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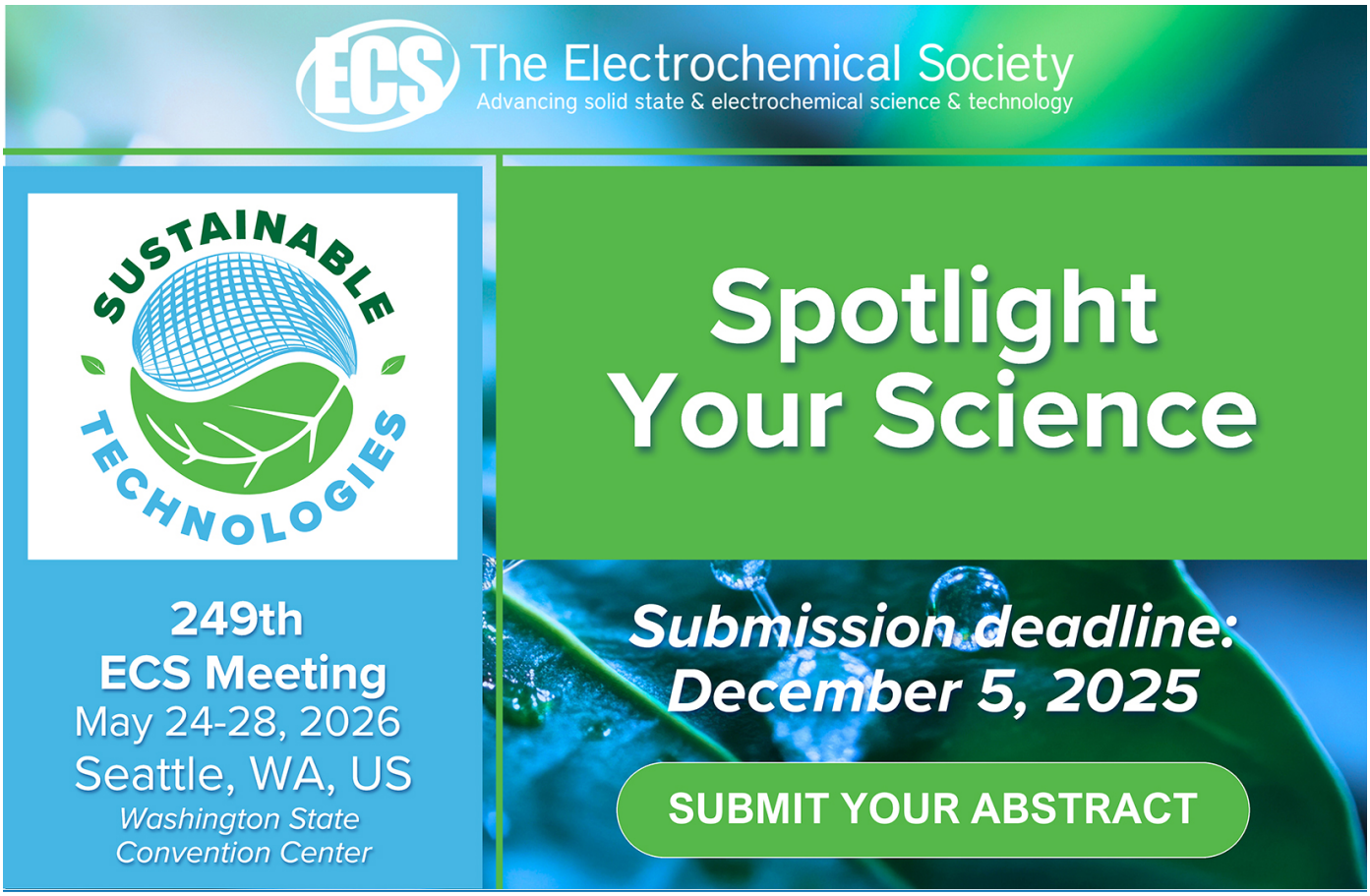

