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BIOPLASTIC (POLY -3-HYDROXYBUTYRATE) PRODUCTION BY LOCAL PSEUDOMONAS AERUGINOSA ISOLATES UTILIZING WASTE COOKING OIL

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ABSTRACT

Disposal of domestic wastes, such as waste cooking oil (WCO), without pretreatment, contributes to the deterioration of the environment and creating sever problems. Synthetic plastic poses threat to the environment, because it is non-biodegradable are now accumulate in the environment at great millions of tons per year. Bioplastic are polymers such as Poly -3-hydroxybutyrate (PHB), which has gained importance since it can be easily degrade in nature. The present study was aimed to isolate a promising microbial producer of (PHB), and optimization experiment to evaluate the best environmental and physiological factors that lead to maximum (PHB) production.

Seventy Pseudomonas species were isolated from different contaminated soil and water samples. The isolates thereafter were screened for (PHB) production using Sudan black and Nile blue as indicators stains to investigate the bacterial isolates for PHB production. It was found that 50 of them were capable of producing PHB. The isolate Pseudomonas aeruginosa Dw_7 exhibit higher production of PHB, therefore it's selected for further studies. . Pseudomonas aeruginosa D_7 was showed maximum production after 48 hours of growing on WCO yielding 1.74g/l of dry weight and 0.62g/l of PHB. Deferent WCO were tested as the sole carbon sources, the isolate yielded a relatively good dry weight 1.99g/l with 0.75 of PHB yielding 37% (PHB/dry weight of biomass) when grown on corn oil waste with initial concentration of 2%. Ammonium sulphate was found to be the best nitrogen source which raises the production of dry weigh biomass to 2.67 g/l with 1.03 g/l of PHB (yielding 39 %). The highest (PHB) yield was observed as an intracellular product in Pseudomonas aeruginosa

D_{w7} accumulating as high as 4.32 g\l dry weigh with 2.30 g/l of PHB (yielding 53.2 %) in C\N ratio of 30:1 in culture medium. Using WCO as a carbon source for synthesis of PHB is an economic process, turns polluting wastes into a valuable biodegradable product. This renewable source of waste oil can thus be exploited as low cost materials for PHB production.

KEYWORDS: Bacteria; Poly-3-hydroxybutyrate; Oil waste; Bioplastic.

INTRODUCTION

Environmental pollution by the disposal of synthetic polymers (i.e. conventional plastics) e.g. Polypropylene (PP) is a growing problem. Since the 1940s plastics have been replacing glass, wood, metal and other constructional materials in numerous applications.[24] The use of plastics is widespread within our society; this is primarily due to the favorable thermal and mechanical properties of plastics making it a stable and durable material.[24] The extensive global use of plastics has contributed heavily to environmental pollution; as plastics are not always properly discarded or recycled and consequently persist within the environment. Bioplastics have been researched for many years as an alternative to synthetic plastics. Some of the major naturally occurring bioplastics are cellulose, starch, poly (lactic acid) and polyhydroxyalkanoates (PHAs). These materials hold further advantages over synthetic plastics as they are biodegradable and are produced from renewable sources; they are storage materials that accumulate by various bacteria as energy and carbon reserve materials. They are biodegradable, environmentally friendly, and also biocompatible bioplastics. Unlike petrochemical-based plastics that take several decades to fully degrade, PHAs can be completely degraded within a year by variety of microorganisms into CO_2 and water. It has wide applications in different areas such as medicines, long term dosage of drugs, cosmetic world, cosmetic containers, shampoo bottles, insecticides, fertilizers, packing materials. Bacterial cells accumulate PHAs as intracellular food and energy reserve as a response to nutrient limitation or imbalance in the environment as a means of preventing starvation when essential nutrients are limited.[27] PHA degrades naturally and completely into carbon dioxide and water under aerobic conditions and into methane under anaerobic conditions.[8] A wide variety of microorganisms are able to naturally accumulate PHB as intracellular energy storage materials under an excess of carbon source and conditions of limiting nutrients such as oxygen, nitrogen and phosphate. The objectives of this study were to isolate potentially poly-3-hydroxybutyrate (PHB) producing bacterial isolates from natural environment,

screening the isolated cultures for production of (PHB) and enhancing production process by optimizing growth conditions.

