Rapid Detection and Identification of Bacteria in Milk Products by Matrix-Assisted Laser Desorption/Ionization Tandem Mass Spectrometry (MALDI-MS/MS)

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It is essential to be able to identify potentially dangerous pathogens. Although *Bacillus thuringiensis* bacteria are not harmful to humans, a closely related bacteria *Bacillus anthracis*, which is known as anthrax, is capable of causing death [1]. Anthrax exposure has been documented on various occasions; a highly publicized instance occurred 2001, and of the twenty-two people infected, five died [1,10, 11]. It took multiple days to confirm that the anthrax exposure took place [11]. Since such a long period of uncertainty is counterproductive for effective treatment of those exposed, a fast and reliable method for detection is needed. Having the ability to identify anthrax spores and other pathogens is of great importance.

The method that we are using is fast, relatively cheap, does not create much waste, and most importantly, the results are reliable and can be supported with a high level of confidence. Through our research, we expect to be able to readily identify bacteria in the *Bacillus* genus through matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy (MS). A highly used technique in proteomics is peptide mass fingerprinting (PMF). PMF can be used when certain proteins are species-specific; that is, they are only found in a certain species. We can use these species-specific proteins to identify the source of a protein.

Peptide mass fingerprinting is a technique used to identify proteins with ease. In this method, a protein is digested with a protease [8]. A protease is an enzyme that breaks down proteins into smaller peptides [12]. There are many different types of proteases; each breaks a protein at a specific amino acid. For my research, we use the protease trypsin, which breaks proteins at lysine (K) and arginine (R). After the tryptic digestion, the next step is to determine the peptides through mass spectroscopy. Mass spectroscopy provides the molecular weights of a molecule with very little sample needed. A sample is bombarded by high-energy electrons, which break the molecules into fragments [12]. A plot is made by graphing the abundance versus mass of the fragments. The molecular weights of the fragments and the protease are then used to screen in a database to determine the unknown protein from which the fragments originated. Peptide mass fingerprinting provides a distinct signature of a protein; an amino acid sequence can be obtained from daughter ions in a mass spectrum, these masses are then matched against a database to identify the protein [5]. When we ran our digested peptides and fragmented peptides through the database, we received a positive match for *B. thuringiensis* spores.

After testing the method and showing that the results were accurate, the next step was to test how the results were affected when we place the spores in an organic matrix instead of water. We spiked the *B. thuringiensis* spores into milk to see if we could replicate the same results that we got from placing the spores in water. Attempts to rapidly identify *Bacillus thuringiensis* sbsp. kurstakin with proteomics through MADLI-MS proved to be difficult in milk even though it had been possible in DI water. Species-specific peptide peaks seen in .1 M KOH after a 20-minute tryptic digestion were not seen when digested with milk nor when digested spores were spiked into milk. It

is believed that ion suppression took place and that the milk proteins hindered the *B. thuringensis* peptides from ionizing. Instead of focusing on peptides for identification, the focus shifted towards using peaks that were seen in DI water, KOH and milk. It is possible that a lipopeptide discovered in 2000 can be used as a biomarker for the *Bacillus* genus but not for species-level identification [7].

Bacillus thuringiensis spores have demonstrated antifungal properties; during sporulation, it produces insecticidal δ–endotoxin proteins and is often used as an active ingredient in insecticides [7 & 9]. The kurstakin lipopeptide, which was noted to be washed off spores with multiple organic solvents, is likely located on the outside of the spores [6 & 7]. Kurstakins has also been detected in six strains of *Bacillus thuringiensis* and *Bacillus cereus* [2]. While kurstakins is not species-specific, it may be a particular to the *Bacillus* genus. Through our future work, we intend to show whether kurstakins is specific to the *Bacillus* genus. If it is, our work could be used as a quick check for food producers to check whether *Bacillus* bacteria are in their products.

