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**DETECTING BACTERIA BY DIRECT COUNTING
OF STRUCTURAL PROTEIN UNITS
BY THE INTEGRATED VIRUS DETECTION SYSTEM (IVDS) AND
MASS SPECTROMETRY**

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PREFACE

The work described in this report was started in September 2004 and was completed in June 2005.

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DETECTING BACTERIA BY DIRECT COUNTING OF STRUCTURAL PROTEIN UNITS BY THE INTEGRATED VIRUS DETECTION SYSTEM (IVDS) AND MASS SPECTROMETRY

1. INTRODUCTION

This report explores the direct counting of "hair-like" structures, as shown in Figure 1, specific for gram-positive bacteria. These structures are also found on many other classes of bacteria and lead to the interesting possibility of determining a direct method for analysis by counting their numbers and determining a concentration. Indications show that these structures are intact after removal from the cell and are sufficiently different from species to species of bacteria to indicate a bacteria's type, if not its actual identity [1-2]. Their detection would represent a new approach to bacteria detection and identification. This report documents the detection of the bacterial structures using the physical nanometer counting methodology in the Integrated Virus Detection System (IVDS) and Electrospray Ionization-Mass Spectrometry (ESI-MS).

Bacteria are completely different types of microorganisms than viruses and are a magnitude larger in size. Bacteria are classified in their own scheme. They have cell walls, and they are organized into cellular components and generally are considered to be among the self-sustaining organisms. Viruses are sometimes not even considered to be alive in this sense and have a completely different life cycle. This difference has made some use detectors difficult to construct. The "hair-like" structures found on all classes of bacteria, however, open the possibility of using a physical virus detector for use in detecting and quantifying these unique structures that behave like a particle when separated from the bacterium. The ESI-MS will identify the detected proteins.

The IVDS can differentiate particles from 5-950 nm in size. Bacteria are generally 0.5-1 μm wide and 2-3 μm long, well outside the physical ability of IVDS. However, there are interesting features on the surface of a bacterium that are in the proper size range for the IVDS to characterize. The bacteria surface is complicated, from many points of view, and more complex than the surfaces of ordinary cells. Gram-negative bacteria, named because of their inability to retain crystal violet-iodine complex stain, have rigid surface appendages called "pili." These "hair-like" structures are around 7 nm in diameter and vary in length, up to 25 nm for the longer flagellate [3], which are other nanometer-sized structures that can be attached to the surface of bacteria. Pili are composed of structural protein subunits called "pilins." Some structures have only one structural protein unit, while other pili are more complex and have several. These pili consist of a precise helical arrangement of one or more types of protein and as indicated may have different lengths for different bacteria. Choudhury [4] and others discuss crystal complexes associated with pilin subunits. Cell lysis breaks the cell into components. Lysis can be achieved by changes in pH, temperature, and sonic treatment or by chemical means. The pili can then be treated as any nanometer particle, separated, and counted. Different pili proteins for different bacteria species can be expected. Evidence suggests that the IVDS can indeed "see" these virus-sized bacteria components, and thus detect bacteria.

Recent advances in the field of proteomics, where it is desirable to obtain a comprehensive mapping of the expressed proteins in an organism, and provide a promising strategy to characterize bacteria using their protein biomarkers [7-11]. Mass spectrometry is widely used in proteomic fields for characterizing various microorganisms by either identifying as many protein components as possible biomarkers [9] or identifying one or several protein component(s) in a complex mixture [10-11]. Various MS techniques had been extensively used to characterize bacteria rather than viruses [10-12]. Many factors attributed to this phenomenon (e.g., the significant number of protein biomarkers encoded in bacteria, and thermal stability bacterial proteins upon introduction into the ionization source of the MS) [14-17].

Mass Spectrometry had also been used to study the filamentous protein structures found in the pili part of bacterial cells [18-20]. Most studies were oriented toward investigating the post-translation modification of the pili proteins that play an important role in disease carrying from various microorganisms, mainly *Neisseria meningitidis* [21]. However, attempts to investigate the pili proteins in *Bacillus subtilis* using MS or IVDS were not reported in the literature. These studies address the characterization of pili proteins of *Bacillus subtilis* samples that were processed using various sample processing conditions and approaches. Using MS is a complimentary technique to IVDS where accurate and discriminatory characteristics and properties of the biomolecules are revealed.

