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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 severs 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. The isolate DW7 is definitively identified as *Pseudomonas aeruginosa* Dw7 after complete 16S rRNA gene sequences method. Highest accumulation of PHB and DCW were at 30°C, recorded 0.65 g/L and 1.78 g/l respectively with deletion of surface tension to 28mN/m with yield of PHB ranged between (35.6 and 36.5 %). Different WCO were tested as the sole carbon sources. The highest cell growth was observed on the polymer samples produced from corn oil, followed by the polymer produced using sunflower oil. The isolate yielded a relatively good dry weight 1.78 g/l with 0.72 of PHB yielding 40%

(PHB/dry weight of biomass) when grown on corn oil waste with an initial concentration of 1%. Ammonium sulphate was found to be the best nitrogen source with initial concentration at 1gm/ l which raises the production of dry weight biomass to 2.6 g/l with 1.33 g/l of PHB (yield 51%). Optimum oil concentration was 3% with best DCW, highest PHB accumulation and yield which were 3.7, 1.98 and 53 % respectively. The highest PHB yield was observed as an intracellular product in *P aeruginosa* D_w7 accumulating as high as 4.32 g\l dry weight 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: *Pseudomonas aeruginosa*; 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. 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 (Poirier *et al*, 1995). 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 (Reema *et al*, 2013).

PHA degrades naturally and completely into carbon dioxide and water under aerobic conditions and into methane under anaerobic conditions (Doi *et al*, 1992).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 or plastic 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 of 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 (Bänziger and Tobler, 2009).

Rapid screening of PHB producing Bacteria

For Screening of PHB production the method of (Burdon, 1946) 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 100 x magnifications and contrast face. The dark blue coloured granules into cells were taken as positive for PHB production (Burdon, 1946).

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°C 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. 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 (Muheim and Lerch, 1999).

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 [(Ostle and Holt1982; Spiekermann, 1999)].

Cell Dry Weight Measurement: After incubation, samples were centrifuge for 15min at 6000 rpm. The pellet were washed twice with sterile deionized water and dried for 24h at 60°C. The total bacterial dry weight was determined (Yuksekdag *et al*, 2004).

PHB production

All the Sudan Black B positive isolates were subjected to quantification of PHB production as per the method of (John and Ralph,1961) The screened isolates were grown in Erlenmeyer flask 1% of cooked sun flower oil .The flasks were incubated at pH7, Temo.30°C in shaken incubator (150 rpm) 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 2 h to break the cell wall of bacteria. (Grothe *et al*, 1999). The supernatant was obtained by centrifugation at 13,000rpm for 10 mints 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 10 ml of chloroform to the mixture in a hot water bath at 60°C. The weight of the dried chloroform extract was thus determined [(Arnold *et al*, 1999; Belma *et al*, 2002)].

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 (Vedaraman and Venkatesh, 2011).

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 (Rehm, 2010).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 (Law and Slepeky, 1961).

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 42° 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 (Lopez, 2009).

Optimization of PHB Production

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 (Kim ,2000).The trace element solution contained (per litter 0.5 n HCl): 5.56 g FeSO$_4$.7H$_2$O, 3.96 g MnCl$_2$.4H$_2$O, 5.62 g CoSO$_4$.7H$_2$O, 0.34 g CuCl$_2$.2H$_2$O, 0.58 g ZnSO$_4$.7H2O, 0.60 g H$_3$BO$_3$, 0.04 g NiCl$_2$.6H$_2$O and 0.060 g Na$_2$MoO$_4$.2H$_2$O (Doi et al,1992), supplemented with 1% 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 below, dry weight of biomass, PHB content and surface tension were determined.

Effect of incubation time

To determine the effect of incubation period, the liquid MSM pH7 supplemented with 1% of sunflower oil as waste cooking oil (WCO) were inoculated and flasks were incubated at 30ºC and 150 rpm under different incubation period (24, 48 ,72, 96 and 120 h).

Effect of pH

To determine the optimum pH, experiments were carried out in liquid MSM with different pHs (5, 6, 6.5, 7, 7.5 and 8). Flasks were inoculated and incubated in a rotary shaker at 30°C and 150 rpm for 48 h.

