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Species Assignment to A Polyhydroxybutyrate-Producing Microbacterium Isolate from Egypt

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Abstract – Poly (3-hydroxybutyrate) is a natural polymer belonging to the family of polyhydroxyalkanoates, which accumulates in a wide variety of bacterial strains as reserve food material under imbalanced growth condition. Poly (3-hydroxybutyrate) is a biodegradable alternative to traditional petrochemical plastics such as polypropylene and polyethylene. The *Microbacterium paraoxydans* (WA81) is a local isolate producing large amounts of Poly (3-hydroxybutyrate) which was confirmed by Sudan black B stain and transmission electron microscopy. Moreover, this isolated was further characterized by bacteriological and molecular methods such as sensitivity to antibiotics, plasmid profile and MALDI-TOF-MS. This local isolate contained a 10kb single plasmid and was identified as *Microbacterium paraoxydans* DSM 15461 using MALDI- TOF –MS technique

Keywords – PHB , PHA, *Microbacterium* , MALDI-TOF-MS, biodegradable plastic.

I. INTRODUCTION

Over the years, petrochemical plastics had become one of the most important products with different applications all over the world. Their versatility, outstanding technical properties and relatively low price contributed to their great success. Nowadays, plastic has universal applications, as it is part of many industries such as the manufacture of cars, computer equipment, household appliances, construction, sports, entertainment equipment, packages and even medical applications, so it is clear that the plastic material has become indispensable. However, plastics are environmental unfriendly products being a source of nuisance and pollutant, as they do not biologically degraded **[1].**

Due to the environmental impact and the rise in crude oil prices, as a result of an expected end of oil reserves, the need for alternative materials is increased. Therefore, numerous studies have been conducted to achieve proper alternatives to petroleum-based materials such as polyhydroxyalksnotes (PHAs) which are very promising and biodegradable new materials for replacement of petroleum-based plastics **[2]**. Additionally, PHAs extracted from bacterial cells show physical and mechanical properties that are similar to polypropylene **[3].**

PHAs have advantages over petrochemical plastics such as they are natural, renewable, biocompatible and biodegradable; as many micro-organisms have the ability to degrade these macromolecules enzymatically. PHAs are polyester groups produced and accumulated naturally by a large number of bacteria as intracellular granules in response to unfavorable growth conditions and imbalance of nutrient **[4].** Poly(3-hydroxybutyrate) (PHB) is the most widely studied and best characterized member of PHAs **[5]**. One of the important variables that has restricted the widespread use of PHAs as a biodegradable commodity plastic is the high

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production cost. Improving PHA production strategies will lead to a reduction in costs, which means a widespread use of PHA in everyday life **[6]**.

In order to make the production process commercially viable, many strategies have to be followed simultaneously such as, the using of cheap substrates, improving the cultivation strategies and an easier down-streaming processes methods **[7].** Also for efficient production of PHB at a low cost novel microorganisms were sought to achieve the production goals. Among various PHB producing organism *Microbacterium* sp. was recently reported to possess the ability to produce PHB with significant amount **[8], and [9].**Characterizing the Egyptian isolate *Mircrobacterium* WA81 as producer of would be help in improving culturing properties and production process of PHB.

II. Materials and methods

Bacteria and growth condition

PHB- producing bacterium, *Microbacterium* WA81, was obtained from the Bacteriology and Molecular Biology Laboratory at Faculty of Science, Mansoura University. The bacterial isolate was maintained on nutrient agar slants, stab and was stored at 4°C for daily usage, while, long term storage was done at -80°C in 50% glycerol. The bacterial isolate was grown in mineral salt medium consisted of: 2.0 g/L (NH4)2SO4, 2.0 g/L KH2PO4, 0.6 g/L Na2HPO4, 0.2 g/L MgSO4.7H2O, 20 mg/L CaCl2, 10 mL/L trace metal solution, and 0.1 g/L yeast extract was added. The trace metal solution consisted of: 1.3 mg/L ZnSO4.7H2O, 0.2 mg/ L FeSO4.7H2O, 0.6 mg/L (NH4)6Mo7O24.4H2O and 0.6 mg/ L H3BO3. Fructose was used as a carbon source (40g/L) for PHB production media and 10g/L g/L for inoculum development. Fructose and mineral salt solutions were sterilized separately and aseptically mixed together at room temperature prior to inoculation. The pH of the resulting broth was adjusted to 7.0 with 2N NaOH/2N HCl **[10].**