2. EXPERIMENTAL PROCEDURES

2.1 IVDS.

A sample of *Bacillus subtilis* was obtained from Dr. Deborah Kuzmanovic, Geo-Centers, Inc., Gunpowder Branch, APG, MD. A 0.5-mL aliquot of the stock solution was passed in and out of a 22-ga. needle to break the pili from the surface of the bacteria. The shearing action of repeated passages through the needle removes pili from bacteria [5]. The sample was centrifuged at 12,000 rpm (10,000 g) for 25 min to pelletize the large bacterial cells. The supernatant was collected and diluted 1:100 with 20 mM ammonium acetate for examination in the IVDS (designated BS2). Figure 2 shows the peak associated with the bacterial pili between 10 and 12 nm.

The BS2 sample was filtered by the Ultra Filtration (UF) subsystem of the IVDS using a 100K-Da filter (for details on the IVDS and its different subsystems, the reader is referred to a companion report [6]). The filtration system's purpose is to remove from the solution any material (e.g., growth media, salt molecules, and proteins) with a molecular weight smaller than the weight (100K Da in this case) for which the filtration system is set, and leave a concentrated pili solution. The filtered sample was diluted 1-10 times with 20 mM ammonium acetate before analysis with the IVDS. Figure 3 shows the ultrafiltered bacterial sample (designated BS3) with the peak at ~12 nm.

The original sheared sample was also ultrafiltered, before dilution, using a 100K-Da filter and analyzed with the IVDS. After filtration, the filtered sample was diluted 1:10 with 20 mM ammonium acetate before analysis with the IVDS. Figure 4 shows the ultrafiltered neat bacterial sample with the peak at ~12 nm (designated BS1).

2.2 MS.

2.2.1 ESI-MS Parameters.

An ion trap mass spectrometer (LCQ-Deca, Thermo Finnigan-USA) equipped with an ESI ion source was used. The mass spectrometer was operated under the control of the X-Caliber program with a manual deconvolution algorithm. Spectra were collected in the positive ion mode. Three microscans were used with a maximum ion injection time of 200 ms. The ESI spray voltage was maintained at 4 kV. The capillary voltage was maintained at 23 V, and the temperature of the ion transport tube was 190 °C. The mass spectrometer was calibrated to achieve a ± 2 Da resolution using a mixture of cytochrome c, BSA, insulin, and myoglobin proteins.

2.2.2 Automated Deconvolution Algorithm Analysis.

The automated in-house deconvolution algorithm was developed to provide a filtered mass list instead of a conventional peak-at-every-mass output for the Biological Sample Processing System-Mass Spectrometry (BSPS-MS) analysis of bacterial extracts. A detailed procedure for the automated in-house software will be presented elsewhere. Briefly, the in-house software deconvolutes the bacterial protein masses through analysis of a raw mass spectral file. Mass range, isotope peak width, S/N threshold, and maximum number of returned peaks, and user input parameters were selected prior to the start of the deconvolution process. The software identified LC peaks and deconvoluted their corresponding average mass spectra to generate a list of masses. The total deconvolution process takes 10 min for a 60-min BSPS-ESI-MS analysis time. The deconvolution process is interfaced with relational database management software to update the in-house database with experimental bacterial protein masses.

3. RESULTS AND DISCUSSIONS

3.1 IVDS.

The sample of *Bacillus subtilis* that was sheared and shown in Figure 2, displays the pili protein at ~10-12 nm next to a very large peak, <10 nm, that results from cellular and growth media contamination. The large contamination peak was removed by ultrafiltration of the sample. The ultrafiltration molecular weight cut-off (MWCO) was 100K Da that allowed the removal of growth media and salts while retaining the pili fragments. After ultrafiltration, the peak showing the pili in solution was more pronounced and not masked by other contaminants.

To determine if the 1-100 dilution before ultrafiltration and IVDS analysis affected the pili, the neat sample was ultrafiltered after shearing and centrifuging. As shown in

Figure 4, the results from the IVDS does not show any significant difference from the diluted and ultrafiltered sample shown in Figure 3.

3.2 MS.

MS Results.

The unsurpassed sensitivity of ESI-MS analysis to biomolecules is an ideal technique to use to determine the molecular weight of intact proteins and their identity. The expansion and extensive genomic and proteomic databases provide relevant information for the identity of the biomolecules present in the analyzed sample in this study. Applying Liquid Chromatography-MS (LC-MS) in characterizing biomolecules originating from various parts of the bacterial cell has been successful in numerous studies [9-13]. In this study, the *Bacillus subtilis* samples underwent different sample processing approaches. A *Bacillus subtilis* sample was sheared, and centrifuged without any further filtration. This sample was labeled BS1. The other *Bacillus subtilis* samples were exposed to similar sample processing that included shearing of the bacterial cells followed by centrifugation and dilution, and finally molecular weight cut-off using a 100K-Da membrane to remove cellular debris and large particulates. These samples were labeled BS2 and BS3. Figure 5 shows the LC-MS analysis result of the BS1 sample. The upper chromatogram represents the total ion chromatogram of BS1, and the lower spectra are the mass spectra of the dominant proteins detected in this sample by the ion trap MS. It was observed that the peaks appearing toward the end of the TIC resulted from the presence of a large amount of buffer in the sample. This is reflected in the nonsymmetrical peak shape, which is characteristic of a mixture of nonproteinaceous molecules. Upon examining the mass list generated from the LC-MS analysis of BS1, the above mass spectra represents protein characteristic of the pili and flagella parts of the bacterial cell. Comparison of the deconvoluted protein molecular weights with the public database, Swissprot*, resulted in matching with outer coat and extracellular proteins that are associated with the pili and flagella parts of the bacterial cell [22]. The molecular mass lists of the bacterial protein are listed in Table 1. This table also enlists the accession number of the identified protein of LC-MS analysis of BS1 from the Swissprot* public database.

