Biodegradability of microbial synthesized Poly-β-hydroxy-butyrate produced from *Pseudomonas aeruginosa* Dw7 local isolate

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Abstract

Environmental pollution caused by plastics produced from petrochemicals is a source of concern, as it is not biodegradable as well as high poisonous. Studies have therefore been directed to polymers which have biodegradability with characteristics similar to traditional plastics. Poly-β-hydroxy-buty (PHB) is a member of the polyhydroxyalkonates family that is manufactured by many bacterial isolates as a compound within cells and store energy under certain conditions relaying on increasing the carbon source and reducing other nutrients such as nitrogen. The use of biodegradable polymers has been increasing in recent years, specifically in the field of medical applications as these materials not only serve the desired purpose, where it can be disposed of by the body due to their biodegradable nature.

Pseudomonas aeruginosa Dw7 local isolate was identified as PHB producing isolate in a previous study. Different polymer properties were studied. The contribution of A. niger to the microbial degradation of PHB film in soil was studied too. To evaluate the activity of this isolate, the clear zone around the colonies was measured; it was established by recording 7 mm of clear zone around the fungal colonies in 7 days of incubation at 30°C. The results showed 100% of degradation after 40 days indicating the high loss in PHB content in the soil as an open system. Antibacterial activity test was accomplished using disk diffusion method against gram positive and negative bacteria. The results showed that antibacterial compounds of PHB extracted by hypochlorite solution and chloroform are active against selected bacterial isolates which demonstrated inhibition zone ranging between 8-40 mm as a sign of antibacterial efficacy.

Keywords: *Pseudomonas aeruginosa*, Degradation, Poly-β-hydroxy-buty rate, bioplastic.

Introduction

There has been great concern in the development and production of biodegradable plastics in response to many challenges related with plastic waste and its pernicious effects in the environment. These have led to the research and development on sustainable, compatible and biodegradable plastic materials, the bio-plastic.[12]

The most important consequences of using bioplastics are that they can be produced from renewable sources and they are environment friendly. These so-called biodegradable polymers or bioplastics were first isolated as lipid-like intracellular storage granules from *Bacillus megaterium*.[16,22] Many researches have reported that production of PHA is enhanced when bacteria are grown in exceedingly suitable carbon source and limited other nutrients sources such as nitrogen, phosphorus and Sulfur[20]. Several studies have shown the relationship between the effectiveness of certain enzymes, the disintegration of the compound and changes in different bonds[24]. Many bacterial isolate were isolated from various soils, lake water, activated sludge and air capable of growing on PHB, as the sole source of carbon and energy. All bacteria utilized a wide variety of monomeric substrates for growth; most of the strains were restricted to degrade PHB and copolymers of PHA. Fungi play a considerable role in degrading polyesters as they are predominantly involved in the decomposition of organic matter in the soil[11].

Fungi are an important part of degrading microorganisms playing a most active function in the degradation of organic matter and in the element cycling. PHA possess unparalleled chemical and physical properties like strong ultraviolet absorption or antimicrobial activity in the pH range of 7–8 even in the absence of light, therefore PHA is widely used for applications such as optical devices and antimicrobials. Recently, attention has been paid to the process of introducing antimicrobial agents with packaging materials which increases their ability to resist pathogenic bacteria give rise to damage to food products.[1]

Studies have been conducted to find preservatives of degradable PHB in the environment and keep food healthy at the same time.[26] The films prepared from PHB have anti-microbial activity of bacteria causing damage and food corruption, comprising of *Escherichia coli, Salmonella typhimurium, Shigella flexneri* in addition of *Staphylococcus aureus*. The study included the biodegradability of the PHB and antimicrobial activity after extracting by suitable solvents.

Material and Methods

Pseudomona saeruginosa Dw7 was grown in MSM medium at pH 7 supplemented with 3% of cooked corn oil at 30° in Fermentation vessel (bioreactor) with 5-liter volume and speed of agitation were kept at 20 % of air saturation and 500 rounds /minutes. Samples of 25 ml were withdrawn from the liquid culture (fermentation vessel) after 60 h.