MATERIALS AND METHODS

Collection of Samples

Different soil and water samples were collected from Hydrocarbons contaminated soils, oil sewage from Al-Dora refinery \Baghdad, sewage sludge agricultural .The sample transfer in sterile bottles and stored in a refrigerator at 4°C.

Isolation of Pseudomonas sp

The isolation of isolates was converted by additive 1g o collected sample ,in 9 ml of sterile distilled water ,then sterile dilutions were made up to 10^{-5}.Thereafter 0.1ml of each samples were directly pour plated on to Luria-Bertani (LB) agar plates supplemented with 1%glucose. The plates were then incubated at 30°C for 2-3 days. Colonies with different morphology, color, pigmentations etc. were isolated in pure form and maintained on slants and stored at 4°C.

Primary identification of Pseudomonas sp. isolate

All pure bacterial isolate were cultured in Cetrimide medium (pH7) by streaking 0.1ml overnight growth of isolate on agar plates, and then incubated at 30°C for 24h. Growth of isolate on the agar plate indicates positive results.

Screening for PHB producing bacteria

The presence of intracellular PHB granules was confirmed with the aid of staining with Sudan black B and Nile blue A.[3]

Rapid screening of PHB producing Bacteria

For Screening of PHB production the method of[7] was followed with modifications, as described briefly, PHB producing bacteria was detected using the lipophilic stain Sudan black B. Sudan black stain was prepared as a 0.3% solution (w/v) in 60% ethanol. Smears of PHB producing bacteria were prepared on glass slides and heat fixed. Samples were stained for 10 min with Sudan black solution, rinsed with water and counter-stained with 0.5% safranin for 5 s. Stained samples were observed under oil immersion at 1000x magnification. The dark blue coloured granules into cells were taken as positive for PHB production.

Qualitative screening of Pseudomonas sp. isolates for PHB production

A total of 50 bacterial isolates were qualitatively tested for PHB production using Sudan Black B dye. For rapid screening of PHB producers, on carbon rich nutrient agar medium contained (w/v) (glucose 1%, beef extract 0.3%, peptone 0.5%, and sodium chloride 0.8%, agar 1.5%). Medium was autoclaved and poured into petri plates and allowed for solidification. The plates were inoculated with bacterial isolates. The plates were incubated at 30^0C for 24-72 hrs. Ethanolic solution of (0.02%) of Sudan black was spread over the colonies and the plates were kept undisturbed for 30 minutes.[22] They were then washed with 98% ethanol to remove the excess stain from the colonies. The dark blue colored colonies were taken as positive for PHB production.[22]

Staining of Bacterial isolates Producing PHA using Nile blue A

Staining of cultures, grown under PHA accumulation conditions, was performed with Nile blue A using a modified method.[23,32]

Cell Dry Weight Measurement: After incubation, samples were centrifuge for 15min at 6000rpm. The pellet were washed twice with sterile deionized water and dried for 24h at 60°C. The total bacterial dry weight was determined.[36]

PHB production

All the Sudan Black B positive isolates were subjected to quantification of PHB production as per the method of[16] The screened isolates were grown in Erlenmeyer flask 2%of cooked sun flower oil. The flasks were incubated at pH7, Temo.30°C in shaken incubator (150rpm) for72 h.After end of incubation. Bacterial cells containing the polymer were pelleted at 10,000 rpm for 10 min. and the pellet washed with acetone and ethanol to remove the unwanted materials. 5ml of 0.4% Sodium hypochlorite was added to dry cell biomass and was incubated at 37°C for 2h. to break the cell wall of bacteria.[11] The supernatant was obtained by centrifugation at 13,000rpm for 10mins and was transferred into separating funnel for extraction. Cell lipids and other molecules (PHB) present were extracted by adding 5ml of 96% ethanol and 5ml of 96% acetone. PHB was thereafter extracted by adding 10ml of chloroform to the mixture in a hot water bath at 60°C. The weight of the dried chloroform extract was thus determined.[1,5]

Yield of PHA accumulation (%) = Dry weight of extracted PHA (g/L) / DCW (g/L) × 100%.

