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Characterization of Bacterial Lysates by Use of Matrix-Assisted Laser Desorption–Ionization Time of Flight Mass Spectrometry Fingerprinting

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Abstract

Bacterial lysates have for long been used to boost the immunological response to Respiratory Tract Infections (RTI) both in children and adults. They are prepared by growing bacteria usually associated with RTI, followed by chemical or mechanical disruption to prepare single bacterial lysates that are combined in the final product.

Despite the wide range of applications, one drawback for their universal use is the difficulty to assure consistency in their composition given their particular form of preparation; thus there is a need for alternative analytical methods that ensures batch composition consistency.

Here, we demonstrate that MALDI-TOF MS provides reliable and reproducible mass spectral fingerprints for bacterial lysates of S. pneumoniae, H. influenzae, K. pneumoniae, Staphylococcus spp. We also found that mechanical disruption provides markedly better-defined fingerprints. Analysis of the formulated Polyvalent Bacterial Mechanical Lysate (PBML) also showed a characteristic spectrum.

Overall, we found that mechanical lysis coupled to MS analysis allowed for accurate and highly sensible detection of key proteins in each bacterial lysate, a method that can be used to standardize batch-to-batch product composition. Applying this methodology to the production pipeline shall result in better products expanding the acceptance of these cost-effective tools to prevent respiratory tract pathologies.

Keywords: Respiratory infections; bacterial lysates; mass spectrometry.

Abbreviations: MALDI-TOF MS: Matrix-Assisted Laser Desorption–Ionization Time of Flight Mass Spectrometry Mass; RTI: Respiratory Tract Infections; PBML: Polyvalent Bacterial Mechanical Lysate; PBL: Polyvalent Bacterial Lysates; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; OD: Optical Density; TFA: Trifluoroacetic acid.

Introduction

Polyvalent Bacterial Lysates (PBL) has been widely used for decades for prevention of respiratory diseases [1]. They contain whole inactivated microorganisms or defined cellular components from different bacterial strains frequently implicated in upper and lower RTIs i.e. *Staphylococcus aureus, Streptococcus viridans, Streptococcus pneumoniae, Streptococcus pyogenes, Klebsiella pneumoniae, Klebsiella ozaenae, Moraxella catarrhalis, Hemophylus influenzae* [2]. The emergence and up rise of antibiotic resistant bacteria, has posed new problems to the burden and effective control of these pathogens, and there is a need for new treatment modalities that can respond to this situation. Many infectious diseases that were once easily treatable with antibiotics are now a serious health threat because use of antibiotics may lead to even more resistant infectious strains [3].

Because of the clinical relevance of respiratory infections and the importance of the development of new immunization antigens, bacterial lysates used individually or as combination of them (polyvalent) have recently gained new interest due to their capacity to induce a range of effects on the immune system by reducing the level of colonization [4,5]. Although their safety levels are considered acceptable, as are their anti-infective properties, there have been claims that fuller understanding of their mechanism of action is needed, as well as a detailed description of their effects. Consequently an extensive chemical characterization of the antigens to obtain high reproducibility between the preparations and an understanding of their chemical properties may provide better comprehension of their mechanism of action and therefore enhance their safety [2,6].

Bacterial lysates can be obtained by progressive alkaline hydrolysis or by high pressure cell disruption (mechanical disruption). The latter leads to well-conserved antigenic structures avoiding the denaturalization produced by the use of chemical products [7]. There are many different brands of bacterial lysates currently being marketed and several patents describing their preparation and their chemical characterization, that mainly rely on classic laboratory methods such as SDS-PAGE electrophoresis, protein and carbohydrate content among others [7,8,9]. At present considerable effort is being devoted to improve available methods for bacterial lysates characterizations. The introduction of matrix-assisted laser desorption/ionisation

(MALDI-TOF) has proven a significant progress in this field [10,11,12]. The technique allows the acquisition of unique mass signatures for each microorganism and is thus ideal for the characterization of bacterial lysates [13]. MALDI-TOF technique is used to produce a single charged ion (the "soft ionization" method) which preserves proteins integrity. Briefly, the analyte is deposited on a target plate embedded in a crystalline matrix that absorbs laser energy. Once ionized and desorbed sample molecules are analyzed by a mass analyzer (a component of mass spectrometer) and the mass spectrum of the sample sequence can be achieved based on the ratio of molecular weight to charge (m/z) [12,13,14].