Cell dry weight measurement: After incubation, sample was subjected to centrifugation for 15min at 6000rpm. The pellets were washed twice with sterile deionized water and dried for 24h at 60°C. The total bacterial dry weight was determened.[27]

Extraction of Polyhydroxybutyrate: One gram of dry cells powder was mixed with 100 ml of 3% hypochlorite solution for 1 h at 30°C. The digest cell material was then recovered by centrifugation at 6000 rpm for 10 min. After centrifugation three phases appeared, the upper phase represented the hypochlorite solution, the middle phase contained the non PHB cell material and undisrupted cells while the bottom phase was chloroform containing PHB. The bottom phase was carefully removed by separating funnel and the PHB was recovered by precipitation with hexane.[8]

Biodegradability test of PHB

Fungal isolate (*Aspergillus niger*): Fungal isolate for the study of PHB degradation capability was obtained from Department of Biotechnology /Ministry of Science. It was a local isolate from soil contaminated with oil wastes. This fungal isolate was identified as *Aspegillus niger* and selected for its high polyaromatic hydrocarbons degrading activity and was maintained on PDA slants.

Degradation in solid medium: The polymer degrading ability of the fungal isolate *(Aspergillus niger)* was determined by different techniques. Fungal isolate was tested for PHB degradation in Basal medium and supplemented with 0.01% peptone and 0.01% yeast extract and 0.2% of PHB.[15] The plates were inoculated and incubated at room temperature (30°C ± 2°C) for 7-12 days. Degradation of the polymer by fungal isolate was detected by the formation of a clear zone surrounding the growth and the extent of degradation was measured from the width of the clear zone formed.

Degradation in liquid medium: The degradation of the polymer in liquid medium was determined by growing the fungal isolate in the assay medium as in BSM above without agar and supplemented with 0.05 % w/v of the polymer.[17]

After inoculation, the culture was incubated at 30°C with 120 rpm/min, in rotatory shaker for 14 days. The residual of the polymer after incubation was determined according to the method defined by Law and Slepecky[14] after extraction with chloroform and evaporation to be dry.

Calculation of residual PHB was done as below:

% of residual PHB = PHB weight g/l after incubation periods / initial weight of PHB*100.

The amount of the dry biomass was determined to calculate the growth of the isolate as below:

A volume of 100 ml from culture media was centrifuged with speed 10000 rpm at 4^0C for 20 minutes in cooling centrifuge, supernatant discarded and the biomass was washed 2-3 times with distilled water, collected and then re-centrifuge under the same conditions. The cells were placed in sterile weighted plates and the dry weight of the cells was estimated after drying at 80˚C for 24 hours.[3]

Degradation of PHB film in soil

Preparation of a biopolymer film: Totally 500 mg of PHB and 4.25 ml of glycerol as plasticizer and 0.25 gm saccharin for to create the desired bond between molecules was dissolved in 50 ml chloroform. The mixture was evenly distributed into petri dish. The dish was maintained at 30ºC to allow complete evaporation of chloroform to form PHB film in the petri dish. Vacuum drying was further applied to completely remove any possible solvent remained in the film.[9]

Degradation experiment: Two disinfected and pre-weighted 5 gm film sheet of PHB (1cm x 3 cm x 0.05 mm) were buried in 25 g of freshly collected soil in petri dish (90 mm diameter). Disinfection was done by washing in 70% ethanol for 1 h and rinsing twice with sterile distilled water. The dishes were kept open and incubated at room temperature up to 60 days after inoculated with pure isolate of *A. niger* as inoculum.

