Bio plastic production from Bacillus strains isolated from Golestan province's landfill soil

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Background: Annual Global production of plastic waste is in millions of tons, which are durable and take hundreds of years to degrade. Bioplastics are a class of plastics from vegetable oil, starch, cellulose, or bacterial derivatives which distinguishes them from other plastics. These plastics can replace all other plastic productions.
Methods: 70 samples were collected from waste recycling locations, culture and strain purification was done on Nutrient agar and indigo blue dye was used to identify PHB producing strains. The microbes were further incubated on Minimal Davis media for two days at 37. The samples were placed on a slide and colored, isolates were selected based on color intensity and used for bioplastic production.
Results: the results demonstrated that the Bacillus bacteria produces a considerable amount of poly-3-hydroxy butyrate in both soil and sewage which is useful for the production of bioplastics in the laboratory and industrial scales.
Conclusion: According to the results, bioplastic production from the bacterial isolate's Bacillus strains is possible.
Keywords: Bioplastic; polyhydroxybutyrate; polyhydroxyalkanoate; bacterial isolate

INTRODUCTION

Modern times provide certain benefits and at times cause irreparable damage to mankind, e.g., Plastic bags and disposable containers. According to prior research Iran solely produces more than three million tons of plastic annually. Most of these plastics are durable and take hundreds of years to degrade on their own. Today plastics act as an important synthetic Material in different industry sectors. The wide application and their increasing production cause major environmental problems which have caused the United Nations Environment Program to declare them one of the significant major pollutants. The term biodegradable refers to substances that are simply degraded by living organisms to their original constituents and therefore do not remain in nature (1). Bioplastics are a group of plastics that are with masses originating completely or partially from vegetable oil, starch, cellulose, or bacterial derivatives and differ from other plastics which originate from hydrocarbon fossil fuels like oil and gas. In the last decade scientists and researchers have proposed biodegradable substitute materials, e.g.: 1)Aliphatic polyesters: Polyhydroxyalkanoates (PHAS like: poly-3-hydroxybutyrate (PHB), Polyhydroxyvalerate, Polyhydroxyhexanoate (PHH)); Polylactic acid (PLA); Polybutylene succinate (PBS), polycaprolactone (PLC) 2)Polyanhydride 3)Polyvinyl alcohol 4)Cellulose esters 5)Starch derivatives Use of oxidizing additives in olefinic plastics .Most bacteria like Bacillus, Pseudomonas, Cupriavidus spp., Aeromonas spp., Have been evaluated for PHB production in industrial scales (2, 3, 4, 5). Multiple bacterial species like Actinobacillus, Azotobacter, Agrobacterium, Rhodobacter, and Sphaerotilius are under evaluation for their ability to produce PHB from organic waste. For an industrial scale of PHB production bacteria like Cupriavidus spp., Aeromonas spp., Pseudomonas spp., and Bacillus spp. Have been widely used (2, 3, 4, 5, 7). One of the hypothesized issues for using starch as a feedstock for bioplastic is its competition with food sources. It is hypothesized that this will cause the food source to further divide which will be detrimental for poor regions of the world (6). Production and use of plastic are growing globally (8). Plastic containers and packaging are usually disposable after a single use and not only do they cause problems by entering the oceans (9-13). They further occupy landfills, exacerbate CO2 production after waste burning which consumes fossil fuels and affects the environment and animal lifecycles. The cost of big-scale bioplastic production is high, estimated at 1.3-4 euros per kilogram. Since bioplastic production in industrial scales is not economically feasible this practice will not be widespread. Bioplastics will inevitably reduce CO2 emissions compared to traditional plastics. Therefore, biodegradable plastics are environmentally friendly and can replace the entire plastic industry in the future.

METHODS

Sampling and isolation of bacteria from organic wastes

40 soil samples and 30 sewage samples were acquired from landfills in Gorgan city. After sample collection they were moved to the laboratory. Samples were diluted and spread on solid Nutrient agar media and subsequently incubated at 37 for 24 hours. After sufficient growth was achieved using a loop, bacteria were picked and streaked onto the Nutrient agar and incubated at 37 for 24 hours to acquire a single colony and colonies like Bacillus were chosen.