Furthermore the technique can quickly identify few bacterial cells present in unknown cellular suspensions or from complex mixtures [13,14]. The ability of technique to rapidly and accurately discriminate between bacterial species has become a revolution in the clinical microbiology and has demonstrated the power of this tool [15,16,17].

Nevertheless, it has been reported that only a small fraction of the proteins primarily separated by SDS PAGE are identified during the MALDI TOF analysis. The reason for such protein behavior is has been explained due to the variation in the ionization properties of the proteins, the limited energy available for proteins ionization process or the presence of non ionizable impurities [18]. However the robustness of the technique has been demonstrated for a wide range of organisms [15,19,20].

The aim of this work was to combine high pressure lysis (mechanical disruption) of bacteria with the MALDI-TOF MS technique, to obtain spectral protein profiles of bacterial lysates and to develop a standard and reproducible protocol for the characterization of these bacterial lysates.

Currently, the work in this field is focused on investigations about the reproducibility of these spectra [19]. The technique can also be used in conjunction with other chemical and biochemical techniques to detect key proteins in complex mixtures as bacterial lysates given that proteins are considered to be the most reliable mass spectrometry biomarkers [15,19,21].

Material and Methods

Monovalent bacterial lysates of Streptococcus pneumoniae (ATCC® BAA334™), Haemophilus influenza (ATCC® 19418™), Klebsiella pneumoniae subsp. pneumoniae (ATCC® 10031™), and Staphylococcus aureus subsp. aureus (ATCC® 25923™), were used for the experiments described in the present work. Hartman serum, inorganic salts glucose, amino acids and vitamins were from Sigma–Aldrich.

MALDI matrix 3, 5-Dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid) and peptide calibration standard mix were purchased from Bruker Daltonics (Billerica, MS, USA).

The total protein concentration of the lysate was estimated by the Bicinchoninic Acid Method [22]. The carbohydrate content was estimated by the Phenol-Sulfuric acid method and SDS-PAGE electrophoresis analysis was done according to [23,24].

The cultivation media for *Staphylococcus aureus, Klebsiella pneumoniae, Haemophilus influenza* was performed in a standard prepared medium containing: sodium chloride (2g/L), dibasic sodium phosphate (2g/L), sodium acetate (0.5g/L), vegetable soy peptone (40g/L), inosine 0,1 g/L) and glucose(6g/L). Both Hemin and nicotinamide adenine dinucleotide (NADH) in a final concentration of 25mg/ml each was added for *Haemophlius influenzae* cultivation. *Streptococcus pneumoniae* was grown in a chemically defined medium previously reported by Texeira [25]. Furthermore the cultivation of *S. pneumoniae* and *H. influenzae* was performed at 37°C in 5% CO_2 atmosphere.

Preparation of bacterial lysates

Overnight cultures of each individual bacterium were grown for 12 h at 37°C at 200 rpm. Turbidity was verified and the optical density (O.D) at 600 nm was monitored. 1/100 dilution was made and the culture was reincubated for 4 hours at 37° C until O.D values between 1,0 to 1,2 were reached. For *Haemophilus influenzae* the dilution after 12 h cultivation was 1/50. At the end of the cultivation time the bacterial suspension was centrifuged, afterwards the pellet was resuspended in 100 mL of Hartmann serum and the lysis was performed.

Mechanical lysis: The bacterial suspension (100 mL) was applied twice to a homogenizer EmulsiFlex-C3 with constant flow-through capacity of 3L/hr. The homogenizing pressure was adjusted between 500 and 30000psi or 35 and 2000bar. The polyvalent bacterial lysates (PMBL) were prepared by mixing 250 µg/mL protein of each monovalent bacterial lysates.