Methods and Procedures

Ten milligrams of *Bacillus thuringiensis* spores were prepared in 100mM KOH at pH 13. They were shaken for 1 minute and allowed to sit for 19 additional minutes so that the δ-endotoxin, *B. thuringiensis'* protein toxin, will solubilize [11]. The pH of the spores was adjusted to eight with .1% triflouroacetic acid. A 100µL aliquot of immobilized trypsin (Lot # NJ178775A) in 100mM of NH_4HCO_3 was prepared and centrifuged for three minutes at 10,000 X g; the supernatant was then removed. The trypsin was washed with NH_4HCO_3 two additional times. The trypsin was added to the spores and allowed to digest for 20 minutes. The digestion was ended by centrifuging the mixture for three minutes at 10,000 X g. The peptides were spotted on a MALDI plate after being desalted with a C_{18} zip tip. The MALDI matrix, consisting of 70% acetonitrile, 1% trifluoroacetic acid and 10mg alpha-cyano-4-hydroxy-cinnamic acid, was then spotted on top of the sample.

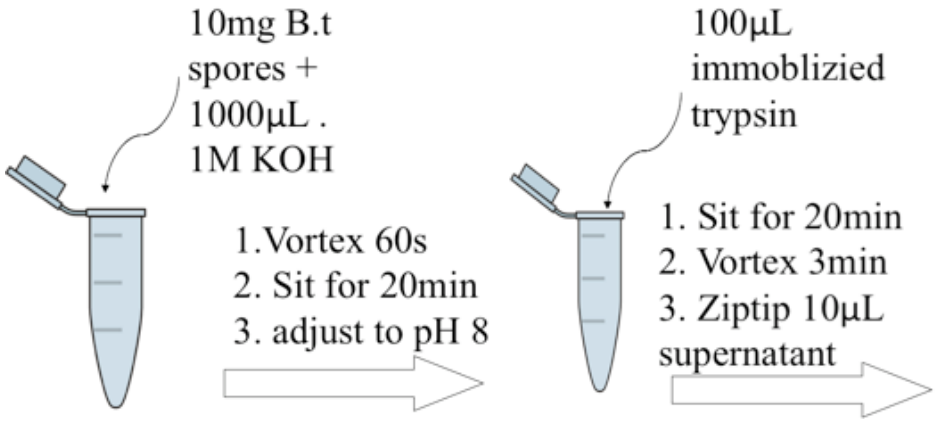

Diagram 1: Flowchart representation of trypsin digestion
For end result, see Figure 1 below.

Out of 233 species-specific peptides, 56 of them proved to be specific the *B. thuringensis* toxin. Of these 56, at most thirteen of them have been present in our tryptic digestions [11]. The peaks and their respective fragments were run through the MASCOT database. A significant match to *Bacillus thuringiensis'* insecticidal crystal protein was found with a protein score of 146 and an expect value of $4.1e^{-08}$. The very same digestion was spiked into a sample of milk in 4:1 ratio. No digestion peaks were seen in the mass spectrum. When trypsin was added simultaneously to the spores and the milk, milk proteins were seen; however, no spore peptides were seen. This is attributed to ion suppression. In order to overcome these problems, chromatography would most likely be needed. While this would aid in solving the ion suppression dilemma, it would slow down the process of detection. Relying on tryptic peptides for identification in milk became problematic. Instead of focusing on digestion peaks, the direction shifted to a peak seen from the spores that was visible in the milk.

A peak at 924.3 had been visible in every spectra of tryptic digestions. This peak had been identified as a lipopeptide, called kurstakin, in 2000 and has been further explored since its discovery [7]. It is possible that it is a biomarker for the *Bacillus* genus as it has been located in other species such as Bacillus cereus [2]. Hathout identified four $[M+H]^+$ ions *m/z* 879, 893, 893 and 907 which differed by 14Da which led to the belief that they were homologous lipopeptides [9]. Each lipopeptide differs by the fatty acid chain to which it is attached. The four proposed fatty acid chains are methyl-decanoic, dodecanoic, 10-methylundeaconic and 11-methyldodeanoic acids. The amino acid sequence of the lipopeptide is Thr-Gly-Ala-Ser-His-Gln-Gln [6]. Another isoform of kurstakins has a lactone ring connecting serine and the C terminus of the ending glutamine [bechet].