Macroscopic and microscopic examination:

The purity of the bacterial culture was checked by Gram's staing from a single colony grown for 48h on LB agar medium **[11].** Sudan balck stain was used for dectection of PHB granules in cytoplasm of *Microbacterium* WA81 cells **[12],** these cells were grown for 48 h in PHB production broth containing 40g/L fructose **[10].**

Identification of PHB-producing bacterium using MALDI-TOF-MS

Microbacterium WA81 strain was plated on nutrient agar medium and incubated for 48 h at 37°C. One colony was picked and deposited on the MALDI-TOVF-MS target plate (Bruker Daltonics, Bremen, Germany) in three or four replicates to minimize random effect. Two microlitres of matrix solution (saturated_ cyano-4-hydroxycinnamic acid, 50% acetonitrile, 2.5% trifluoroacetic acid) were then added and allowed to co-crystallize with the sample. Analysis was performed in a MALDI-TOF-MS spectrometer (337 nm) (Autoflex; Bruker Daltonics) with the FLEX control software (Bruker Daltonics). Positives ions were extracted with an accelerating voltage of 20 kV in linear mode. The spectra were analyzed in a mass-to-charge ratio (*m*/*z*) range of 2000–20 000. The profiles were compared and analyzed and finally mass spectrometric data were analyzed using ClinProToolsTM 2.0 to find PVL biomarker candidates. Generating the classification model in ClinProToolsTM 2.0 was performed using Genetic Algorithm. Recognition capability and cross-validation (i.e. the measure of the reliability used to predict the accuracy of the model) were calculated by the software with percentage values **[13].**

Antimicrobial Susceptibility Test

The local isolate bacterium *Microbacterium* WA81 was examined for its antibiotic sensitivity according to the standard disk diffusion method **[14].** The bacterium was inoculated onto Mueller Hinton agar plate and the antibiotic sensitivity was performed using antibiotic discs obtained from Oxoid co. [England]. Amoxicillin/clavulanic acid 20/10 mcg (AMC -30), Ampcillin /sulbactam 10/10 mcg , Azithromycin 15mcg (AZM-15), Cefadroxil 30 mcg (CFR-30), Cefotaxime 30 mcg (CTX-30), Ceftazidime 30mcg(CAZ-30), Cephalexin 30mcg (CL-30), Cephradine 30 mcg (CE-30), Cephradine 30mcg(CE-30), Ciprofloxacin 5mcg (CIP-5), Clindamycin 2mcg (DA-2), Gentamycin2mcg (DA-2), Levofloxacin,Ofloxacin 5mcg(LEV-5), Penicillin G 10 mcg (P-10), Rifampin 5mcg (RA-5) and Tetracycline 30mcg (TE-30) were used.

Plasmid profile

Plasmid DNA was extracted by Zyppy Plasmid Miniprep Kit according to the manufacture instructions. Asingle colony was inoculated in LB medium and incubated at 37°C for (24-48) h then 600µl of bacterial culture grown in LB medium was added to a1.5 microcentriguge tube,100µl of lysis buffer was added and mixed by inverting the tube 4-6 time for 2 minutes the solution changed from opaque to clear blue due to complete lysis then 350 µl of cold neutralization buffer was added and mixed throughly by inverting the tube 2-3 times until the solution turned to yellow and a yellowish preciptate was formed. The mixture was centrifuged for 3 min at 13,000 rpm at room temperature and the supernatant was carefully transferred to zymo-spin colum ,centrifuged for 15 s and and the flow-through was discarded. The DNA was washed twice first with 200µl of endo wash buffer,centrifuged at 13,000 rpm for 30 S min and the flow through was discarded then with 400µl of zyppy wash buffer, centrifuged at 13,000 rpm for 1 minute and the flow through was discarded .Finally, DNA was eluted by placing the zymo-spin column in a new sterile 1.5 ml micro-tube by 30 µl of zyppy elution buffer. The extracted DNA was loaded onto agarose gel and visualized using gel documentation system **[15]**.