Surface tension determination

Surface tension measurements were made for samples and control by the Du Noüy-ring method using a surface tension balance at room temperature. A platinum wire ring was placed into the solution and then slowly pulled through the liquid-air interface. Stabilization was allowed to occur until the standard deviation of 10 successive measurements was less than 0.4 mN/m between two consecutive measurements; the platinum ring was rinsed with water and acetone and allowed to dry. All the measurements were made on cell-free broth (in duplicate) obtained by centrifuging the cultures at 12900 x g for 20 min.[35]

Quantitative analysis of PHB by Spectrophotometry

Dried chloroform extract of PHB samples 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 to converts the product Crotonic acid (add extra sulfuric acid if reading is high) The absorbance was taken at 235nm using sulfuric acid (99%) as blank in UV Spectrophotometer.[28] Crotonic acid powder was dissolved into sulfuric acid and standard solution of 0.1µg of Crotonic acid/ µl of sulfuric acid was prepared. Working STD solution of, 10, 20, 30, 40, 110µg/3m of sulfuric acid were prepared. Blank was prepared by adding 3 ml of sulfuric acid. The absorbance was read at 235nm. Standard curve of concentration versus absorbance was plotted.[19]

Morphological, biochemical and molecular characterization of selected isolate

The isolate was morphologically characterized by observing the standard microbiological methods. The biochemical characterization of the isolate was done by series of biochemical tests including carbohydrate fermentation, oxidase, Catalase test and Growth at 41°C.

Genomic Characterization of Bacterial isolate

Genomic DNA extraction from bacteria: DNA extraction was carried out following the CTAB method .Amplification reaction for was per formed according to.[20]

Optimization of PHB

The optimization for maximum PHA production by selected isolate was carried in 250-ml Erlenmeyer flask containing 50 ml of mineral salt medium (MSM).The mineral salt medium composition was as follows (per litter distilled water): 4 g Na2HPO$_4$, 1 g KH$_2$PO$_4$, 0.2 g MgSO$_4$. 7H$_2$O, 0.05 g CaCl$_2$.2H$_2$O, 3 g NH$_4$NO$_3$, 0.2 g NACl 1 g glucose and 1 ml of trace element solution.[22] The trace element solution contained (per litter 0.5 n HCl): 5.56 g

$FeSO_4.7H_2O$, 3.96 g $MnCl_2.4H_2O$, 5.62 g $CoSO_4.7H_2O$, 0.34 g $CuCl_2.2H_2O$, 0.58 g $ZnSO_4.7H2O$, 0.60 g H_3BO_3, 0.04 g $NiCl_2.6H_2O$ and 0.060 g $Na_2MoO_4.2H_2O$ [18] supplemented with 2% of cooking waste oil. The flasks were incubated at pH7, temperature 30°C in shaking incubator (150 rpm) for 72 h. Several cultural parameters were evaluated to determine their effect on biomass and PHB production. The optimized value for each parameter was selected and kept constant for further experiments. Several cultural parameters like effect of incubation time, pH, and temperature effect of oil and nitrogen source were evaluated to determine their effect on biomass accumulation and PHA production. In each experiment above, dry weight of biomass, PHB and surface tension were determined.

Effect of Carbon Sources for PHB production

250 ml conical flasks containing 50 ml of modified MSM with different cooking waste oils as a carbon sources. The Substrates considered were olive oil, sunflower oil, sesame oil corn oil and glycerin.

Effect of nitrogen source on PHB production

To investigate the effect of nitrogen with different source on the PHB production capability of isolate , cultures were grown in MSM containing different sources of nitrogen included (NH_4Cl,NH_4SO_4, Pepton and Yeast extract) at concentration of o.5g/l was carried out. Thereafter the best nitrogen source was selected then the best source (ammonium sulphate) was tested with different concentration ranging for (0.2 to1.5) g/l on PHB production in the present of corn oil as carbon source.

Effect of oil source concentration on PHB production

The effect of initial oil concentration included(0.5,1,2,3,3.5,4)% on PHB production was carried out in MSM medium containing different concentration of the best oil source(corn waste oil) The flasks were incubated at pH7. at 30°c in shaking incubator (150rpm) for 48 h. after end of incubation, dry cell weight, PHB was quantified.

Effect of C: N ratio on PHB production

Using the best C and N sources were done included (10:1, 20:1, 25:1, 30:1and35:1). After inoculation and incubated on a rotary shaker (150 rpm) at 30°C. After 48 h, PHB yields were extraction and quantified as mentioned before. Duplicates from each treatment were used.