To show that the source of the 924 peak seen in our digestions was kurstakin, undigested spores were placed in .1M KOH and a solution containing NH_4HCO_3 buffer adjusted to pH 7. A matrix blank was also prepared; no peak at 924 was observed. While no peak at 924 was seen in undigested milk nor digested milk, alpha casein peaks were observed in milk digested with trypsin.

In .1M KOH, hydrolysis of the lactone occurs; the gain of water shifts the peak to 924.27. The sequence of kurstakins was identified as R-CO-Thr-Gly-Ala-Ser-His-Gln-Gln [7]. When the two glutamine are allowed to hydrolyze overnight, the peak shifts to 927, as they are converted to glutamic acids.

After fragmenting the 924.27 peak, we matched the sequence of the lipopeptide to five peaks. The peak at 778, b_6, corresponds to R-CO-Thr-Gly-Ala-Ser-His-Gln. Each R group is a straight chain fatty acid consisting of twelve carbons ($C_{12}H_{25}$). The peak at 650 results from the loss of the two glutamines. The sequence Gly-Ala-Ser-His-Gln-Gln corresponds to the peak at 627 while the peak at 499 originates from the sequence Ser-His-Gln-Gln. The last peak, seen at 412 comes from His-Gln-Gln.

In the buffer solution, a peak at 906.36 was seen. This represents the closed form of lipopeptide before hydrolysis of the lactone. The peak was fragmented and some of the resulting fragments were the following: 760, 632, 609, 481 and 412. After hydrolysis of the lactone, the addition of 18Da shifts the peaks to the ones labeled as b_4, b_5, y_1, y_2, y_3 in Figure Three, respectively. The only peak that does not shift is the 412 (y3) peak; this fragmented sequence does not contain the lactone. Since

the peak presented itself without the trypsin, it cannot be a tryptic peptide. This supports the conclusion that the peak comes from kurstakin.

Procedures:

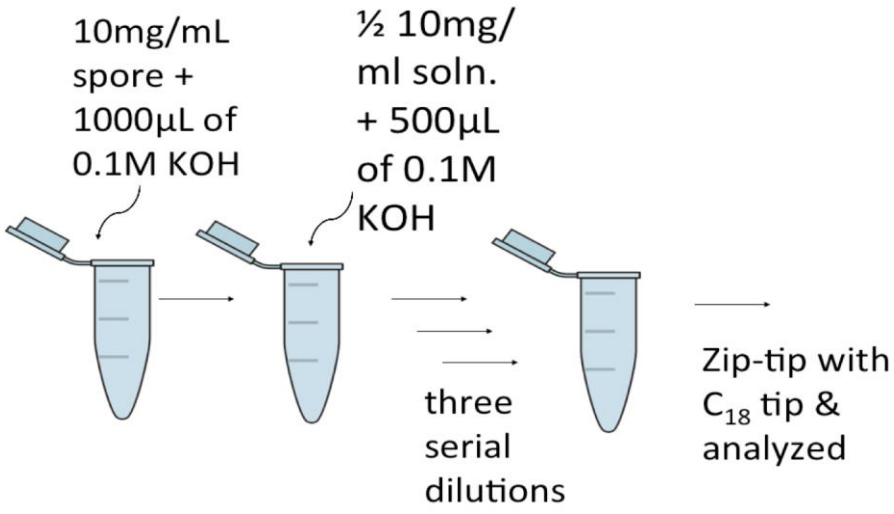

Diagram 2: Flowchart representation of dilutions in KOH

Undigested Spores in Milk. Ten milligrams of *Bacillus thuringiensis* spores were added to 1000µL of milk. The molarity of the sample was adjusted to 100mM with 6M NaOH. The spores were vortexed for one minute and the pH was lowered to below 4 with HCl. While some samples were centrifuged for 3 minutes at 10,000 X g, no significant difference in the results were found when the sample was not centrifuged. The spores were then zip tipped and spotted on a MALDI plate.