Effect of temperature

To determine the effect of temperature, liquid MSM supplemented with 1% of WCO (sunflower oil) and pH of 7 was incubated at 25, 30, 35, 37, 40 and 45ºC under 150 rpm/min for 48 h.

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 (150 rpm) 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

Sample collection and isolation of bacterial isolates

In the present study twenty eight soil and water samples were collected from six diverse locations including those such as oil factory waste water, hydrocarbons contaminated soils, oil sewage and plastic wastes contaminated soils, at different periods from February to May 2016. Bacterial isolates were isolated by spread plate and streak plate methods. One gram of soil sample was dispensed in 10ml of sterile distilled water, then mixed vigorously and 1ml from this sample is taken and added to another tube contain 9 ml sterile distilled water to get a dilutions.

For the isolation of organisms, 0.1ml of each dilution was plated onto a nutrient rich medium by spread plate method for the propagation of microbial growth. The plates were incubated at 30°C for 24-72 hours. Colonies with different appearance like orange, red, white, yellow etc. were isolated, purified and maintained as pure cultures on nutrient agar slants. Out of these samples, a total of 195 bacterial isolates as indicated in Table (1) were isolated. Maximum numbers of cultures were collected from samples of activated sludge, hydrocarbon contaminated sites, oil waste and detergent waste, and minimum numbers of isolates were collected from soil samples from gardens of different sites.

Table 1: Profile of the sites where from sampling carried out to obtain *P. aeruginosa.*

S. No.	Source of sample	Number of bacterial isolate	Number of *Pseudomonas aeruginosa* isolate	Identification symbols of isolate
1	Hydrocarbons contaminated soils near electric generators\AL-Gadiriya	35	11	Hs
2	Hydrocarbons contaminated soils near petrol station \AL-Karrada	36	12	Ps
3	Oil sewage from Al-Dora refinery	44	14	Os
4	Detergent waste water from oil factory \Baghdad	23	12	Dw
5	Activated sludge from AL-Kadimiyaee;' sewage water	27	12	As
6	Different soil samples from plastic waste contaminated sites from Baghdad.	30	9	Wp

Primary identification of *Pseudomonas aeruginosa* isolates

All the bacterial isolates were cultured on selective -differential media that support *Pseudomonas* growth (Cetrimide agar medium). A total of 195 pure of bacterial isolates were tested for their ability to grow on solid Cetrimide medium. Growth of isolate on Cetrimide agar plate indicates positive results as *P. aeruginosa* isolates. 70 bacterial isolates (66) % of total number representing were able to grow on Cetrimide selective medium fig (1) and Table (1).All bacterial isolates were positive for Cetrimid test were screened for PHB production.

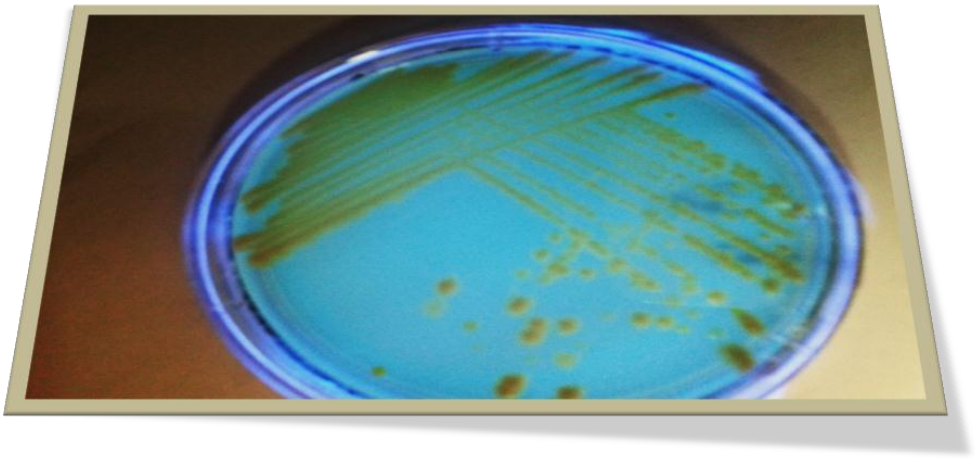

Figure 1: *P. aeruginosa* morphology on Cetrimide solid medium.