MALDI-TOF analysis

MALDI-TOF measurements were conducted on a Microflex LR MALDI-TOF (Bruker Daltonics, Billerica, MS, USA) with a 337 nm nitrogen laser operated in positive ion lineal mode with delayed extraction and optimized in the m/z range of 0 to 20 kDa. Calibrations were performed with a peptide calibration standard mix (Bruker Daltonics). The laser was fired 100 times at each of ten locations for each sample well on a 96 well plate for a cumulative 1000 shots per sample well taken at 30% intensity.

At the time of analysis, 1 µl of each of the bacterial suspension was purified through a Zip Tip containing C18 and immediately after mixed with 1 µl of matrix solution (sinapinic acid 10 mg/ml in sterile H2O with 1% TFA) at ratios of 1:1. The sample and matrix mix was spotted onto a 96 well stainless steel plate and allowed to air dry for 15 minutes at room temperature.

Results and Discussion

There is a need to better characterize, standardize and control the bacterial lysates extracts in order to make them safer, more effective and longer lasting.

Polyvalent Mechanical Bacterial Lysates (PMBL) containing bacterial lysates derived from pathogenic bacterial strains namely *Klebsiella pneumoniae, Haemophilus influenzae, Staphyilococcus aureus* and *Streptococcus pneumoniae* as well as each monovalent lysate were subjected to MALDI TOF-MS analysis in order to identify proteins markers to be used for characterization.

Several research groups have demonstrated that MALDI-TOF is a useful technique to produce protein profiles after cellular extraction and purification [26,27,28].

In the present work each individual bacteria were grown in specific growth media, vegetable-based medium or in the case of S. pneumoniae a chemically defined medium afterwards filtered to remove larger cellular debris [25]. Thus, the resulting bacterial lysates only contained soluble molecular components.

Mechanical lysis of bacteria was selected as the method to lyse the cells, because it has the advantage to preserving the antigenic structure, as opposed to chemical methods like alkaline lysis which produce changes in protein structure [29]. All the lysates (individual or polyvalent) antigenic patterns were analyzed by SDS gel electrophoresis, protein and carbohydrate content and by a mass spectrometry. The matrix selected for the identification of the bacterial lysates proteins was Sinapinic Acid (SA), one of the most predominantly used organic matrices, which demonstrated to provide an effective ionization of the bacterial lysates proteins [30]. The spectra patterns obtained were evaluated on the basis of signal strength, resolution and reproducibility. These criteria are all essential for effective fingerprinting [19,28].

Figure 1a, b, c and d show representative spectral fingerprints of Staphylococcus aureus, *Haemophilus influenzae, Streptococcus pneumoniae* and *Klebsiella pneumoniae* by MALDI-TOF MS. The individual spectra profiles observed are the result of mechanical disruption which favors well-conserved protein structure which can be used as ideal markers for the characterization. The signals in the range between 4000 and 20000 Da were previously attributed to be highly cellular conserved housekeeping proteins or cell wall associated proteins and consequently being used as protein markers for identification of bacterial species [19]. Furthermore Krishnamurthy and collaborators reported that disrupted intact cells mixed with sinapinic acid (MALDI matrix) and subjected to direct matrix assisted laser desorption ionization (MALDI-MS) analysis released biomarkers in greater abundance and provide their molecular masses [28]. The protein pattern information is rapidly generated and the technique has clear advantages over the laborious SDS-PAGE technique.

Under the current experimental conditions, most spectral peaks observed were in the mass range of *m/z* 5000-12000 Da. Figure 1 a showed *Haemophilus influenzae* three possible biomarkers centered on around *m/z* 7181, 9123 and 12336 respectively with a high intensity in a reproducible manner. Hagg, et al. reported the use of MALDI/TOF-MS as a technique for the rapid identification and speciation of *Haemophilus influenzae* [31]. The mass spectral fingerprints reported by the authors differ between whole cells and those that have been lysed. In the present work the *m/z* protein values are coincident but not identical with the *m/z* protein values found by the authors for their lysates, a possible explanation for the differences could be due to the lysis method used [31]. Furthermore, in our experiments we found consistency in the patterns obtained from three independent grown samples, suggesting that MS spectrum profile of *Haemophilus influenzae* is reproducible and reliable and thus may be useful for characterization purposes.

