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Characterisation of a novel naturally-modified biogenic calcium carbide-containing exopolysaccharide produced from Pseudomonas aeruginosa strain S16 from soil in Nigeria, West Africa

I. K. M. Okorie* and A. A. Ogunjobi

Department of Microbiology, University of Ibadan, Nigeria

***Corresponding Author**
I. K. M. Okorie, Department of Microbiology, University of Ibadan, Nigeria.

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Abstract

Antibiotic resistance, that occurs globally, has led to a reduction in the efficacy of many contemporary conventional antibiotics. The aim of this study was to characterise an exopolysaccharide, produced from Pseudomonas aeruginosa strain S16, accession number OQ734845, that was observed to be effective against two antibiotic-resistant strains, namely Klebsiella pneumoniae and Staphylococcus aureus SO183, in another study. HPLC and MS of the exopolysaccharide, confirmed the presence of glucose, rhamnose, xylose arabinose, D-ribose, galactose, mannose and inositol. The phenol-sulfuric acid method, quantified the carbohydrate sugars concentration, as 0.0060 g/L. SEM showed that the exopolysaccharide possessed a sparsely flaky surface, with globular projections. EDX detected nitrogen (48.00%); carbon (20.10%); phosphorus (12.6%); sodium (8.00%); oxygen (6.00%); calcium (2.30%); chlorine (1.20%); magnesium (0.89%); zinc (0.67%); potassium (0.44%), and manganese (0.33%). FTIR spectroscopy, showed oxygen-manganese-oxygen bonds, and the internal bonds of the following component compounds: magnesium oxide, zinc oxide and calcium carbide. FTIR spectroscopy also showed the following functional groups: carboxyl, imine/oxime, amide/peptide, nitro, halo, and anhydride. The exopolysaccharide produced from Pseudomonas aeruginosa strain S16, is a novel, heteropolysaccharidic, naturally-modified, biogenic calcium carbide-containing, exopolysaccharide. The multiple chemicals in the exopolysaccharide, likely give it the potential for a range of biological activities. From available documented research, this is the first report of an exopolysaccharide naturally modified by biogenic calcium carbide, and sodium, magnesium, potassium, and manganese ions.

Keywords: Characterisation, Naturally-Modified, Novel Biogenic Calcium Carbide-Containing Exopolysaccharide, *Pseudomonas aeruginosa* Strain S16, Antibiotic Resistance

1. Introduction

Antibiotic resistance occurs when a targeted bacterium, no longer responds to the drugs designed to kill it, or to arrest its growth. These alterations by bacteria, of their metabolic activities, in order to attenuate, or completely stop, the effectiveness of an antibiotic (CDC, 2013), has led to research, for effective antibacterial agents. Exopolysaccharides have been found to possess antibacterial activity, in addition to other useful properties (Angelin and Kavitha, 2020).

Exopolysaccharides (EPSs) are large molecules that are extracellularly excreted as a firmly bound sac, or as a loose, lightly-bound layer of goo (Angelin and Kavitha, 2020). Exopolysaccharides are chiefly, straight-chained secondary metabolites of certain microorganisms, whose molecules possess pendant chains of different measurements and spatial formations, that are set at regular intervals (Gundogan, 2014). Exopolysaccharides are polymeric compounds, secreted outside the cell. As a whole, they possess replicating sequences of a small quantity of linked monosaccharides. (Derdak et al., 2022).

Exopolysaccharides are also, the swiftest-produced, microbial extracellular polymeric substances, because of their pivotal role, in cell adhesion and the protection of the bacterium (Rana et al., 2020).

Exopolysaccharides are the most frequently-occurring, extracellular polymeric substances in biofilms (Nwodo et al., 2012), and are basically responsible, for the gooey nature, that characterises microbes that aggregate on hard surfaces; most exopolysaccharides are mucoid in texture (Panchal et al., 2022). Exopolysaccharides can be classified based on where they are located, their chemical configurations, the weight of their molecules, their chemical bonds and their functions (Panchal et al., 2022). Capsular exopolysaccharides are firmly affixed to the cell surface, while free exopolysaccharides, are secreted into their immediate vicinities (Panchal et al., 2022). Exopolysaccharides can either be homopolysaccharides, or heteropolysaccharides, with respect to their chemical configuration (Panchal et al., 2022).

Homopolysaccharides have the same repeating monosaccharide unit, a molecular mass of 10^7 Daltons, and are subdivided into four types, namely: α-D-glucans; β-D-glucans; fructans and polygalactans, based on the linking molecular bonds, and the nature of the monomers present (Panchal et al., 2022). Examples of homopolysaccharides are cellulose and chitin (Osemwegie et al., 2020). Heteropolysaccharides are exopolysaccharides, that have recurring L-rhamnose, D-galactose and D-glucose moieties (Sanalibaba and Çakmak, 2016). They may also possess N-acetyl-galactosamine, N-acetyl-glucosamine or glucoronic acid (Panchal et al., 2022); hyaluronan is an example of a heteropolysaccharide (Osemwegie et al., 2020).

Functionally, exopolysaccharides could be sorptive, structural, surface-active or redox (Rana et al., 2020). Sorptive exopolysaccharides are composed of polymers that bear electric charges; these polymers help in sorption to other equally-charged molecules that partake in interactivity at the surface of the bacterial cell (Rana et al., 2020). Structural exopolysaccharides serve as a support to the bacterial cell framework. Examples include chitin and cellulose. Cellulose is documented as the most bountiful polysaccharide on the earth (Seddiqi et al., 2021). Surface-active exopolysaccharides are a group of exopolysaccharides that have exopolysaccharides that are made of amphipathic particles, as one of its members (Nwodo et al., 2012).

By strict definition, exopolysaccharides are composed of varied proportions of certain recurring monosaccharide moieties (Kazou, 2022). Consequently, the presence of molecules/particles or compounds other than monosaccharides, or a removal of an element or elements in a molecule or molecules of a monosaccharide, modifies the pre-existing architecture. Any given particle has undergone molecular modification, if that particle is a registered particle with a well-reported profile, and has been subsequently, subjected to a change - to whatever degree - in its configuration, in order to improve its efficacy as a therapeutic agent (Nadendla, and Yemineni, 2023). However, the molecular modification of a compound can occur if the bound atoms of the compound have been altered physically without an alteration of their chemical makeup (Li et al., 2015). With respect to exopolysaccharides, molecular modifications can occur naturally, for example, exopolysaccharides produced by bacteria can be altered via the actions of enzymes such as transferases, to execute such functions as protection, biofilm generation and destructiveness (Whitfield et al., 2015). The molecular modification of an exopolysaccharide can also be performed by man; in this regard, it is performed to increase the inherent capacity of the exopolysaccharide to effect predetermined changes in biological systems (Chaisuwan et al., 2020). This synthetic molecular modification of an exopolysaccharide, can be achieved through the use of elements or complexes that enable the addition or removal of functional moieties (chemical molecular modification) (Liu et al., 2023); through the use of biotic substances such as enzymes (biological molecular modification) (Whitfield et al. 2015; Razvi et al., 2023) and via the application of an extrinsic force to degrade molecular bonds in the exopolysaccharide and thereby reduce its molar mass (physical molecular modification) (Chaisuwan et al., 2020).

The use of many chemicals, moieties or compounds to modify a given compound, often in a bid to enhance its inherent capacities and its resultant uses, describes the process of multi-chemical or multiple chemical molecular modification (Chen J. et al. 2022). These enhancements that result from multiple chemical modification, have been observed to be greater than those that occur from the use of a single element, moiety or compound, as a chemical modifier (Chen J. et al. 2022). Nevertheless, these modifications may result in an actual loss of the capacity of the compound to exert any measurable effect in a given medium (Gimeno et al., 2019). In addition, although molecularly modifying a therapeutic agent, may lessen the adverse reactions associated with the lead compound (Nadendla and Yemineni, 2023), the analogues produced can be more harmful to man and his environs, than the lead compounds were, as has been noted to occur even with minute molecular modifications of aldehydes (LoPachin and Gavin, 2014).

Nitrogenous exopolysaccharides are externally-secreted polysaccharides that contain nitrogen. Examples of naturally-occurring nitrogenous polysaccharides are chitosan and chitin (Peter, 1995); chitosan is formed when nearly half of the acetyl compounds in chitin, is hydrolysed (Díaz-Montes, 2022). These nitrogen-containing polysaccharides are tough, because of their denseness and the crystal subunits that are present (Song et al., 2012). Nitrogenous exopolysaccharides do not dissolve in water that consists of an equal clustering of hydrogen and hydroxide ions (Shirvan et al., 2019). They can be used to heal injured skin (Jayakumar et al., 2010) and can be used as a backbone for building human tissues (Jayakumar et al., 2011; Ishikawa et al., 2007).

Phosphorylated exopolysaccharides are extracellularly-excreted polysaccharides that possess phosphates that are attached to the linked monosaccharides in their structures. A polysaccharide is phosphorylated when the hydroxyl groups in its linked simple sugars, are chemically replaced by phosphorus that is bound to oxygen atoms (Zhou and Huang, 2021). Phosphorylation enhances the measurable, observed characteristics of the polysaccharide, and the reactions that result when it comes in contact with other chemical substances (Xia et al., 2021). Therefore, phosphorylated exopolysaccharides can exhibit antioxidant (Guo et al., 2013) and antineoplastic (Ye et al., 2013) properties. They have also been reported to possess the capacity to strengthen the immune system (Han et al., 2016).