On the other hand, the LC-MS analyses of BS2 and BS3 samples showed significant resemblance between their TIC plots and the dominant bacterial protein identified. It is interesting to note that the TIC plots in Figures 6(a) and 6(c) are characterized by a dominant peak that has the same retention time observed in the LC-MS analyses of these bacterial extracts. This dominant peak is an indication of significant expression of this protein compared to the other observed proteins. The mass spectra of this dominant peak revealed that the same protein was found for both samples with better S/N observed in BS2 than in BS3. The difference in S/N ratio is because BS3 was diluted 100 fold more than BS2. The peak identity revealed that it is a coat protein in *Bacillus subtilis*, which is an indication of the effectiveness of the sample processing approach in isolating desired parts of the bacterial cells, in this case, the pili. It is also worth mentioning that this coat protein was observed in the BS1 sample but with lower peak intensity than those observed in the TIC plots of BS2 and BS3 samples.

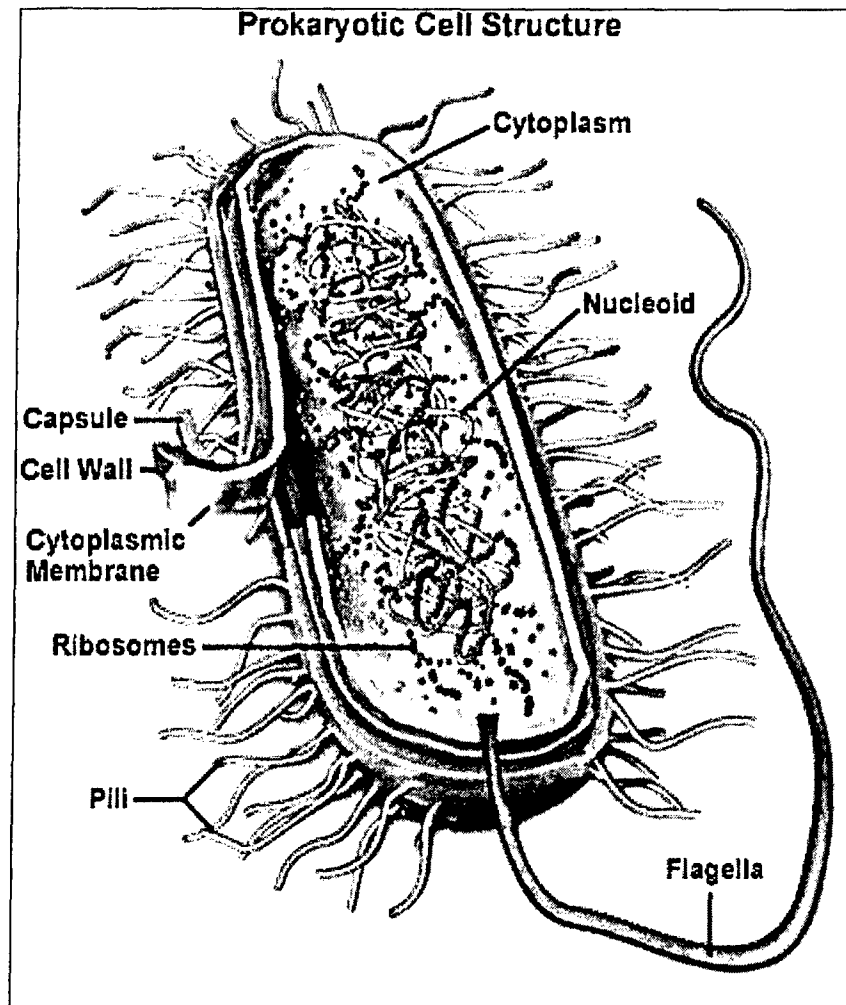
In addition, LC-MS data were investigated to determine data reproducibility and the effect of sample processing on the LC-MS analyses of *Bacillus subtilis* extracts. Figure 6