Inoculum of *A niger* was prepared by growing the fungal isolate in 8 ml of BSM containing 0.1 g/l yeast for 72 h. The soil was moistened with 1 ml of the culture solution. The film sheets were removed aseptically and washed in sterile 0.25% (w/v) Tween 20 solution. The film sheets were dried to a constant weight by placing in an oven for 1 h at 80°C.[18] The petri dish was incubated at room temperature (open system) and sterilized distilled water content added to the dishes. The degradation of the film was conventionally evaluated in terms of weight loss. The degradation rate % was determined as below:

Degradation rate % (Loosing PHB) =
(Weight of initial PHB film - Weight of final PHB film/ Weight of initial PHB film *100

Results and Discussion

Degradation of PHB in solid medium: A reliable way to isolate true PHB degraders is to use the clear-zone technique[18] which involves inoculation of the isolates onto mineral agar that contains PHB as sole carbon source. Clear zones are formed around colonies of microorganisms with

PHB depolymerase activity on the surface of the agar medium, as a result of polymer degradation. Degradation ability of A. *niger* to PHB was visualized by the presence of a clear zone around the colonies on solid medium plate.

Relatively high rate of degradation was determined by recording 7 mm cm of clear zone around the fungal colonies in 7 days of incubation at 30°C.

Clear zone obtained after growth on assay agar by fungal isolates was shown in fig. 1. In comparison with growth of *A. niger* on same medium without addition of PHB under same conditions, results showed that the growth of fungi was weaker and without clear zone around the colonies. The fungal isolates showed a high capability of utilizing PHB as growth substrate and produced a clear-zone on the assay. The extracellular hydrolyzing enzymes secreted by fungus hydrolyze the polyesters in the agar medium thereby producing zones of clearance around the colony.[19]

Degradation of PHB in liquid medium: Degradation of PHB was recorded after growing *A. niger* in liquid minimal media with the PHB as a sole carbon source. The dry biomass of *A. niger* was attained by these isolates through different incubation time per day. Growth was measured by drying the fungi. The residual PHB left over in the medium after growth was measured to calculate the residual of PHB. Fig. 2 showed dry biomass and residual PHB after growth in assay medium.

It was established that *A. niger* isolate grew well in the assay media showing good PHB degradation capabilities and offered the best polymer degradation capabilities after 12 days, since it produces highest biomass 2.03 g/l leaving less residual PHB in the medium recorded to be 16%.

Degradation of biodegradable PHB is necessary in order to formulate guidelines regarding their appropriate use. *A. niger* presented good results in degradation and reduction to 20 % of the produced PHB in liquid culture, probably due for production at least two different enzymes, which are depolymerase and either lipase or cutinase.[15] A number of mesophilic fungi belonging to the genus *Aspergillus* have been found to be responsible for degrading PHAs in soil and aquatic environments.[12]

PHB degradation in soil (natural environment): Prepared PHB blend sheet film was applied in petri dishes containing of 25 g of soil and inoculated with *A. niger* then incubated at room temperature. The test piece of PHB blend sheet lost weight during incubation period was used to determine the ability of *A. niger* to degrade PHB in soil as open system.

The degree of weight loss increased through incubation in environment in which the polymer sheet was incubated. The rate of degradation of the PHB films was 25.6% after 7 days of incubation and 50.4% of degradation was noticed within 14 days.

The PHB films were found to be degraded into fine pieces in 21 days resulting into 80.5% loss in weight of PHB film which was indicating the importance of this fungal isolate in PHB degradation (figure 3). Kulkarni et al[13] observed that the rate of degradation of the PHB film was 12% and 23.6% within 8th week respectively in soil containing 15% and 20% moisture while in the soil having 25% and 30% moisture. PHB films were found to be degraded into fine pieces in 6 weeks resulting into 95% loss in weight of PHB film.[13]