Chlorine is a yellow-green, non-metal chemical, with a proton number (Z) of 17, a melting point of -100.98 °C, a boiling point of -33.97 °C, and exists in all three states of matter (Schmittinger, 2008). The element chlorine, possesses 24 variants out of which, the variants with atomic masses of 35 and 37 are the only non-radioactive ones; the former comprises approximately three quarter, and the latter, a quarter, of existing variants (Berglund and Wieser 2011; Bonifacie, 2018). Chlorine belongs to the halogen (salt-formers) group of the periodic table of elements (Cleaves, 2011). Chlorine very readily combines with both carbon-based and non-carbon-based compounds; and is found as a molecule comprising only two atoms, in the natural world (NRC-SAEGL, 2004). Chlorine in the chloride ion form (Cl^-), is an essential chemical in the bodies of both humans and animals; and is used as a nutriment that is required in large amounts, in plants that possess vascular bundles (Uvarov et al., 2013; Colmenero-Flores, 2019; Marunaka, 2023).

Chloride ions were discovered, in vitro, to activate or be needed for, the increase in the cell number and size of 11 bacteria (*including Aeromonas hydrophilia, Escherichia coli* and *Proteus mirabilis*), in solutions containing high quantities of sodium ions (Roeßler et al., 2003). Humans use chlorine in the form of sodium chloride, for preparing and conserving food (Kendrick, 2018). Chlorine is also used, by man, for manufacturing drugs, fabrics and resins, and for disinfecting water (Tundo, 2012; Kendrick, 2018). Though chlorine kills many microorganisms, certain bacteria such as *Mycobacterium avium* are impervious to concentrations of chlorine employed for disinfection (Miyamoto et al., 2000). Some strains of *Pseudomonas aeruginosa* can withstand the disinfectant action of chlorine when meted out in sub-standard, water-disinfection concentrations (Shrivastava et al., 2004).

Sodium is a metal element with a nuclear charge number (Z) of 11, a relative atomic mass of 23, a boiling point of 883 °C, and a liquefaction point of 94.80 °C (Dsoke &Sabi, 2023). The metal sodium, is argent, soft, easily beaten and stretched into fine-gauge wires, and readily reacts with other elements (Guo, 2009; Isac-García et al., 2016). Sodium composes 2.6% of the mass of the lithosphere and therefore is the fourth most bountiful substance present (Guo, 2009). Sodium is present in vegetation where it is reported not to be extremely important for the metabolism of the majority (Maathuis, 2014). In sharp contrast, sodium is reported to be absolutely necessary for the regular performance of the activities of the organ-systems in man and beast, where it is the major positively-charged ion in the body fluids that bathe the cells (McCaughey, 2019). A compound formed by the complexing of sodium to a polysaccharide backbone, that is composed of alternating β-d-mannuronic acid (M) and α-l-guluronic acid (G) sugar moieties, viz, sodium alginate, exists in the natural world (Razavi et al., 2014). Additionally, sodium ions can couple to exopolysaccharides in the soil; in so doing they lower the soluble salts present and make water uptake by vegetation less arduous (Singh and Jha, 2016; Bhagat et al., 2021).

Potassium, is a pearlescent, yielding, metal chemical that possesses a nuclear charge number (Z) of 19, a relative atomic mass of 39.0983, and the proclivity to lose its single electron to form its cation (Russo et al., 2018; Prohaska et al., 2022). Potassium is one of the elements that most readily takes part in chemical reactions (Gad, 2014). In the lithosphere, potassium is the 7th most bountiful element; natural deposits of potassium include potassium chloride ("sylvin"), and potassium magnesium sulphate ("langbeinite") (Moreno-Rojas et al., 2016). Potassium is the major positively-charged ion inside the cells of virtually all organisms, where it ensures the proper functioning of the cells, by maintaining the voltage across the plasmalemmas (Palmer, 2015; Korolev, 2021). The production of bacterial exopolysaccharides, can be increased, in experimental conditions, by potassium ions (Almansoory et al., 2020). It has been observed that both potassium and sodium display similar characteristics in the manner in which they react with other elements (Guo, 2009).

Zinc is a metal chemical with a nuclear charge number of 30, and whose non-radioactive forms, possess an atomic weight of 65.38 Daltons (Barak and Helmke, 1993). The element zinc is bluish-white, with a silvery-grey lustre, possesses a hexangular Bravais lattice, and is not found unbound, in the lithosphere and in saltwater (Kuklik and Kudlacek, 2016). The unbound nature of zinc in the natural world, occurs because the zinc atom achieves a stable electronic configuration only when it loses two electrons; this oxidation number of 2+ attracts a commensurate anion and forms a complex (Barak and Helmke, 1993). No organism produces zinc, however, zinc is required in minute amounts, in all living things, for the proper functioning of the cells and the maintenance of cellular activities (Frassinetti et al., 2006). Zinc oxide ultrafine particles, have been shown, under experimental conditions, to bring about or inhibit the production of exopolysaccharides, depending on the bacterial species and strains involved (Raliya et al., 2014; Hayat et al., 2022).

Magnesium is a yielding, slate, metal element that is at the 12th position on the periodic table, with a nucleon number of 24.31, and that readily combines with other elements (Griffin, 2003). The chemical magnesium is the 8th most bountiful element in the world, and comprises about 1.93% and 0.13% of the lithosphere and the high seas, respectively (Kojima, 2000). Magnesium, together with silicon, is abundant in cosmic dust; in this space, intergalactic magnesium exists in the cationic form, due to the loss of one or both valence electrons by interplanetary particle collisions (Gurzadyan, 1977; Rogantini et al., 2020). In addition, the magnesium that is found in the ionosphere and the middle atmosphere of the planet, emanates from the burning off, as it were, of elements that compose the surfaces of faxed stars (Plane et al., 2015; Bones et al., 2018). Magnesium is also found in every cell of lifeforms (Maguire and Cowan, 2002). However, the magnesium found in living things, is obtained, only from external sources, as a vital nutrient (Fiorentini et al., 2021; Ishida et al., 2023). In man, the main foods that provide magnesium include greens and kernels (He and Tsinovoi, 2017). Ingested magnesium is subsequently involved in such processes as the formation of polypeptides, deoxyribonucleic acid and ribonucleic acid; the maintenance of proper systolic function of the heart, and the protection of the human body against pathogens and noxious substances (Al Alawi et al., 2021).

Although animals such as livestock can obtain magnesium from what they are normally fed, a predetermined measure of magnesium, is often specifically added, to prevent hypomagnesemia and to enhance fecundity and an increase in numbers (Gaál et al., 2004; Pinotti et al., 2021). Vegetation takes up magnesium from the ground and this process is influenced by conditions prevalent within and external to both interfaces (Ishfaq et al., 2022). In both photosynthetic and non-photosynthetic plants, magnesium is key to maintaining certain important intrinsic processes that promote efficient cellular activities, and overall plant maturation (Ahmed et al., 2023). Moreover, for plants that manufacture their own food, magnesium is the central building block of the chlorophyll molecule (located within specialized plastids), that traps electromagnetic radiation that is needed to integrate atmospheric carbon dioxide into carbon-containing molecules such as carbohydrates (Cakmaka and Kirkbyb, 2008; Ishfaq et al., 2022).

Magnesium is crucial for maintaining efficiency in the cellular processes that occur in microorganisms and in promoting microbial cell maturation (Walker, 1994). Furthermore, under experimental conditions, magnesium has been observed to be crucial for the binary fission of rod-shaped bacteria (Webb, 1948). In microorganism such as bacteria, the control of magnesium cations that enter their cells, determines the degree of susceptibility to certain drugs designed to kill them, or to inhibit their growth (Dong-yeon et al., 2019). Although the particles of magnesium are loosely packed, the metal magnesium, as a whole, is strong; these contrasting characteristics of lightweightness and strength, make magnesium quite distinctive, and together with its amalgams, has caused it to be more frequently utilised in certain spheres of human endeavour such as medicine, pharmacy, technology, aeronautics and car manufacturing (Tan and Ramakrishna, 2021; Prasad et al., 2022).

Magnesium cations can increase the production of exopolysaccharides from certain fungi (Tang et al., 2008). With regards to man and animals, magnesium and manganese cations function distinctively in vivo; however, ex-vivo, both cations have been observed to be swappable, in certain biochemical reactions they participate in (Foradori et al., 1967).

Manganese (Mn) is a metal weighing 54.9 and is at the 25th position on the periodic table (Al-Fartusie. and Mohssan, 2017). In addition, in the lithosphere, it is the 12th most bountiful element, therefore constituting 0.1%. of this zone (Al-Fartusie. and Mohssan, 2017). It is extremely reactive in the soil (Li et al., 2021), and forms both inorganic and organic complexes (Gad and Pham, 2014). Manganese is present in the air, and water (Gad, 2005). The principal means of entry into man, are fortified foods, and water drawn from wells (Harischandra et al., 2019). Manganese performs the work of an enzyme co-factor in all the cells of the body where it is found in minuscule quantities (Harischandra et al., 2019).