Primary screening of *P.aeruginosa isolates* for PHB production

For the rapid detection and isolation of PHB-producing bacteria, primary screening of isolates were carried out in Sudan black solid medium. Among 70 bacterial isolates tested which were 24 hours old cultures, 50 isolates, (71%) were positive for PHB production. The bacteria positive for PHB production were selected by observing the granules under 100x oil immersion and contrast phase microscopic testing, the positive result were dark black condensed granules of PHB were observed inside cells surrounding pink cytoplasm as shown in table (2) and fig(2).

Table 2: Screening of *Pseudomonas aeruginosa isolates* on selective medium and its relative PHB production under microscopic test using Sudan black B stain.

Identification symbols of isolate	Number of Pseudomonas aeruginosa isolates	Number of Sudan black positive isolates
Hs	11	**3**
Ps	12	**5**
Os	14	**11**
Dw	**12**	**12**
As	12	**10**
Wp	9	**9**

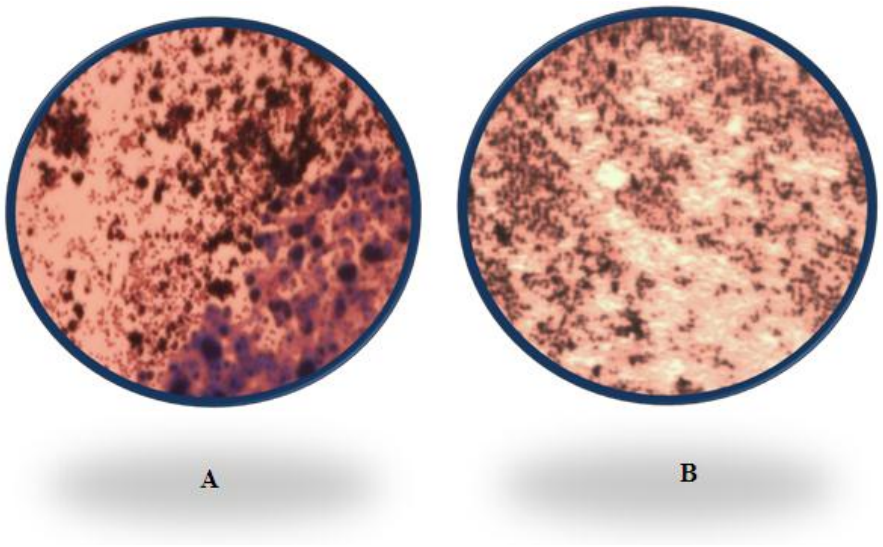

Figure 2: Sudan black B stains of PHB granules (black section) in *P aeruginosa* isolates observed under contrast phase objective microscope (A) and (B) 100x oil immersion.

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 (Du *et al*, 2001; Steinbüchel, 2001). PHB was accumulated as inclusion bodies so that the dry cell weight of bacterial cells was composed of PHB (Tabandeh and Vasheghani, 2003). The genus Pseudomonas is well known for its metabolic versatility and genetic plasticity. The species of Pseudomonas, in general, grow rapidly and are particularly renowned for their ability to metabolize an extensive number of substrates, including toxic organic chemicals, such as aliphatic and aromatic hydrocarbons. Ayub *et al*. (2004) isolated *Pseudomonas* sp. 14-3, a strain from Antarctic environments that accumulated large quantities of polyhydroxybutyrate (PHB) when grown on octanoate. Sujatha and Shenbagarathai. (2006) isolated PHB producing bacteria from different locations such as garden soil, tannery effluents, sewage sludge and field soil. They obtained higher PHB positive strains from sewage sludge and tannery effluents compared to other sources.Verma *et al*. (1995) found 19 *Bacillus* isolates with high florescence on Nile blue A dye and three isolates showed significant accumulation of PHB granules which appeared as blue black droplets using Sudan Black B staining.

Qualitative screening of *P aeruginosa sp.isolates* for PHB production

Viable colony method was used to detect PHB production. Isolates were grown on enriched medium showed black-blue coloration when stained with Sudan black B (table 2), a

preliminary screening agent for lipophilic compounds. Results show that out of 50 isolates, fourteen bacterial isolates were strong PHB producers (data not shown) as detected by Sudan black, after screening on solid agar medium using Sudan black (Figure 3). 14 isolates of P. *aeruginosa* were obtained and further screened as described below.