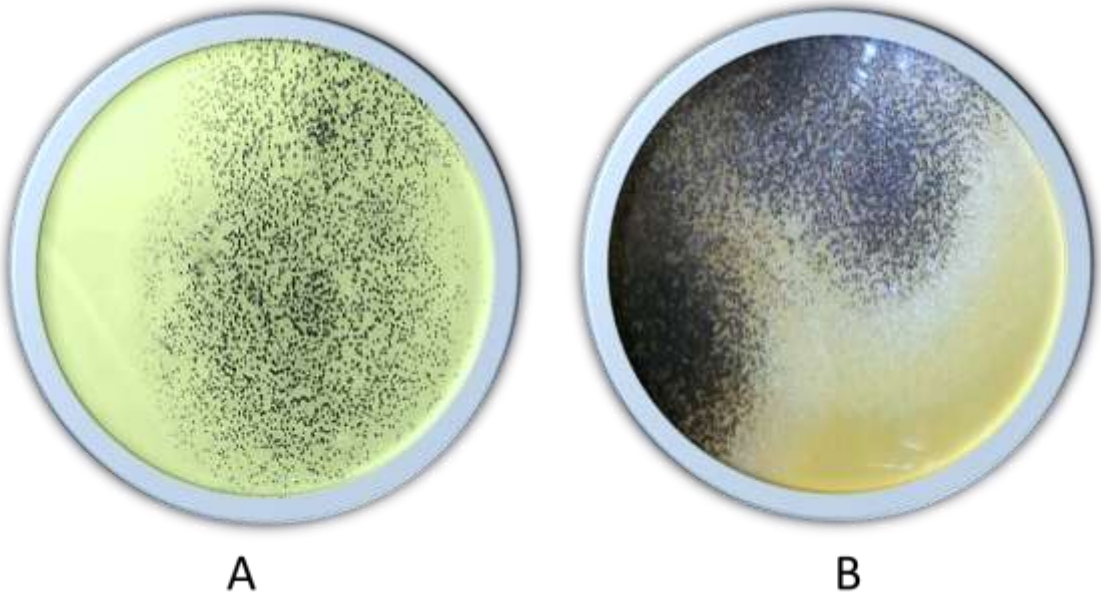

Figure 1: PHB degradation assay by clear zone: (A): growing *of A. niger* on medium without PHB (negative control), B: growing of *A. niger* on PHB with clear zone detecting degradation of PHB.

However, the results in fig. 3 showed that 100% of degradation was observed after 60 days indicating the high bioactive decomposition of PHB blend sheet film content in the soil as open system. Similar observation was noted by Wang et al[24] which confirms biodegradable nature of PHB. Visual observation in fig. 4 showed differences in the appearance of sheet through incubation periods as a result of breakdown pattern and evidence of PHB exposed to microbial attack in soil conditions.

PHB films were found to be degraded into fine pieces in 40 days resulting into 80.2% and 100% loss in weight of PHB films.[24] Food packaging products made of standard PHB have been shown to undergo effective biodegradation in garden soil for periods of up to 60 days and thus can act as environmental-friendly substitute for petroleum-based plastic.[4]

Antibacterial activity of PHB: The antibacterial activity of different extraction fractions of the PHB was tested by the Disk Diffusion method against four pathogenic bacteria. Hypochlorite solution and chloroform extract found to be the most active agent against pathogenic which recorded highest inhibition zone around the four sensitive bacterial strains ranging from 6 to 40 mm inhibition zone as in fig. 5 and table 1.

Antibacterial activity of PHB was assayed by Disk Diffusion method against two gram positive (*Bacillus cereus*, *Staphylococcus aureus*) with inhibition zone of 40 and 38 mm respectively and two-gram negative bacterial strains (*Eschericheia coli*, *P. aeruginosa*) with inhibition zone of 8 and 37 mm respectively. Study of the antimicrobial activity showed that PHB produced by bacterial isolates is high promising antimicrobial materials.[4,21]