Chemically, manganese possesses 11 oxidation states ranging from 3- to 7+ (USEPA, 1984; Röllin and Nogueira, 2011). Although the oxidation state of 2+, is the one present in most natural complexes (DCCEEW, 2022,), both oxidation states of 2+ and 3+, are of key relevance in living systems (Röllin and Nogueira, 2011). Synthetic manganese composites, have been used by man in such industries as pyrotechnics, glass-production, alloy-making, and textiles (DCCEEW, 2022). Manganese carbonate, an organic manganese composite, has been used for livestock feeds manufacturing, paint making, and in medicine and pharmacy (DCCEEW, 2022). Manganese is crucial to the proper functioning and maintenance of the cells of human beings, beasts, and plant life (O'Neal and Zheng, 2015).

Manganese is required for cellular metabolism (Erikson et al., 2005). In addition, the inadequate intake of manganese in, or its over-exposure to human beings, leads to a range of illnesses that involve body systems like the reproductive, the respiratory and the central nervous systems (Keen et al., 2013; Harischandra et al., 2019; DCCEEW, 2022). Whereas, demonstrable signs from a lack of manganese in the human body, are hardly ever observed (Erikson et al., 2005), disease states from manganese overexposure are observed, and result more frequently, from manganese-inspiration from polluted air, or as an occupational hazard (Williams et al., 2012). In animals, manganese deficiency leads to signs and symptoms such as crippling in pigs (Soetan et al., 2010) and retarded maturation of the fetus of young female cows (Hansen et al., 2006), while its excess is mainly seen as an iron deficiency disorder (Keen and Zidenberg-Cherr, 2003).

Manganese deficiency in plants is often cryptic and may later on, show signs of deficiency (Alejandro et al., 2020) such as light-coloured, spotted leaves and yellowing of the areas of the leaf between its veins (Schmidt et al., 2016). Yellowed leaf tissue and specks of dead leaf tissue are the most frequently observed tell-tale signs of plant manganese toxicity (Millaleo et al., 2010).

Calcium carbide is a gunmetal mineral compound, with a molecular formula of CaC_2, a formula mass of 64.10 and a mass per unit ratio of 2.22 g/cm^3 (Pratnaik, 2003). Pristine calcium carbide is uncoloured (Ropp, 2013). Calcium carbide has a fusion point of 2160 °C, a boiling point of 2300 °C (in an environment devoid of reactive gases), and undergoes hydrolysis with the release of heat (Ropp, 2013; Pratnaik, 2003). From the outset, calcium carbide has been produced mainly in factories, by reacting calcium carbonate with pre-heated coal, at extremely elevated temperatures of between 2000 to 2200 °C (Wilson, 1895; Ropp, 2013; Teong and Zhang, 2020).

Calcium carbide can, however, also be obtained by combining calcium oxide with incompletely combusted biowastes ("biochars"), instead of pre-heated coal, and this effectively reduces both kinetic rate and activation energy to about 300 seconds and 500 °C, respectively; it also reduces production expenditure (Kim et al., 1979; Teong and Zhang, 2020). Within the calcium carbide compound, the calcium cation (Ca^{2+}) ionically bonds with the carbide/acetylide anion (C_2^{2-}), that was formed by a covalent, triple-bond linkage between the two carbon atoms present (Rodygin et al., 2016). Elemental calcium on its own, is a lustrous, alabaster metal with a nuclear charge number of 20, a nucleon number of 40.0, and readily combines with other elements, because of its two slackly-attached, outer electrons; it is also found bound in the natural world in both the lithosphere and the seas (Pratnaik, 2003).

Carbon, on the other hand, is a non-metal, possesses a nuclear charge number of 6, a nucleon number of 12 (carbon-12 variant); and displays the property of catenation, in addition to that of combining with the particles of certain other elements it comes in contact with (West, 2008; Baowan et al., 2017). Carbon is also present in all the three matrices of the environment (air, soil and water), among which it is reprocessed (Pulselli, 2008). Whereas both calcium and carbon are vital nutrients for all lifeforms, the element carbon plays a much more pivotal role: it is the fundamental component of all lifeforms (Miller et al., 2001; Theobald, 2005; Remick and Helmann, 2023). Nonetheless, both elements, calcium and carbon, were combined, under certain experimental conditions, to form pristine calcium carbide that was subsequently employed for scientific research (Knapp and Ruschewitz, 2001; Rodygin et al., 2016).

In addition, some studies have observed that calcium carbide possesses crystallographic diversity at different temperatures (Rodygin et al., 2016). The major commercial applications of calcium carbide are the manufacture of calcium carbamide and ethyne gas (Ropp, 2013). Calcium carbamide is used as plant food and as a toxicant (Greenwood and Earnshaw, 1997), while ethyne gas (acetylene) is commonly used to produce carbon-based substances, and as a heat source for combining materials and for machining (Speight, 2019). However, the practice of employing ethyne gas (acetylene) produced from calcium carbide, to imitate the natural produce-maturation property of its homologue ethene (ethylene), in a bid to combat potential food scarcity in some nations, comes with attendant severe health hazards to humans and beasts (Iyare et al., 2020; Okeke, et al. 2022; Bhandare and Malode, 2023).

The phrase "biogenic substances", describes substances that are components, metabolic products and exocytotic outputs, of flora and fauna (Greeshma, 2020). A biogenic substance can either be composed of only one type of atom ("biogenic element"), or a complex of biogenic elements ("biogenic compound") (CPBCE, 1990). Examples of biogenic elements include oxygen, carbon and nitrogen (CPBCE, 1990). Some types of biogenic compounds include biogenic amines (de Jong et al., 2011), vitamins (Syairah and Mutalip, 2020), cellulose (Barbará, et al., 2023), biogenic volatile organic compounds (Janyasuthiwong et al., 2022), and biogenic hydrocarbons (Fuentes et al., 2007). Calcium carbide, has been reported as man-made and not natural (Ropp, 2013; Wang et al., 2024). Therefore, it is apparent that the occurrence of "biogenic calcium carbide" is largely unknown or undocumented.

The procedures employed in characterising a compound, generally aid in determining the exact nature of the compound. High performance liquid chromatography (HPLC) is a frequently used scientific procedure that sorts out, names and ascertains the quantity of the chemicals that compose a substance (Gupta et al., 2022). The process of HPLC involves the introduction of an aliquot of the substance under investigation, into a mobile phase; this blend of chemicals is driven through a column that houses a stationary phase (Ali, 2022). The components of the substance are sorted out, based on their varied masses, and their interactions with both stationary and mobile phases (Ali, 2022; Sadapha and Dhamak, 2022) in time. The retention time is a measure of the period between the introduction of the substance into the HPLC device and the identification of the separated components of the substance by the HPLC detectors (Ali, 2022).

The measurand from the HPLC, can be further analysed using Mass spectrometry (MS). Mass spectrometry is a very effective evaluative technique that ascertains the type and quantity of the component ions in a measurand, as a result of the dissimilarities in their mass-to-charge ratios (Rockwood et al., 2018). The mass spectrometer causes the ionisation of the measurand, and the disassociation of the measurand ions, in an electromagnetic field; the disassociated ions are subsequently detected and interpreted as a mass spectrum (Reusch, 2013). Mass spectrometry therefore, makes qualitative (elemental composition) and quantitative (elemental concentration) characterisation of a compound, possible (Ho et al., 2003); it can be applied in such fields as medicine (Zhang et al., 2020), material science (De Bruycker et al., 2020), astronomy (Arevalo and Danell, 2022) and archaeology (Hendy, 2021).

A detailed image of the surface of a compound, generates valuable information that aids in the characterisation of the compound, and a scanning electron microscope (SEM) has the capacity to achieve this. In contrast to light microscopy that employs electromagnetic energy, scanning electron microscopy utilises a highly charged beam of electrons (Mohammed and Abdullah, 2018), to create a greatly enlarged, detailed representation of a limited area of the surface topography of a compound. The interplay of the electron beam from the SEM, with the atoms of the compound, produces backscattered electrons, with energy dissipation (Nanakoudis, 2019). On the other hand, the interplay of the SEM electron beam, with the surface of the compound, produces secondary electrons, without energy dissipation; both electron types are utilised in the construction of the surface image of the compound (Nanakoudis, 2019).

EDX employs an energy dispersive spectroscope as an elemental analyser that utilises the X-ray radiation released when the highly-charged electron beam, from an SEM, hits the compound under investigation (Raval et al., 2019). It is a tool used in research, that determines the elements or chemicals that compose a compound (Ismail et al., 2019). EDX also calculates the relative amount of a single chemical or element in the compound (Nasrollahzadeh et al., 2019). The EDX detectors display the result of the interaction of the SEM X-rays with the compound, as a spectrum (Raval et al., 2019).

SEM and EDX as a unit, has been used in industries and various professions. In the construction industry, for example, it has been employed in the discovery of effective eco-friendly alternatives to conventional cement (Allison et al., 2015). In addition, in medicine, it has been used to discover pathological elements in human tissues (Scimeca et al., 2018).