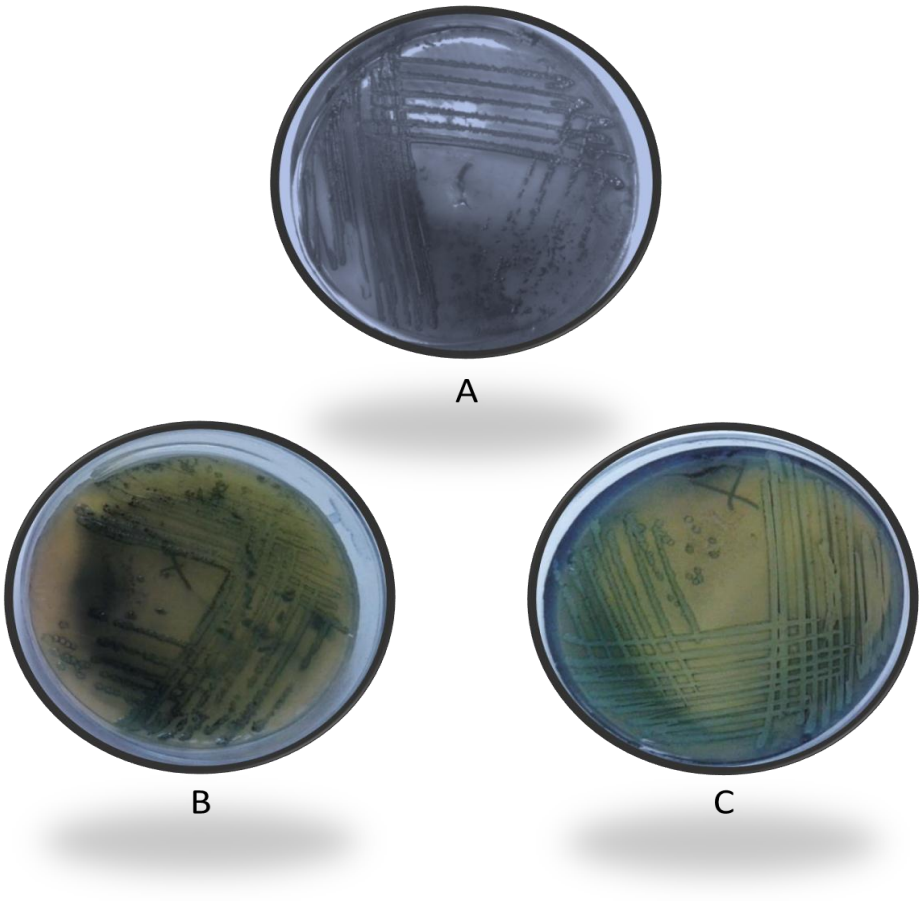

Figure (3) Strongly Sudan black B stained colonies as showing in A taking symbol (+++), medium stained colonies as showing in B taking symbol (++), poorly stained colonies as showing in C taking symbol (+).

Nile blue A plate assay

All PHB-accumulating colonies were confirmed for PHB production by subjected to screening by Nile blue A staining. All isolates tested showed bright orange fluorescence on irradiation with UV trans-illuminator at a wavelength of 312 nm in Nile blue A as shown in figure (4) .The results obtained was similar to that observed by Lopez *et al*,(2009) and Teeka *et al*,(2010) when they used the same method of screening the potential PHB-producing bacteria from the soil.

