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Effects of Extraction Methods and Age of Cells on the Whole-cell Protein Patterns of *Lactobacillus*

[1,2]C.C. Sieo, [2]B.T. Wong, [1,2]N. Abdullah and [1]Y.W. Ho
[1]Institute of Bioscience
[2]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

Abstract: Whole-cell protein fingerprinting has been used in various studies for identification, characterization and differentiation of bacteria. It has been observed that the methods of extraction of the whole-cell protein and age of the cells used were varied in several studies. Thus, in this study, the effects of different extraction methods and age of cells on the whole-cell protein patterns were evaluated. The whole-cell proteins from *Lactobacillus reuteri* C1, C10 and C16, *Lactobacillus salivarius* I24 and *Lactobacillus gallinarum* I16 and I26 were extracted from 6, 12, 18 and 24-h-old cells by using lysozyme treatment, glass beads and sonication. A comparison of the whole-cell protein profiles generated showed that extraction by lysozyme treatment produced the most complete profile, with protein bands at high intensities. Glass beads and sonication could only extract major proteins. The age of cells also affected the whole-cell protein profile. Different strains were found to produce optimum results at different cell ages when extracted with the three methods.

Key words: Age of cells, glass beads, lysozyme, sonication, whole-cell protein

INTRODUCTION

Classification and identification are among the most important aspects in the taxonomy of microorganisms. Many of the microorganisms were classified and identified based on their physiological and biochemical characteristics. Although this technique has been proven to be a useful and indispensable tool, it is regarded as time-consuming and limited in terms of both its discriminating ability and accuracy (Sánchez *et al*., 2003). More recently, protein expressions have been applied to study the microorganisms. In particular, typing schemes of the microbial proteins via electrophoresis of the generated protein fingerprint profiles has become a general method for comparison and differentiation of bacterial strains.

The protein typing schemes rely on the presence of a relatively small number of major proteins within the organism to allow for visual differentiation. The protein profiles may be generated by using sub-cellular fractions such as their membrane extracts, or their specific groups of proteins, e.g., bacterial penicillin-binding proteins, for analysis. However, the preparation process of these proteins is tedious and complicated. Alternatively, as an easy way, the whole-cell protein lysate was recently being used for the analysis. In fact, this is preferred compared to the earlier mentioned techniques as it is fairly fast and easy and when performed under highly standardize conditions, could give a good

Corresponding Author: Dr. Sieo Chin Chin, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Tel: +603-89466702 Fax: +603-89430913

level of taxonomic resolution at species or subspecies level. Good correlation between the results of the numerical analysis of protein patterns and DNA/DNA hybridization for differentiation of bacteria has also been reported (Chen *et al*., 2007).

The whole-cell protein fingerprinting technique has been applied successfully in various studies. Millership and Want (1992) suggested that the whole-cell protein fingerprinting is much simpler and more useful for microorganisms e.g., *Mycobacterium tuberculosis*, which requires to be processed under containment. Others have also applied this technique for the identification of lactic acid bacteria from spontaneous fermentation of Almagro eggplants (Sanchez *et al*., 2003), differentiation of *Candida albicans* from oral cavities of healthy children (Boriollo *et al*., 2003), analysis on changes in the composition of microflora on vacuum-packaged beef during chiller storage (Sakala *et al*., 2002) and identification of *Helicobacter callitrichis* (Won *et al*., 2007). However, it has been observed that the methods of extraction of the whole cell proteins were varied among those studies. Cultures of 3-48 h were used and the whole cell proteins were extracted by lysozyme treatment, glass beads vortexing or sonication (Gatti *et al*., 2001; Sakala *et al*., 2002; Flemming *et al*., 2007; Svec *et al*., 2007). It is not known whether cells of different ages and different extraction methods would generate different protein profiles. Therefore, this study was undertaken to analyse and compare the profiles of the whole-cell protein extracted from cells of *Lactobacillus* spp. with different ages and by different extraction methods.

MATERIALS AND METHODS

Source and Maintenance of Lactobacillus Isolates

L. reuteri C1, C10 and C16, *L. salivarius* I24 and *L. gallinarum* I16 and I26 (Jin *et al*., 1996) were grown in MRS broth at 39°C under anaerobic condition provided by GasPak Anaerobic Systems. Routine maintenance of the isolates was carried out by transferring 1% (v/v) of the culture into fresh MRS broth every 48 h. When not in use, the isolates were stored at -80°C in MRS broth containing 15% (v/v) glycerol. Analysis of the whole cell protein pattern of the cells was carried out at the Institute of Bioscience, Universiti Putra Malaysia.