Undigested Spores in KOH. Ten milligrams of *Bacillus thuringiensis* spores were added to 1000µL KOH. The molarity was adjusted to 100mM with HCl. The spores were vortexed for one minute and the pH was lowered to below 4 with HCl. While some samples were centrifuged for 3 minutes at 10,000 X g, no significant difference in the results were found when the sample was not centrifuged. The spores were then desalted with a C_{18} zip tip and spotted on a MALDI plate.

MASCOT Search. Species-specific peptide peaks that matched the tryptic digestion and the resulting fragment peaks were ran through the NCBInr database using the MASCOT MS/MS ion search (matrixscience.com). The peptide tolerance was set to ±0.6 Da while the MS/MS tolerance was set to ±0.6Da. Modifications that were allowed were carbamidomethyl (C) and oxidation (M). The taxonomy group was restricted to Bacteria (Eubateria) and no missed cleavages were allowed.

Dilutions in Milk. Ten milligrams of spores were placed in 1000µL of milk and 17µL of 6M NaOH was added to bring the concentration of NaOH to approximately .1M . To create a 5mg/mL solution, 500µL of the 10mg/ml solution was removed, placed in a separate vial and diluted to 1000µL with milk. 6M NaOH was again added to the solution to adjust the concentration back to .1M. Other dilutions were performed in the same manner;

the resulting concentrations were 2.5mg/ml, 1.25mg/ml, 0.625mg/ml and 0.3125mg/ml.

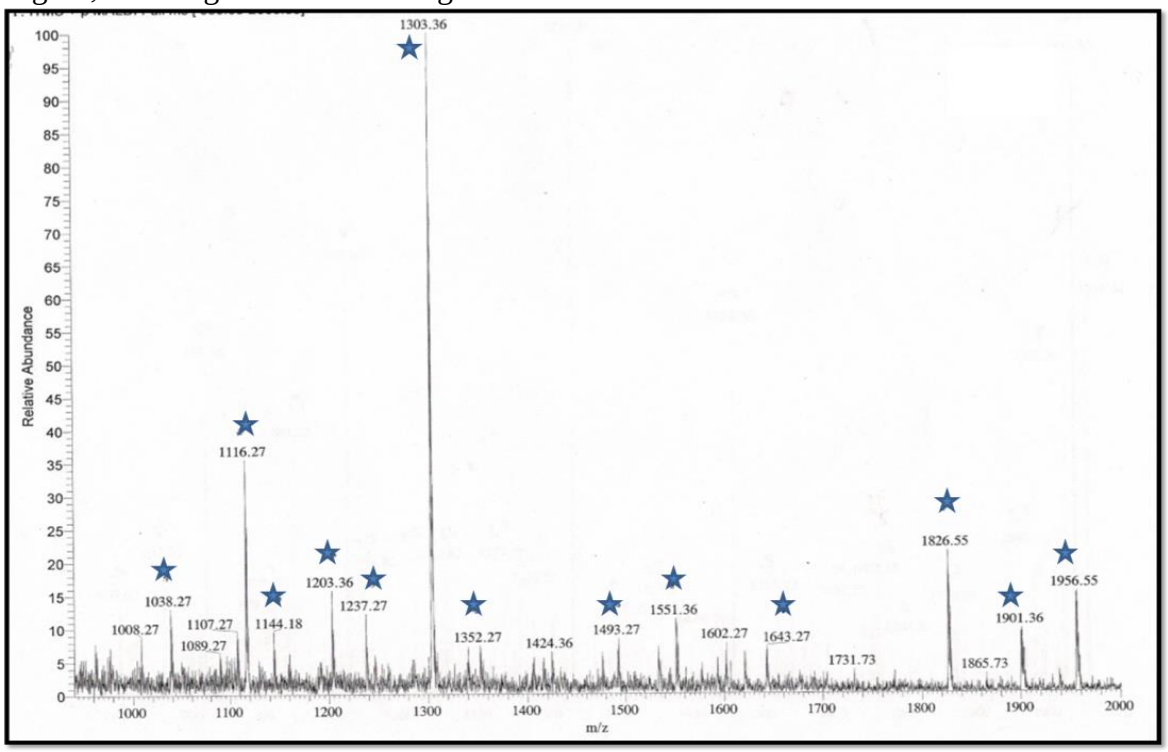

Figure 1: A tryptic digestion of the spores; the starred peaks are species-specific peptides.