Bacteria analysis as plastic biodegradation agent and biofertilizer

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Abstract. Several bacteria have been observed as tolerant to the plastic waste according to previous studies. However, its ability as in biodegradation of plastic and biological fertilizers has not been previously tested. Therefore this research is intended to test the potential ability of these bacteria as in biodegradation of plastic waste and biological fertilizer by using Gas Chromatography-Mass Spectrophotometry (GC-MS) technique. According to GC-MS analysis results on SP6 isolates as plastic-tolerant bacterial models, have obtained 5 specific secondary metabolites which act as biodegradation of plastic with the composition as follows: Malic acid ($C_4H_6O_5$) 1.85%, Xanthine ($C_5H_4N_4O_2$) 1.08%, Myo inositol ($_6H_{12}O_6$) 1.32%, γ Glutamyl alanine ($C_8H_{14}N2O_5$) 0.85% and Ribitol 5 phosphate ($C_5H_{13}O_8P$) 1.30%. In addition, the isolate was also able to synthesize several phytohormones- an indicator for potential use as biofertilizer- with the composition as follows: α Aminobutyric acid3 ($C_4H_9NO_2$) 1.20%, Indoleacetic acid ($C_{10}H_9NO_2$) 1.85%, Kinetin ($C_{10}H_9N_5O$) 1.19%, Kinetin glucoside ($C_{16}H_{19}N_5O_7$) 1,19 % and Benzyladenine 9 glucoside N6 ($C_{18}H_{21}N_5O_5$) 1.31%. It is therefore, the bacterium has a double potentiality not only to degrade plastics, but also to be used as a bio-fertilizer.

1. Introduction

Extensive plastic use poses a severe environmental threat to terrestrial and marine ecosystems due to the nature of its waste which can hardly be degraded and generally disposed in a large scale. Because of high production and extensive use of plastic, its disposal becomes a major problem. The accumulation of plastic products in the environment has a negative impact on wildlife, wildlife habitat, land, waterways and oceans. Chlorinated plastic resulted from this process can also affect the groundwater ecosystem. Methane gas, a greenhouse gas produced during the degradation process is a very strong gas associated mainly with global warming [1]. In the ocean, lution-type of plastic may kill marine mammals through direct consumption, because it is thought to be food. Some studies have discovered that all species, including small zooplankton, most fishes, seabirds, and all sea turtles can easily swallow plastic bits and junk items such as cigarette lighters, bottle caps, and even light plastic bags. Sunlight may exacerbate the sea water containing plastics as it may eventually break down large polymer compounds into smaller carbon compounds and made it available for zooplankton and other small marine animals.

Plastic waste such as Poly-ethylene (PE) can interfere with hormonal growth, growth of children, cancer, sperm count reduction, and weakened infertility and immunity. On the other hand, Poly Vinyl Chloride (PVC) may cause skin disease, bronchitis infection, deafness, vision problems, and some digestive problems primarily related to the liver. Other dangerous toxic chemicals released during PVC

degradation (i.e. mercury, dioxin and phthalates) can span lifelong health problems that cannot be overcome. When PVC plastic is produced or burned, it releases dioxin, the group containing most synthetic chemicals that can cause cancer and jeopardize the immune and reproductive systems. Besides, Bisphenol A is believed as the main cause for cancer by damaging the immune system; cause for early puberty and trigger the development of chronic diseases such as diabetes and obesity [2].

Synthetic polymers (plastics) are considered as major environmental pollutants in the form of solid wastes that are difficult to degrade. This problem has been becoming a main concern especially for researchers in the scope of polymers' biodegradation. Environmental factors such as heat, light, humidity, chemical conditions, and biological activity can break down polymer carbon chain bonds. The formation of structural homogeneity and new functional groups may also occur during polymer degradation process [3].

Depending on the nature of the agent, polymer degradation has been classified as photo-oxidative, thermal degradation, ozone-induced degradation, mechano-chemical degradation, catalytic degradation, and biodegradation. Thermo degradation is the degradation of polymers by heat energy with the support of atmospheric oxygen and is known as thermo-oxidative-degradation. The initial stage of degradation is the process of breaking up macromolecular bonds to produce radical sites. This generally involves changes to the molecular weight of polymers. Meanwhile, the presence of light in the degradation process is called photo-degradation. Its mechanism involves light energy absorption by the appropriate group of compounds entering polymer molecules. By this way smaller fragments conversion will be obtained after the cutting of polymer molecules in the exact position of the chain. Thus, degraded polymers entail both the chain-responsive and additional chain of photo responsive groups [4]. Polymer photo-degradation in the meantime uses UV light for both degradation and oxidation processes. During UV degradation, UV light is used to alter the final product. Plastic is transformed and broken down by the use of heat energy during oxidation process. However, many synthetic polymers are identified to be resistant to chemical and physical degradation process. Both thermal and physical degradation methods reduce the molecular weight of the plasma and are left to decompose.