Whole-cell Protein Extraction

The whole-cell protein of the cells was extracted by using three different methods (lysozyme treatment, glass beads vortexing and sonication). The preparation of the cells for extraction was standardized for all the procedures. The cells were prepared by inoculating 1% (v/v) of a 24 h inoculum into 10 mL of MRS broth. After 6, 12, 18 and 24 h of incubation, the cells were harvested by centrifugation at 3000 x g for 10 min at 4°C. The cell pellet was washed twice with sterile distilled water and finally resuspended in sterile distilled water to OD_{600} 2.0. The prepared samples were then subjected to different whole-cell protein extraction procedures.

Extraction by Lysozyme Treatment

The extraction was carried out according to the method described by Kato *et al.* (2000) with slight modifications. The cells were spun down and suspended in 0.8 mL of 0.15 M Tris/HCl buffer, pH 6.8. Then 100 μL of 10 mg mL^{-1} lysozyme was added and incubated for 90 min. This was followed by the addition of 100 μL of SDS sample buffer [modified to contain 0.5 M Tris/HCl pH 6.8, 10% (w/v) SDS, 20% (w/v) glycerol, 1% bromophenol blue, 10% (w/v) 2- mercaptoethanol]. The suspension was heated at 100°C for 10 min (instead of 5 min) and then centrifuged at 13000 x g for 10 min at 4°C to remove the remaining whole-cell or non-proteinaceous debris which could interfere with the analyses (Coakley *et al*., 1977). The supernatant obtained was stored at -80°C until next use.

Extraction by Glass Beads

The extraction method was as described by Hébert *et al.* (2000) but with slight modifications. The cells were spun down and suspended in 1 mL of SDS sample buffer (composition modified as mentioned above). Then 0.3 g of glass beads (0.10-0.11 mm, Sigma) was used to disrupt the cells on a vortex mixer for 6 min. The suspension was heated at 100°C for 10 min, before it was centrifuged at 13,000 x g for 10 min at 4°C. The supernatant obtained was stored at -80°C.

Extraction by Sonication

The method used was slightly modified from that described by Vogel *et al.* (1994). The cells were spun down and suspended in 0.9 mL SDS sample buffer (composition modified as mentioned above). The suspension was sonicated for 1 min (instead of 3 min) on ice with a Soniprep 150 sonicator (United Kingdom), using an exponential microprobe. The process was repeated three times with 30 sec intermittence. Subsequently, 0.1 mL of 20% (w/v) SDS solution was added and the mixture was heated for 10 min at 95°C. The mixture was centrifuged at 13,000 x g for 10 min at 4°C. The supernatant obtained was stored at -80°C.

Electrophoresis of the Whole-cell Protein

The whole-cell protein was analysed on discontinuous gels in vertical slab apparatus (Bio-Rad Mini-Protein® 3 Cell). The extracted samples were heated for 10 min at 95°C and were loaded into 0.75 mm thick gels with 12% separating gel and 4% stacking gel. The gel was stained with Coomassie Blue.

RESULTS

Extraction of the Whole-cell Protein from Cells of Different Ages by Various Extraction Methods

The whole-cell protein profiles with different intensities were obtained from cells of different age and extracted with different methods. In lysozyme treatment, the intensities of the protein bands for all the isolates, except *L. gallinarum* I16 and I26, were found to be reduced with age. For *L. gallinarum*, the highest intensity bands were obtained in the 12- and 18-h-old cultures. For the other isolates, young cells of 6- h-old produced the clearest protein profiles, as shown by the representative gel in Fig. 1. The effectiveness of glass beads in the whole-cell protein extraction was strain dependent. Clearest protein profile was obtained at different age of cells for different isolates. For instance, the clearest protein profiles were obtained in 6-h-old *L. reuteri* C1 and C10, 12-h-old *L. reuteri* C16 and *L. salivarius* I24 (Fig. 2) and 18-h-old *L. gallinarum* I16 and I26. When extracted by sonication, the clearest protein profiles were obtained in 6-h-old *L. reuteri* C1 and C16, *L. salivarius* I24 and *L. gallinarum* I26, 12-h-old *L. reuteri* C10 and 18-h-old *L. gallinarum* I16 (a representative gel is shown in Fig. 3).