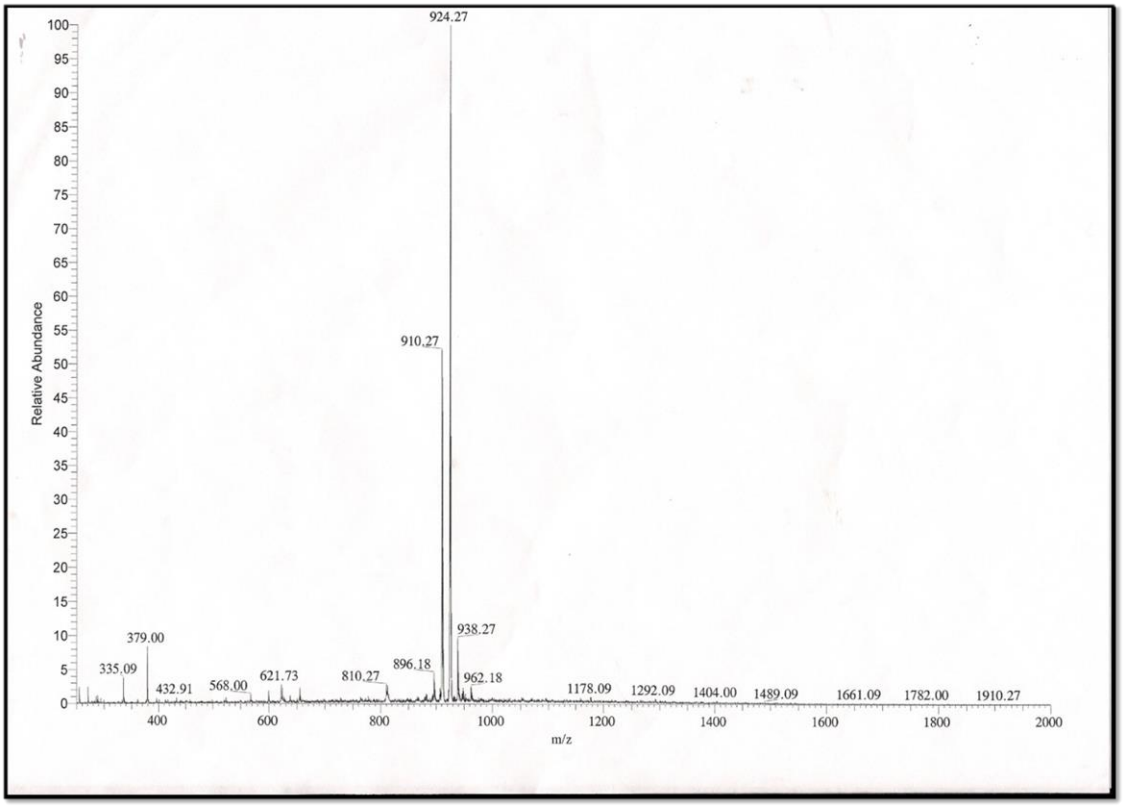

Figure 2: The spectrum of Bacillus thuringiensis spores after hydrolysis of the lactone by .1M KOH.