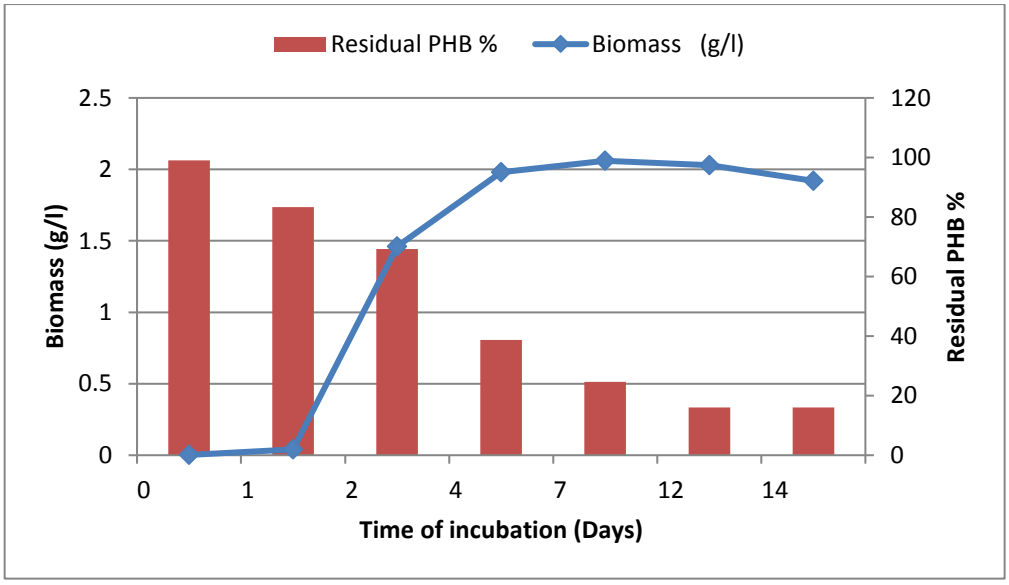

Figure 2: Degradation of PHB assay in liquid medium after 14 days of incubation with *A niger*