On the other hand, biodegradation is a degradation process involving the role of living organisms to break down organic matters. This is sought to be a natural mechanism for the loss of most chemicals released into the environment. Biodegradation uses microorganisms to stimulate abiotic degradation involving physical, chemical or enzymatic actions [5]. Microorganisms have a pivotal role in degradation process and through altering synthetic and natural polymers. Polymers are not used directly by microorganisms where most biochemical processes take place. Plastic degradation is a very slow process influenced by environmental factors, including pH, UV and temperature. Hence, for this regard microorganisms have developed specific to use materials such as plastic polymers as their sources of carbon and energy [6].

According to Konduri *et al*. [7], biological degradation of polymeric substances requires enzymatic action preceding next few steps. The most important type of this enzymatic polymer division is a hydrolysis process. Chemical bonds such as glycosidics, esters, and peptide relationships are exposed to hydrolysis through nucleophilic attacks on carbonyl carbon atoms. In general, the biological degradation is much triggered by the mutual presence of enzymes and microorganisms, biotic availability of polymeric structures and suitable abiotic factors.

In addition, abiotic factors such as pH, temperature and humidity can have an impact to the rate of hydrolysis reactions during degradation process. Increased in temperature and humidity can cause an increase in the rate of hydrolysis reactions and microbial activity. In a high humidity condition, hydrolysis reactions become faster resulting in more chain cuts and leading to increased locations available for microorganisms to attack polymer chains, thus inducing a faster degradation [8]. Besides biotic factors, extracellular enzymes produced by different microorganisms may have active sites of different shapes and are therefore able to degrade certain polymers. As an example, fungi *Aspergillus niger* and *Aspergillus flavus* produce enzymes that digest aromatic polyesters deriving from 6-12-carbon monomers more easily than those produced from other monomers [9].

Raziyafathima *et al*. [10] states that polymer degradation kinetics rely on whether the environment is humid air, dry air, soil, landfills, composting environment, wastewater, fresh water, or the marine environment. Every environment has a concentration profile of its own characteristics of important factors: water, oxygen, other chemicals, sunlight and destructive microorganisms. Conformation flexibility plays a vital role in polymer bio-degradation. The more flexibility the polymer has, the more easily accessible to the microbes. In general, microorganisms can only digest parts with low molecular weight, which are taken into cells and then converted into metabolites. Therefore, microbes and water can easily access the smaller molecules rather than larger ones. The addition of commanders into polymer structures increases the irregularity of polymer chains causing reduction in crystallinity of polymers and may surge accessibility to microbes and water.

Microorganisms such as bacteria and fungi are commonly involved in plastic degradation which may differ from each other and have their own optimal growth conditions. Biodegradation is commonly known as series of hydrolysis process catalyzing by enzymatic and non-enzymatic hydrolysis [11]. During degradation, the exoenzymes of microorganisms break down complex polymers into short chains or smaller molecules, i.e. oligomers, dimers, and monomers, which are small enough (soluble in water) to pass through a semi-permeable outer bacterial membrane and then becoming as a source carbon and energy. The initial process of solving this polymer is called de-polymerization. Environmental conditions are also important to determine polymer degradation pathways. Complete polymer decomposition produces organic acids, CH_4, CO_2, and H_2O [12].

Another point to consider is that, some bacteria resistant to plastic waste has potentiality to secrete hormones such as Indole Acetic Acid (IAA), Giberelin (GA) and Kinetins. These hormones are mainly associated with the growth of the plants through regulating in ethylene production, thus the bacteria secreting these widely known as plant growth promoting rhizobacteria (PGPR) [13]. The PGPR mutually interacts with the plants through stimulating their growth and health and acting as biological fertilizer, hence increasing crop productivity. Vejan *et al.* [14] has extensively reviewed the role of PGPR associated with different types of crop. This is mainly related to the beneficial impact of PGPR that can influence on crop productivity and ecosystem functioning especially as in bioremediation and bio-fertilization process.