PHB production evaluation of isolates

A total of 70 samples, comprised of 40 samples from soil and 30 sewage bacteria were cultured on Nutrient agar and assessed for PHB production capacity. For identification of soil and sewage isolated bacteria and to characterize their PHB production capacity indigo dye was used. Bacterial isolates were maintained for 2-3 days at 37on the Minimal Davis media (Merck, Germany). A loop of bacteria was picked and placed on sterile slides, heat dried, and colored with indigo dye. The dye remained on the samples for 20 minutes at room temperature and then slides were washed. Afterward, the slides were dried for a few minutes and observed under a fluorescent microscope under a 490 nm wavelength emission. Plastic-producing strains had a yellow/orange color and were selected for bioplastic production aged on color intensity.

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Identification of PHB producing isolates

Multiple tests were done for this purpose like the Gram coloring test (to determine whether the bacteria are Gram-positive or negative), electron microscope imaging (to determine morphology), and various biochemical tests (motility, citrate use, gelatin hydrolysis, catalase test, starch hydrolysis to determine their sources for growth).

Bio plastic extraction

Selected isolated were grown for 3 days at 37 and agitation rpm of 150 on a rotary shaker incubator. Afterward, bioplastic extraction was done using the sodium hypochlorite chloroform method. A volume of 5 ml from the culture was centrifuged at 10000 g for 10 minutes and the supernatant was discarded. The precipitated material was solved in 2.5 ml of 4% sodium hypochlorite and 2.5 ml of hot chloroform and incubated at 37 for 1 hour to degrade it. This suspension was centrifuged at 1500 g for 10 minutes. The lower plastic containing phase was retrieved with chloroform and hot chloroform extraction continued and precipitated with ethanol and acetone (1:1). This precipitate is dried at 30 to acquire colorless plastic crystals.

RESULTS

Identification of bacterial isolates from soil and sewage

In total 11 isolates (4 soil isolates and 7 sewage isolates) were selected from 70 samples (40 soil samples and 30 sewage samples) were isolated and cultured on Nutrient agar media and various biochemical tests were done to identify the PHB producing bacteria from soil and sewage (Table 1 and 2).

Result	Gelatin hydrolysis	Starch hydrolysis	Citrate	Motility	Catalase	Gram Stain	Cell Shape	Number of Isolates
Bacillus sp	+	+	+	+	+	+	Rod	S1
Bacillus sp	+	+	+	+	+	+	Rod	S2
Bacillus sp	+	+	+	+	+	+	Rod	S3
Bacillus sp	+	+	+	+	+	+	Rod	S4

Table 1: Biochemical tests for identifying different soil isolates

Result	Gelatin hydrolysis	Starch hydrolysis	Citrate	Motility	Ctalase	Gram Stain	Cell Shape	Number of isolates
Bacillus sp.	+	+	+	+	+	+	Rod	W1
Bacillus sp.	+	+	+	+	+	+	Rod	W2
Bacillus sp.	+	+	+	+	+	+	Rod	W3
Bacillus sp.	+	+	+	+	+	+	Rod	W4
Bacillus sp.	+	+	+	+	+	+	Rod	W5
Bacillus sp.	+	+	+	+	+	+	Rod	W6
Bacillus sp.	+	+	+	+	+	+	Rod	W7

Table 2: Biochemical tests for identifying different sewage isolates

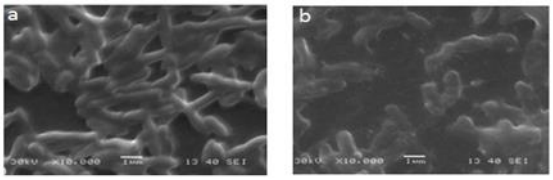

Figure 1: Electron microscopy pictures from sewage isolates that produce PHB (figure a, isolate W2), soil (figure b, isolate S3)

Based on the results of various biochemical tests and images obtained from electron microscopy of isolates isolated from wastewater and soil, Bacillus was identified.