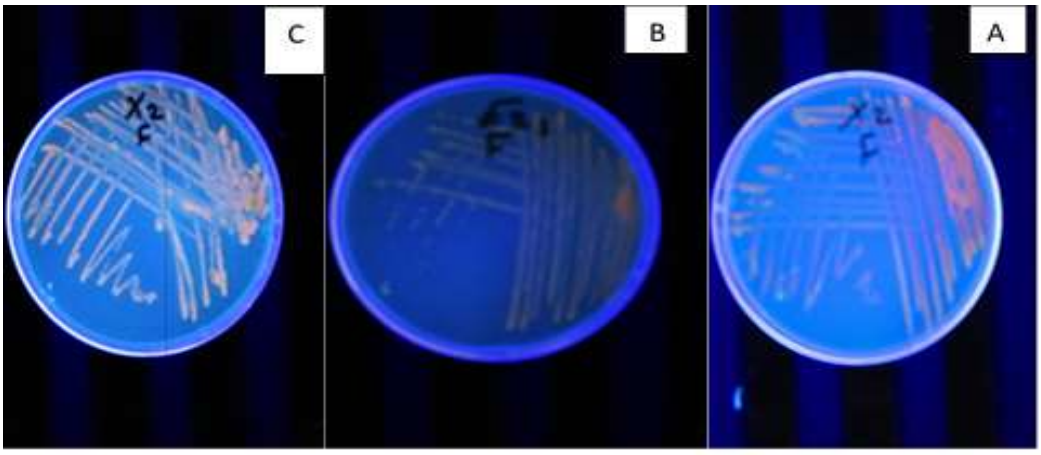
RESULTS AND DISCUSSION

Isolation and screening of PHA-producing isolates

Table (1) Primary screening of *Pseudomonas aeruginosa* isolates for PHB production

Source of samples	Number of *Pseudomonas aeruginosa* isolates	Number of Sudan black positive isolates	Identification symbols of isolate
Hydrocarbons contaminated soils near electric generators\AL-Gadiriya	11	3	Eh
Hydrocarbons contaminated soils near petrol station \AL-Karrada	12	5	Eh
Oil sewage from Al-Dora refinery	14	11	SD
Detergent waste water from oil factory \Baghdad	12	12	Dw
Activated sludge from AL-Kadimiya sewage water	12	10	Ac
Different soil samples from gardens in Baghdad University	5	5	Gs
Different soil samples from gardens in Ministry of Science and Technology	4	4	Gs

A wide variety of bacteria are known to accumulate PHA granules intracellularly as an energy reserve material. Microbial species from over 90 genera have been reported to accumulate approximately 150 different hydroxyalkanoic acids as polyhydroxyalkanoate polyesters granules.[10,33] The present study 70 bacterial isolates were obtained from different soil and waste samples were screened for PHB production. For the rapid detection and isolation of PHB-producing bacteria, primary screening with 0.02% alcoholic solution of Sudan black B, viable colony method[32] was used. Among 70 bacterial isolates tested which were grown on enriched medium showed black-blue coloration table (1) when stained with Sudan black B fig. (1), a preliminary screening agent for lipophilic compounds. Under contrast phase microscopic testing, the positive result was dark black condensed granules of PHB were observed inside cell surrounding pink cytoplasm (fig.2).



Figure(3)Pink-orange florescence under UV light by PHB producer isolate Dw7 with Nile blue A staining by viable colony method(A-high ,B-medium C-low) intensively

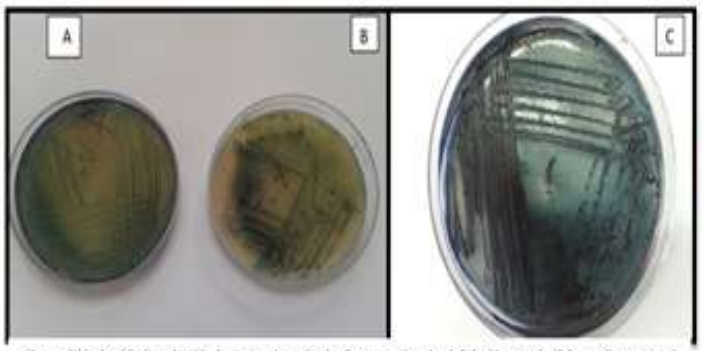


Figure: (1)Sudan black-stained isolates Poorly stained colonies as showing in(A)taking symbol(+), medium stained colonies as showing in (B) taking Symbol (++), strongly stained colonies as showing in (C) taking symbol(+++)

