medium on volumetric yield of fourteen different model enzymes. The improvement ranged between 2 and 31 times and a protein yield above 1 g of purified enzyme per litre of culture was achieved with six different enzymes. The increase of volumetric yield was mainly due to higher cell density, yet significant number of enzymes exhibited also per cell basis yield improvement. We conclude that novel semi-defined medium EnPresso B provides significant and robust improvement of protein yields in shake flasks.

**Acknowledgments**

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**Competing interests**

Authors declare no competing interests.

**Authors’ contributions**
L.C. carried out all laboratory experiments. L.C., J.D. and R.C. jointly designed experiments and interpreted the results. L.C. and R.C. drafted the manuscript. All authors have read and approved the manuscript.

References


Papanenophytou CP, Kontopidis G. Statistical approaches to maximize recombinant protein expression in *Escherichia coli*: a general review. *Protein Expr Purif* 2014;94:22–32.


Siurkus J, Panula-Perala J, Horn U *et al.* Novel approach of high cell density recombinant bioprocess development: Optimisation and scale-up from microlitre to pilot scales while maintaining the fed-batch cultivation mode of *E. coli* cultures. *Microb Cell Fact* 2010;9:35.


Table 1. Cultivation parameters of cultures and comparison of volumetric/relative yields of microbial enzymes HLDs obtained in semi-defined EnPresso B and complex LB medium.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Vector</th>
<th>OD(_{600}) at harvesting</th>
<th>Yield per litre of culture (mg)(a)</th>
<th>Volumetric yield improvement</th>
<th>Relative yield (mg/L/OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EnPresso B</td>
<td>LB</td>
<td>EnPresso B</td>
<td>LB</td>
</tr>
<tr>
<td>DadB</td>
<td>pET24a</td>
<td>44.2 ± 3.3</td>
<td>2.5 ± 0.1</td>
<td>2718 ± 513</td>
<td>365</td>
</tr>
<tr>
<td>DatA</td>
<td>pET21b</td>
<td>58.7 ± 4.4</td>
<td>4.5 ± 1.8</td>
<td>57 ± 6</td>
<td>30(^b)</td>
</tr>
<tr>
<td>DbeA</td>
<td>pET21b</td>
<td>56.4 ± 3.8</td>
<td>6.4 ± 0.1</td>
<td>784 ± 34</td>
<td>40(^c)</td>
</tr>
<tr>
<td>DbjA</td>
<td>pET21b</td>
<td>39.6 ± 0.9</td>
<td>4.3 ± 0.8</td>
<td>1572 ± 112</td>
<td>50(^d)</td>
</tr>
<tr>
<td>DhaA</td>
<td>pET21b</td>
<td>42.3 ± 2.6</td>
<td>4.7 ± 1.0</td>
<td>2049 ± 14</td>
<td>120(^d)</td>
</tr>
<tr>
<td>DhlA</td>
<td>pET11a</td>
<td>46.2 ± 1.4</td>
<td>2.8 ± 0.2</td>
<td>1598 ± 222</td>
<td>525</td>
</tr>
<tr>
<td>DmbA</td>
<td>pET21b</td>
<td>49.9 ± 4.9</td>
<td>4.0 ± 0.3</td>
<td>56 ± 7</td>
<td>13</td>
</tr>
<tr>
<td>DmlA</td>
<td>pET21b</td>
<td>45.1 ± 2.3</td>
<td>5.5 ± 0.3</td>
<td>982 ± 227</td>
<td>340</td>
</tr>
<tr>
<td>DmmA</td>
<td>pET24a</td>
<td>46.5 ± 4.6</td>
<td>5.5 ± 1.1</td>
<td>2471 ± 166</td>
<td>150(^d)</td>
</tr>
<tr>
<td>DmsA</td>
<td>pAQN</td>
<td>40.8 ± 1.2</td>
<td>5.3 ± 0.7</td>
<td>437 ± 80</td>
<td>50</td>
</tr>
<tr>
<td>DmxA</td>
<td>pET21b</td>
<td>51.2 ± 1.4</td>
<td>5.5 ± 0.1</td>
<td>2292 ± 130</td>
<td>390</td>
</tr>
<tr>
<td>DpcA</td>
<td>pET21b</td>
<td>23.4 ± 0.7</td>
<td>3.2 ± 0.2</td>
<td>883 ± 29</td>
<td>30(^e)</td>
</tr>
<tr>
<td>DppA</td>
<td>pET28a</td>
<td>33.2 ± 0.3</td>
<td>5.5 ± 1.4</td>
<td>650 ± 78</td>
<td>250</td>
</tr>
<tr>
<td>LinB</td>
<td>pET21b</td>
<td>50.8 ± 7.5</td>
<td>4.0 ± 1.2</td>
<td>347 ± 61</td>
<td>50(^d)</td>
</tr>
</tbody>
</table>