Effect of Extraction Methods on the Whole-cell Protein Profiles

To determine the effect of extraction methods on the whole-cell protein profiles, the clearest (distinctive) protein profiles of all the *Lactobacillus* isolates obtained through different extraction methods (described above) were compared. For all the isolates, extraction by lysozyme treatment produced the most number of bands with higher intensity when compared to the other extraction methods (Fig. 4 shows the whole-cell protein profiles of *L. reuteri* as an example). A comparison of the protein profiles also showed that different banding profiles were produced for different species of

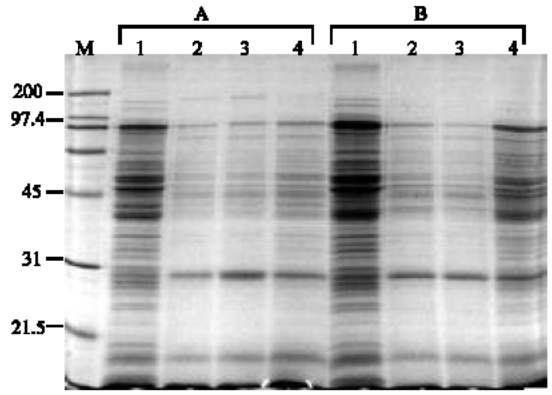

Fig. 1: SDS-polyacryalamide gel of the whole-cell proteins extracted from cells of different ages of *L. reuteri* C16 (A) *L. salivarius* I24 (B) by lysozyme treatment. Lane M: Molecular mass standards (in kilodaltons); Lanes 1-4: 6, 12, 18 and 24-h-old cells, respectively

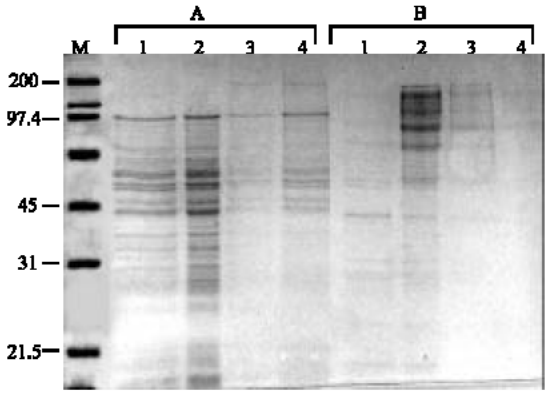

Fig. 2: SDS-polyacryalamide gel of the whole-cell proteins extracted from cells of different ages of *L. reuteri* C16 (A) *L. salivarius* I24 (B) by glass beads. Lane M: Molecular mass standards (in kilodaltons); Lanes 1-4: 6, 12, 18 and 24-h-old cells, respectively

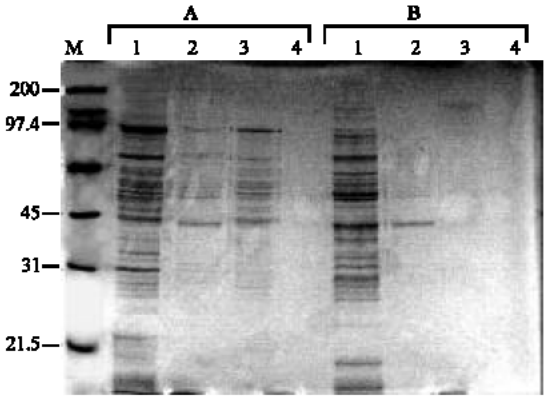

Fig. 3: SDS-polyacryalamide gel of the whole-cell proteins extracted from cells of different ages of *L. reuteri* C16 (A) and *L. salivarius* I24 (B) by sonication. Lane M: Molecular mass standards (in kilodaltons); Lanes 1-4: 6, 12, 18 and 24-h-old cells, respectively