In the case of *Staphylococcus aureus* Figure 1 b two peaks centered on around m/z 5500 and 7000 showed high intensity, a result similar to that reported by Bernardo and colleagues who reported two specific peaks m/z 5500 and m/z 7000 for S. aureus bacterial lysate obtained by MALDI TOF MS in a reflector mode [32]. Another study reported that nine of S. aureus clinical isolates shared m/z values very near those m/z values found by Bernardo [33]. In the present work these results were reproducible for *S. aureus* preparations supporting the idea that these proteins could be used as biomarkers for bacterial lysates characterization.

Figure 1 c shows the *Klebsiella pneumoniae* protein profile. *Klebsiella pneumoniae* bacterial lysate showed reliable protein pattern accuracy with m/z 7332, m/z 8621 and m/z 9142. All three peaks were previously found in *Klebsiella pneumonia* clinical isolates [33]. In that study there were reported stable fingerprint spectra for *Klebsiella pneumoniae* with a few more proteins peaks than the ones observed here, the difference could be explained due the extraction procedure carried out or the protonization efficiency of the proteins.

The bacterial lysate of *Streptococcus pneumoniae* Figure 1 d gave rise to ions near m/z 6200, m/z 6900 and m/z 8800 in MALDI-TOF mass spectra. Unfortunately there are not many reports in the literature using *S. pneumoniae* mechanically disrupted lysates for MALDI analysis, to be compared with the protein pattern found here. However there are some groups which used modified approaches for sample preparation of *S. pneumoniae* isolates to investigate differentiation of species [34]. The spectrum acquisition of *S pneumoniae* bacterial lysate presented here consistently showed presence of three peaks which proved to be specific for the strain used.