\(^a\)The values represent the yield of purified enzyme per litre of cell culture.

\(^b\)Hasan et al. (2011)

\(^c\)Chaloupkova et al. (2014)

\(^d\)Koudelakova et al. (2013)

\(^e\)Drienovska et al. (2012)
Table 2. Relative content of soluble overexpressed microbial enzymes in the cell free extracts and ratio of relative protein content in a soluble and an insoluble fractions of cultures prepared using semi-defined EnPresso B and complex LB medium determined by densitometric analysis of gels after SDS-PAGE.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction of soluble protein (in %) determined in cell free extract(^a)</th>
<th>Ratio of relative quantities of the protein in a soluble and an insoluble fraction(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EnPresso B</td>
<td>LB</td>
</tr>
<tr>
<td>DadB</td>
<td>75.3</td>
<td>72.5</td>
</tr>
<tr>
<td>DatA</td>
<td>8.9</td>
<td>4.1</td>
</tr>
<tr>
<td>DbeA</td>
<td>80.1</td>
<td>43.3</td>
</tr>
<tr>
<td>DbjA</td>
<td>53.4</td>
<td>15.4</td>
</tr>
<tr>
<td>DhaA</td>
<td>83.9</td>
<td>37.5</td>
</tr>
<tr>
<td>DhlA</td>
<td>87.9</td>
<td>79.1</td>
</tr>
<tr>
<td>DmbA</td>
<td>32.7</td>
<td>31.5</td>
</tr>
<tr>
<td>DmlA</td>
<td>36.1</td>
<td>64.4</td>
</tr>
<tr>
<td>DmmA</td>
<td>79.9</td>
<td>74.4</td>
</tr>
<tr>
<td>DmsA</td>
<td>21.4</td>
<td>10.8</td>
</tr>
<tr>
<td>DmA</td>
<td>58</td>
<td>36.2</td>
</tr>
<tr>
<td>DpcA</td>
<td>73.9</td>
<td>70.5</td>
</tr>
<tr>
<td>DppA</td>
<td>70.5</td>
<td>74.5</td>
</tr>
<tr>
<td>LinB</td>
<td>32.5</td>
<td>35.6</td>
</tr>
</tbody>
</table>

\(^a\)Measured in triplicates with the standard error less than 10%.

\(^b\)Calculated as a ratio of relative protein content in a soluble fraction (cell free extract) and an insoluble fraction solubilized with 8 M urea. The higher the number is, the better improvement of protein solubility in EnPresso B medium has been achieved.
Table 3. Melting temperatures and specific activities of microbial enzymes HLDs expressed in semi-defined EnPresso B and complex LB medium. The specific activity was determined with 1,2-dibromoethane at 37°C in 100 mM glycine buffer (pH 8.6) unless stated otherwise.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Melting temperature (°C)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific activity&lt;sup&gt;b&lt;/sup&gt; (µmol s&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt; of enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EnPresso B</td>
<td>LB</td>
</tr>
<tr>
<td>DadB</td>
<td>54.1</td>
<td>54.5</td>
</tr>
<tr>
<td>DatA</td>
<td>47.8</td>
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<td>53.6&lt;sup&gt;j&lt;/sup&gt;</td>
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<sup>a</sup>Measured in triplicates with the standard error less than 5%.

<sup>b</sup>Measured in triplicates with the standard error less than 10%.

<sup>c</sup>Measured with 1-bromobutane.

<sup>d</sup>Measured at 25 °C.
Hasan et al. (2011)

Chaloupkova et al. (2014)

Sato et al. (2005)

Koudelakova et al. (2013)

Koudelakova et al. (2011)

Drienovska et al. (2012)