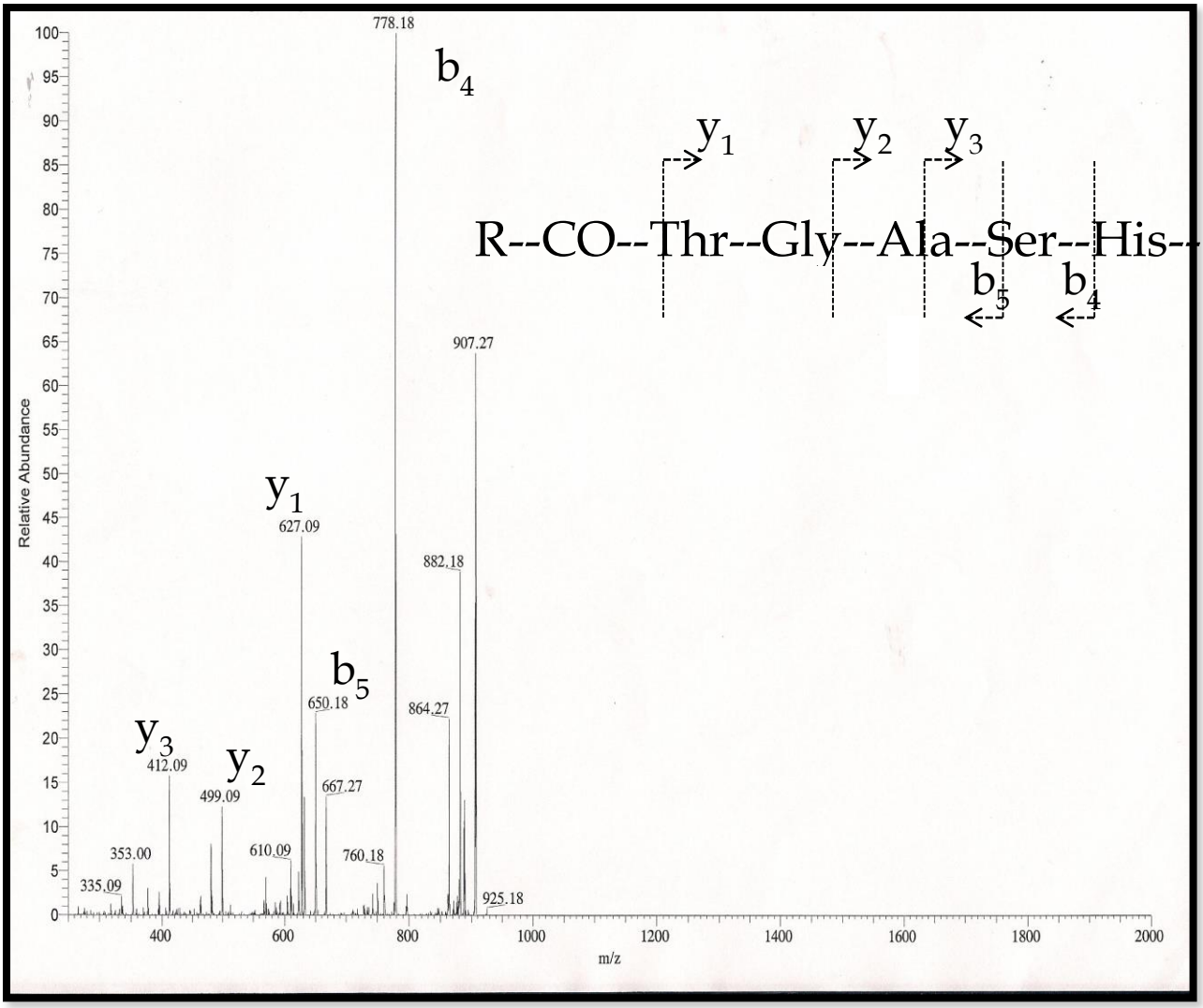

Figure 3: A fragment of the 924.3 peak after hydrolysis of the lactone. The sequence of kurstakins is shown along with several identifiable peaks.

Conclusion and Discussion

While the kurstakin peak was readily identifiable in a 10mg/ml solution of *B. thuringiensis* spores, it was important to find out if the signal was still strong in dilute solutions. We created five solutions by serial dilution of a solution containing 10mg of spores in 1ml of .1M KOH. The resulting concentrations were 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml and 0.3125mg/ml. In all solutions, a peak at 924 was seen. When the same dilutions were performed in milk, only the 10mg/ml and 5mg/ml spectra had a peak at 924. This demonstrates that, while kurstakin is more detectable in DI water, identification in dilute samples of milk is also possible. Further research will include efforts to determine if kurstakin is present in other *Bacillus* species.