The results of previous studies have obtained several bacterial isolates that are tolerant of plastic waste. However, in this study, its ability has not been analyzed in plastic remodeling and its ability as biological fertilizer. Therefore, this study is intended to examine deeper secondary metabolites that play a role in their ability to remodel plastic and their ability to synthesize phyto-hormones as biological fertilizers. Analysis of secondary metabolites was carried out using Gas Chromatography-Mass Spectrophotometry (GC-MS) with helium carrier gas and absolute methanol extractant. Therefore, a double-capability of bacterial isolates will be obtained, namely as plastic-degrading bacteria and biological fertilizers.

2. Materials and Methods

2.1. Bacteria Isolation

The bacterial isolates used in this study were the results of previous studies isolated from soil contaminated with plastic waste. From 14 isolates obtained in the previous study, 2 representative isolates were taken, namely S5 isolates were used as a representative of plastic waste sensitive bacteria and S6 isolates as tolerant bacterial isolates.

2.2. Chemicals and media

The chemicals used include: absolute methanol for extraction of extracellular secondary metabolites, minimum M63 media with composition of 100 mM KH_2PO_4, 75 mM KOH, 15 mM $(NH_4)_2SO_4$, 0.16 mM $MgSO_4$, 3.9 µM $FeSO_4$ and 10 mM D-glucose. Besides that, Luria broth (LB) media also consists of yeast extract 5 g/l, tripton 10 g/l and NaCl 5 g/l, tryptophan, physiological salt (NaCl), glucose,

alcohol, agar bacteria, yeast extract, polyethylene glycol (PEG), polyvinyl alcohol (PVA), as described by Juhi *et al*. [15].

2.3. Plastic Biodegradation Test with PEG and PVA Polymers
Selected bacterial isolates from previous studies S5 and S6 were tested for their ability to degrade plastic by using PEG and PVA plastic polymer carbon sources. The bacteria were grown on a minimum standard M63 medium with PEG and PVA supplementation as a source of carbon equivalent to 40% of the source intake of 10 mM glucose carbon at. The growth of bacteria in using PEG and PVA as a carbon source was observed using spectrophotometric at wavelength of λ 600 nm as described by Juhi *et al*. [15].

2.4. Test of ability as biological fertilizer
At this stage, both S5 and S6 isolates were grown on a minimum medium of M63 + 100 ppm L-tryptophan. The two isolates were grown on an incubator shaker temperature of 37 oC, shaking speed of 125 rpm, for 24 hours. Bacterial culture was then harvested by centrifugation at 4000 rpm for 5 minutes; the supernatant was extracted with absolute methanol of 1: 1 v/v by vortex for 3 minutes, then concentrated with absolute methanol cold 1: 1 v/v and slowly shaken. Metabolites which were coagulated with cold methanol were then centrifuged at 6000 rpm for 5 minutes. Supernatant was slowly poured and the cell pellets obtained were re-suspended with methanol until concentration reaching 50 times v/v. The metabolite solution from concentration was then used as GC-MS analysis material as described by Vejan *et al.* [14].

2.5. Analysis of Extracellular Metabolites
The composition analysis of extracellular metabolites was carried out using GC-MS (Gass Chromatography-Mass Spectrophotometry) as described by Bolten *et al*. [16] with the following steps:
2.5.1. Isolation of extracellular metabolites. The isolate culture was harvested by 4000 rpm centrifugation for 5 minutes. The supernatant obtained was extracted with absolute methanol of 1: 1 v / v by vortexing for 3 minutes, then concentrated with cold methanol absolute 1: 1 v / v and slowly shaken. Coagulated metabolite was further centrifuged at 6000 rpm for 5 minutes. Supernatant was slowly poured and the cell pellets obtained were re-suspended with methanol until the concentration reaching 50 times v/v. The solution of the metabolite from the concentration was then used as an analysis material for GC_MS [17].

2.5.2. Analysis of cell metabolites. The metabolites were analyzed using GC-MS type Shimadzu GCMS QP 2010 SE with ZB-AAA column (10 mL x 0.25 mml D. Helium carrier gas with a flow rate of 0.5 ml / minute, and pressure 27.4 kPa. Initial GC oven temperature at 120 ^{o}C and 5 minutes after injection increased at a speed of 5 ^{o}C / minute until it reached 320 ^{o}C and held for 30 minutes [18, 19]. Sample solutions were then inserted using micro-injector at 1- 2 µl.