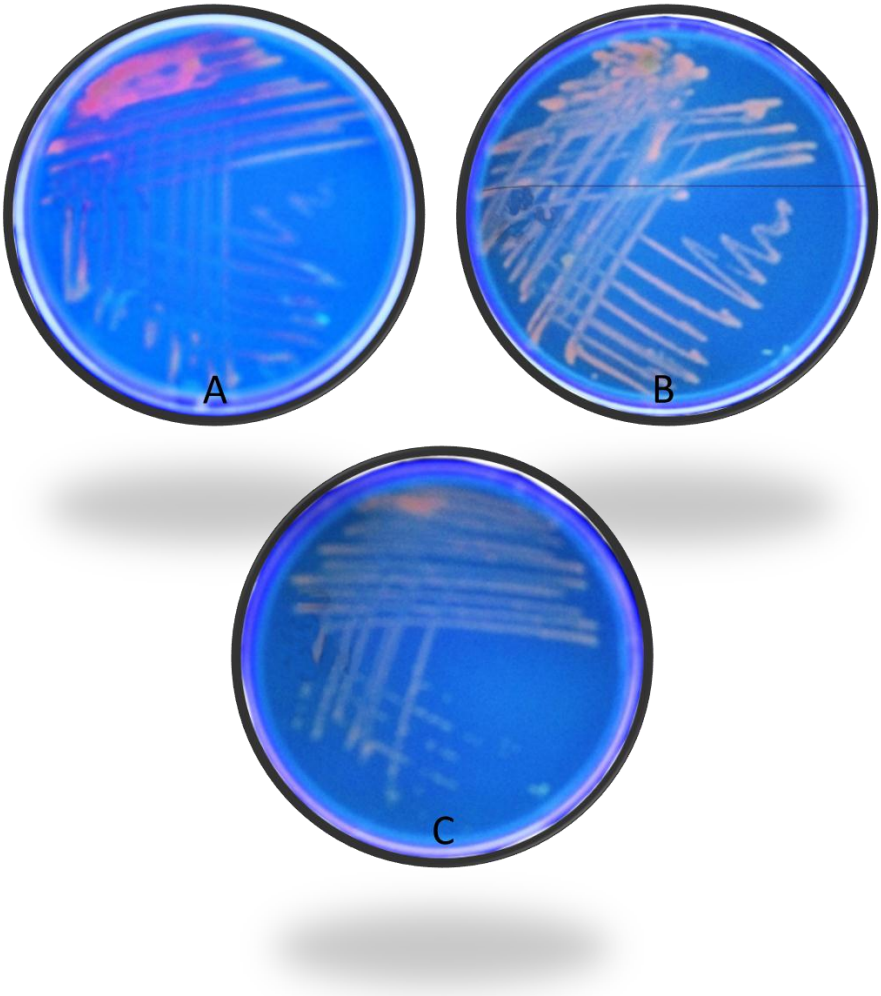

Figure (4) pink- orange fluorescence under UV light by PHB producer (A: High fluorescence, B: Moderate fluorescence and C: Weak fluorescence).

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 Ostle and Holt,(1982) shown in figure (4) and table (3) which refers to that out of 50 isolates ,14 showed excellent staining by Sudan black in Plate assay method and confirmed by Nile blue A staining.

Screening of PHB Production in Liquid Media

The highest positive isolates for Sudan black and Nile blue A were selected for further screening in liquid submerged fermentation in Minimal salt medium (MSM) supplemented with 1% of sunflower cooking waste oil as sole carbon source . Since deep frying is popular, there is a potential to turn this waste resource into a useful biomaterial. It was chosen to use

sunflower oil, since it is common frying oil in Iraq. In other regions of the world the nature of frying oil can differ. The PHB from the isolates was extracted by the hypochlorite and chloroform method (Singh *et al*, 2011)**.** As shown in table (3) the content of PHB in cell dry weight was determined. It was ranged from 10-110 mg/l depending on standard curve by quantitative with spectrophotometric assay using H_2SO_4 according to (Slepecky and Law, 1960b; Kuniko et al., 1988). Cell growth was monitored by measuring dry cell weight. The most active isolate Dw7 was the potential PHA producer which was found to be a significantly higher PHB producer compared to the other fourteen (14) isolates .The isolate Dw7 showed maximum PHB production 0.36 g/l of biomass, yielding 100 mg/l of PHB/biomass in enriched fermentation medium. Thereafter the isolates which exhibit highest yield and PHB production was selected for further identification and optimization studies.

Table 3: PHB production using sunflower waste cooking oil by bacterial isolates.

Bacterial isolate	Cell dry weight(g/l)	PHB content mg/l
Hs_3	0.23	60
Hs_4	0.34	75
Ps_2	0.08	45
Os_9	0.02	15
Os_{34}	0. 17	75
Os_{42}	0. 22	10
Dw_7	**0.36**	**100**
Dw_9	0.32	80
Dw_5	0.03	15
Dw26	0.03	15
Wp_3	0.06	20
Wp_8	0.06	20
Wp_9	0.07	15
Wp_{12}	0.06	25

Identification of isolate Dw7

Morphological, Biochemical identification

Morphological, Biochemical and Physiological tests of selected isolate was done by using Bergey's manual of determinative bacteriology (Holt et al, 2009). The isolate Dw_7 forms round, smooth, bluish green colonies with entire margins and convex elevations and the isolate was gram negative, exhibiting rods with single arrangements figure (5).