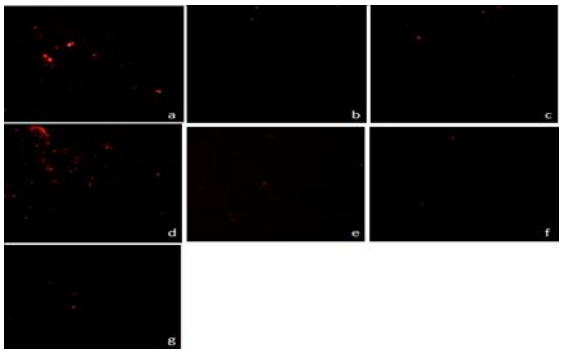

Figure 2: Fluorescence microscopy images of PHB-producing soil isolates

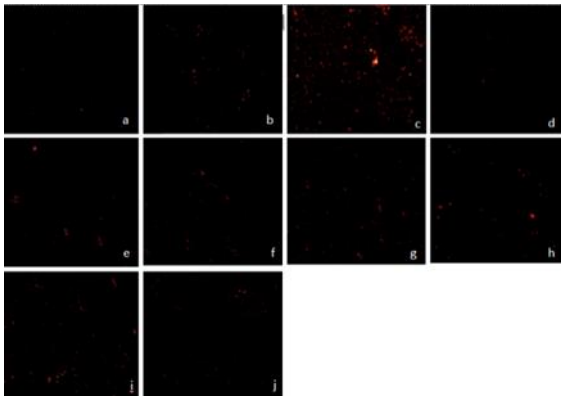

Figure 3: Bacterial extracted PHB crystals from soil origin (a) and sewage (b).

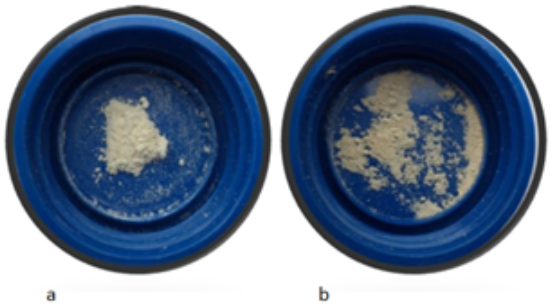

Figure4

PHB production quantity of isolated bacteria

The soil and sewage isolated bacteria were assessed for PHB production using indigo dye coloring, and under fluorescent microscopy, the PHB-producing bacteria appeared bright orange. Isolates W2-W5 in their respective order produced the highest amount of PHB according to

fluorescent microscopy (figure 2). Most of the isolates acquired from soil had PHB production capacity.

DISCUSSION

By comparing the PHB production capacity of the isolates from soil and sewage it becomes apparent that the sewage originating bacteria are superior for PHB production.

Saito and Doi isolated Comamonas acidovorans DS-17 from activated sludge and found that this bacterium produces copolymers of 3-hydroxybutyrate and 4-hydroxybutyrate in 30 and restrictive growth conditions which corroborates our findings. In the studies of Hewery, Hekmet, Rohini, Mohapatara it is reported that many Bacillus strains can produce PHB in high amounts inside their cytoplasm under nutrient starvation up to 97-96% of their dry mass. According to the results of this study, Bacillus strains had the highest potential which is affirmed in our study. Rohini et al. (2006) reported the production and identification of PHB from Bacillus thuringiensis and reported the highest amount of dry mass (3.9-4.1 g/l) in the presence of 1% glycerol and the lowest (1.28 g/l) in the presence of 1% lactic acid. Additionally, they demonstrated that the highest amount of PHB was produced in the presence of glycerol and ethanol and acetic acid did not elicit production of PHB. In the current study, the sodium hypochlorite chloroform extraction was used which does not match their findings.

Nayer, Sankharek, and Prasertesan showed that many effective elements on the production of PHB by environmental isolates exist such as Nutrients such as carbon, nitrogen, amino acids, and metallic ions. Carbon is the most restrictive nutrient amongst others for PHB production and therefore it is important for enhancing PHB production, this disagrees with our findings.

Behagovati reported production, identification, and improvement to PHB, Bacillus originating bioplastic and reported Bacillus cereus to be the most potent strain for PHB production with 22.1% and 40% after 48 and 72 hours, after this the sea isolated bacteria have 5% and 33% production after48 and 72 hours. Additionally, this study stated maltose as the most efficient carbon source for PHB production.

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