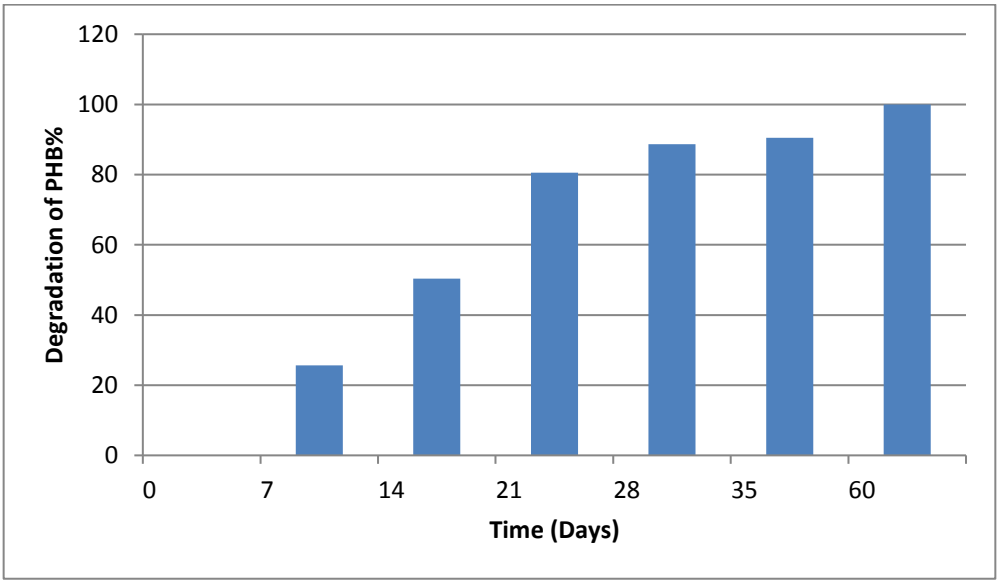

Figure 3: Lose of PHB weight after exposing PHB film to *A. niger*

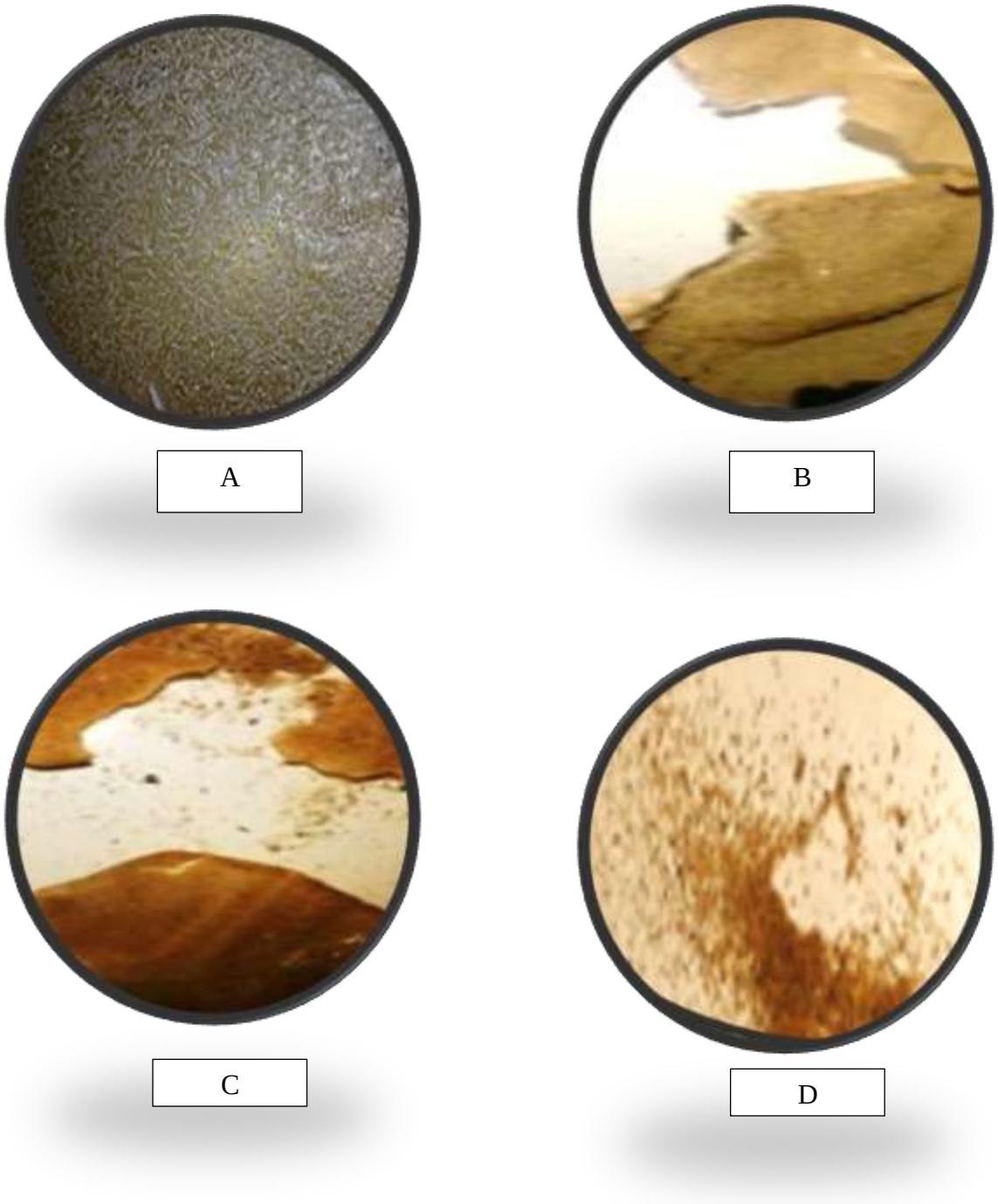

Figure 4: PHB film degradation after different periods (days) of exposure to *A. niger* in soil as open system
A: at zero time, B: after 7 days, C: after 14 days and D: after 40 days

Viegelmann et al[23] observed a polyhydroxylated fatty acid which belonging to glykenin antibiotics to have antibacterial activity against methicillin-resistant *S. aureus*.

The antibacterial compounds were identified as the monounsaturated fatty acid hexadecenoic acid (palmitoleic acid; C16:1 n-7) and the relatively unusual polyunsaturated fatty acid such as hexadecatrienoic acid (C16:3 n-4). Both are active against gram-positive bacteria further inhibitory to the growth of the gram-negative marine pathogen. These free fatty acids warrant further investigation as a new potential therapy for drug-resistant infections because they are active at micro-molar concentrations, highly active against multidrug-resistant *S. aureus.*[6] Compounds with a chain-length of 11 to 15 are most active though some of the general properties relating the activity of fatty acids to their antimicrobial action are similar to those of amine compounds.[5]