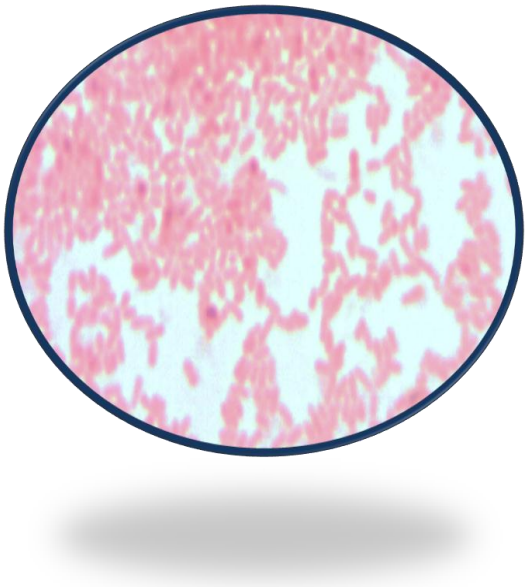

Figure (5) *P aeruginosa* isolate appearance under oil emersion after gram stained.

The gram negative property was further confirmed by growth on Cetrimide agar. The isolate Dw7 was a motile gram-negative rod-shaped bacterium. Activities of cytochrome oxidase and catalase were present production of pyocyanin as Blue green colonies were observed after 24 hours on Cetrimide agar table (4). The isolate was grown on McConkey and has the ability to ferment many sugars including Glucose, Fructose, Raffinose, Sucrose and Maltose in addition of its ability to grow at 42°C. In order to produce PHBs, the most commonly produced form of PHA, scientists typically use gram-negative strains because they can utilize cheap carbon sources and produce up to 80% of the cell's dry weight in PHB (Lopez *et al*., 2009).

Table 4: Chemical and morphological characterization to identify the potent bacterial isolate for PHB production.

Test	Results
Gram stain	–
Oxidase	+
Catalase	+
Motility	+
Growth at 42° C	+
Utilization of carbohydrates	
Glucose	+
Fructose	+
Sucrose	+
Raffinose	+
Maltose	–
Production of pyocyanin on Cetrimide agar	+

16SrRNA sequences

For further characterization, almost complete 16S rRNA gene sequences were determined. Genomic DNA extraction was carried out according to the method of Chen and Wu (2005a) and Pawar (2012).Extraction was performed by phenol chloroform method .Phenol is a strong oxidizing reagent and thus, is used directly to disrupt the cell wall in order to release the genomic DNA from the bacterial cell .The purification of gDNA was carried out by chloroform to isolate the genomic DNA from RNA and different proteins. Table (5) shown the value of purity and concentration of genomic DNA which extracted from Dw7 isolate. Results indicated that the sample was uncontaminated with proteins, phenol or polysaccharides. The high purity of the sample confirmed by absorbance ratio at (260/280) and at (260/230) for the gDNA extracted from isolate which was recorded to be more than 1.7 and 1.8 respectively.

Table 5: Concentration value and purity of genomic DNA.

Isolate	DNA concentration µg/ml	Ratio of 260/280 nm	Ratio of 260/230 nm
Dw7	5	1.90	2.1

Agarose gel electrophoresis was performed for Dw7 isolate DNA sample figure (6).The appearance of a sharp band of high molecular weight indicated the presence of genomic DNA for bacterial isolate, the single and pure band in each line confirm the purity of DNA.The PCR product obtained from the bacterial isolate after amplification were analyzed on standardized 0.8 agarose gel electrophoresis, 10 µl from reaction mixture was loaded in wells with negative control, positive control and DNA ladder. Reaction for bacterial isolate represented in figure 7) confirmed that the bacterial isolate was *P. aeruginosa* Dw7. These result agreed with the identification results obtained from the classical identification of the isolate. Dw7 isolate was definitively identified as *P aeruginosa* Dw7.