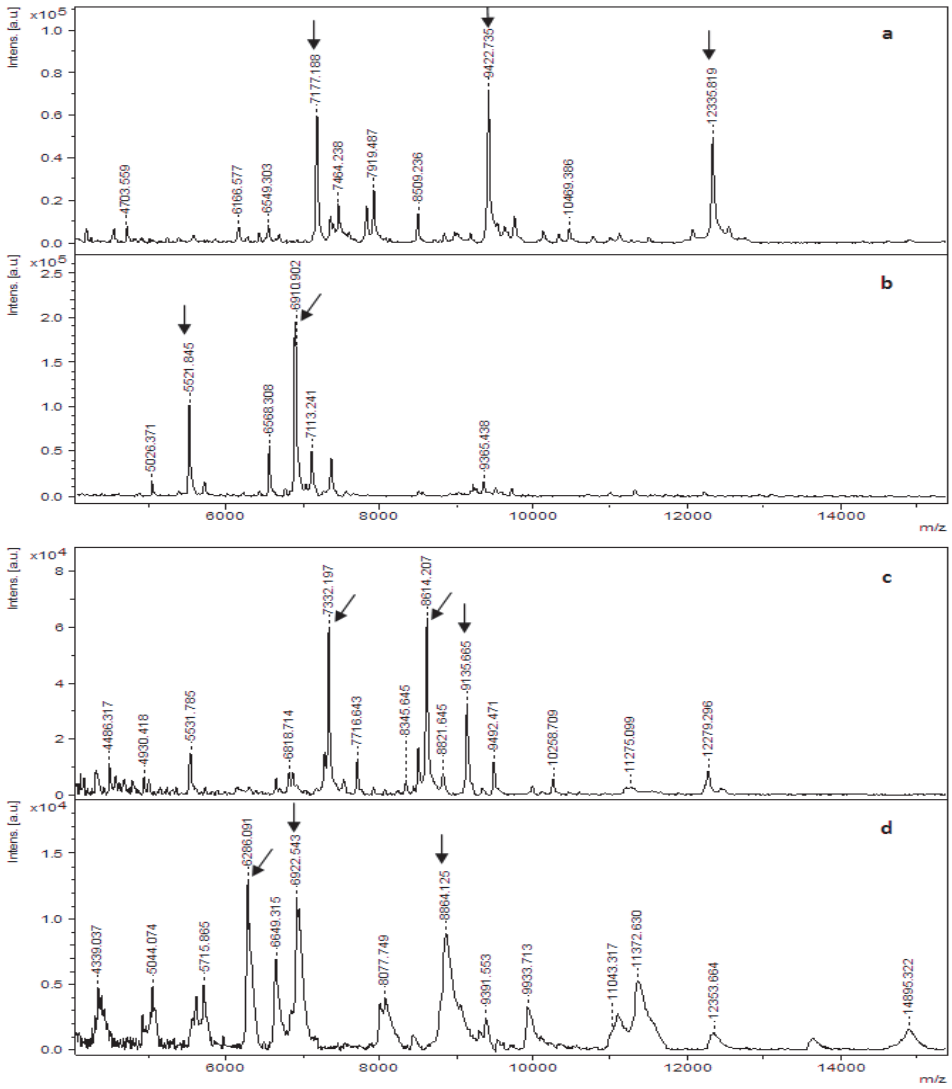

Figure 1: Representative MALDI-TOF mass spectra high intensity peaks are shown in a reproducible manner of; a) *Haemophilus Influenzae* bacterial lysate, m/z 7177, 9422, 12335; b) *Staphylococcus aureus* bacterial lysate, m/z 5521, 6910; c) *Klebsiella pneumoniae* bacterial bacterial lysate, m/z 7332, 8614, 9135; d) Streptococcus pneumoniae bacterial lysate, m/z 6286, 6922, 8864.

One of the factors that affect the obtained spectra is reproducibility. Some authors reported early that reproducibility is dependent on the MALDI-TOF standardization for experimental conditions such as the matrix used the sample: matrix ratio, the culture medium and growth conditions among others, although other studies have shown that a subset of peaks was conserved in the spectra obtained despite different experimental conditions [33,35]. The spectra pattern obtained in the present study for all the strains has shown high reproducibility. As shown in Figure 2, the mass spectral comparison of replicates of Klebsiella pneumoniae grown under same conditions on different days exhibited a high degree of similarity, generates a stable spectrum and provided evidence that these protein signals might serve as signature markers as previously reported [19,28].

Furthermore these ribosomal proteins are well represented and can explain the feasibility to use MALDI-TOF for bacterial identification and bacterial lysates characterization even without the standardization of experimental conditions. However, it is worth underlining that to optimize the reproducibility, a standardization of sample preparation should be established.

On comparing and analyzing the results obtained by MALDI-TOF MS with those from the conventional techniques SDS-PAGE Figure 3, it was noticed more proteins abundance that the respective MALDI spectra pattern, the discrepant results may be explained due to the deficiency of proton transfer. Ion suppression can arise from impurities that are less ionizable such as salts which can affect the quantity of charged ions hitting the detector [18]. A small pipette tip ''Zip-Tip'' filled with reverse phase sorbents (C18) was used to overcome this partial ionization suppression and improve the fingerprints obtained.