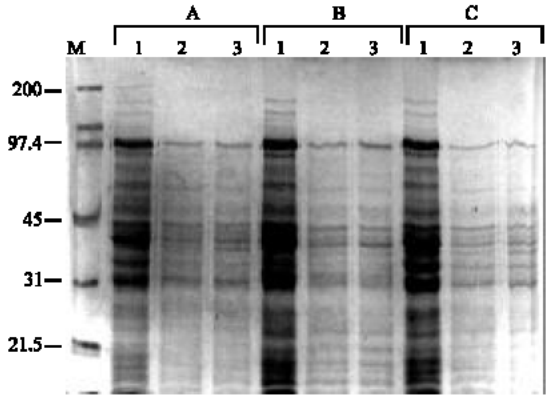

Fig. 4: SDS-polyacryalamide gel of the whole-cell proteins of *L. reuteri* C1 (A), *L. reuteri* C10 (B) and *L. reuteri* C16 (C) extracted by lysozyme treatment (Lane 1), glass beads (Lane 2) and sonication (Lane 3). Lane M: Molecular mass standards (in kilodaltons). The whole-cell protein profiles shown were obtained from 6-h-old cells, except for the whole-cell protein of *L. reuteri* C10 extracted by sonication [Lane B(3)] and whole-cell protein of *L. reuteri* C16 extracted by glass beads [Lane C(2)], in which 12-h-old cells were used

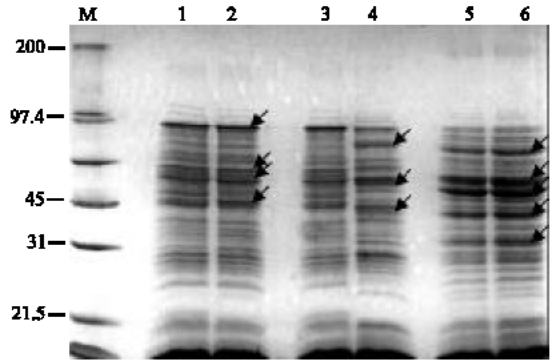

Fig. 5: SDS-polyacryalamide gel of the whole-cell proteins of *L. reuteri* C1 (Lane 1), *L. reuteri* C10 (Lane 2), *L. reuteri* C16 (Lane 3), *L. salivarius* I24 (Lane 4), *L. gallinarum* I16 (Lane 5) and *L. gallinarum* I26 (lane 6) extracted by lysozyme treatment. Lane M: Molecular mass standards (in kilodaltons). The whole-cell protein profiles were obtained from 6-h-old cells, except for *L. gallinarum* I16 and I26, in which 12-h-old cells were used. Major protein band for respective *Lactobacillus* species

Lactobacillus, regardless of the extraction methods used. For instance, when extracted by lysozyme, major protein bands with sizes 83.2, 60.2, 56.2 and 47.9 kDa were produced for *L. reuteri* (Fig. 5). For *L. salivarius*, major protein bands with sizes 91.2, 53.7 and 45.7 kDa were observed. Major protein bands with sizes 67.6, 56.2, 48.9, 41.7 and 33.1 kDa were observed for *L. gallinarum*. Different distinctive profiles were produced for different species when different extraction method was used (Fig. 6 and 7). Thus, all the methods could be used to differentiate the *Lactobacillus* to different species.

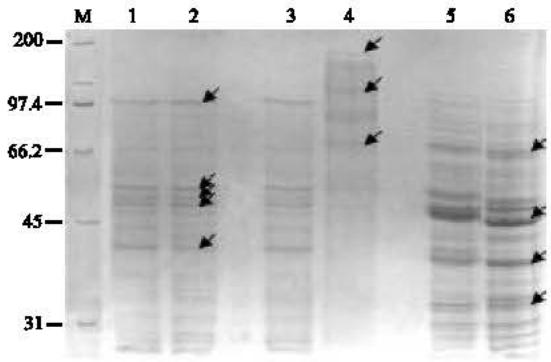

Fig. 6: SDS-polyacryalamide gel of the whole-cell proteins of *L. reuteri* C1 (Lane 1), *L. reuteri* C10 (Lane 2), *L. reuteri* C16 (Lane 3), *L. salivarius* I24 (Lane 4), *L. gallinarum* I16 (Lane 5) and *L. gallinarum* I26 (Lane 6) extracted by glass beads. Lane M: Molecular mass standards (in kilodaltons). 6-h-old *L. reuteri* C1 and C10, 12-h-old *L. reuteri* C16 and *L. salivarius* I24 and 18-h-old *L. gallinarum* I16 and I26 were used. Major protein band for respective *Lactobacillus* species