Cellular protein profile

Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyzed the total cellular peotein of *Microbacterium* WA81 as described by **[16]** and protein bands were visualized and documented under the visible light using gel docomentation system.

III. Result

Microscopic examination

The bacterial colonies were bright yellow, smooth and sticky. Gram's staining showed that the bacterial cells were positive, small coryneform rods shape. Moreover, bluish black, ovoid or spherical granules of PHB resulted after Sudan black B stain were accumulation in the cytoplasm of the examined bacterial cells.

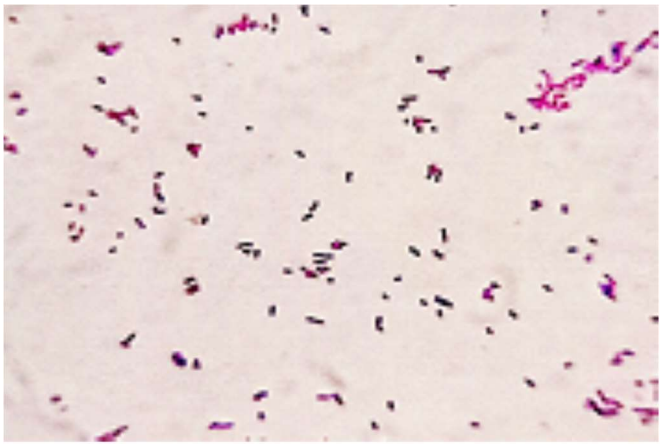
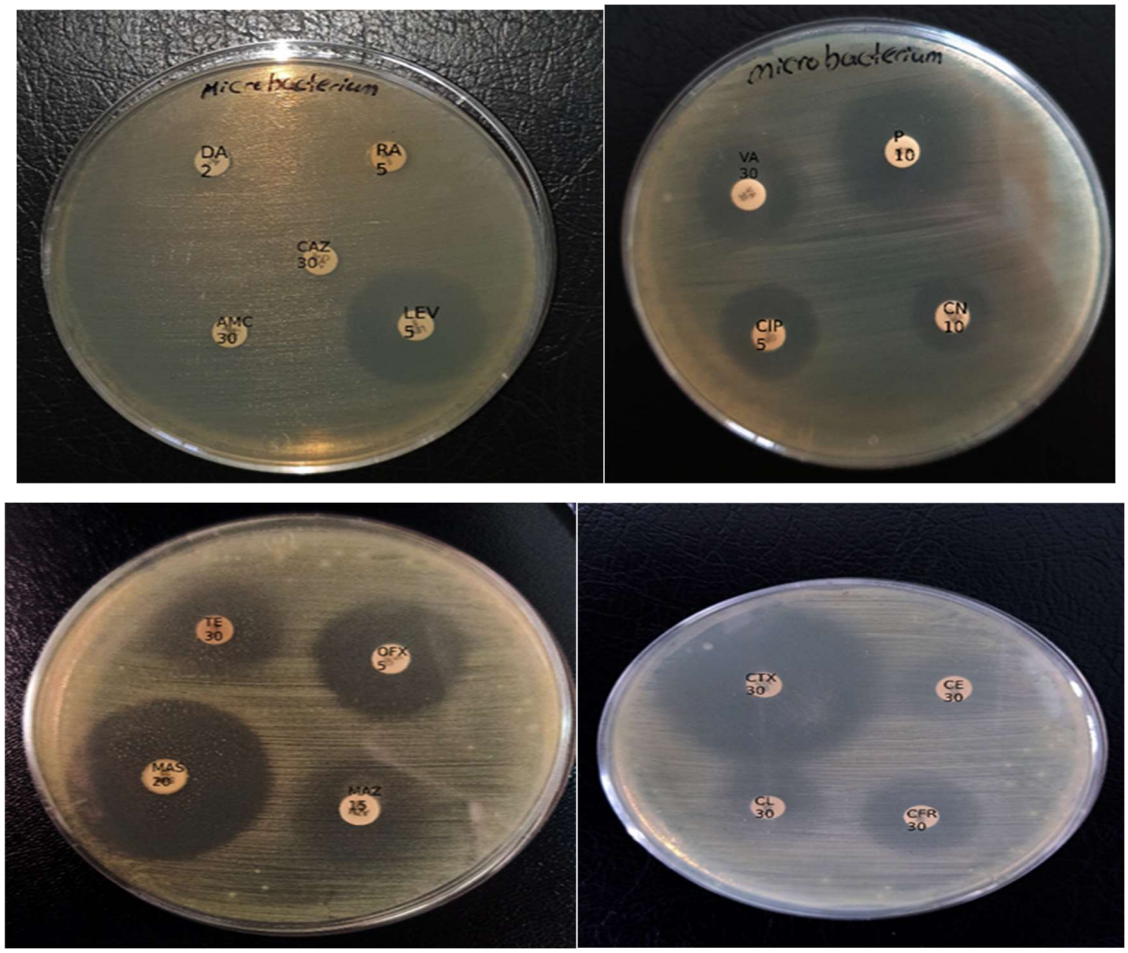


