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ARTICLE

A comparative study on optimisation of protein extraction methods for *Saccharomonospora azurea*

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ABSTRACT To establish the optimal cell disruption and protein extraction protocol for achieving the most efficient whole-cell protein extraction of *Saccharomonospora azurea*, four commonly used methods (X-Press, bead-vortexing, freezing-throwing and TCA/acetone/phenol extraction) were compared. Total protein content, as well as 1D and 2D SDS-PAGE protein patterns were assayed in the extracts to study the efficacy of these methods. Accordingly, of the four methods the X-Press proved the most effective for all initial weight (maximum 21.523 ± 0.23 mg/ml protein) followed by TCA/acetone/phenol method (maximum 13.682 ± 0.15 mg/ml protein), while the effectiveness of the two other methods were substantially inferior (maximum 3.188 ± 0.03 mg/ml protein). The analysis of protein gels proved that the X-Press method revealed a protein pattern characterised by the presence of the highest number of protein bands (on average of 52 and 385, on 1D and 2D gels, respectively). The TCA/acetone/phenol extraction provided similar effectiveness for only 100-300 mg initial bacteria mass, whereas bead-vortexing produced maximum 35 and 227 separated protein bands, on 1D and 2D gels, respectively. It can be stated that of the four methods the X-Press was the most effective one for all initial weight of bacteria, while the TCA/acetone/phenol method provides interpretable results for the 100-300 mg weight-range of bacteria. **Acta Biol Szeged 61(1):45-50 (2017)**

KEY WORDS
bead-vortexing
freezing-throwing
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Introduction

The spread of multi-resistant bacteria inspires researchers to search for new effective antibiotics. The primycin complex first described by Vály-Nagy et al. (1954) produced by *Saccharomonospora* sp. seems promising in tackling this problem. Re-investigated of the efficacy of this antibiotic, Feiszt et al. (2014) clearly demonstrated that primycin possesses high activity against the most frequent Gram-positive pathogens including some multi-resistant strains, without the development of remarkable resistance. Due to its great properties, a wider range of medical applications would be possible upon our better understanding of the regulation of synthetic processes of this "new-old" antibiotic. As first step, to get deeper insight into the bioactive natural products metabolism of *Saccharomonospora azurea* SZMC 14600, whole-genome sequencing was performed (Csepregi et al. 2012). Since proteomics represents a dynamic view of expressed genes, the proteomic approach combined with genetic studies provides a more comprehensive insight into the regulation of secondary metabolism to identify proteins associated with primycin production. In this context, the reliable knowledge of protein distribution is essential.

Efficient protein extraction highly depends on the quality of cell disruption. There is a great diversity of techniques including physical (bead-mill, French press, ultrasonic vibration) and chemical (detergents, lysozyme, osmotic shock) methods as well as the combination of them available for protein extraction of different types of samples and for various purposes (Wilson and Walker 2000; Islam et al. 2004; Singh 2013; Alam and Ghosh 2014; Malafaia et al. 2015; Tiong et al. 2015), therefore, it is a challenge to find a technique that can produce high yields and the greatest possible number of proteins in the sample analysed. Presently, we know of no comparative studies about the total protein distribution of *S. azurea*, consequently no currently available the most effective methods can be associated with this bacterium.

Therefore, in this study we compare four different commonly used total protein extraction methods including X-Press, bead-vortexing, freezing-throwing and the TCA/acetone/phenol technique to achieve a highly efficient isolation of total proteins for protein profiling of *S. azurea* SZMC

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14600. The effectiveness of the studied methods was evaluated by total protein content, 1D and 2D SDS-PAGE.