shows the reproducibility of the molecular weight of the deconvoluted proteins generated from the LC-MS analyses of the *Bacillus subtilis* extract samples. The masses generated for these samples were the averages from three replicate LC-MS analyses per each sample. The statistical correlation study showed that a higher number of common masses were observed between BS2 and BS3 than those observed with BS1. Figure 7 also reflects the relevance of comparing the mass lists generated from differently processed bacterial extract samples because as the correlation factor among samples increases, it indicates a resemblance in the sample processing approach as evident in this case. Samples BS2 and BS3 were processed under similar conditions with the only difference being in the dilution as compared to BS1. In addition, this observation was supported upon comparing the deconvoluted mass lists generated for BS2 and BS3, in which over 99% of the masses were matching with each other as shown in Figures 7 and 8.

Comparing the correlation in the masses among all samples is presented in Table 2, where the correlation factor of 99% match is observed between BS2 and BS3, as compared with 80-81% match with BS1. Overall, the LC-MS provided a confirmatory technique on the data obtained from the IVDS analysis. The similarity between the IVDS and LC-MS analysis of the *Bacillus subtilis* samples is observed. The similarity in comparing the IVDS data among the *Bacillus subtilis* extract samples was also observed within their corresponding LC-MS analyses.

4. CONCLUSIONS

The Integrated Virus Detection System (IVDS) is well suited for rapid physical measurements of the submicron pili of bacteria after mechanical removal. The pili were shown to be from *Bacillus subtilis* through mass spectrometry analysis. It may be possible, with further experimentation, to correlate the rapid identification of bacterial groups with the straightforward counting methodology of the IVDS.



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Figure 1. Bacterial cell constituents

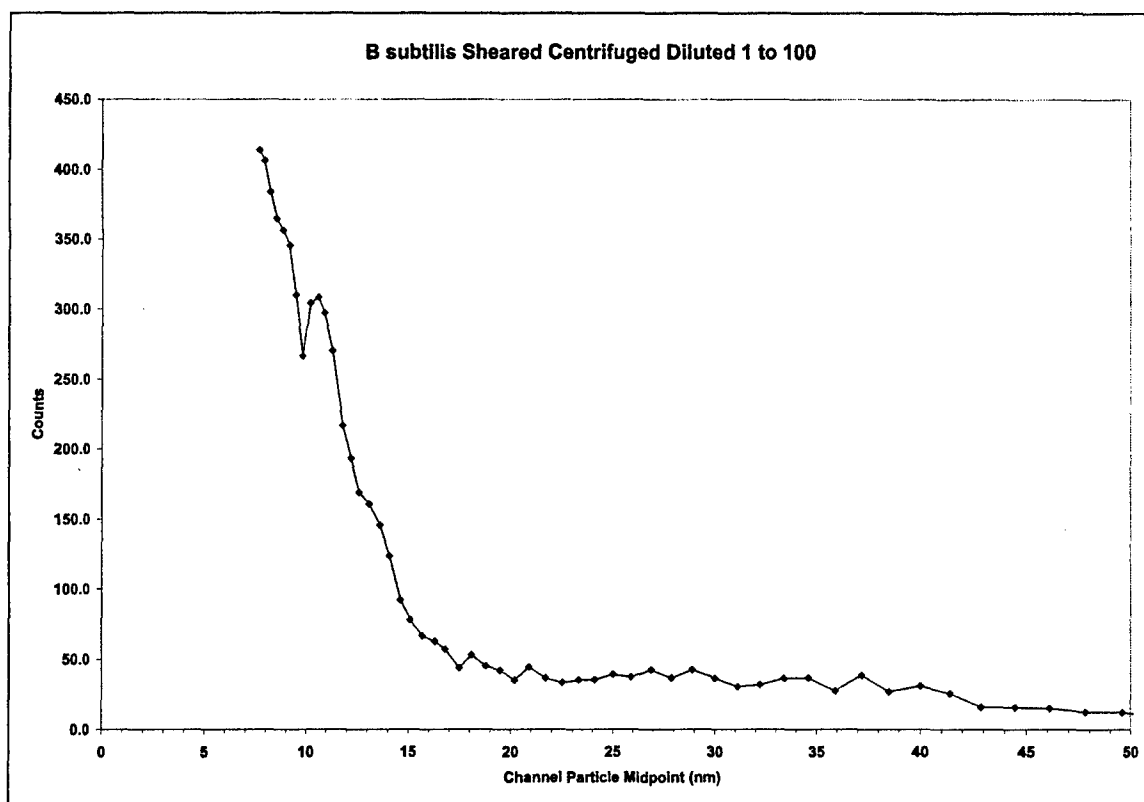


Figure 2. Bacterial Pili sheared from *B. subtilis* (3 scan average, BS2)