Fig. 1. Sudan black B stain showing the bluish spherical granules of PHB in cytoplasm of *Microbacterium* WA81 cells

Antimicrobial Susceptibility Test:

The result showed that *Microbacterium* WA81 was resistant to RNA synthesis Inhibitors (rifampin), macrolides (Clindamycin and Azithromycin), Amoxicillin/clavulanic acid 20/10 and Ceftazidime, while it was susceptible to fluoroquinolones (levofloxacin and ciprofloxacin), Ofloxacin, natural pencillin (penicillin G), Cefotaxime and Ampcillin /sulbactam 10/10 as shown in table 1 and figure 2



Figure(2):Antibiogram of Microbacterium WA81 grown on Muller Hinton agar.

Table (1)1: Antibiotic sensitivity of *Microbacterium WA81* to different antibiotics

Antibiotic	Zone diameter (mm)	Interpretation
Amoxicillin/clavulanic acid 20/10	-	R
Ampcillin /sulbactam 10/10	31mm	S
Azithromycin	-	R
Cefadroxil	20mm	I
Cefotaxime	35mm	S
Ceftazidime	-	R
Cephalexin	18mm	I
Cephradine	-	R
Ciprofloxacin	20mm	I
Clindamycin	-	R
Gentamycin	15mm	R
Levofloxacin	26mm	S
Ofloxacin	23mm	S

Penicillin	23mm	S
Rifampin	8mm	R
Tetracycline	18mm	I

Plasmid profile of *Microbaterium* WA81 :-

Analysis of the plasmid DNA extracting from the local strain *Microbacterium* WA81 showed a single plasmid higher that 10,000 bp **Fig. 3.**

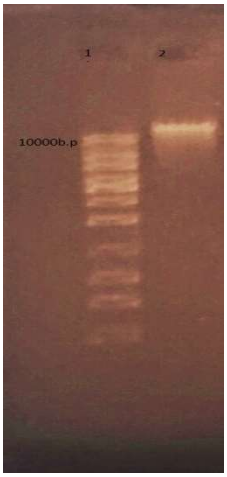


Fig (3): Plasmid profile of polymer-producing Microbacterium WA81 (lane 2) and lane 1: Molecular weighr marker (10000Bp to 100 Bp),

Protein profile using SDS –PAGE

Total cellular protein fractionation of the local isolate *Microbacterium* WA81 was analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) as shown in **Fig. 4**.