Materials and Methods

Bacterial cultures were maintained according to the industrial primycin fermentation processes (Juhász et al. 2011). *S. azurea* SZMC 14600 freeze-dried stock cultures maintained at -80 °C were used to directly inoculate 50 ml of pre-fermentation medium (PF) containing (w/v): 3% soy flour, 4.2% water soluble starch, 0.36% NaCl, 0.6% $CaCO_3$ and 0.5% (v/v) sunflower oil (pH 8). PF culture was grown for 2 days at 37 °C in an orbital shaker at 200 rpm. Thereafter, 1 ml suspension of bacterial cells was used to inoculate 35 ml of main fermentation medium (MF) containing (w/v): 4% soy flour, 4% water soluble starch, 0.3% NaCl, 0.5% $CaCO_3$, 0.6% sunflower oil, 0.3% stearic acid and 0.1% KH_2PO_4 (pH 9.5). The MF cultures were incubated at 28 °C for 5 days. The cultures were harvested by centrifugation (9000 g, 4 ºC, 15 min). Soluble proteins were extracted from 100, 200, 300, 500, and 1000 mg fresh weight of bacterial mats.

TCA/acetone/phenol method

The whole-cell protein was extracted according to the method described by Wang et al. (2006).

X-Press method

The frozen (-20 °C) bacterial mats in 5 ml phosphate buffer (PBS: 0.1 M, pH 7.4, 10 mM PMFS) were forced through an orifice and subjected to very high hydraulic pressures in X-Press Disintegrator Type X25 (Ab Biox; Göteborg, Sweden) appliance.

Bead-vortexing method

The modified method of Sánchez et al. (2003) consisted of adding 250 mg of glass beads (diameter 425-500 µm) to the bacteria mats suspended in 5 ml SDS treatment buffer (0.0625 M Tris, pH 7.4, 2% (w/v) SDS, 10% (v/v) glycerol, 10 mM PMFS) and vortexing (Tissue Lyzer, Quiagen) for 4 min (30 s vortexing/30 s in ice) at the maximum setting. After cooling on ice and centrifuging (13600g, 15 min, 4 °C), supernatants were used for further experiments.

Freezing-throwing method

The cells were destroyed by freezing and thawing five times, using liquid nitrogen (Kajiwara et al. 2003). Proteins were extracted by 5 ml lysis buffer (7 M urea, 1 mM-os PMFS, 4 % (w/v) CHAPS, 2% (v/v) Triton X-100, 5 % (v/v) 2-mercaptoethanol, 0.5 M EDTA). After centrifugation, (13600 g, 15 min, 4 °C), supernatants were used for further experiments.

The purified proteins were stored at -20 °C. For further studies protein extracts from different methods and initial mass of bacteria (100 mg, 200 mg, 300 mg, 500 mg, and 1000 mg) were diluted to the same volume. Protein concentration was measured by the Bradford method (Bradford 1976).

Each extraction was performed in triplicate. A statistical analysis was performed using one-way analyses of ANOVA. Values are reported as mean ± SD (standard deviation). The level of significance was adopted at $p<0.05$.

SDS-PAGE

The gel electrophoresis of whole cell protein extracts was performed according to Laemmli (1970) on vertical slab gels (8.6 x 6.7 x 0.1 cm) in a Mini Protean Tetra Cell gel electrophoresis apparatus (Bio-Rad Laboratories) using 12% separating gel (pH 8.8) and 5% stacking gel (pH 6.8). The running buffer contained 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS (pH 8.35), 5 µl and 10 µl of samples and 10 µl of protein molecular weight standard (Precision Plus Protein Unstained standard, Bio-Rad) were applied. Electrophoresis was performed at a constant current of 120 V for two hours. At the end of electrophoresis, the gels were visualized by staining with Coomassie Brilliant Blue (10% (v/v) acetic acid, 45% (v/v) methanol, 0.25% (w/v) Coomassie Brilliant Blue R-250) with constant shaking followed by decolorization with a solution of 10% (v/v) methanol and 10% (v/v) acetic acid.