Spectroscopy is the analysis of the interplay that results from the impingement of light energy on a substance; the particles of the substance assimilate, release and disperse the light energy (Sasikala et al., 2021). Fourier transform infrared (FTIR) spectroscopy is a form of spectroscopy that is based on the infrared light-absorptive property of a majority of the particles in a substance; the frequencies of escaping infrared radiation are usually recorded at the range of 4000-400 cm^{-1} (Mishrah et al., 2018). Each molecule in a compound possesses its own infrared wavenumber and therefore can be differentiated (Undavalli et al., 2021). As a result, FTIR can detect the functional groups in a substance (Sravan Kumar et al., 2015). FTIR, however, does not characteristically detect sodium, potassium and chloride ions because these ions do not absorb infrared light in the mid-infrared region of the spectrum of electromagnetic radiation, that is used for FTIR spectroscopy (Chruszcz-Lipska et al., 2022).

The quantity of sugars in a compound, can be calculated by utilizing chemical analytical techniques. The phenol-sulfuric acid method calculates the concentration of all the sugars in a compound (expressed in milligrams /litre), by using the combined reactions of phenol and sulfuric acid on the compound, and utilizes a glucose curve, in interpreting the results obtained (Chaplin and Kennedy, 1986).

Antibiotic resistance, that occurs globally, has led to a reduction in the efficacy of many contemporary conventional antibiotics. The search for more effective antibacterial agents, or potent antibiotic alternatives, inspired the production of an exopolysaccharide from *Pseudomonas aeruginosa* strain S16, accession number OQ734845 in a biocontrol activity study conducted as a Master's project (Okorie, 2023). The bacterial DNA sequence was deposited in the National Centre for Biotechnology Information (NCBI) GenBank database under its accession number. The aim of this present study was to characterise this exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, using SEM and EDX; HPLC and MS; FTIR and the phenol-sulfuric acid method.

2. Materials and Methods

A small quantity of the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, was introduced into a sterile bottle, and taken for some analytical procedures, in order to detect the elements, it possessed. The analytic methods used were High-performance liquid chromatography (HPLC) with mass spectrometry (MS); scanning electron microscopy (SEM) with energy dispersive x-ray spectroscopy (EDX); Fourier transform infrared spectroscopy (FTIR) and the phenol-sulfuric acid method.

2.1 High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS)

High performance liquid chromatography (HPLC) of the exopolysaccharide, was performed to analyse the monosaccharides, present in the secondary metabolite. This procedure was performed through the use of an Agilent 1100 series HPLC System (Agilent Technologies, California, USA), equipped with a diode array detector (DAD) and a reversed-phase C-18 Zorbax Eclipse extradense bundle (XDB-C18) column with specifications: 250 × 4.6 mm, 5 µm particle size, and 300 Å pore size. An Agilent 1100 Series LC/MSD benchtop mass spectrometer, merged with the HPLC device, was used to measure the mass-to-charge ratio of the monosaccharides.

With reference to Sharma et al. (2020), Anumula (1994), Guzzetta (2001), and the Agilent 1100 Series High Value System User's Guide from Agilent Technologies (1999), the separation of the monosaccharides was carefully carried out. Degassing of the mobile phase was performed through the use of HPLC-grade isopropanol, before the purification process was started. The HPLC reverse phase solvent system, comprised solvent A (HPLC-grade water combined with 0.1% v/v formic acid) and solvent B (HPLC-grade acetonitrile and 0.1% v/v trifluoroacetic acid) (Guzzetta, 2001). An HPLC injector set of instructions was activated to allow multiple injections of the same samples to be executed (Huber, 2010).

The end-product of the HPLC was directed into an Agilent 1100 Series LC/MSD (liquid chromatography/ mass selective detector) benchtop mass spectrometer. Ionisation of this end-product (sample), preceded separation of its ions, in an electromagnetic field, generated by the spectrometer; this was based on the ionic mass/charge (m/z) ratios differences of the dispersed ions (Reusch, 2013). Subsequently, the separated sample ions were qualified and their masses calculated; in this manner, the technique of ionic capture, by both ultraviolet and mass-selective detectors, produced the chromatogram (Reusch, 2013).

The final result of both HPLC and MS was displayed as a blend of spectral displays called a total ion chromatogram.

2.2 Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDX)

A JEOL JSM-7600F Scanning Electron Microscope (SEM), (Tokyo, Japan), showed the physical configurations of the surface topography of the exopolysaccharide. In addition, the inbuilt energy dispersive X-Ray spectroscope, revealed the elements that made up the exopolysaccharide.

The manual for SEM sample preparation by Core Facilities (Facilities, 2021), was used as a reference. A calculated amount of the exopolysaccharide sample was fixed with glutaraldehyde and formaldehyde (primary fixation). Thereafter, secondary fixation with osmium tetroxide was performed, before dehydration of the fixed sample using ethanol, was performed. Drying of the sample ensued. Mounting of the treated exopolysaccharide was subsequently performed, before fixation on a rigid specimen stub, and transference into the specimen slot. Finally, sputter-coating with platinum, and subsequent cutting of the exopolysaccharide sample into appropriate sections, to be analysed by the scanning lens of the SEM, was performed.

The composition of the sample was ascertained, using an elemental analyser, the EDX, embedded in the SEM. A beam of high-powered electrons, from the SEM device, interacted with the surface of the exopolysaccharide, forcing it to emit X-rays, from its surface atoms (Thambiratnam et al., 2020). These emitted X-rays, were detected by the X-ray detector of the EDX, and interpreted into a spectrum, that displayed the name of the elements in the exopolysaccharide, and their respective percentage concentrations (Thambiratnam et al., 2020).

2.3 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was utilised to characterise functional groups present in the exopolysaccharide. A Perkin Elmer Spectrum 100 Series 3000 MX spectrometer was used for this analysis. The exopolysaccharide sample, was prepared using potassium bromide (KBr) plates. With reference to an FTIR guide from NanoScience Technology Centre (NTSC) (2008), the computerized display of the FTIR device, was switched on, and its sample holder was wiped clean, using acetone. A set of computer commands, was entered into the FTIR device, to properly input the name of the sample, the range of the scan, and the number of the scan. Thereafter, relevant computer commands were executed, to record the spectrum generated. The spectroscopic software, Win-IR Pro Version 3.0, was used to process and analyse this result, after which the results were saved, the computer was logged off, the device was shut down, and the sample holder was cleaned.

The infrared (IR) spectra were interpreted using IR Spectra Table and Charts from Sigma-Aldrich Company (Aldrich, 2019), and FTIR spectra. Results-analyses, from Aksnes and Aksnes (1963), Reddy et al. (2012), Abadi et al. (2015), Trivedi et al. (2015), Ashok et al. (2016), Vijaykumar et al. (2016), Smith (2017), Nandiyanto et al. (2019), Cuevas-Acuña et al. (2020) and Hasan et al. (2023).

2.4 The Phenol-Sulfuric Acid Method

To ascertain the concentration of all the sugars present in the EPS, the phenol-sulfuric acid method was utilised, with glucose as the standard (Chaplin and Kennedy, 1986). This concentration, was then expressed in milligrams/litre. The total sugar quantity, was calculated in the manner described. A volume of the exopolysaccharide (0.1 mL), was initially dissolved in 2 mL of distilled water. Subsequently, 6% phenol measuring 0.1 mL, and 95% (v/v) sulfuric acid, measuring 5 mL, were swiftly added to the dissolved EPS. The mixture was shaken, before it was left to stand for 10 minutes, at room temperature. Afterwards, a reading of the optical density of the mixture, at 490 nm, was performed, in triplcate. The experimental blank was a mixture of two millilitres of distilled water, with the exact, same, quantity of 6% phenol and 95% (v/v) sulfuric acid, that was used for the exopolysaccharide. The optical density of the blank was also measured. A glucose standard curve was needed for the quantification test, therefore, the glucose for this curve, was prepared in the manner described by Dubois et al., (1956) with some modifications. Glucose (0.1g), was dissolved in 1 L of distilled water, to produce the stock solution.

Ten millilitres of the stock solution was then added to 90 mL distilled water, to produce 100 mL of diluted stock solution. Five different volumes of diluted stock solution, namely, 0.1 mL, 0.2 mL, 0.4 mL, 0.8 mL and 1.0 mL, were poured into their corresponding-labelled, sterile bottles. Varied concentrations of glucose, in each sterile bottle, were subsequently achieved, by adding the necessary volume of distilled water. to obtain 3 mL volumes of the reagents namely: 0.1 mL of 6% phenol, and 5 mL of 95% (v/v) sulfuric acid, were quickly added, to each of these concentrations, and the resultant mixtures, were left to stand for 10 minutes, at room temperature. The optical density at 490 nm, of each glucose concentration, was measured. Next, the standard glucose curve was plotted from the best-fit-line, that was achieved, by recording the different glucose concentrations in mg/L, on the x axis, and recording their respective optical densities, on the y axis. A graph of the EPS optical densities, was also likewise plotted.

The following mathematical equation was obtained from the standard glucose curve:

$y = mx + c$

where:

y = the optical density (absorbance) at 490 nm;

m = the mass;

x = the concentration of the exopolysaccharide sugars and

c = the mathematical (velocity) constant

(Jain et al., 2017).