Analysis of the gel photo indcated various protein bands with different molecular weights (114.107,103,99,95,83,75,64,59,56,48,41,34,31,28) KDa

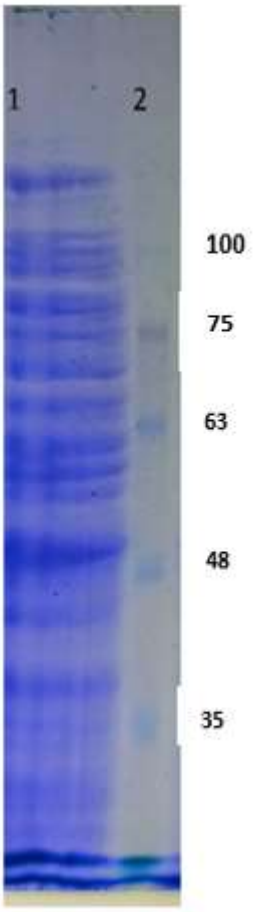


Figure (4): Total cellular proteins analysis of local Microbacterium WA81 (lane 1), protein marker (lane 2)

Identification of PHB producing bacterium using MALDI-TOF-MS

MALDI-TOF-MS directly analyzed the profiles of bacterial proteins PHB producing bacteria. The procedure provides a unique mass spectral fingerprint of the microorganisms. This method requires that the biopolymer molecules normally present in the condensed phase be converted into intact, isolated ionized molecules in the gas phase. Then, ions are separated according to their molecular weight after migration in an electric field. Each molecule detected is characterized by the molecular mass (m), the charge (z), the ratio mass/charge (m/z), and the relative intensity of the signal. The measured range of 2,000 to 20,000 Da is displayed. The characteristic mass peaks are predominantly ribosomal proteins. Subsequently, the integrated MALDI software matches the pattern with entries of a database

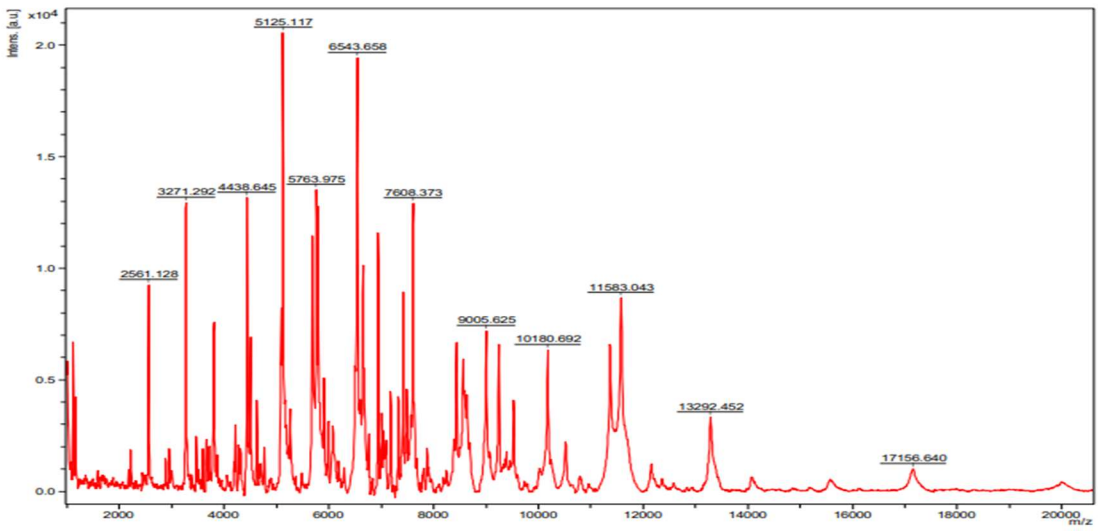


Figure (5). MALDI-TOF mass spectrum of Microbacterium.

