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ISOLATION AND CHARACTERIZATION OF *PSEUDOMONAS PUTIDA* PRODUCING BIOPLASTIC (POLYHYDROXY ALKANOATE) FROM VEGETABLE OIL WASTES

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ABSTRACT

The current study aimed isolation and characterization of local bacterial isolates capable of production Poly hydroxy alkanoate (PHA) using oil residues as a single carbon source. Many polluted environmental sites were used as sources of isolation of bacterial isolates.

The collected samples were processed by serial dilution followed by spread plating in nutrient agar plates to get isolated colonies. Bacterial isolates were tested for (PHA) granules production using Sudan blank and Nile blue stains, nine of fifty isolates could produce PHA. Production of the polymer of bacterial isolates was found to be ranging from 21% - 57 % (w/w) of the biomass. The highest PHA yield was observed in *Pseudomonas putida* SD12, which was diagnosed according to diagnostic methods, i.e., colony morphology, gram staining, and then was confirmed by new gram-negative identification card (Vitek 2 NGNC). PHA accumulating was 2.80g/L, amounting to 57.20% (w/w) of cell dry weight. Waste frying oil was the best source of carbon which gave better results for PHA production than pure oil. PHA production was 2715 ppm with comparison of 1223 mg /3mlwhen used pure oil as a carbon source when incubated at 30°C after 48 hours.

Key words: *Pseudomonas putida*, poly hydroxy alkanoate, bioplastic, waste oil

INTRODUCTION

Synthetic plastic is one of the human inventions developed into many different industries and commodities in human's life [Sudesh and Iwata 2008]. They are designed to be of high performance and stability to make it resistant to chemical and natural conditions.

it has been a form of large plastics as industrial polymers since 1940, as it has been used as an alternative to glass, wood, other structural materials and even metals in many industrial, [Poirier *et al*, 1995, Lee, 1996). Bio-based materials such as polynucleotides, polyoxoesters, polyamides, polysaccharides, polythioesters, polyanhydrides and polyisoprenoids potential candidates for substitution of synthetic [Steinbuchel, 2001]. An important family of biomaterials is which are usually built from hydroxy-acyl–CoA derivatives through different metabolic pathways.

The term polymers represent compounds produced by microorganisms under different environmental conditions and are chemically non-related [Poirier *et al*,1995, Degeest *et al*,2001; Sudesh and Iwata 2008]. Bio-plastics vary in their basic structure, structure of molecules and physical properties depending on their microbial origin, and most of them are characterized as biodegradable and biocompatible, making it extremely important in terms of biotechnology.

Bio plastic is a special type of biomaterial. They are polystyres, produced by a number of microorganisms that develop under certain environmental conditions and under different nutrients. [Madison and Huisman, 1999; Akiyama *et al*., 2003]. Polyhydroxy alkanoate (PHA) is one of these biomaterials, which belongs to the group of polyoxoesters has received intensive attention because it possesses biodegradable thermoplastic properties [Madison and Huisman, 1999]. Polyhydroxy alkanoates (PHAs) are bacterial products are characterized by natural, renewable and biologically active polymers. Plastic materials with similar characteristics to plastics and petrochemicals can be substituted for these materials in many applications. (Philip *et al*. 2007). Poly-p-hydroxybutyrate (PHB) is a polymeric ester which functions as an energy and carbon reserve in prokaryotic cells. PHB exists as discrete inclusions granules in the cell (Robards. 1973). They can be exploited by many species (biodegradable) and do not cause toxic effects in the host, giving them a significant advantage with respect to other conventional industrial products [Steinbuchel and Fuchstenbusch, 1998; Zinn and Egli, 2001].

Due to their thermal properties and biodegradra-Bility, these materials have gained industrial importance and have been studied extremely over the past two decades. PHAs are produced by bacteria under unbalanced growth conditions and the nutrient depletion necessary for energy and growth [Lee,1996; Albuquerque et al., 2007].