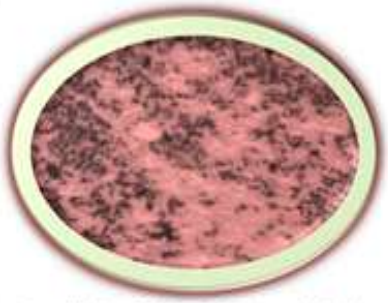


Figure (2) Sudan black B stain of PHB granules (black section) on *P. aeruginosa* DW7 observed under 100X oil immersion contrast phase objective

Confirmed for PHB production by subjected for screening by Nile blue A staining. All isolates tested showed bright orange fluorescence on irradiation with UV trans-illuminator at a wavelength of 460nm in Nile blue A as shown in (fig. 3). The results obtained was similar to that observed by [20;34]they used the same method of screening the potential PHB-producing bacteria from soil, .Also Ramachandran and Abdullah, 2010 observed the orange fluorescence colonies formed on nutrient-rich medium under ultraviolet light (UV) which indicated the presence of PHB producers. Their fluorescence intensity increased with increase in PHB content of the bacterial cells. Based on the intensity of the fluorescence were observed in the Nile blue staining method as followed by.[23]

Production of PHB in MSM medium

The highest positive isolates for Sudan black and Nile blue A were selected for Screening in liquid submerged fermentation in MSM medium supplemented with 2% of sunflower cooking waste oil. The PHB from the isolates was extracted by the hypochlorite and chloroform method[30] as described earlier. Three potential PHA producers were obtained out of fifty isolates were screened and the isolate Dw_7 showed maximum PHB production 0.36 g/l of biomass, 0.14g/l of PHB/l yielding 45%PHB/biomass in enriched fermentation medium. Thereafter the isolates which exhibit highest yield and PHB production was selected for further identification and optimization studies.

Identification of isolate Dw_7

Morphological, Biochemical and Physiological tests of selected isolate was done by using Bergey's manual of determinative bacteriology.[14] The isolate Dw_7 forms round, smooth, bluish green colonies with entire margins and convex elevations. The organism is gram negative, exhibiting rods with single arrangements. The gram negative property was further confirmed by growth on Cetrimide agar the selected isolate was classified up to species level

using the morphological and biochemical characteristics and was identified as Pseudomonas aeruginosa. For further characterization, almost complete 16S rRNA gene sequences were determined. DW_7 isolate is definitively identified as P. aeruginosaDw_7 (fig. 4).

Figure (4): *Pseudomonas* isolate with 341F and 907Rprimers set: The first lines positive control *P. aeruginosa*, M:

Optimized conditions for PHB production

Effect of incubation time on PHB production

The PHB production of P. aeruginosa Dw_7 was followed for 120 h by growing in 250 mL Erlenmeyer flask at an interval of 24 h. After 24 h of incubation, the biomass began to increase and reached maximum value on the second day of incubation. The production of PHB increased up to 48 h (0.62 g/L) and, thereafter, got reduced (0.50 g/L after 72 h) (Figure 5). This reduction in PHB production after 72 h may be due to lack of micronutrients as well as an increase in the metabolites that might have negative effect on the PHB production, the observation was supported by[36]. Reduction in surface tension was observed every day for a period of five days. P. aeruginosa has been reported for the production of biosurfactant [12; 15]. As the number of bacterial cells increased the requirement of carbon source for growth increased and production of biosurfactant increased resulted in access of bacteria to oil carbon source.

Effect of incubation temperature and pH on PHB production

From analysis it is clear that pH 7 was favorable for PHB production by P. aeruginosa Dw_7 in oil waste containing medium. The current observation was in agreement with [6]. Maximum PHB production of o.66 g/L was recorded at 30∘C after48 h. The increase of temperature beyond 40∘C has negative impact on PHB production (fig. 7). The decrease in PHB production at high temperature could be due to low PHB polymerase enzyme activity[9]. Results shown that the growth conditions including pH, temperature plays an important role

in production rate of PHB which also related to its biomass. As the biomass increases the bacteria also starts accumulating PHB.