Table (2): Molecular weight, intensity, and Area under the peak to the protein content of Microbacterium WA81

Molecular Weight (m/z)	Intensity	Area
2561.128	9254	64297
3271.292	12943	118497
4438.645	13169	150745
5125.117	20559	355988
5763.975	13517	173839
6543.658	19441	307891
7608.373	12915	258686
9005.625	7187	329181
10180.692	6340	317511
11583.043	8677	583849
13292.452	3326	251700
17203.777	647	11178

The local bacterial isolate was identified as *Microbacterium paraoxydans* DSM 15461 DSM after automatic comparison of generated spectrum with the MALDI-TOF database. The degree of similarity to the reference spectrum is represented by a score value. Identification results with score values above 2.0 are considered to be correct for determination of the respective species

Table (3) : Computer display of identification results using MALDI-TOF-MS

Sample Name	Organism	Score	NCBI Identifier
Micro	Microbacterium paraoxydans DSM 15461 DSM	2.04	33882

The capability for recognition of species distinctions within the complex spectrum of bacterial diversity is necessary for progress in microbiological research.

In clinical settings, accurate, rapid and cost-effective methods are essential for early and efficient treatment of infections. Characterization and identification of microorganisms, using, bottom-up proteomics, or “proteotyping”, relies on recognition of species-unique or associated peptides, by tandem mass spectrometry analyses, dependent upon an accurate and comprehensive foundation of genome sequence data, allowing for differentiation of species, at amino acid-level resolution. In this study, the high resolution and accuracy of MS/MS-based proteotyping was demonstrated, through analyses of the three phylogenetically and

taxonomically most closely-related species of the Mitis Group of the genus *Streptococcus*: i.e., the pathogenic species, *Streptococcus pneumoniae* (pneumococcus),and the commensal species, *Streptococcus pseudopneumoniae* and *Streptococcus mitis*. To achieve high accuracy, a genome sequence database used for matching peptides was created and carefully curated. Here, MS-based, bottom-up proteotyping was observed and confirmed to attain the level of resolution necessary for differentiating and identifying the most-closely related bacterial species, as demonstrated by analyses of species of the *Streptococcu*s Mitis Group, even when *S. pneumoniae* were mixed with *S. pseudopneumoniae* and *S. mitis*, by matching and identifying more than 200 unique peptides for each species.

IV. DISCUSSION:

Speciation of microorganisms, including bacteria, is one of the cornerstones in the fields of microbiology and infectious diseases. Bacterial nomenclature thus provides the foundation from which the functionality of the microbe can be predicted such as economic importance, the host-parasite disease relationships, and epidemiological investigations; as published by a recent correspondence in *Clinical Infectious Diseases***[17].** In this report they highlighted problems concerning the potential misidentification of bacteria when commercial identification systems were used. However, this concern is only the tip of an iceberg of a potentially larger problem with more important ramifications. Concerns regarding appropriate methods to identify bacteria in published reports will become an increasingly difficult issue in the future unless standards are developed for the description of new taxa, reporting of DNA sequences into databases, and the submission of case reports involving unusual (rare) bacterial species. With the advent of the availability of automated molecular techniques to identify bacteria (e.g., 16S rDNA sequencing), more species are being described based upon examinations of five or fewer strains and a limited number of differential biochemical characteristics **[18]**. Therefore, reliance on a single identification system, phenotypic or genotypic, to identify an organism provides more opportunity for misidentifying bacterial species, and if the trend described above continues, the risk will become even greater in the future.