2.5.3. Identification of Metabolites. The metabolite constituent molecules will be separated from each other by different retention times, and then the molecule is read by the detector and determined by AMDIS software (Automated Mass Spectral De-convolution and Identification System). Data obtained in the form of compound names, molecular formulas, molecular weight, relative concentration (%) and similarity index. The data obtained from the GC-MS analysis were then analyzed by the NCBI database alignment system and to determine specific metabolites having a role in degradation of plastic polymers and metabolites that belong to phytohormones [20].

3. Results and Discussion

3.1. Potential Biodegradation Test for Plastic Polymers

Test the potential biodegradation of plastic polymers with a growth curve approach on media of at least M63 + PEG or PVA polymers 40% of DL intake of 10 mM glucose as a carbon source (Fig. 1).

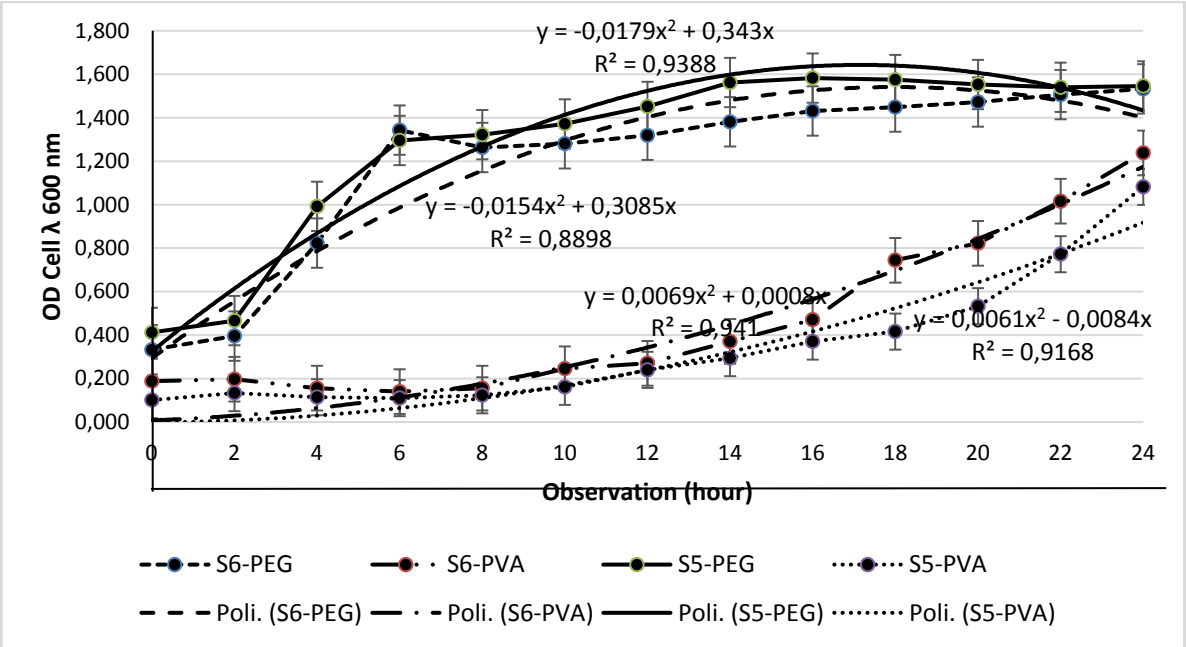

Figure 1. Growth curve of isolates S5 and S6 in M63 + PEG or PVA 40% substitution of DL glucose 10 mM.

From the growth curve it appears that isolates S5 and S6 have different growth patterns in both M63 + 40% PEG and M63 + 40% PVA media. This indicates that S5 and S6 isolates are different bacteria in using PEG and PVA polymers as carbon sources with different biodegradation systems. Biodegradation is controlled by various factors including the polymer characteristics, organism types, and the nature of pretreatment. Polymer characteristics such as crystallinity, molecular weight, mobility, functional group types, and substituents present in their structure, and plasticizers or additives added to polymers all play important roles in the degradation process [21]. Polymer biodegradability is mainly influenced by two factors such as exposure conditions and characteristic features of the polymers. Further conditions of exposure can be categorized as abiotic and biotic factors. The main chain cutting of photo-degradation reduces the amount of average molecular weight, which gives greater accessibility to polymer chains by microorganisms [2]. The microbes can then easily hydrolyze or utilize smaller plastic molecules. In the case of aliphatic aromatic polyester, breaking down of the main and cross-linking chains can be observed as the result of photo-degradation. This can affect plastic films in two ways, firstly through the mechanism of Norrish I and Norrish II which causing major random chain cuts [22].