Correlating the values, the exact concentration of the exopolysaccharide sugars was obtained from the formula:

$OD_{exopolysaccharide} = 0.3492x + 1.2234$

where:

$OD_{exopolysaccharide}$ = the average optical density of the exopolysaccharide sugars

3. Results

High-performance liquid chromatography (HPLC) and mass spectrometry (MS); scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS or EDX); Fourier transform infrared (FTIR) spectroscopy and the phenol-sulfuric acid method, were employed, to characterise the exopolysaccharide

3.1 High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS)

Figure 1 showed the total ion chromatograph of the HPLC and MS performed on the exopolysaccharide. The different masses of the simple sugars, that corresponded to different retention times, were compared to the relevant standard for identification. There were many peaks for each simple sugar compound, because of the use of different injection rates in the HPLC, and the possibility of stereoisomers. Also, the total number of peaks for each kind of carbohydrate sugar, was shown. The exopolysaccharide from *Pseudomonas aeruginosa* strain S16, consisted of monosaccharide moieties of glucose, rhamnose, xylose, arabinose, D-ribose, galactose, mannose and inositol. There was a total of seventeen recorded peaks, with glucose possessing four peaks (4, 8, 10 and 17); rhamnose, three peaks (1, 13 and 14); xylose, two peaks (3 and 15); mannose, two peaks (5 and 9); arabinose, two peaks (6 and 16) and galactose, two peaks (11 and 12). Ribose and inositol, had one peak each (peaks 2 and 7, respectively). Glucose and galactose are stereoisomers.

3.2 Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Analysis/Spectroscopy (EDS/EDX)

Plate 1 showed the SEM image for the exopolysaccharide from *Pseudomonas aeruginosa* strain S16. The SEM image, was a high-resolution image of the scanned surface of a narrow stretch of the surface of the exopolysaccharide, and revealed a sparsely flaky surface, with globular projections. The X-Ray energy emitted by the SEM electron beam, was captured and analysed by the inbuilt EDX. The EDX recorded the elemental compositions of the exopolysaccharide, as a percentage of its total weight, with the following results: nitrogen (48.00%); carbon (20.10%); phosphorus (12.6%) sodium (8.00%); oxygen (6.00%); calcium (2.30%) chlorine (1.20%) magnesium (0.89%); zinc (0.67%); potassium (0.44%) and manganese (0.33%). The carbon and oxygen contents, indicated the presence of carboxylic functional groups present in sugars.

3.3 Fourier Transform Infrared (FTIR) Spectroscopy

The FTIR result of the exopolysaccharide from *Pseudomonas aeruginosa* strain S16, at 4000- 400 cm^{-1} frequency range, is shown on figure 2. The functional groups of the exopolysaccharide from *Pseudomonas aeruginosa* strain S16, were determined by interpreting the peaks in both the functional group and the fingerprint regions of the FTIR spectrum. The FTIR showed a strong absorption peak at 3271.83 cm^{-1}, that corresponded to O-H stretching of a carboxylic acid functional group and N-H stretching in an amide/ peptide bond. The peak at 2346.92 cm^{-1} correlated closely with vibrations from the stretching of a C-H bond in the carbene, methylene. The absorption peak at 1639.89 cm^{-1}, was medium, and was from C=N stretching of an imine or oxime functional group.

This medium absorption peak at 1639.89 cm^{-1} was also attributable to C=O vibrational frequency, found in both an amide/ peptide bond, and in simple sugars. The strong absorption peak at 1542.49 cm^{-1} was generated by N-O stretching, of a nitro-compound; it also alluded to N-H bending and C-N stretching present in an amide/ peptide bond. The fingerprint region (below 1500 cm^{-1}) of the exopolysaccharide, had more absorption peaks than the functional group region. The absorption peak at 1377.94 cm^{-1}, emanated from the stretching of a phosphoryl functional group. The absorption peak at 1084.47 cm^{-1}, was strong, and correlated closely with the vibration from the C-O stretching of aliphatic ethers. The strong absorption peak at 1043.98 cm^{-1}, resulted from, the stretching of CO-O-CO bonds, in anhydrides, while the strong absorption peak at 876.83 cm^{-1}, was from a C-H bond, and indicated the presence of 1,2,4 trisubstituted; 1,3-disubsituted or 1,2,3-trisubstituted compounds.

The strong peak at 582.98 cm^{-1}, was from C-Br or C-I bending, of halo compounds or/ and from out-of-plane bending of a C=O (carbonyl) group, in an amide/ peptide bond. This strong peak at 582.98 cm^{-1} also fell within the 550-670 cm^{-1} FTIR ambit, characteristic for magnesium oxide (MgO) stretching vibrational frequency and correlated with vibrations from oxygen-manganese-oxygen bonding. The absorption peak at 413.99 cm^{-1}, was attributable to the stretching vibrational frequency from zinc oxide (ZnO) and correlated closely with vibrations from a Ca-C bond in calcium carbide.

3.4 The Phenol-Sulfuric Acid Method

The phenol-sulfuric acid method quantified the carbohydrate sugars concentration as 0.0060 g/L.

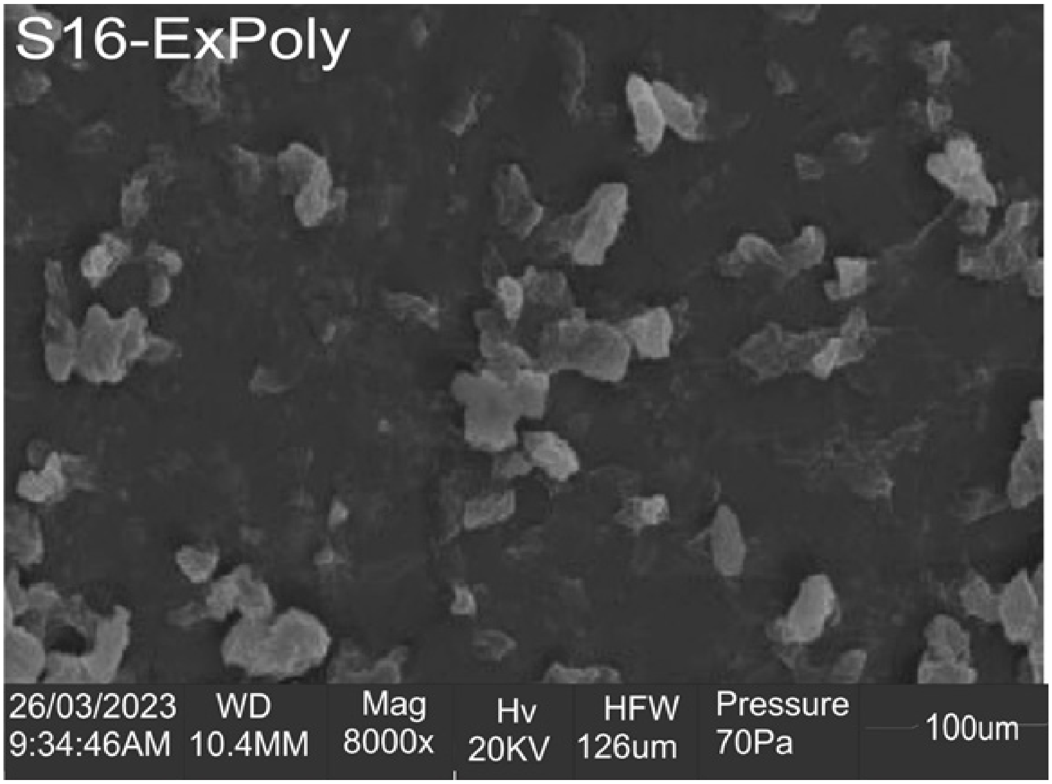
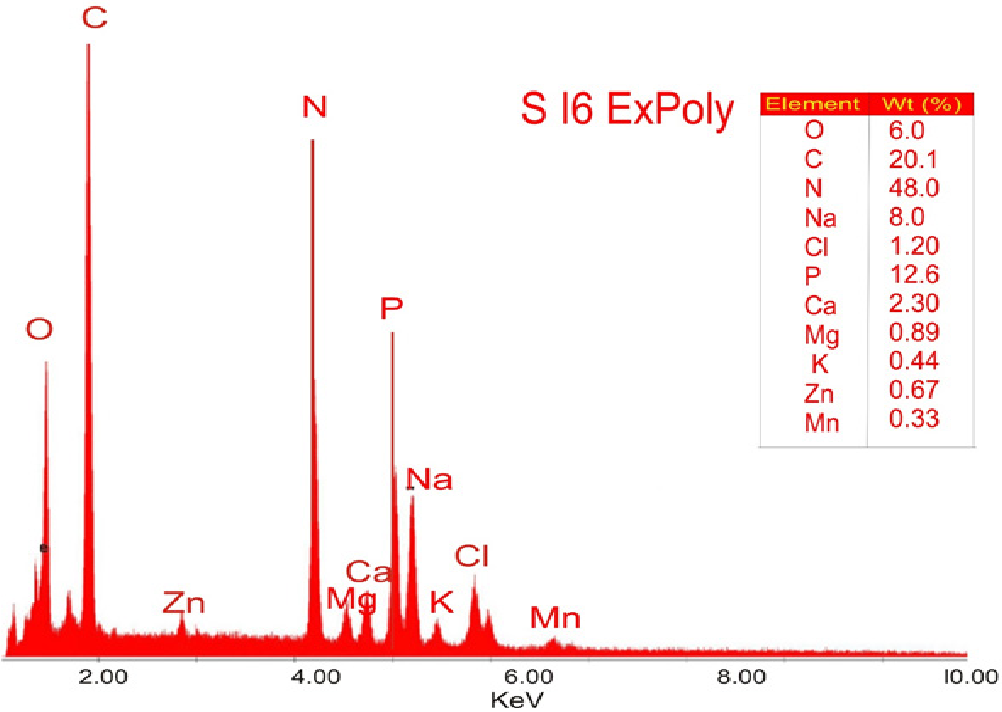