A local bacterium belonging to the genus *Microbacterium* WA81 was recently isolated and recognized for its ability to produce the economically and environmentally important compound the PHB by a colleagues at the Bacteriology Lab, Mansoura Faculty of Science, Mansoura University, Egypt **[9]**. She did not speciate it, so, it was important to characterize it at the species level before being deposited in any culture collection. This isolate of *Microbacterium* showed resistance to a quite large number of antibiotics such as rifampin, macrolides, amoxicillin/clavulanic acid 20/10 and Ceftazidime while showing sensitivity to penicillin G, ampcillin /sulbactam 10/10, while another strain of *Microbacterium paraoxydans* sensitive to pencillin,ampcillin,ciprofloxcina and vancomycin **[19]**. *Microbacterium paraoxydans* (WA81) was senstive to fluoroquinolones (levofloxacin and ciprofloxacin), Ofloxacin and Cefotaxime. At the molecular level, it harbered a single plasmid with a molecular size estimated to be larger than 10kb, which appear to be necessary for this isolate to survive in its environmental niche with limited nutrient supply **[20]**.

MALDI-TOF-MS technology identified the local PHB-producing bacterium to the species level as *Microbacterium paraoxydans;* which turned out to be identical to an existing strain in the German Culture Collection under the number 5461 DSM with score 2.04. According to the databases of the MALDI instrument, any score value above 2.0 validates the species identity **[21]**. The accuracy of the MALDI-TOF-MS for bacterial identification is now accepted the by the scientific community and qualified it to be used in clinical diagnosis of bacterial pathogens worldwide **[13]**. This technique depended on comparing peptide mass fingerprint (PMF) of unknown organism with the PMFs contained in the database, or by matching the masses of biomarkers of unknown organism with the proteome database. In PMF matching, the MS spectrum of unknown microbial isolates is compared with the MS spectra of known microbial isolates contained in the database. For species level identification of microbes, a typical mass range m/z of 2–20 kDa is used, which represents mainly ribosomal proteins along with a few housekeeping proteins The characteristic pattern of highly abundant ribosomal proteins, which represent about 60–70% of the dry weight of a microbial cell, in the mass range of 2–20 kDa **[22].**

Currently, MALDI-TOF MS technology is quickly not only replacing traditional methods for identifying microorganisms in the clinical laboratory, but establishing new trends in speed and costs of the identification process of bacteria and fungi. This technology is still limited because of the inherited library of spectra generated for microbes; i.e. the databases. These databases containing spectra of known organisms is highly important for the identification of species with similar phenotypic, genotypic, and biochemical properties. Moreover, its importance in clinical settings should lead to improvements in diagnosis and hence administering the appropriate therapy. These limitations also reflect on poor discrimination between species, as well as misidentifications. Overall, MALDI-TOF MS has revolutionized bacterial identification for research laboratories and infectious disease diagnosis and clinical care.

In conclusion, speciation of the PHB-producing bacterial isolate (Microbacterium paraoxydans) established the novelty of the isolate because it was not known to produce PHB and describe in the literature. Moreover, it has led to the discovery of its resistance to multiple antibiotics. Future perspectives in this field could discover antibiotic modifications by degrading enzymes, the detection of resistance mechanism determinants through proteomic studies and the analysis of modifications of target sites, such as ribosomal methylation. The quantification of antibiotics is suggested as a new approach to study influx and efflux in bacterial cells. The results of the presented studies demonstrate that MALDI-TOF MS is a relevant tool for the detection of antibiotic resistance and opens new avenues for both clinical and experimental microbiology [23]

REFERENCE

[1] Zinn, M., B. Witholt, and T. Egli, *Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate.* Advanced drug delivery reviews, 2001. **53**(1): p. 5-21.

[2] Ibrahim, M. and A. Steinbüchel, *Zobellella denitrificans strain MW1, a newly isolated bacterium suitable for poly (3-hydroxybutyrate) production from glycerol.* Journal of Applied Microbiology, 2010. **108**(1): p. 214-225.

[3] Amaro, T.M., et al., *Prospects for the use of whey for polyhydroxyalkanoate (PHA) production.* Frontiers in Microbiology, 2019. **10**: p. 992.