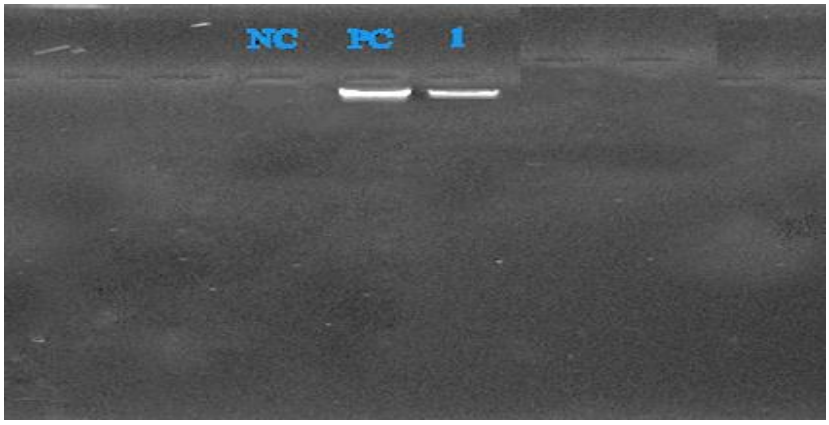

Figure 6: Agarose gel electrophoresis analysis of genomic DNA. Lane 1: extracted gDNA from *Pseudomonas aeruginosa* Dw7, PC: positive control, NC: negative control.

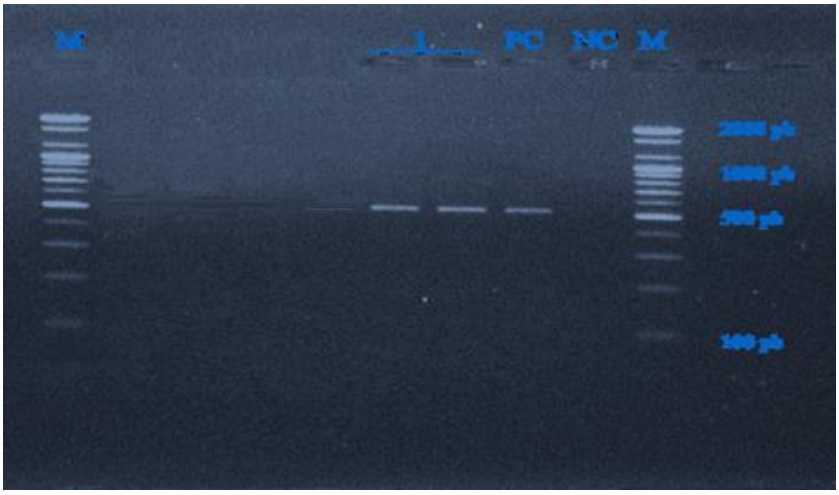

Figure 7: Agarose gel electrophoresis analysis of PCR reaction for DNA extracted from pure culture of *Pseudomonas aeruginosa* Dw7.Lane M DNA ladder; NC :negative control PC: positive control, Lane 1:DNAfrom selected isolate(duplicate).

Many *pseudomonads* belonging to the rRNA homology group have the ability to produce PHA polymers from a number of different substrates (Huisman et al. 1989).

Optimum conditions for PHB production

Effect of incubation time on PHB production

The PHB production of *P aeruginosa* Dw7 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 1.74 g/l of dry cell weight yielding (35.5%) of PHB which is agree with Singh et al ,(2009)and, thereafter, got reduced (0.50 g/L after 72 h) (Figure 8). 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 (Yuksekdag et al, 2004).