2D electrophoresis

Isoelectric focusing of rehydrated protein samples (250 µg) were performed on 7 cm IPG Strips (pH 3-10, Bio-Rad) at 250 V for 15 min (rapid voltage ramping), at 4000 V for 1 h (linear voltage ramping), at 4000 V (rapid voltage ramping) up to 15 000 Vh . The IPG strips were equilibrated in 2% (w/v) DTT containing buffer (6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris-HCl, pH 8.8), for 20 min. 2D electrophoresis were performed on 12.5% (w/v) SDS-PAGE gels, using the Bio-Rad Mini-PROTEAN Tetra Cell vertical electrophoresis system. Runs were carried out at room temperature for 10 min at 50 V and successively for 1 h at 200 V. Gels were stained with Coomassie Brilliant Blue (Sigma-Aldrich) as described above.

Gel images were captured by AlphaImager high performance gel documentation system (Protein Simple, Alpha Innotech, San Leandro, CA) and analysed using Prodigy 1D and SameSpots 2D Software package (Nonlinear Dynamics) according to the instructions of manufacturers.

Table 1. Protein recovery from *S. azurea* (SZMC 14600) cells using different extracting protocols. Values (mg/ml protein) are reported as means ± SD of three independent experiment (n = 3).

Mass of bacteria	X-Press	TCA/acetone/phenol	Bead-vortexing	Freezing-throwing
100 mg	3.284 ± 0.36	12.693 ± 0.	1.531 ± 0.02	0.572 ± 0.04
200 mg	7.010 ± 0.06	13.682 ± 0.15	2.153 ± 0.03	0.547 ± 0.06
300 mg	8.062 ± 0.07	12.997 ± 0.14	2.764 ± 0.03	1.863 ± 0.21
500 mg	12.368 ± 0.16	2.325 ± 0.03	3.188 ± 0.03	1.634 ± 0.18
1000 mg	21.523 ± 0.23	2.146 ± 0.03	3.034 ± 0.04	1.561 ± 0.21

Table 2. Number of protein bands on SDS-PAGE gels obtained from *S. azurea* (SZMC 14600) applying different extraction methods and initial weight of bacteria for extraction. Values are reported as means ± SD of three independent experiment (n = 3).

Number of protein bands					
Initial weight of bacteria	100 mg	200 mg	300 mg	500 mg	1000 mg
X-Press	27 ± 4	48 ± 6	53 ± 6	52 ± 6	52 ± 5
TCA/acetone/phenol	42 ± 6	51 ± 5	53 ± 5	12 ± 3	11 ± 3
Bead-vortexing	16 ± 4	25 ± 4	35 ± 4	37 ± 5	35 ± 5

Results

In order, to monitor the optimal cell disruption and protein extraction protocol for achieving the maximal release of total protein from *S. azurea* SZMC 14600 four methods were compared. As shown in Table 1, the protein recovery strongly depended both on one hand methods used and the initial mass of bacteria. Accordingly, of the four methods the X-Press was effective for all initial weights of bacteria, while TCA/acetone/phenol method did not provide usable results for the higher mass (500 mg, 1000 mg). The effectiveness of two other methods was significantly lower, to the extent that the freezing/throwing technique proved to be completely inadequate, therefore, the results of this protocol was no longer estimated.

The changes in protein pattern obtained from *S. azurea* SZMC 14600 by different extraction protocols were evaluated by SDS-PAGE. Figure 1 shows representative Coomassie Brilliant Blue staining gels of the whole-cell protein extractions. The SDS-PAGE analysis revealed that the extractions presented good quality proteins, with well-defined bands