Microorganisms obtain energy by catalyzing energy-producing chemical reactions that include breaking chemical bonds and removing electrons from contaminants. This type of chemical reaction is known as an oxidation-reduction reaction where organic contaminants are oxidized-the technical term for electron loss; consequently, chemicals obtaining electrons are reduced. Contaminants are called electron donors, while the others are called as electron receivers [12]. The resulting energy from the transfer of electrons is then "invested," along with several electrons and carbon from contaminants to produce more cells. This will induce a process called aerobic respiration by destroying organic compounds with the help of oxygen. In aerobic respiration, microbes use oxygen to oxidize parts of

carbon in contaminants to carbon dioxide (CO_2), with the remaining carbon used to produce new cell masses [10].

Oxidative degradation is the main mechanism for plastic degradation. This mechanism reduces the molecular weight of the material. Extracellular and intracellular enzymes produced by microbes converting polymers into monomers, dimers, and oligomers. The by-products produced during conversion into microbial cells can be used as an energy source. Different types of microorganisms are responsible for the degradation of various groups of plastic. A certain bacterium can constantly synthesize all the enzymes sought for degradation or the others can synthesize enzyme to metabolize the activity or whenever thermodynamically beneficial as needed [11]. Balasubramanian *et al.* [23] reported that environmental factors (physical and chemical) play a major role in initiating HDPE degradation and also supporting microorganisms to reduce PE (HDPE).

3.2. Specific Metabolic Analysis that Acts as a Plastic Degrading Bacteria

The results of the analysis of specific secondary metabolites based on the GC-MS test obtained 6 compounds in S5 isolates and 5 compounds in S6 isolates (Table 1).

Table 1. Result analysis GC-MS of specific extracellular metabolites that act as biodegradation of plastic polymers.

Metabolites Compound	Control: S5 (plastic sensitive isolate)	Treatment: S6 (Plastic tolerant isolate)	Composition (%)	Function
Formic acid	√	-	0,82	Regulates the process of metabolic acidosis, inhibits oxidase in cytochrome thereby reducing the concentration of energy that can affect cell function
α Ketoglutaric acid	√	-	1,27	Stimulates protein synthesis and inhibits protein degradation
Galactosamine	√	-	2,44	amino acids as precursors in the biochemical synthesis of proteins and lipids
Diaminopimelic acid	√	-	2,14	Is one of the main constituents of peptidoglycan in bacteria
Thiamin	√	-	0,92	Regulates intracellular glucose use and inhibits the use of glucose in cell proliferation
Glutahione	√	-	1,16	Acting as a cofactor in several enzymes and antioxidants.
Malic acid	-	√	1,85	Acting as an energy source needed for the metabolism of living things
Xanthine	-	√	1,08	Acting as a conjugate in the degradation of adenosine monophosphate to uric acid.
Myo inositol	-	√	1,32	Acts as a metabolite inhibitor
γ Glutamyl alanine	-	√	0,85	Is an enzyme natural substrate
Ribitol 5 phospate	-	√	1,30	Acting as a precursor in the synthesis of ribitol-teichoic acid from gram-positive bacteria cell walls, whereas acting as a polysaccharide in gram-negative bacteria

The difference in the number of extracellular synthesized compounds is due to the different genetic constructions of the two isolates to deal with the given environmental stress by deactivating several genes and activating other genes to increase the efficiency and effectiveness of the isolates. Resistance by synthesizing secondary metabolites is one of the adaptation mechanisms due to chromosomal, transposon and plasmid differences in each bacterium [24]. According to Yuka *et al*. [25], microorganisms overcome environmental stresses by involving enzymatic mechanisms that are controlled by resistant genes in plasmids, transposons or chromosomes. One of the resistance mechanisms in bacteria against environmental contamination is the presence of RND proteins (Resistance, Nodulation, Cell-Division) that regulate the transport of pollutant compounds through the cell membrane.