Plate 1: Scanning Electron Microscopy (SEM) Micrograph of An Exopolysaccharide from *Pseudomonas aeruginosa* Strain S16
Key: S-16 Expoly: exopolysaccharide from *Pseudomonas aeruginosa* strain S16; WD: working distance; Mag: magnification; Hv: high voltage; HFW: horizontal field switch; MM: millimetres; KV: kilovolts; µm: micrometres; PA: Pascals



Element	Wt (%)
O	6.0
C	20.1
N	48.0
Na	8.0
Cl	1.20
P	12.6
Ca	2.30
Mg	0.89
K	0.44
Zn	0.67
Mn	0.33

Figure 1: Energy Dispersive X-Ray Analysis Spectrum for Exopolysaccharide from *Pseudomonas aeruginosa* Strain S16

Key- O: oxygen; C: carbon; N: nitrogen; S: sulphur; P: phosphorus; Na: sodium; Ca: calcium; K: potassium; Mn: manganese; KeV (x-axis): energy of X-rays; y-axis: peak intensity (counts); Wt (%): percentage weight: S16Exopoly- exopolysaccharide from *Pseudomonas aeruginosa* strain S16.

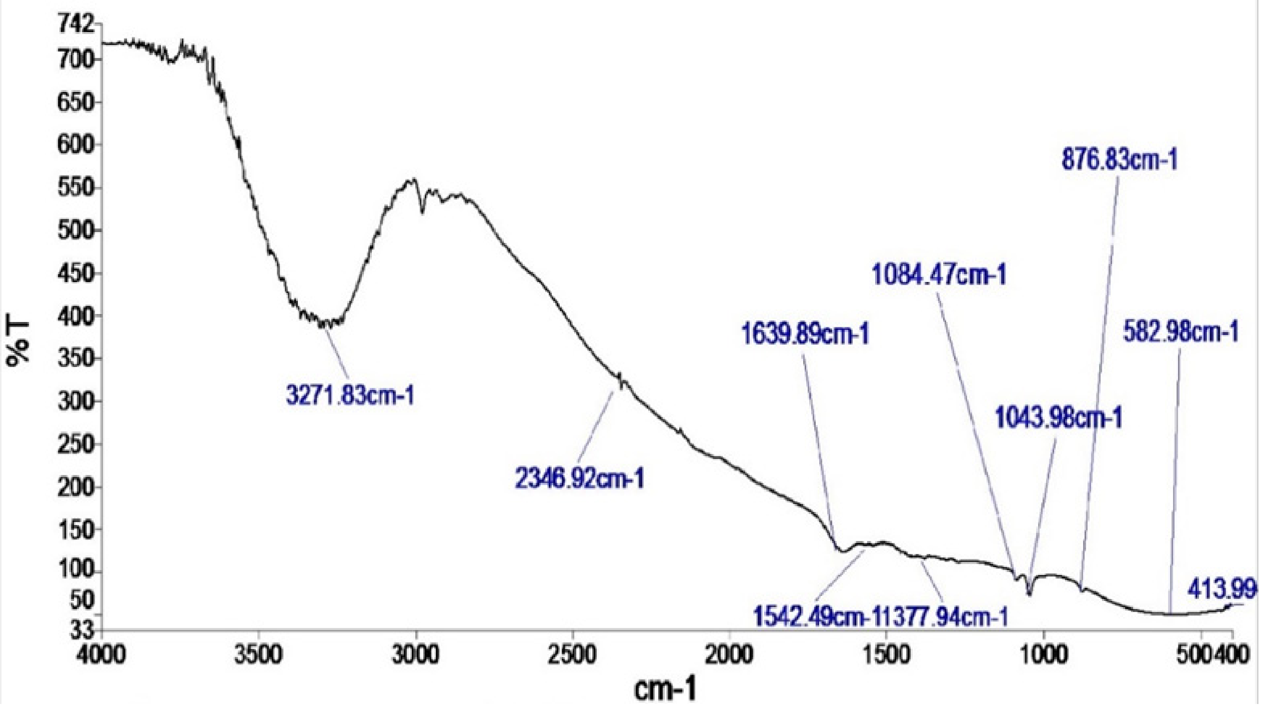


Figure 2: FTIR Spectrum For Exopolysaccharide from *Pseudomonas aeruginosa* Strain S16
Key: y-axis- absorbance (2-log%T); x-axis-wavelength measured in cm^{-1}

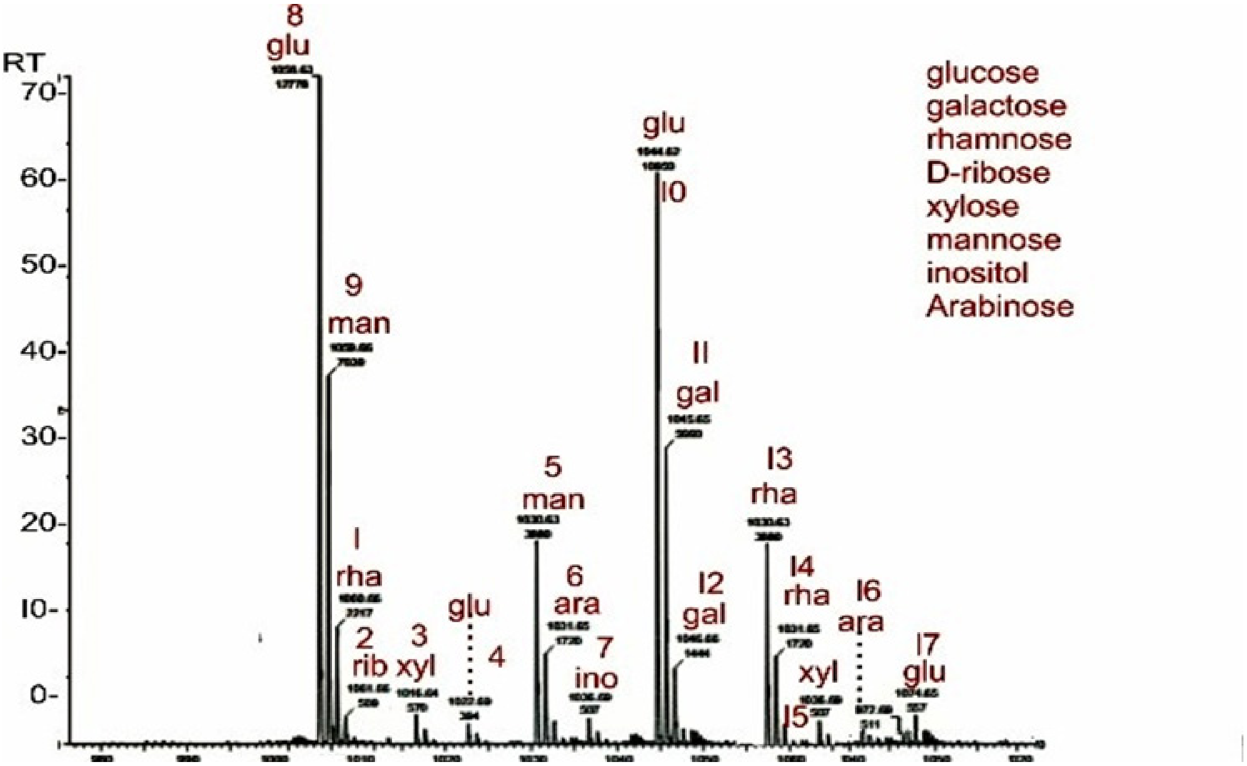


Figure 3: Total Ion Chromatogram of Exopolysaccharide from *Pseudomonas aeruginosa* Strain S16
Key: RT: retention time: s: seconds; glu: glucose; xyl: xylose; man: mannose; rha: rhamnose: rib: D-ribose; gal: galactose; ara; arabinose; ino; inositol

4. Discussion

The physical properties, chemical components, chemical bonds and functional groups of the exopolysaccharide from *Pseudomonas aeruginosa* strain S16, were determined using scanning electron microscopy (SEM) with energy dispersive x-ray spectroscopy (EDS or EDX); Fourier transform infrared (FTIR) spectroscopy; high-performance liquid chromatography (HPLC) with mass spectrometry (MS) and the phenol-sulphuric acid method.

Scanning electron microscopy (SEM) of the exopolysaccharide from *Pseudomonas aeruginosa* strain S16, revealed a sparsely flaky surface, with globular projections, while energy dispersive x-ray spectroscopy (EDS) revealed a relative abundance of nitrogen (48.00%); carbon (20.10%); phosphorus (12.6%) sodium (8.00%) and oxygen (6.00%). The elements, calcium (2.30%); chlorine (1.20%); magnesium (0.89%); zinc (0.67%), potassium (0.44%) and manganese (0.33%) were also present.