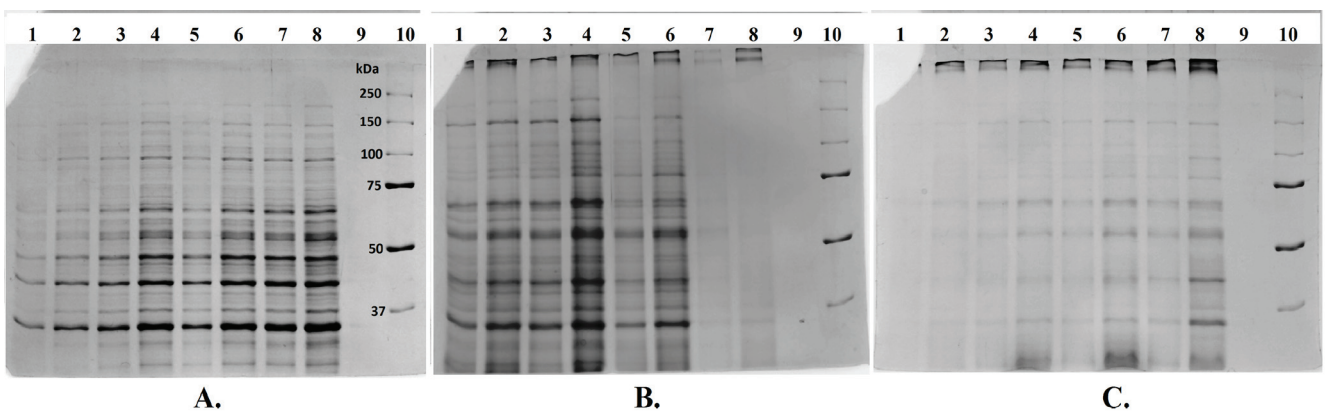

Figure 1. Whole cell protein patterns obtained by SDS-PAGE for *S. azurea* SZMC 14600 by using different homogenisation and protein extraction protocols. Lane 1 and 2: 100 mg initial mass of bacteria (5 µl and 10 µl, respectively). Lane 3 and 4: 200 mg initial mass (5 µl and 10 µl, respectively). Lane 5 and 6: 300 mg initial mass of bacteria (5 µl and 10 µl, respectively). Lane 7: 500 mg initial mass of bacteria (5 µl). Lane 8: 1000 mg initial mass of bacteria (5 µl). Lane 10: molecular weight standard (Precision Plus Protein Unstained, 5 µl; Bio-Rad). A: X-Press; B: Bead-vortexing; C: TCA/acetone/phenol.