The number and size of the granule, the composition of the monomer, the molecular structure, and the physical and chemical properties vary according to the producer organism [Murray *et al*., 1994; Ha. and Cho, 2002]. Focusing on the importance of supporting research in the production of biological products will enrich the wealth of all mankind. This study examined the production of polyhydroxy alkanoate by local bacterial isolate isolated from soil samples; their diversity in soil makes them a suitable area for exploit as potential producers of PHA. In this work we studied PHA recovery from *Pseudomonas putida* Sd12 which possesses tendency to utilize corn oil and it has been reported previously. That waste frying oil are desired feed stocks for PHA production because they are also inexpensive in comparison with pure oil and other carbon sources, such as sugar. In this paper we also detected PHA production using private stains.

MATERIALS AND METHODS

Samples collection: Soil sample, sewage sludge and detergent waste were collected from different sites in Baghdad Isolation and purification of bacteria. Sequence of series of selected environental models was carried out and plated on nutrient agar medium plates. Plates are incubated at 30° C for 48 hours. Isolated colonies were purified to obtain a single colony and maintained a slant droplet at 4 ° C.

PHA producers screening: Sudan Black B dye was used for qualitatively test for PHA production as rapid screening of PHA producers. Nutrient agar medium supplemented with 1% glucose was autoclaved and poured into petri plates. After solidification, plates were inoculated with the bacterial isolates. The plates were incubated at 30°C for 24-48 hrs. To remove the excess stain from the colonies they were washed with 98% ethanol. The dark blue colored colonies represented positive for PHA production.

Detection of PHB with Nile blue: Positive isolates for Sudan black B were further screened by Nile blue A, a more specific stain, which is considered a more rapid and sensitive, Carbon-rich nutrient agar medium supplemen-ted with 0.5 µg/mL Nile blue A and inoculated with bacterial isolates and growth of the cells occurred in the presence of the dye. After Nile blue a staining, bright orange fluorescence on irradiation with UV light was shown from PHA accumulating colonies, and their fluorescence intensity increased with the increase in PHA content of the bacterial cells. The isolates which showed bright orange fluorescence were selected as PHA accumulators.

Production media: For PHB production, the bacterium was grown in basal medium modified by Gamal et al., (2012) called productive medium containing (g/L): $(NH_4)_2SO_4$ 1.0, KH_2PO_4 1.5, $Na_2HPO_4.12H_2O$ 9.0, $MgSO_4.7H_2O$ 0.2, 1 mL of trace elements solution ($FeSO_4.7H_2O$ 10, $ZnSO_4.7H_2O$ 2.25, $CuSO_4.5H_2O$ 1.0, $MnSO_4.4H_2O$ 0.5, $CaCl_2.2H_2O$ 2.0, $Na_2B_4O_7.10H_2O$ 0.23, 1.0 ml of waste frying oil as the sole carbon source. 100 ml media was prepared, autoclaved and used for PHB production by the organisms. The pH was adjusted to 7.0 before sterilization. Experimental conditions: Batch experiments were performed in shake flasks for production of the biopolymer was quantified in shake flasks as batch experiments. Flasks were inoculated with the bacterial isolates and incubated for 48 h at 30°C, 120 rpm. Then the PHB produced was extracted using chloroform method and quantified using crotonic acid standard graph. Extraction and quantitative analysis of PHA: Sample was taken from the production media. Centrifuged at 10,000 rpm at 4˚C for 20min. The supernatant was discarded and 0.1ml of acetone was added to the pellet and keep till it dries. After the acetone is dried 1:1 of 500µl of NaClO and chloroform was added (NaClO cleaves the cell wall and chloroform dissolves PHA). Mixture was mixed thoroughly and kept for about 30min. Then centrifuged at 10,000 rpm for 30min at 4˚C for 20min. Three layers were formed: top NaClO, middle cell debris and bottom chloroform with PHA. The chloroform layer was pipetted into glass bottles. Kept out till the chloroform dries. Then 5ml of sulfuric acid was added into the bottles and kept in hot air oven at 100˚C for 1hr which converts it to Crotonic acid. The absorbance was taken at 235 nm using sulfuric acid as blank in UV Spectrophotometer [John and Ralph, 2001].