The difference in the number and types of synthesized extracellular metabolites also reflects differences in the number and enzymes involved in dealing with plastic stress. According to Sivan [26], the relative amounts of various enzymes produced by microorganisms vary with species and even between strains of the same species. Enzymes are very specific in their action on the substrate, thus different enzymes help in the degradation of various types of plastics. The Laccase enzyme can assist in the oxidation of PE (poly ethylene) and reduce the average molecular weight of PE by 20% and 15%.

Some other strains capable of reducing PE are *Brevibacillus spp.*, *Bacillus spp*., in which proteases are responsible for degradation [26]. Papain and urease are two proteolytic enzymes found to reduce PU medical polyester. Polymers degraded by papain are caused by hydrolysis of urethane and urea bonds which produce free amine and hydroxyl groups. The enzymes responsible for the degradation of various types of plastics describe the substrate that uses plastic as a source of carbon and energy and helps in the integration processes. Microbial enzymes induce the rate of biodegradation of plastic very effectively without causing damage to the environment [10].

3.3. Analysis of Specific Metabolites that Act as Phytohormone

The analysis of extracellular metabolites with GC-MS obtained 5 types of phytohormones in both 5S control and 6S treatment with different synthesis patterns and concentrations (Table 2).

Table 2. Results of analysis of GC-MS metabolites that act as phytohormones.

No.	Metabolites Compound	Chemical formula	Composition (%)		Function
			Control	Treatment	
1	α-Amino-butyric acid	$C_4H_9NO_2$	1.15	1.20	Phytohormone pre cursor
2	3 Indole-acetic acid	$C_{10}H_9NO_2$	1.80	1.85	Phytohormone
3	Kinetin	$C_{10}H_9N_5O$	1.11	1.16	Phytohormone
4	N6 Benzyladenine-9-glucoside	$C_{18}H_{21}N_5O_5$	1.26	1.31	precursor of phytohormone
5	Kinetin glucoside	$C_{16}H_{19}N_5O_7$	1.13	1.19	precursor of phytohormone

Phytohormone is capable of being synthesized by both bacteria is triggered by the addition of tryptophan-phytohormone precursor- in growing media. The use of tryptophan is beneficial in stimulating microbes to meet their energy needs, so that microbes can live and are able to synthesize a number of compounds that act as extracellular phyto-hormones. Rhizobacteria synthesizes Indole Acetic Acid (IAA) groups using different pathways with the known mechanism in auxin synthesis is commonly through the tryptophan pathway [19]. Besides, IAA (auxin) and kinetin hormones induce cell division and proliferation which stimulates plant growth. Phytohormone produced by soil bacteria can affect

plant growth both directly and indirectly by inhibiting pathogenic organisms or as a facilitator in absorbing nutrients from the environment [13].

4. Conclusion

GC-MS analysis results on SP6 isolates as plastic-tolerant bacterial models, have obtained 5 specific secondary metabolites which act as biodegradation of plastic with the composition: Malic acid ($C_4H_6O_5$) 1.85%, Xanthine ($C_5H_4N_4O_2$) 1.08%, Myo inositol ($_6H_{12}O_6$) 1.32%, γ Glutamyl alanine ($C_8H_{14}N2O_5$) 0.85% and Ribitol 5 phosphate ($C_5H_{13}O_8P$) 1.30%. Additionally, the isolate was also able to synthesize several phytohormones with the composition: α Aminobutyric acid ($C_4H_9NO_2$) 1.20%, 3-Indoleacetic acid ($C_{10}H_9NO_2$) 1.85%, Kinetin ($C_{10}H_9N_5O$) 1.19%, Kinetin glucoside ($C_{16}H_{19}N_5O_7$) 1,19 % and Benzyladenine 9 glucoside ($C_{18}H_{21}N_5O_5$) N6 1.31%, which can induce cell division and proliferation so as to stimulate plant growth. Thus the bacteria have a double potential, besides being able to degrade plastic, it also has the potential to be a biological fertilizer. Therefore, these bacterial isolates have the double-potentiality to be used as plastic polymers biodegrading bacteria and bio-fertilizers. The next direction of research needs to be focusing on agronomic test of these isolates for biological fertilizers and on land/water contaminated with plastic waste

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