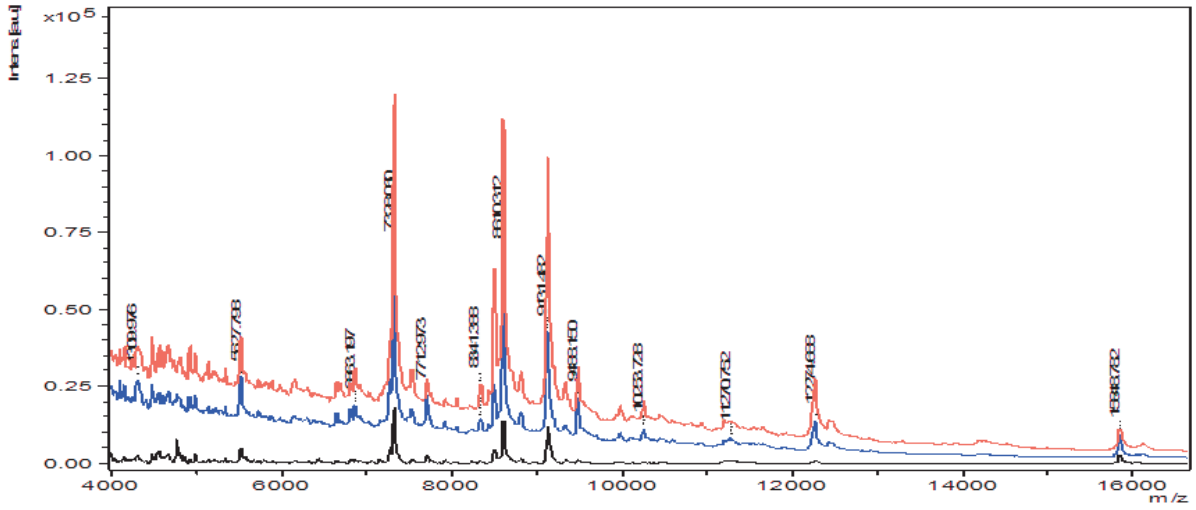

Figure 2: MALDI–TOF MS spectra of *Klebsiella pneumoniae* showing the reproducibility of spectra. Each spectrum represents an independently generated data set of freshly prepared bacterial lysates.

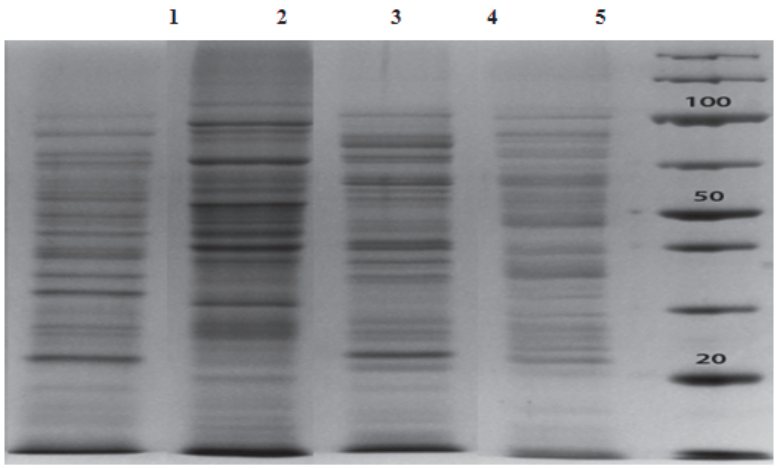

Figure 3: Representative bacterial lysates SDS-PAGE pattern.
SDS-PAGE analysis of mechanically prepared bacterial lysates. **1)** *S. aureus* bacterial lysate **2)** *S. pneumoniae* bacterial lysate **3)** *K. pneumoniae* bacterial lysate **4)** *H. influenzae* bacterial lysate. **5)** Thermo protein standard Molecular Weight 5-250 kDa

In Figure 4 the protein profile of the mechanically polyvalent bacterial lysate is shown. The instrument detected 89 protein peaks in the range of m/z 4000 and 20000 of which the most outstanding were at around on at m/z 7360, m/z 8800, m/z 9540 and m/z 12340. The remarkable reproducibility of the method allowed the measurement of proteins consistently expressed at a high level of abundance, and these biomarkers could be used for the characterization of the bacterial lysates.

To extend the bacterial lysate characterization both protein and carbohydrate content were measured. The average protein and carbohydrate content for all the bacterial lysates were in the range of 0.5 ± 0.019 mg/mL to 1.3 ± 0.019 mg/mL for protein and in 0.4 to 0.5 mg/mL for carbohydrates respectively. These values are in agreement to those found in the literature mainly in the patents which previously reported preparation and characterization of bacterial lysates [7,8,36].