[4] Vazquez, G.J., M.J. Pettinari, and B.S. Mendez, *Evidence of an association between poly (3-hydroxybutyrate) accumulation and phosphotransbutyrylase expression in Bacillus megaterium.* International Microbiology, 2003. **6**(2): p. 127-129.

[5] Lee, S.Y., Y. Lee, and F. Wang, *Chiral compounds from bacterial polyesters: sugars to plastics to fine chemicals.* Biotechnology and bioengineering, 1999. **65**(3): p. 363-368.

[6] Li, R., H. Zhang, and Q. Qi, *The production of polyhydroxyalkanoates in recombinant Escherichia coli.* Bioresource technology, 2007. **98**(12): p. 2313-2320.

[7] Grothe, E. and Y. Chisti, *Poly (β-hydroxybutyric acid) thermoplastic production by Alcaligenes latus: behavior of fed-batch cultures.* Bioprocess Engineering, 2000. **22**(5): p. 441-449.

[8] Shivakumar, S., *Accumulation of poly (3-hydroxybutyrate) by Microbacterium barkeri DSM 20145.* Turkish Journal of Biology, 2012. **36**(2): p. 225-232.

[9] Osman, Y.A., et al., *Molecular Characterization of a Poly-β-Hydroxybutyrate-Producing Microbacterium Isolate.* International Journal of Applied Sciences and Biotechnology, 2015. **3**(2): p. 143-150.

[10] Khanna, S. and A.K. Srivastava, *Statistical media optimization studies for growth and PHB production by Ralstonia eutropha.* Process Biochemistry, 2005. **40**(6): p. 2173-2182.

[11] Beveridge, T.J., *Use of the Gram stain in microbiology.* Biotechnic & Histochemistry, 2001. **76**(3): p. 111-118.

[12] Moreno, N., et al., *Bioprospecting and characterization of poly-bhydroxyalkanoate (PHAs) producing bacteria isolated from Colombian sugarcane producing areas.* African Journal of Biotechnology, 2007. **6**(13).

[13] Bittar, F., et al., *MALDI-TOF-MS for rapid detection of staphylococcal Panton–Valentine leukocidin.* International journal of antimicrobial agents, 2009. **34**(5): p. 467-470.

[14] Bauer, A., *Antibiotic susceptibility testing by a standardized single disc method.* Am J clin pathol, 1966. **45**: p. 149-158.

[15] Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular cloning: a laboratory manual*. 1989: Cold spring harbor laboratory press.

[16] Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* nature, 1970. **227**(5259): p. 680-685.

[17] Janda, J.M. and S.L. Abbott, *Bacterial identification for publication: when is enough enough?* Journal of clinical microbiology, 2002. **40**(6): p. 1887-1891.

[18] Rosselló-Mora, R. and R. Amann, *The species concept for prokaryotes.* FEMS microbiology reviews, 2001. **25**(1): p. 39-67.

[19] Laffineur, K., et al., *Bacteremia Due to a Novel Microbacterium Species in a Patient with Leukemia and Description of Microbacterium paraoxydans sp. nov.* Journal of clinical microbiology, 2003. **41**(5): p. 2242-2246.

[20] Banu, H. and K. Prasad, *Role of plasmids in microbiology.* J Aquac Res Development, 2017. **8**(466): p. 2.

[21] Wieser, A., et al., *MALDI-TOF MS in microbiological diagnostics—identification of microorganisms and beyond (mini review).* Applied microbiology and biotechnology, 2012. **93**(3): p. 965-974.

[22] Murray, P.R., *What is new in clinical microbiology—microbial identification by MALDI-TOF mass spectrometry: a paper from the 2011 William Beaumont Hospital Symposium on molecular pathology.* The journal of molecular diagnostics, 2012. **14**(5): p. 419-423.

[23] Hrabák, J., E. Chudáčková, and R. Walková, *Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry for detection of antibiotic resistance mechanisms: from research to routine diagnosis.* Clinical Microbiology Reviews, 2013. **26**(1): p. 103-114.