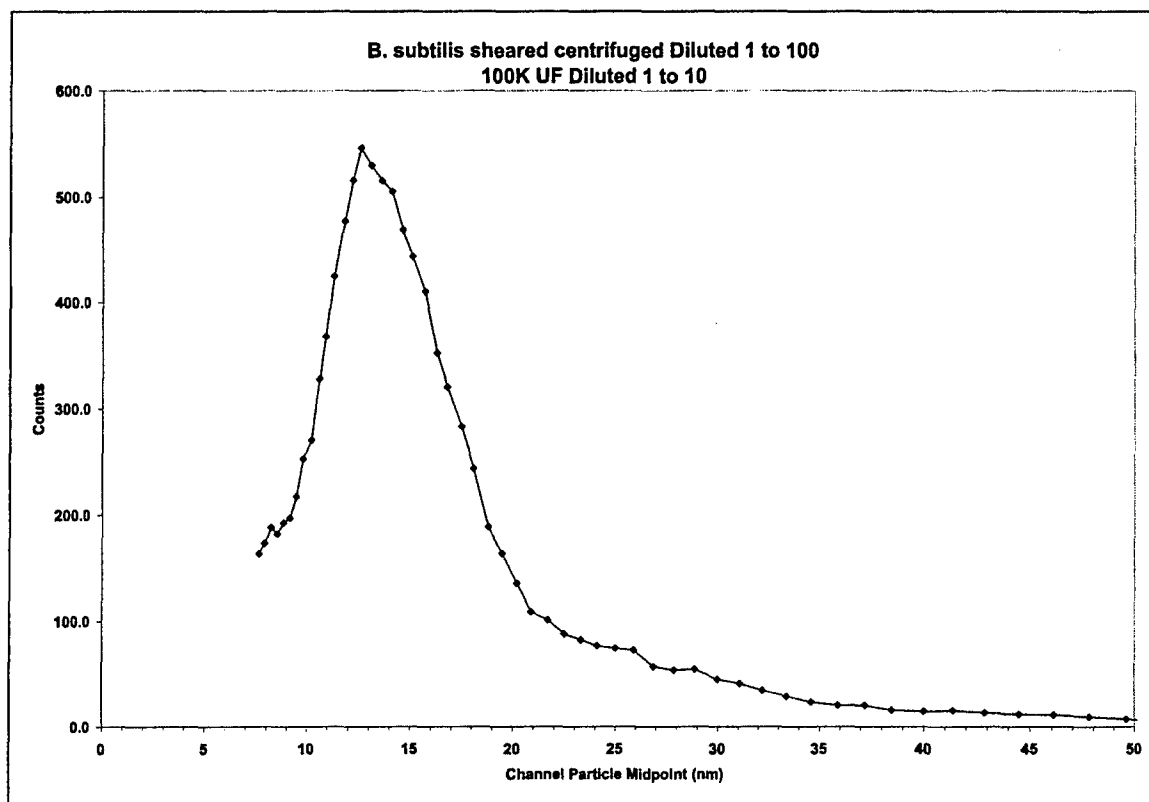


Figure 3. Bacterial Pili ultrafiltered (5 scan average, BS3)