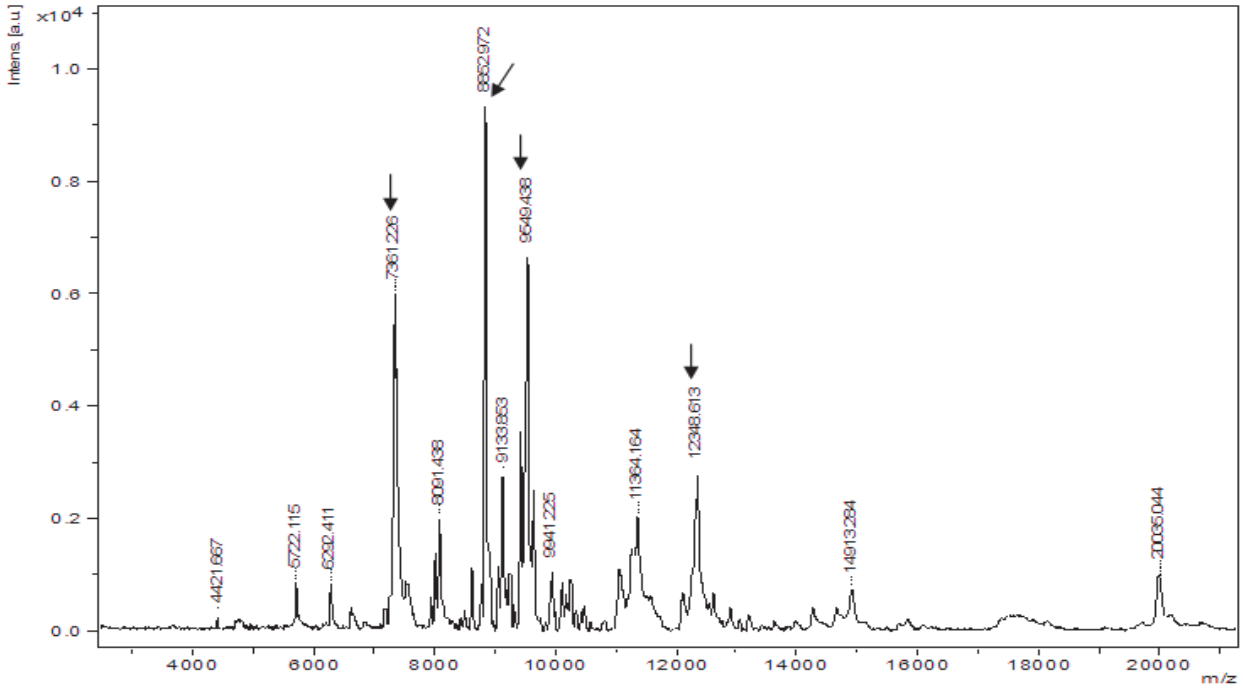

Figure 4: Representative MALDI-TOF spectra of Polyvalent Mechanic Bacterial Lysate (PMBL). MALDI-TOF mass spectra of Polyvalent Mechanical Bacterial Lysate (PMBL) showing four outstanding peaks around m/z 7361, m/z 8852, m/z 9549 and m/z 12348

Conclusions

MALDI-TOF MS analysis of bacterial lysates provided unique spectral patterns for monovalent as well as polyvalent bacterial lysates with high reproducibility. These characteristic fingerprints can thus be used to ensure consistency and reproducibility of bacterial lysate production. Mechanical disruption of bacterial cells using high-pressure lysis, resulted in more precisely defined fingerprints as compared with MS fingerprints obtained from lysates prepared by alkaline lysis, suggesting that mechanical disruption better conserves protein structure in the preparation.

MALDI-TOF MS analysis is simple, rapid and cost-effective, thus the methodology described has shown strong potential to be used as a complementary tool for bacterial lysate characterization. It should be further explored to achieve standard and reproducible protocols in bacterial lysates production, a critical step in the quality control of the final product.

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