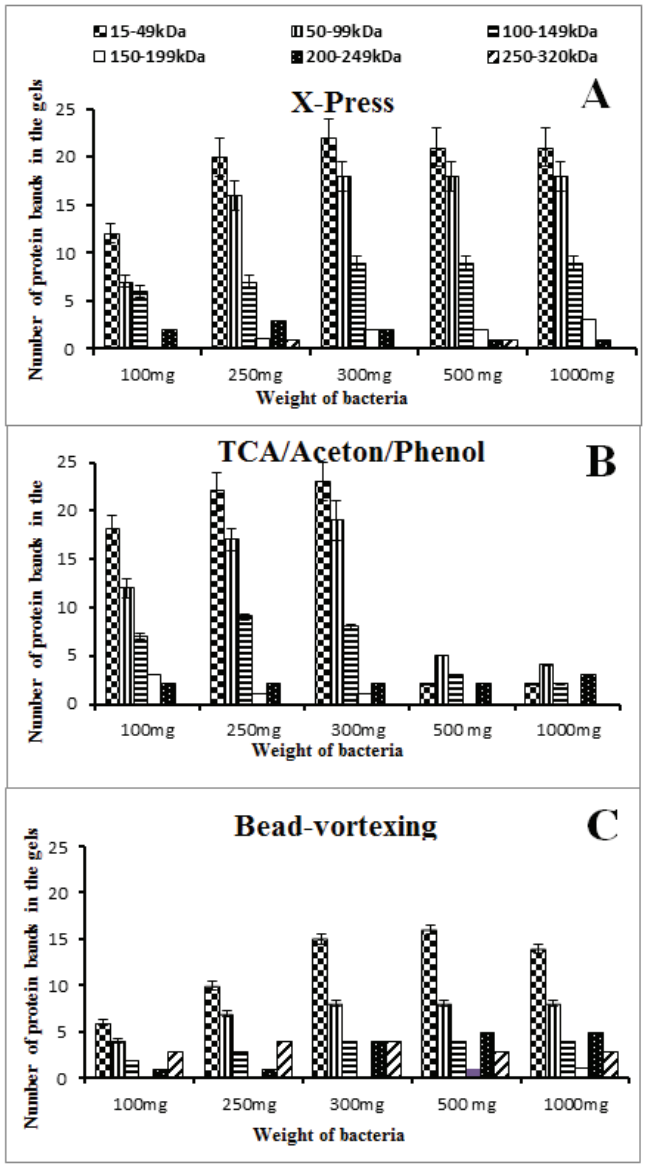

Figure 2. Distribution of separated proteins on the basis of molecular weight in different protein extraction methods applied. Values are reported as means ± SD of three independent experiments (where there was difference in the number of protein bands).

without signs of degradation. Nevertheless, the band intensity of one-dimensional gel electrophoresis, in spite of the same volume of samples provides only limited options for image analyses, it can clearly be seen that the density of bands was in accordance with the protein content of data presented in Table 1.

Beyond the quantitative differences, well-characterised variation in the number of detected bands could be observed amongst the methods (Table 2). The data of qualitative analysis of SDS-PAGE protein pattern clearly demonstrated that the X-Press method gave the bacterial mass-dependent results characterised by the presence of 27-53 protein bands, whereas the TCA/acetone/phenol extraction method allowed for the similar effectiveness for only 100-300 mg bacterial mass, while the bead-vortexing method produced maximum 37 separated protein bands.

Distribution of proteins, based on Mw was also different in the methods compared. Regarding molecular weight, six different groups were created ranging from 15 to 320 kDa (Fig. 2). Of them the proteins of 15-49 kDa proved the highest amount of whole-cell proteins (50%) followed by the 50-99 kDa set (40%) obtained by the X-Press method at all initial bacteria mass (Fig. 2A), and by the TCA/acetone/phenol method using 100-350 mg bacteria (Fig. 2B). The bead-vortexing technique provided conspicuously different molecular weight distribution with a higher rate of the high molecular weight proteins (Fig. 2C).

Moreover, to investigate the effectiveness of the selected protein extraction methods, total protein extracts was separated on the 2D gel covering pH 3-10 and molecular masses of 14 to 116 kDa ranges. Building on the 1D SDS-PAGE results samples from the 300 mg, initial weight of bacteria was compared. As shown in Figure 3, the X-Press and TCA/acetone/phenol methods were obtained a similar, high resolution 2D protein separation, revealing approximately 372 ± 36 and 331 ± 28 spots, respectively, while the bead-vortexing technique provided only 227 ± 25 spots. Most proteins were separated in the molecular mass range of 10-90 kDa. The pH range where the most of protein appeared was pH 4-7, but in this aspect minor differences were observed among the methods used.

Discussion

Appropriate cell disruption and protein extraction method is crucial for proteomic strategy, including the protein yield or outcome of gel electrophoresis. There are numerous methodical studies on optimal release of proteins from bacteria, which concluded for example that the effectiveness of freezing-thawing is strongly dependent on the density of suspensions and on the number of cycles (Benov and Al-Ibraheem 2002), or that the major factor that influenced cell disruption by vortexing is the ratio of beads and the duration of treatment (Velapatiño et al. 2013), as well as the number of passes in French press (Jaschke et al. 2009) and the list goes on. The conclusion of these and other studies suggest that every single experiment needs careful consideration for the selection and adaptation of the appropriate method optimised for the actual organism and the aim of the study (Malafaia et al. 2015). There is no single method for efficient isolation of all kinds of proteins of interest. This statement may