Amer et al. (2021), reported that different exopolysaccharides, have different shapes and contours, probably as a result of the varied methods used in the production of the particular exopolysaccharide. Kanamarlapudi and Muddada (2017), also stated that a uniqueness in physical and chemical characteristics, was another reason for the variation in the surface topography of polysaccharides. Amer et al. (2021) reported an irregular-sized, very compact, and lumpy, exopolysaccharide surface, while Wang et al. (2015), reported a web-like irregular porous surface, of the exopolysaccharide they worked on. The surface of the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, was however, quite similar in description, to that of the exopolysaccharide produced by Yadav et al. (2011), in terms of its flakiness.

Carbon, oxygen, nitrogen, phosphorus, and sodium, were the major elements in the exopolysaccharide from *Pseudomonas aeruginosa* strain S16 and constituted 94.70% of the total elements. Calcium, chlorine, magnesium, zinc, potassium and manganese were also present. Derdak et al. (2022) described exopolysaccharides as compounds possessing repetitive oligosaccharide sequences. Therefore, the existence of elements other than the fundamental carbon, hydrogen and oxygen of simple sugars, as occurred in the exopolysaccharide from *Pseudomonas aeruginosa* strain S16, more than likely, indicated that the exopolysaccharide had undergone natural multiple chemical molecular modification.

The nitrogen (48.00%) and phosphorus (12.60%) in the exopolysaccharide from *Pseudomonas aeruginosa strain* S16 were much higher than the corresponding elements (nitrogen: 10.11%; phosphorus: 4.13%,) in the research conducted by Amer et al. (2021), Conversely, the exopolysaccharide researched by Amer et al. (2021), possessed a much higher composition (42.68%), of oxygen, than was found in the exopolysaccharide from *Pseudomonas aeruginosa* strain S16 (6.00%). These differences in composition, could have been due to species and/ or experimental media differences. Nonetheless, nitrogenous polysaccharides, for example, chitin and chitosan, exist in nature (Peter, 1995). Guo et al (2013) documented that the experimental addition of phosphorus to an exopolysaccharide from *Lactococcus lactis* subsp. *lactis* enhanced its free radical-scavenging property. Therefore, it may be safely assumed that the presence of phosphorus in the exopolysaccharide from *Pseudomonas aeruginosa* strain S16 was contributory to its bioactivity.

A study by Adil et al. (2020), revealed that sodium ions increased exopolysaccharide production from the shiitake mushroom, Lentinus edodes. From this research by Adil et al. (2020), it can be inferred that sodium ions, if already present as an integral part of the chemical composition of a modified exopolysaccharide, may induce an increase in the quantity of the exopolysaccharide produced. A search for documented report on the molecular modification of exopolysaccharides by sodium ions, yielded no result. However, Liu et al. (2023) documented that *Pseudomonas simiae* MHR6 exopolysaccharide (EPS), took up sodium ions and thereby ameliorated saline toxicity in corn. This study by Liu et al. (2023) on corn salinity toxicity, may suggest the possibility that the sodium ions that were present in the exopolysaccharide from *Pseudomonas aeruginosa* strain S16, resulted from their uptake, and were not an integral part of its original chemical composition/ structure. Nevertheless, though sodium cation-bonding with other ions; characteristically undetectable on FTIR (Chruszcz-Lipska et al., 2022), was similarly undetected in the exopolysaccharide from *Pseudomonas aeruginosa* strain S16; documented reports on the existence of the non-synthetic, sodium-modified polysaccharide, sodium alginate, such as those by Solandt (1941), Kamoun et al. (2021), and Guo et al. (2023), raise the very real possibility of the existence of sodium-modified exopolysaccharides, in the natural world.

Chlorine in the exopolysaccharide from *Pseudomonas aeruginosa* strain S16, likely signified the chlorination of the exopolysaccharide, even though chloride ion bonds were characteristically undetected on FTIR (Chruszcz-Lipska et al., 2022). Kanamarlapudi and Muddada (2017) also reported the presence of chlorine in the exopolysaccharide they produced from pure cultures of *Streptococcus thermophilus* (strain CC30), obtained from unpasteurised milk. The review by Cumpstey (2013), that included the chlorination of polysaccharides, served to some degree as reasonable comparison, to decipher the effects of the presence of chlorine in an exopolysaccharide. Therefore, the chlorination of the exopolysaccharide from *Pseudomonas aeruginosa* strain S16, that formed a type of natural chemical molecular modification of the exopolysaccharide, most likely altered its pre-chlorination attributes. Furthermore, the research by Mueller et al. (1996) on native halogen-containing carbon-based compounds, found in deposits associated with certain water bodies, elucidated the effect of the existence of salt-formers in these compounds, on the bioactivities of these compounds. Accordingly, the existence of chlorine in the exopolysaccharide from *Pseudomonas aeruginosa* strain S16, most likely, significantly augmented its inherent bioactivities. On the other hand, it was documented by Luo et al., 2021, that exopolysaccharide production by bacteria, was the major bacterial mechanism by which certain bacteria became impervious to the bactericidal effect of chlorine.

Therefore, though the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16 was likely modified by chlorine ions, it is also quite probable, that the presence of chlorine in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, could have been the result of chlorine uptake by *Pseudomonas aeruginosa* strain S16. Indeed, Shrivastava et al., (2004) reported that *Pseudomonas aeruginosa* strains that were discovered to be impervious to the actions of at least one antibiotic in three or more antibiotic classes, were relatively unaffected by chlorine concentrations below that which gave proper water disinfection.

The strong peak at 582.98 cm^{-1} in the FTIR of the exopolysaccharide produced from *Pseudomonas aeruginosa* strain was almost similar to the FTIR result of 584 cm^{-1} from the magnesium oxide produced from discarded pips of a variety of squash, in the research by Agyapong et al. (2023). This strong peak of 582.98 cm^{-1}, also fell within the 550-670 cm^{-1} FTIR ambit, characteristic for magnesium oxide (MgO) stretching vibrational frequency (Ashok et al., 2016). An extensive search for literature on the molecular modifications of exopolysaccharides by magnesium ions, yielded no result. However, there was documentation on the interaction of magnesium ions with polysaccharides, in certain experimental settings. Wang et al. (2020) demonstrated that in aquatics, the presence of magnesium ions in the process of the high-pressure removal of certain impurities from water, enhanced the binding together of alginate chains, and consequently worsened the natural clogging tendency of the polysaccharide ("fouling").

This synthetic magnesium-polysaccharide interaction, may suggest a mechanism of bioactivity, whereby natural magnesium-containing exopolysaccharides, such as the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, induce "membrane biofouling" and resultant cell death in susceptible nearby organisms, as a strategy for bacterial survival. In addition, Chew and Yang (2016) have reported that, due to the large quantities of electrons that exopolysaccharides possess, they can take up magnesium and calcium cations. However, the FTIR result of 584 cm^{-1} that confirmed the presence of magnesium oxide (Agyapong et al., 2023) in the exopolysaccharide produced from *Pseudomonas aeruginosa* S16, revealed that there was some chemical reaction between magnesium ions and oxide ions of the simple sugars of the exopolysaccharide that resulted in the natural chemical modification of the exopolysaccharide.

The absorption peak at 413.99 cm^{-1} from zinc oxide (Reddy et al., 2012; Vijaykumar et al., 2016) revealed the presence of bound zinc ions in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, and was also a natural chemical molecular modification of the exopolysaccharide. Although documentation on the existence of natural zinc-modified exopolysaccharides could not be found, the study by Dong et al. (2018) on a synthetic *Athelia rolfsii* exopolysaccharides-zinc complex (AEPS-zinc), provided some insight. Dong et al. (2018) documented that the AEPS-zinc complex possessed free-radical-inhibition properties, and was also proposed as a good source for the restoration and maintenance of optimal zinc levels in the human body.

Similarly, the study by Chen Z. et al. (2022) on a new non-synthetic zinc-modified polysaccharide, OZPs-1, extracted from an estuarine mollusc, provided reasonable comparison, from where the possible material characteristics, reactivities and functional activities of the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, could be extrapolated. Therefore, the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16 may exhibit good heat resistance and may utilise hydroxyl and carboxyl moieties for the chelation of zinc, much like was documented for the non-synthetic, zinc-modified polysaccharide, OZPs-1, worked on by Chen Z. et al. (2022). However, unlike was proposed for this non-synthetic, zinc-modified polysaccharide, OZPs-1, worked on by Chen Z. et al. (2022), the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16 may not be suitable for use as a carbon-based replenisher and maintainer of the zinc stores in humans, due to possible potential health concerns from the presence of certain compounds in it. The need for pharmaceutical safety tests for any potential drug candidate cannot be overstated.

The exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, was also naturally modified by potassium ions even though potassium cationic bonds were characteristically undetected by FTIR ((Chruszcz-Lipska et al., 2022). In a study by Almansoory et al. (2020), that investigated varied means of augmenting the generation of exopolysaccharides from a mixture of different bacterial species, the potassium ions released from the addition of potassium chloride salt to the variegated mixture, were observed to produce the greatest quantity of exopolysaccharides when compared with the results observed from other ions that dissociated from their salts. Although no documentation on the existence of a potassium-modified exopolysaccharide, was found, this study by Almansoory et al. (2020), made room for some hypotheses. Chiefly, the chelation of potassium ions by the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, may have been employed by the bacterium as a means of augmenting its exopolysaccharide production in its niche. Additionally, this augmentation of exopolysaccharide *Pseudomonas aeruginosa* strain S16 production may probably have served as a form of enhancement of exopolysaccharide bioactivity, owing to its greater availability.

Manganese was also detected in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, at a concentration of 0.33%. The FTIR of 582.98 cm^{-1} that was attributable to vibrations from oxygen-manganese-oxygen bonding (Hasan et al., 2023), further confirmed the presence of a manganese compound or compounds in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16. This chemical binding between magnesium cations and the oxide anions in the monosaccharides of the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, signaled a natural chemical molecular modification of the exopolysaccharide. Silva et al. (2019) stated that, there are many manganese-containing compounds which can perform the functions of drugs. Examples include, the tetra-substituted manganese (III) phthalocyanines, made by Barut et al. (2016), in their research, which demonstrated, antitumour activities, due to their ability to break up deoxyribose nucleic acid (DNA), amongst other actions. In addition, the bioactivity of isolated manganese (II) compounds, against the biofilms of *Pseudomonas aeruginosa,* was observed, in research conducted by Jabłońska-Wawrzycka et al. (2021).

Brian-Jaisson et al. (2016), reported that, the FTIR spectra, in the range of 900 – 1300 cm^{-1}, were typical for exopolysaccharide-, and nucleic acid-functional groups. Furthermore, the absorption peaks at 1000 to 1100 cm^{-1}, were reported by Lu et al. (2005), to be typical for all sugar derivatives. The FTIR, of the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, had two peaks within the band of 900 – 1300 cm^{-1}, that were strongly indicative of the presence of polysaccharides in the exopolysaccharide, and possibly, of nucleic acids. These peaks were strong at 1084.47 cm^{-1}, from the C-O stretching of aliphatic ethers, and at 1043.98 cm^{-1}, from the stretching of CO-O-CO bonds in anhydrides.

Iqbal et al. (2002), reported that, carboxyl, hydroxyl and nitro groups, were present in the bacterial exopolysaccharide they worked on. This was in agreement with the findings, from the exopolysaccharide produced by *Pseudomonas aeruginosa* strain S16, namely; a strong absorption peak at 3271.83 cm^{-1}, that corresponded to the O-H (hydroxyl functional group) stretching, of a carboxylic acid, and a strong peak at 1542.49 cm^{-1}, generated by N-O stretching of a nitro-compound. Additionally; the review by Noriega et al. (2022) stated that the nitro moiety exhibits a broad range of biological effects that include bioactivities against cancer cells, microorganisms, parasites and high blood pressure in both man and beast. Therefore, the exopolysaccharide produced by *Pseudomonas aeruginosa* strain S16, may display more biological effects than just antibacterial activity

The exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, also possessed imine or oxime functional groups, alluded to, by the medium stretching at 1639.89 cm^{-1}. This stretching, was from a double chemical bond between the carbon and nitrogen atoms, as occurs in imines (Moldoveanu, 2019), and oximes (Lu et al., 2022).

Altamimi et al. (2020) reported that imine compounds exhibited activity against some microorganisms and were blood-material compatible. Surowiak et al. (2020), in addition, stated that oxime compounds displayed activity against inflammation, microorganisms, free radicals and neoplasia. Therefore, the bioactivities of the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16 may mirror these findings.

The medium stretching at 1639.89 cm^{-1}, was also indicative of carbonyl groups. This range was within the 1900-1600 cm^{-1} range, that is typical for, the carbonyl group (Smith, 2017), and was indicative of the presence of carbohydrates (Roberts and Caserio, 1977), in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16. In addition, the strong absorption peak at 3271.83 cm^{-1}, that corresponded to the O-H (hydroxyl functional) group, that is also typical for carbohydrates (Roberts and Caserio, 1977), was also seen in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16.

The absorption at 1377.94 cm^{-1}, fell within the spectral range of 1175 to 1414 cm^{-1}, observed for the phosphoryl functional group (Aksnes and Aksnes, 1963). This confirmed that the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, was phosphorylated. Xia et al. (2021), reported that, phosphorylation markedly alters the capacity of polysaccharides to exert targeted, and measurable changes, in living cells.

The strong peak at 582.98 cm^{-1}, that emanated from C-Br or C-I bending, was indicative of the presence of bromine or iodine respectively (Nandiyanto et al. 2019; (Aldrich, 2019). in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16. However, chlorine was the only halogen detected in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16 on EDX, and at a composition of 1.20%.

Yuan et al. (2019), have reported that, alterations of polysaccharides, by the chemical addition of functional groups, greatly augment the capacity of the polysaccharide, to dissolve in water, and to exert changes, in living cells. Consequently, these modifications, render the polysaccharide, more tailored, towards efficient drug administration (Sinha and Kumria, 2001). These reported attributes for a modified polysaccharide, can also be reasonably conjectured to occur, in a chemically-modified exopolysaccharide, such as the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, that was characterised in this study.

It was both astonishing and alarming, that the presence of calcium carbide was detected in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, as evidenced by the absorption peak at 413.99 cm^{-1}, that correlated closely with vibrations from a Ca-C (calcium carbide) bond. Documented research, such as by Morehead and de Chalmot (1896), Ropp (2013) and Wang et al. (2024), suggested that the compound, carbon carbide, was strictly man-made. However, the discovery of calcium carbide in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, qualified it as being biogenic, and therefore challenged this age-old premise.

This discovery of a source of “biogenic calcium carbide”, also gives rise to speculation that there may be more organisms equipped with the tools for biogenic calcium carbide production, and not just mankind. A blend of research and serendipity, may reveal these species and their scope of biogenic calcium carbide utilisation. Then again, the presence of biogenic calcium carbide in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, raised some concerns. This was because, synthetic calcium carbide poses significant health risks to man and certain animals, due to the presence of such contaminants as arsenic, phosphorus, zinc, manganese, and iron; and the production of an extremely combustible fume, when it comes in contact with moisture (Bandyopadhyay et al., 2013; Iyare et al., 2020; Hassan et al., 2019; Okeke et al., 2022; Ramos et al., 2023).

The exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16 also possessed phosphorus, zinc, and manganese, in addition to certain other metals and non-metals. However, these elements, in conjunction with the biogenic calcium carbide, were observed, ab initio, to be integral parts, of the chemical composition, of the natural multichemical molecularly-modified exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16. Nonetheless, only further research can accurately uncover certain pertinent facts. The bioactivities, toxicity profile and possibility of biodegradability, amongst other inherent characteristics of the biogenic calcium carbide, would have to be determined. Specifically, the net effect of the chemical bonding of phosphorus, zinc, manganese and biogenic calcium carbide, in any organic compound, such as the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, would have to be ascertained to discover if it results in bioactivities that mirror the adverse effects produced by contaminated synthetic calcium carbide. The exact function of the biogenic calcium carbide component of the naturally modified exopolysaccharide, produced from *Pseudomonas aeruginosa* strain S16, fell outside the scope of this study.

The total ion chromatograph of the blend of spectra from high performance liquid chromatography (HPLC) and mass spectrometry (MS), revealed that, the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, consisted of monosaccharide moieties, namely, glucose, rhamnose, xylose, arabinose, D-ribose, galactose, mannose and inositol with varying peaks. This indicated that the exopolysaccharide was a heteropolysaccharide.

The exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, is a novel, naturally-modified biogenic calcium carbide-containing exopolysaccharide. The multiple chemicals present in the exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, likely add to the exopolysaccharide, the potential for a range of biological activities. From available documented research, this is the first report of an exopolysaccharide naturally modified by biogenic calcium carbide, and sodium, magnesium, potassium, and manganese ions.

5. Conclusion

An exopolysaccharide produced from *Pseudomonas aeruginosa* strain S16, accession number OQ734845, that was observed to be effective against two antibiotic-resistant strains, namely *Klebsiella pneumoniae* and *Staphylococcus aureus* SO183 in a biocontrol activity study, conducted as a Master's project, was subjected to some analytical procedures, for its characterisation. The exopolysaccharide is a novel, heteropolysaccharidic, naturally modified, biogenic calcium carbide-containing, exopolysaccharide. The multiple chemicals in this exopolysaccharide likely give it the potential for a range of biological activities, but definitely creates the need to conduct pharmaceutical safety tests before any form of application that would involve humans, animals and the environment. From available documented research, this is the first report of an exopolysaccharide naturally modified by biogenic calcium carbide, and sodium, magnesium, potassium, and manganese ions.

Authors Contribution

I, Ikechukwu Kenneth M. Okorie (Corresponding author), carried out all the work in this study as my Masters (MSc.) research project. Professor Adeniyi A. Ogunjobi supervised my Masters research study.

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Competing Interests

Both authors declare no financial or non-financial competing interest.

Conflicting Interests

Both authors declare no form of conflicting interest.

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