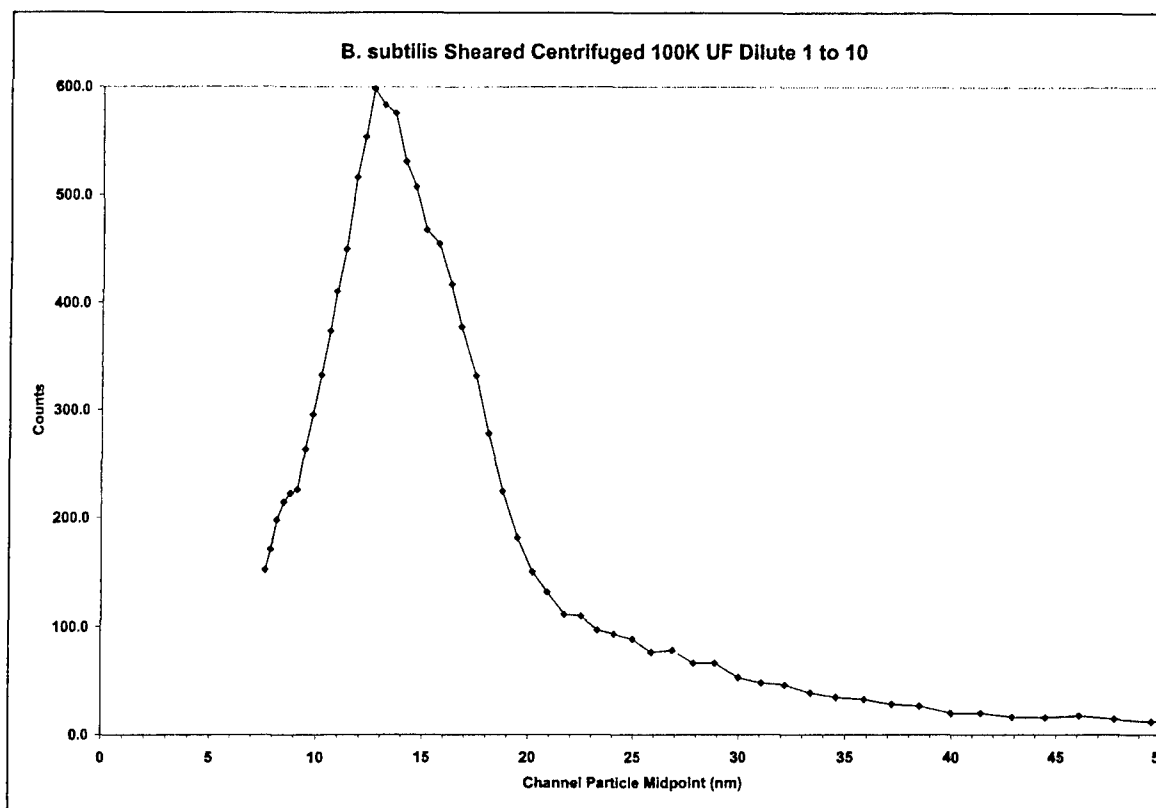


Figure 4. Bacterial Pili ultrafiltered from neat sample (6 scan average, BS1)

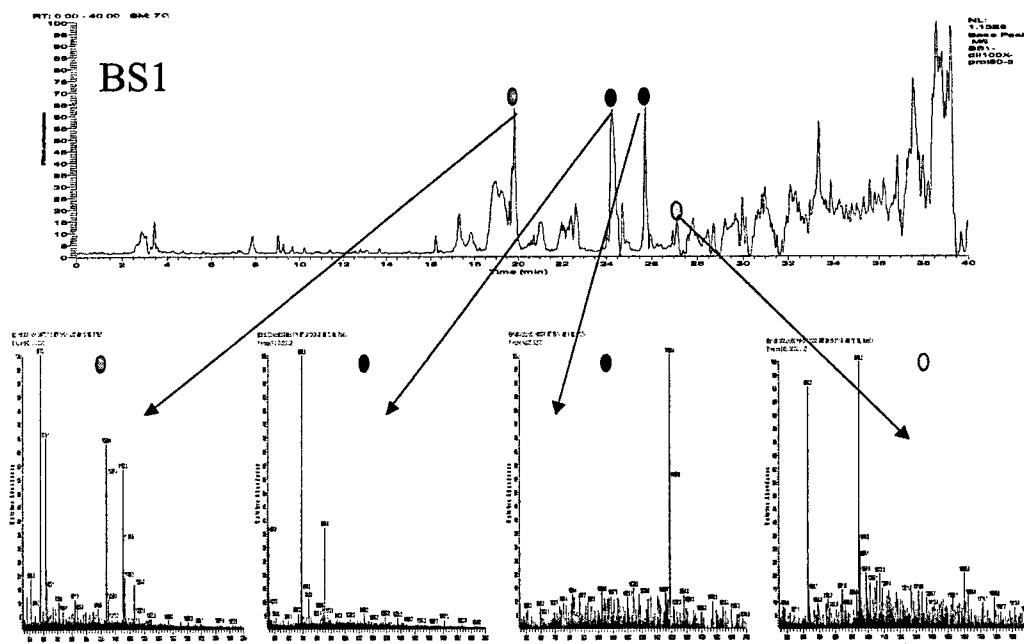


Figure 5. *Bacillus subtilis* sample (BS1) sheared without further filtration. Upper graph represents the Total Ion Chromatogram of its LC-MS analysis. Lower graphs are the mass spectra of the dominant peaks observed in replicate runs, N=3.