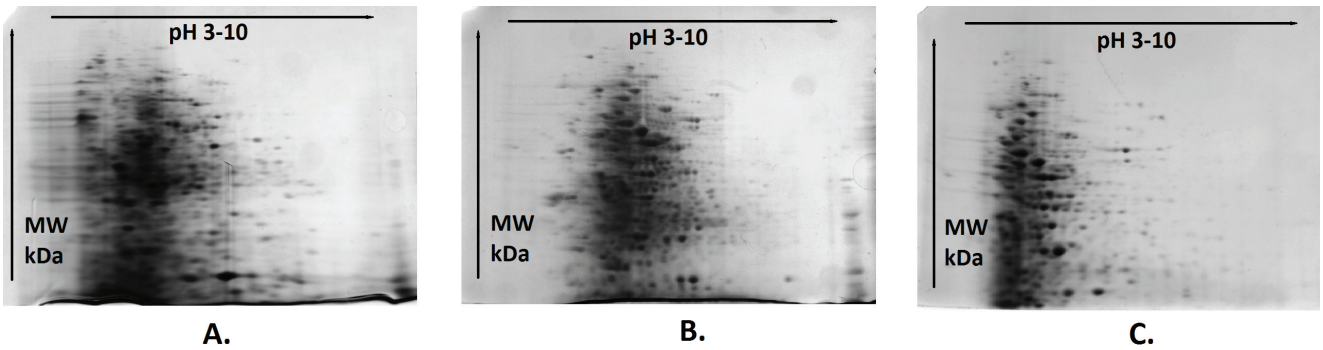

Figure 3. Representative 2D whole cell protein profile of *S. azurea* SZMC 14600 using, A: X-Press; B: TCA/acetone/phenol; C: Bead-vortexing cell disruption and extraction method.

be based on the different chemical composition of cells. In any case, during sample preparation the cell envelope as the main barrier needs to be disrupted to recover the intracellular materials. For Gram-positive bacteria, the main barrier is a thick cell wall, which is mainly composed of peptidoglycan, teichoic acid and polysaccharides. In Gram-negative bacteria, the peptidoglycan cell wall is significantly thinner than in Gram-positive bacteria, which itself is surrounded by an external layer composed of lipopolysaccharides and proteins. Another difference is the presence of the periplasmic layers, which many times thicker than is found in the Gram-positive ones. The composition of cell wall or periplasmic layer may fundamentally determine the cell disruption method (Tan et al. 2011; Cafaro et al. 2014).

In this study four commonly used protein extraction methods were compared to achieve efficient whole-cell protein extraction from *S. azurea* SZMC 14600. To answer the question, in the light of protein content and 1D SDS-PAGE gel results, it can be stated that of methods applied X-Press produced the most reliable productivity independent of the initial weight of bacteria. The bead-vortexing and freezing-throwing techniques proved to be unusable in this respect, while the efficacy of TCA/acetone/phenol method was commeasurable with X-Press, but only for 100-300 mg initial bacteria mass (Fig. 1, Table 1, 2). Furthermore, it can be concluded that the X-Press and TCA/acetone/phenol methods provided similar results with respect to the protein patterns, while the bead-vortexing offered different distribution in molecular mass (Fig. 2). Thus, the latter method is considered inadequate to produce usable images. The results of 2D protein separations showed a good coincidence with these statements. The number of protein spots and the pH region of the greatest protein expression was similar that found in other bacterial species (Wongtrakoongate et al. 2007; Yang et al. 2014). Noticeable, but slight difference among the methods on pH distribution of 2D electrophoresis separated protein spots (Fig. 3) can be attributed to the different concentration of salt or amphoteric components in the extracting solutions which could affect the IEF (isoelectric focusing), the first properties of 2D separation.

In summary, for *S. azurea* SZMC 14600, as a Gram-positive bacterium, the X-Press or TCA/acetone/phenol method proved to be suitable for whole-cell protein extraction, therefore they are recommended for use in proteomic studies. Our results provide a good starting point for further 2D electrophoresis studies combined with MALDI-TOF peptide mass mapping to get more accurate proteomic information on the molecular mechanism responsible for primycin metabolism.

Acknowledgements

The present scientific contribution is dedicated to the 650th anniversary of the foundation of the University of Pécs, Hungary

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