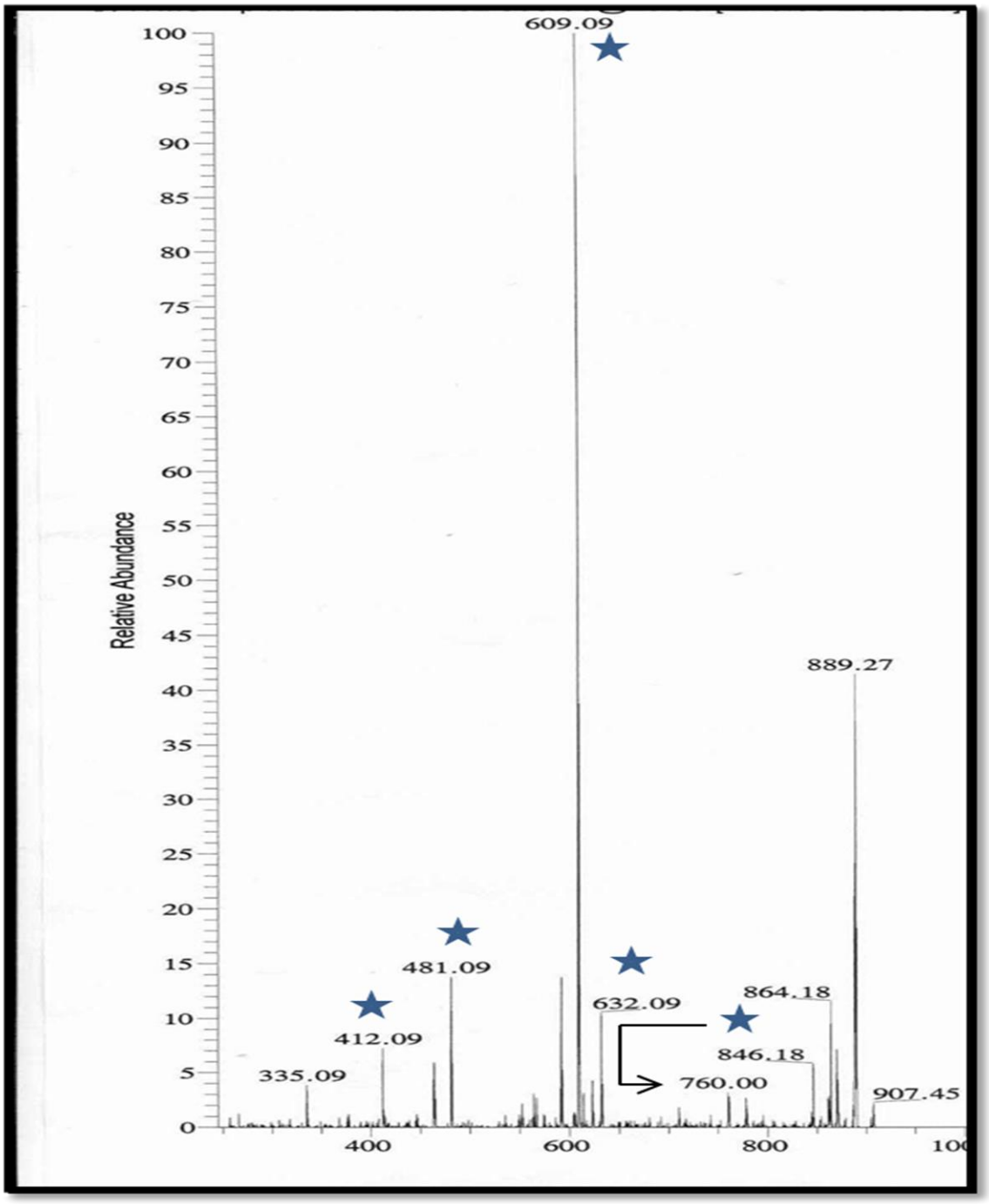

Figure 4: Spores in a NH_4HCO_3 buffer adjusted to ph 7. The starred peaks show the original peaks before hydrolysis of the lactone. Each starred peak, with the exception of the peak at 412, gains 18Da after the addition of water during hydrolysis.

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