Quantification of PHA by Spectrophotometry: Crotonic acid powder was dissolved into sulfuric acid and standard solution of 1mg of Crotonic acid/ 10ml of sulfuric acid was prepared. Working STD of 100, 200, 300… 1000µg/3ml of sulfuric acid was prepared. Blank was prepared by adding 3 ml of sulfuric acid. The absorbance reading took at 235nm. Standard graph of concentration v/s absorbance was prepared [Law and Slepecky, 1999].

RESULTS AND DISCUSSION

A wide variety of bacteria are known as PHA granules accumulators intracellular as an energy reserve material. Over 90 genera of microorganisms have been recorded to accumulate about 150 polyhydroxy alkanoate polyesters granules [Du, 2001; Steinbüchel, 2001]. In the present study, we isolated 50 bacteria from soil, sewage sludge and oil waste. Nine of these isolates were positive for PHA production using Sudan black and Nile blue staining in the plate). After cell staining using Sudan black and safranin, PHA granules were detected inside the cells as black color against red back ground (Figure 1). The isolates which are stained dark, those microbes were selected and screened for producing PHA; they were cultivated in the media containing WFO as standard carbon source. We have noticed that nine bacterial isolates were able to produce major amounts of PHA during growth using the production media mentioned above containing a single carbon source which was corn oil.

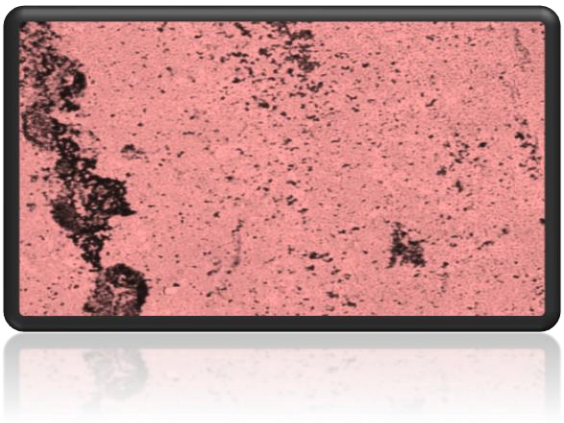

Figure 1: Sudan black B stain of PHA granules (black section) of. *Bacterial isolates* observed under contrast phase

PHA granules exhibited a strong orange fluorescence when stained with Nile blue A. When it is combined with extraction procedures, staining with Nile blue A is a satisfactory test for the presumptive identification of PHA granules in bacterial cells.

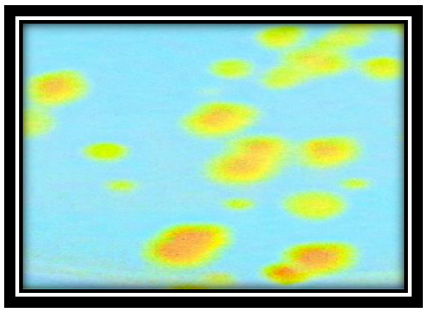

Figure 2: Positive PHA producer bacterial isolates showing fluorescence orange Nile blue on agar plate under UV light

Of all environmental sources, 9 isolates were found to be the most efficient in producing the material after staining it with specialized dyes; it is as follows (Sd4, Sd9, Sd12, Em3, Em6, Em9, Bb2, Bb4 and Bb7). Compared with other isolates, Sd12 was superior, dry cell weight was 4.9g\l whereas PHA reached 2.80g\l yielding 57% of PHA, as shown in Table 1.