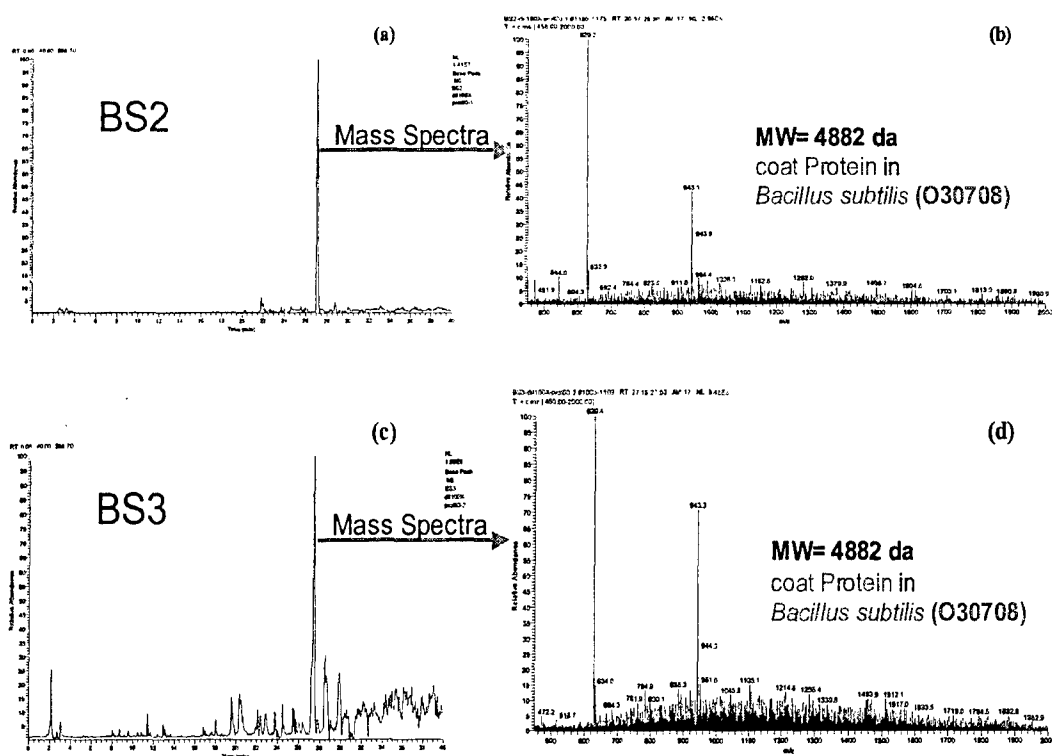


Figure 6. Effect of concentration on the reproducibility of the LC-MS analysis of Pili extracts of *Bacillus subtilis*. (a) Total ion Chromatogram of *Bacillus subtilis* sample (BS2) sheared, centrifuged, and filtered using 100K-da ultrafiltration membrane. (b) Mass spectrum of the most intense peak from the TIC of BS2. (c) Total ion Chromatogram of *Bacillus subtilis* sample (BS3) sheared, centrifuged, and filtered using 100K-da ultrafiltration membrane and 100 fold dilution than that of BS2. (d) Mass spectrum of the most intense peak from the TIC of BS3.