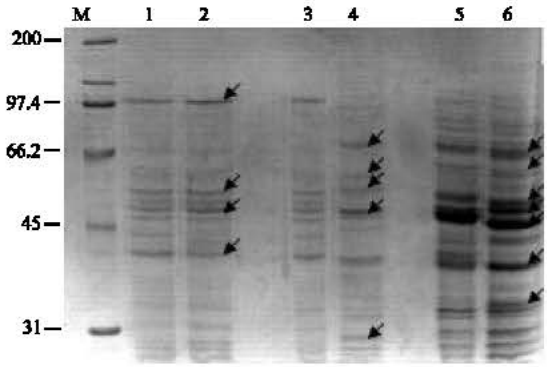

Fig. 7: SDS-polyacryalamide gel of the whole-cell proteins of *L. reuteri* C1 (Lane 1), *L. reuteri* C10 (Lane 2), *L. reuteri* C16 (Lane 3), *L. salivarius* I24 (Lane 4), *L. gallinarum* I16 (Lane 5) and *L. gallinarum* I26 (Lane 6) extracted by sonication. Lane M: Molecular mass standards (in kilodaltons). The whole-cell protein profiles were obtained from 6-h-old cells, except for *L. reuteri* C10 and *L. gallinarum*, in which 12-h-old and 18-h-old cells, respectively, were used. Major protein band for respective *Lactobacillus* species

DISCUSSION

The results of the present study showed that in general, the age of cells affected the whole-cell protein profile, especially those extracted by lysozyme treatment. The intensity of the protein bands was higher in the younger cells than in the older ones. Although most proteins would be expressed along with the maturation of cells, extraction may not be efficient due to interference by the deposited extracellular materials as the cells aged. The presence of extracellular materials may prevent or reduce the actions of lysozyme, which targets on 1, 4-glycosidic bonds between N-acetylglucosamine and

N-acetylmuramic acid in peptidoglycan (Madigan *et al*., 2000). Thus, lysozyme may not be as effective in the older cells. As observed from the results of the present study, the extractions were more efficient in young cells (6 h). However, in older cells, the structure of the cell wall may change and this may ease the extraction of the proteins by lysozyme as compared to actively growing cells. In addition, it is strongly believed that the physical nature of the cells, for instance, the formation of chains or clumps, would also indirectly affect the effectiveness of lysozyme. The efficient extraction in young cells also provides advantage in which analysis could be carried out without long hours of incubation.

As opposed to lysozyme, glass beads break the cells through mechanical rupture. The effectiveness of glass beads in extracting the whole-cell protein would depend on the contact of glass beads with the cells. Thus, the size of the cells may indirectly affect the extraction process. During growth, the cells would elongate to a certain stage before the process stops. The contact of glass beads with the cells may be highest during the elongation process and thus the extraction process would be most effective at this stage. As the growth characteristics of each species differ, it is not surprising to observe that the best protein profile obtained from this method is strain dependent.

In sonication, the cells are lysed through vibration at high frequency. Therefore, the thickness of the cell wall would be the main barrier to extract the whole-cell protein. The presence of extracellular materials may also affect the effectiveness of this method. Thus, for most of the isolates (*L. reuteri* C1, C16, *L. salivarius* I24 and *L. gallinarum* I26), the method was more effective for young cells (6 h).

Among the extraction methods, lysozyme treatment produced the most complete protein profile with major and minor proteins. This may be due to the specific activity of lysozyme which led to the effective lyses of the cells compared to glass beads and sonication, in which the lyses may occur randomly. A comparison on the whole-cell protein profiles among the isolates obtained by the different extraction methods showed that isolates of the same species produced highly similar profiles. Gevers *et al*. (2000) reported that highly similar, if not identical, protein profiles were observed for same species of bacteria. In their study, protein profiles with more than 92% similarity were clustered into same species.

CONCLUSIONS

The results of the present study indicated that the age of the cells and extraction method should be taken into consideration during extraction of the whole-cell protein. In the case of the present study, 12-h-old *L. gallinarum* I16 and I26 and 6-h-old of other isolates produced good banding profiles when extracted by lysozyme treatment. When glass beads were used, 6-h-old *L. reuteri* C1 and C10, 12-h-old *L. reuteri* C16 and *L. salivarius* I24 and 18-h-old *L. gallinarum* I16 and I26 produced better results. By sonication, 6-h-old *L. reuteri* C1 and C16, *L. salivarius* I24 and *L. gallinarum* I26, 12-h-old *L. reuteri* C10 and 18-h-old *L. gallinarum* I16 were recommended to be used. Although the results of the present study were restricted to *Lactobacillus* spp., parameters such as the best cell age and extraction method (which produced the highest number of bands with good intensities) should be determined in order to increase the discriminatory power of this technique.

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