Table 1: production of PHA by selected bacterial isolate

Parameter / Isolates	Biomass g/l	PHA g/l	PHA content %
Em3	2.62	0.90	35
Em6	2.33	0.83	35
Em9	2.24	0.82	36
Sd4	2.65	0.51	27
Sd9	2.05	0.42	20
Sd12	4.90	2.80	57
Bb7	2.52	0.32	21
Bb2	2.34	0.90	39
Bb4	3.32	1.00	30

Chemical analysis should still be performed to confirm the presence of PHA. The ability of bacterial isolates to produce the material is determined by polymer extraction, degradation to crotonic acid, and Check for decomposition products by UV spectra. The results showed that isolation 12 was more efficient in polymer and biomass production using oil residues compared to unused oil. The results showed that the use of oily residues had the effect of increasing the value of biomass and bacterium product compared to the use of unused oil. The biomass of bacterial isolate (Sd-

12) and after 48 hours of growth at 30 ° C was 5.7 g/l using oily residues and the value of the product was 2715 mg/3 ml While the biomass of isolation after 48 and under the same conditions was 4.8 g /l using unused oil while the value of the product was 1223 mg/3.

Table 2: Effect of oil source on PHA production by SD12

Parameter / Time\h	Biomass g\l		PHA mg/3ml	
	Unused vegetable oil	Waste frying oil	Unused vegetable oil	Waste frying oil
0	0.2	0.2	0.00	0.00
24	1.7	2.8	720	1270
48	4.8	5.7	1223	2715
72	2.3	3.6	1000	2120
96	1.2	2.5	630	1100
120	0.8	2.0	320	860
144	0.6	1.6	130	430

Accordingly, PHA accumulation after 48 hours of incubation in bacterial cells was the best Table 2. The synthesis of PHA was observed from the log phase of growth and it keep up late exponential stage where the source of carbon was consumed both for growth and for production of PHA.

Depending on morphological, biochemical tests and Vitek 2 NGNC the highest production isolate SD12 was characterized as *Pseudomonas putida*. Maximum PHA production was determined using 1% of WFO after 48 hours of growth at 30°C comparison with pure oil as carbon source.

Conclusion

The aim of this study was to screen and select suitable bacterial isolates for PHA production. Among the 9 isolated bacteria, isolate SD12 was found to be more efficient for PHA production in the media containing waste frying corn oil as the carbon source with PHA content of 57%.

Although pure corn oil proved to be good carbon source for the growth of the bacteria, but this source of carbon was less efficient in making use of it for PHA production.

Non-solvent-based extraction techniques for extraction of PHA needs to be developed, as it will help to significantly reduce PHA production cost.

Also the cultural parameters have to be optimized with other stress conditions so that the biomass and in turn PHA yield can be increased.

The focus of future research would be to reduce the cost of production as well as improve the quality of the polymers to make it suitable for high cost products.

REFERENCES

Albuquerque, M., M. Eiroa, C. Torres, B.R. Nunes and M.A.M. Reis, Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. *Journal of Biotechnology* 130: 411-421 (2007).

Baumann, P., L. Baumann, M. Mandel, and R. Allen. Taxonomy of aerobic marine eubacteria. *J. Bacteriol*. 110: 402–429 (1972).

Collee, J.G., Duguid, J.P., Fraser, A.G. and P. Marmion, Mackie & McCartney practical medical microbiology. English: *14th ed !996)*.

Degeest, B., F. Vaningelgem and L. Vuyst, Microbial physiology, fermentation kinetics process engineering of heteropolysaccharide production by lactic acid bacteria. *International Dairy Journal* 11: 747-757 (2001).

Du G., Yu J., Chen J. and S. Lun, Continuous production of poly-3-hydroxybutyrate by Ralstonia eutropha in a two-stage culture system. *J Biotechnol*. 88: 59–65 (2001).