Effect of oil and nitrogen source on PHB production.

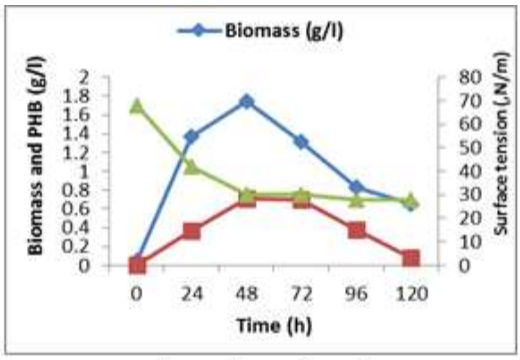


Figure (5) Effect of different incubation period on growth and PHB production by the selected bacterial isolate

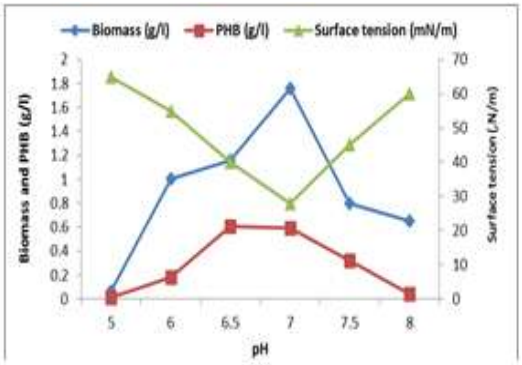


Figure (6) Effect of various pH values on PHB production with *P. aeruginosa* DW7

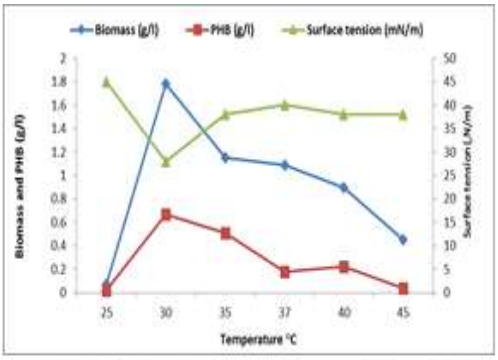


Figure (7) Effect of various temperatures on PHB production with *P. aeruginosa* DW7

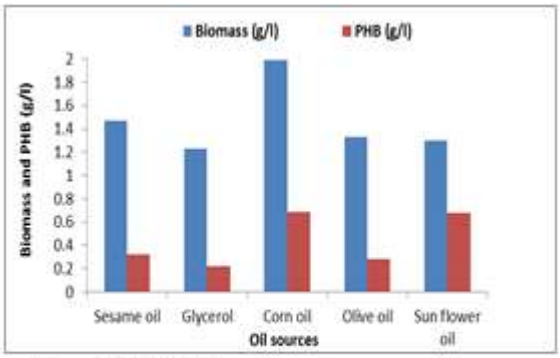


Figure (8) Effect of various oil source on biomass and on PHB yield by the selected isolate

It has been cleared from the results that the maximum PHB was produced with cooking corn oil as a carbon source, it was observed that PHB content reached 0.75 g/l with 1.99 g/l of biomass yielding 37% of PHB (fig. 8). In general, PHB polymer is synthesized by the bacterial cells under limiting growth conditions, when the carbon source is in excess and nitrogen, phosphorus, magnesium, sulfur or oxygen is present in a limiting concentration.[26] Data presented in fig. (9) showed that out of different nitrogen sources tested ammonium sulphate was found to be optimum for maximum PHB production. As shown in fig. (10) the maximum PHB production of 1.33g/l, occurred at 1g/l of ammonium sulphate as a nitrogen source and lower PHB content with high concentration of nitrogen source, This might be due to the fact that PHA accumulation on biomass increased under nitrogen limitations in accordance of already reported work.[4] The effect of different concentration of corn oil on PHB production was studied. The results shown that 45% highest production of 1.98g/l was observed with corn oil concentration of 3% (fig. 11). Results shown that increasing of oil

waste concentration more than 3% led to decrease of PHB production. This finding is in accordance with the work of[17] which referred to that P. aeruginosa where increasing the concentration of oil led to the death of microorganism and decreasing of PHB. Different strains of Pseudomonas have been reported for PHA production using oily substrates.