Membranes of poly (3-hydroxybutyric acid) (PHB) and poly (3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (PHBV) membranes showed antibacterial activity against *E.*

coli, *P.aeruginosa*, methicilin-resstance S. aureus (MRSA) and S. *aureus*.[2] Similar results were observed by Wellington et al[25] who found that polyhydroxylated saturated fatty acids (C20H40O6) produced by *Streptomyces* strain SM8 exhibited higher antimicrobial activity against *B. subtilis*. Also, a mixture of four hydroxyl fatty acids; 7-hydroxy-tetradecanoic acid, 7-hydroxy-pentadecanoic acid, 9-hydroxyhexadecanoic acid and 9-hydroxy-heptadecanoic acid produced by *Streptomyces* sp. TN272 showed antifungal activities against the two tested fungi, *Fusarium* sp. and *Candida tropicalis*.[7]

PHA possess unique chemical and physical properties like intensive ultraviolet absorption or antimicrobial activity in the pH range of 7–8 even in the absence of light, therefore widely used for applications such as optical devices and aantimicrobials.[1] The incorporation of antimicrobial compounds into food packaging materials has recently received considerable attention. Composites with antimicrobial activity restrict the growth of pathogenic and spoilage microorganisms. The product introduced an obvious antibacterial activity against gram negative and gram-positive sensitive strains.

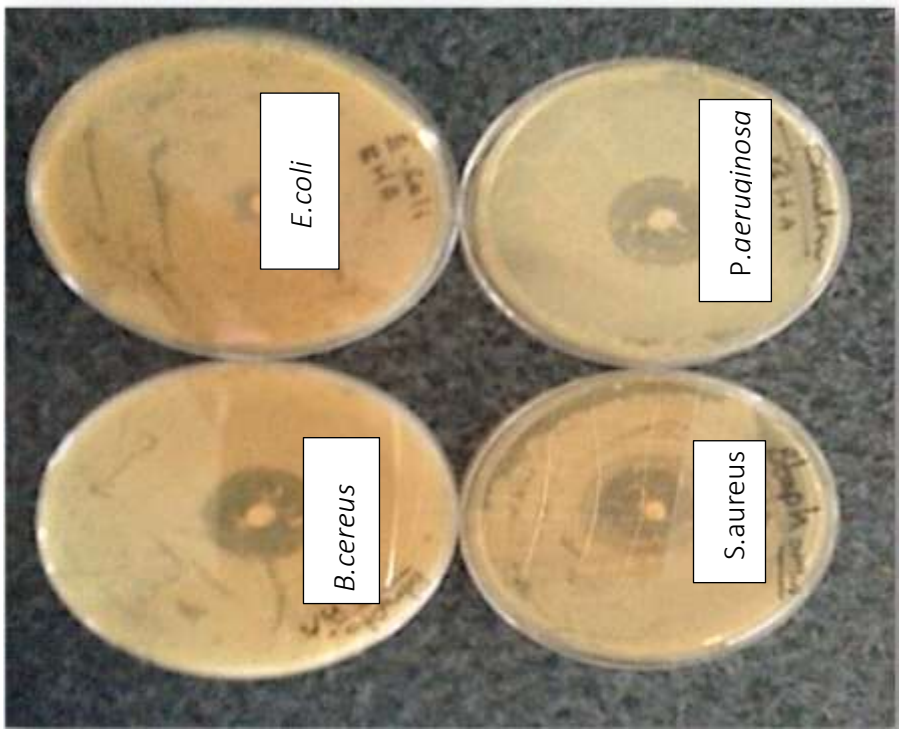

Figure 5: Antibacterial activity of PHB extract from *P. aeruginosa* DW7

Table 1
Antibacterial activity of different extraction fractions of PHB produced by *P. aeruginosa* Dw7

Solvent system	Bacterial isolates	*Staphylococcus aureus*	*Bacillus cereus*	*Escherichia coli*	*Pseudomonas aeruginos*
Acetone and chloroform	Inhibition zone(mm)	20	22	6	22
Hypochlorite solution and chloroform		38	40	8	37
Chloroform		20	24	0	25
Sodium dodecyl sulfate		22	24	0	23

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