Dunlop, W. F., and A. W. Robards, Ultrastructural study of poly-fI-hydroxybutyrate granules from *Bacillus cereus*. *J. Bacteriol*. 114: 1271-1280 (1973).

Gamal R.F., Abdelhady H.M., Hassan E.A., El-Tayeb T.S. and K.A. Aboutaleb, Production of PHAs from waste frying oil by *Pseudomonas fluorescens* S48 using different bioreactor feeding strategies. Egypt J. Microbiol. 47: 130-136 (2012).

Ha, C.S. and W.J. Cho, Miscibility, properties and biodegradability of microbial polyester containing blends. *Programe Polymer Science* 27: 759-809 (2002).

Hahn, S.K., Y.K. Chang, B.S. Kim and H.N. Chang, Communication to the editor: Optimization of microbial poly (3hydroxybutyrate) recovery using dispersions of sodium hypochlorite solution and chloroform, *Biotechnol Bioeng*. 44: 256-261 (1994).

Jeffrey G. Banks and Ron G. Board, The classification of pseudomonads and other obligetory aerobic Gram-negative bacteria from British pork sausage and ingredients. *Syst. Appl. Microbiol*. 4: 424–438 (1983)

Kalaivani, R. and V. Sukumaran, Isolation and identification of new strains to enhance the production of biopolymers from marine sample in Karankura, Tamil Nadu. *Eur. J. Exp. Biol.* 3(3): 56-64 (2013).

Kuniko, M., Y. Nakamura and Y. Doi, New bacterial coployestras produced in *Alcaligenes eutrophus* from organic acids. *Polymer Commun.* 29: 174-176 (1998).

Law J. and R.A. Slepecky, Assay of poly-hydroxy butyric acid. J. Bacteriol. 82:52–55 (1961).

Lee, S.Y., Bacterial polyhdroxyalkanoates. *Biotechnology and Bioengineering* 49: 1-14 (1996).

Mackie and McCartney, Screening and Isolation of PHB-Producing Bacteria in a Polluted Marine. Microbiology, 3th edn; Pp. 54-55 (1989).

Madison, L.L. and G.W. Huisman, Metabolic Engineering of Poly (3-Hydroxyalkanoates): From DNA to Plastic. *Microbiology Molecular Biology Review*, 63: 21-53 (1999).

Madison, L.L. and G.W. Huisman, Metabolic engineering of Poly (3-Hydroxyalkanoates): From DNA to Plastic. *Microbiol. Mol. Biol. Rev*. 63(1):21-53 (19999).

Murray, R.G.E., R.D. Doetsch and C.F. Robinow, Determinative and cytological Light microscopy In Manual of Methods for Genera Bacteriology. *American Society of Microbiology* 10: 21-41 (1994).

Ostle A G and J.G. Holt, Nile blue A as a fluorescent stain for polyhydroxybutyric acid. *Appl. Environ Microbiol*. 44: pp 23841 (1982).

Palleroni, Norberto, Human-and Animal Pathogenic Pseudomonads. The Prokaryotes Volume III, New York: Springer-Verlag (1992).

Poirier, Y., C. Nawrath and C. Somerville, Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. *Biotechnology*, 6: 552-560 (1995).

Steinbüchel A., Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxy alkanoats biosynthesis pathways as a successful example. *Macromol. Bioscien.* 1: 1–24 (2001).

Steinbuchel, A., Perspectives for biotechnological production and utilization of biopolymers: Metabolic engineering of polyhydroxy alkanoate biosynthesis pathways as a successful example. *Macromolecular Bioscience* 1: 1-24 (2001).

Sudesh, K. and T. Iwata, Sustainability of biobased and biodegradable plastics. *Clean Soil, Air, Water* 36: 433-442 (2008).

Zinn, M., B. Witholt and T. Egli, Occurrence, synthesis and medical application of bacterial polyhydroxy alkanoate. *Advanced Drug Review (2001).*