Effect of C: N ratio on PHB production

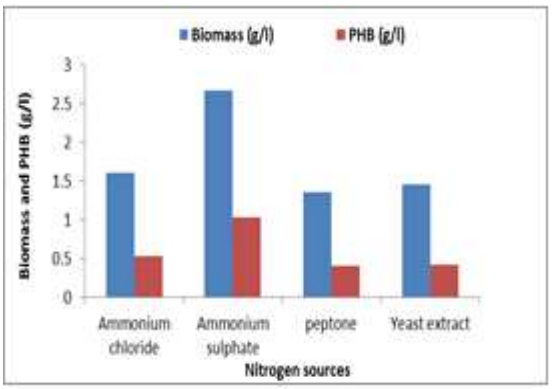


Figure (9) Effect of Nitrogen sources on biomass and PHB Production by selected isolate

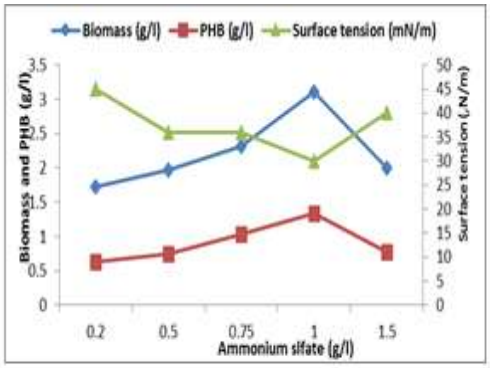


Figure (10) Influence of different concentration of Ammonium sulphate (the best nitrogen source) on PHB yield by the selected isolate

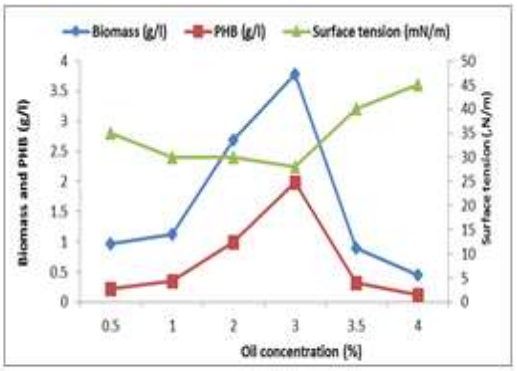


Figure (11) Influence of different concentration of corn oil (the best oil source) on PHB yield by the selected isolate

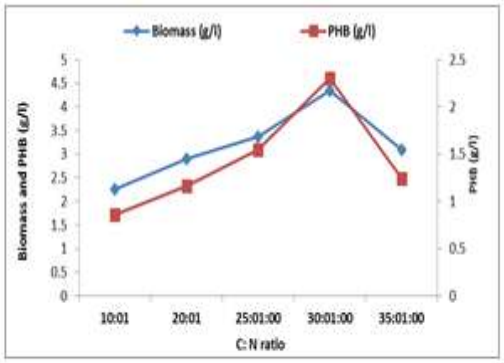


Figure (12) influence of different C: N on PHB yield by the selected isolate

As shown in fig.(12), the best C/N ratio for production was (30/1 w/v). Also ,its observed that the isolate produce maximum PHB when its biomass is as its peak level and slowed down as the biomass is dropped because at this phase of the growth all the nutrients are depleted leading to decrease in PHB content. These results supported by.[7]

CONCLUSIONS

In this study, inexpensive waste cooking oil was utilized as a carbon source to produce PHB. Different bacterial Pseudomonas aeruginosa isolates were isolated from different contaminated soil and water screened for PHB production. The bacterial isolate Pseudomonas aeruginosaDw_7can be exhibit as potential isolate for conversion of corn oil waste into

PHB.Optimization studies revealed an increase in accumulation of PHB in selected isolate with optimizing environmental and cultural conditions, and maximal PHB yield observed at C:N ratio 30:1yield 53% of PHB. Using waste cooking oil is a double benefit for the environment as it enables the production of bioplastics but also reduces environmental contamination caused by disposal of waste oil.

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