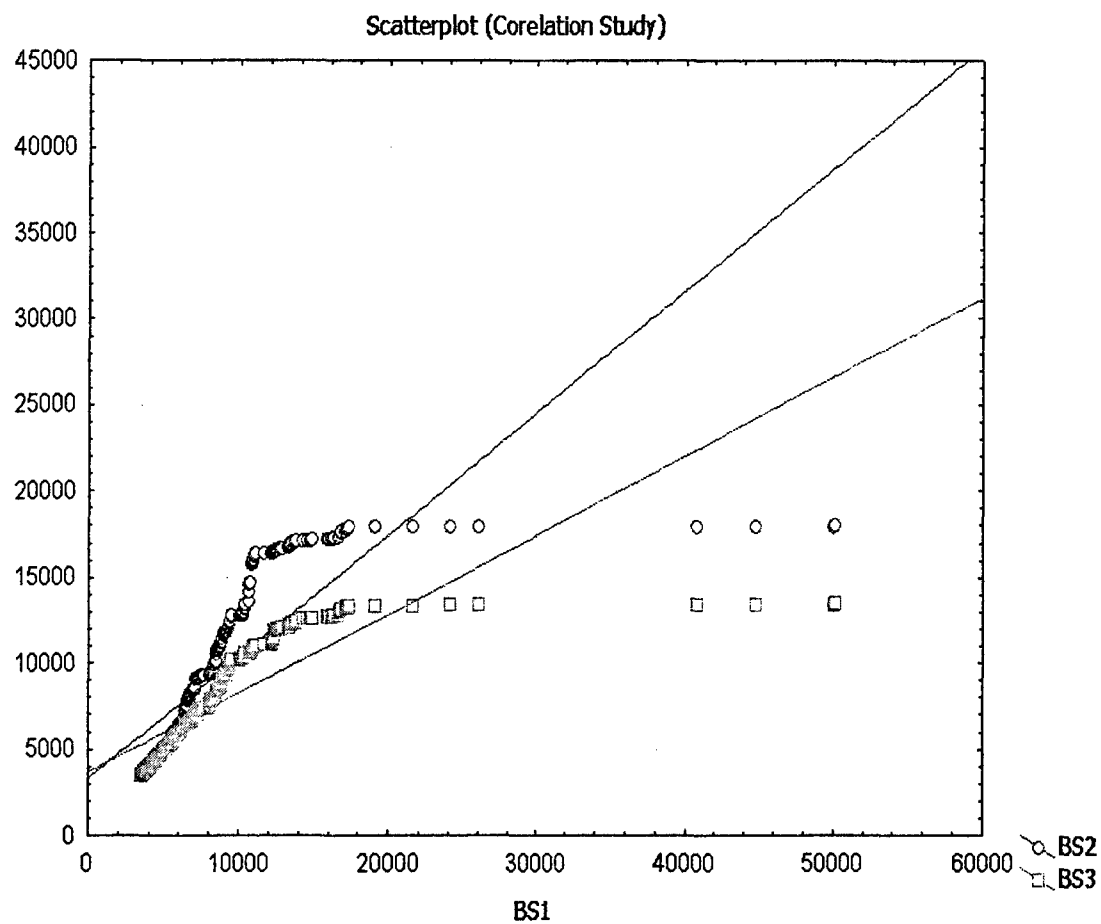


Figure 7. Molecular weight correlation of BS1 vs. BS2 and BS3. Only BS2 and BS3 were exposed to ultrafiltration with molecular weight cut-off of 100K-Da membrane.

Table 1. Dominant Protein Masses from *Bacillus subtilis* Sample 1 (BS1) deconvoluted using in-house algorithm with their corresponding descriptions and accession numbers from their matching with the Swissprot* database.





	MW (da)	Descriptio	Accession Numbe
	13545	Transmembran protein	O05219
	4096	Extracellular protein	Q9R623
	14880	Outer coat	P54520
	4882	Outer coat	O30708

Table 2. Statistical Correlation of the LC-MS Analyses of *Bacillus subtilis* Samples that Underwent Different Sample Preparation Approaches. Marked correlations are significant at $p < 0.05000$.

	BS1	BS2	BS3
BS1	1.00	0.80	0.81
BS2	0.80	1.00	0.99
BS3	0.81	0.99	1.00

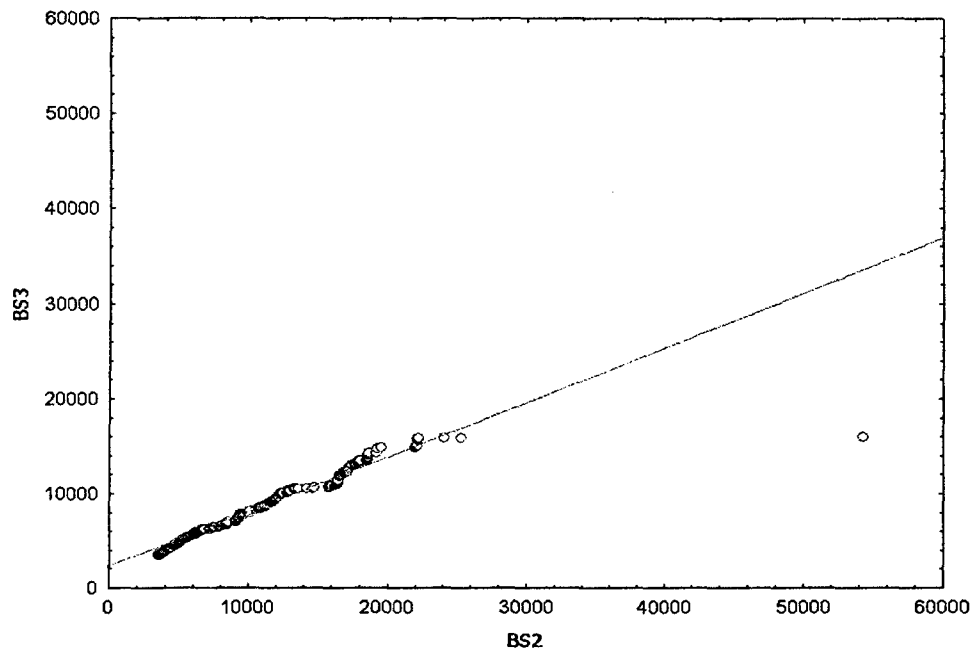


Figure 8. Molecular weight correlation between the protein masses generated from *Bacillus subtilis* samples, BS2 and BS3. Both samples underwent similar sample processing procedures with BS3 diluted 100X more than BS2.

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