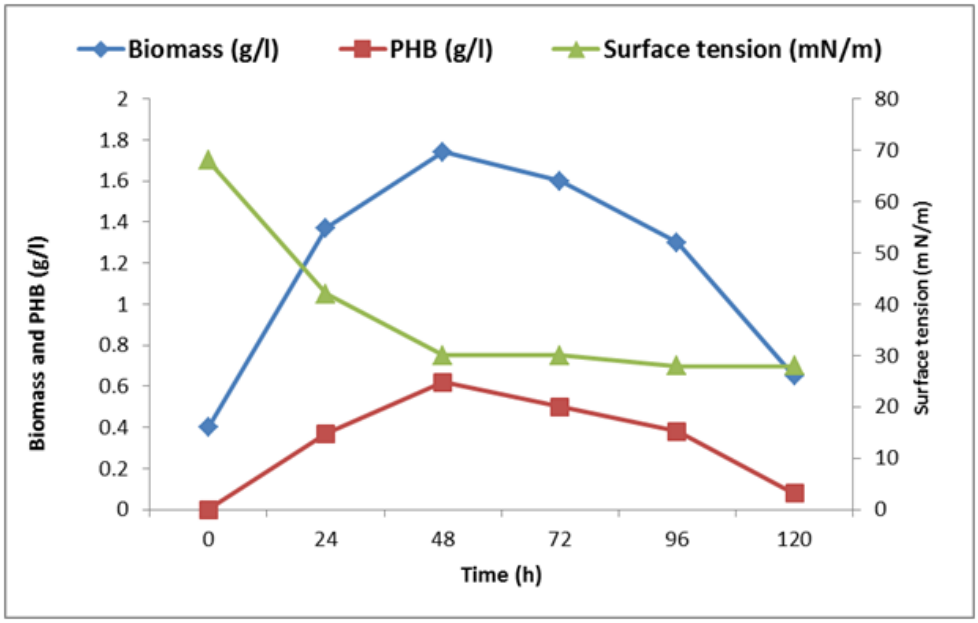

Figure 8: Effect of different incubation period on growth and PHB production by *P. aeruginosa* DW7.

Reduction in surface tension was observed every day for a period of five days. *P. aeruginosa* has been reported for the production of biosurfactant when growing in water immiscible substrate (Hori *et* al, 2002; Haba *et al*, 2007). Studies were carried out to obtain the production of mcl-PHB from *Pseudomonas* species. When grown on glycerol and glucose as carbon sources both *P. putida* and *P. aeruginosa* were able to produce the polymer. *P. putida* accumulated polymer to a maximum of 11.60 % DCW in 48 hrs. *P. aeruginosa* on the other hand accumulated polymer to a maximum of 11.40 % DCW in 48 hrs. The results showed that 48 hours was significantly the best incubation time for PHB production at ($P \leq 0.05$) than other periods of incubation values.

Effect of pH on PHB production

From the result it is clear that pH 7 was favorable for PHB production by *P aeruginosa* Dw7 in oil waste containing medium. Figure (9) shows that the optimal pH for cell growth and PHB production was 7 resulting the highest CDW of 1.76 g/L and PHB production of 0.63 g/L .Deletion of surface tension to 28mN/m and 35.8% yield of PHB occurred at 7. It was found that a significant relationship existed between dry cell weight and PHB production .The current observation was in agreement with Bonartseva *et al*, (1994). The results referred that the best pH value was 7 for PHB and biomass production after 48 h of incubation at 30°C, which was significantly ($P \leq 0.05$) the best value compared with others pH .

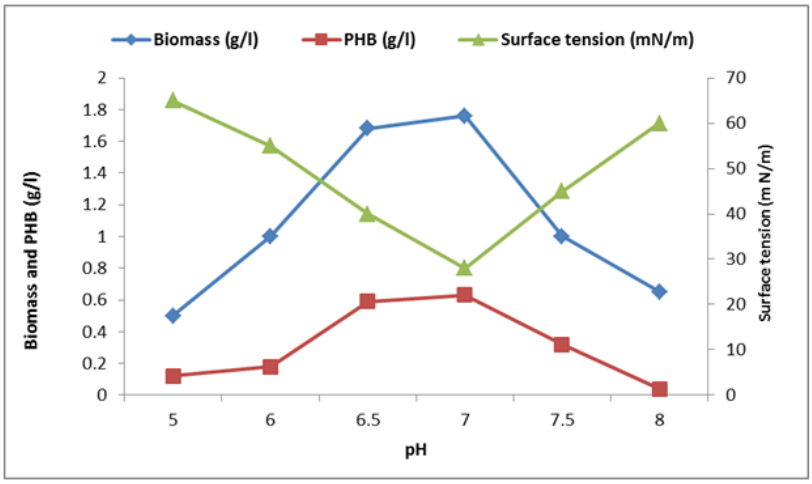

Figure (9) Effect of various pH values on PHB production by *P.aeruginosa* DW7.

Effect of incubation temperature on PHB production

Maximum biomass and PHB production of 1.78 and 0.65 g/L by *P.seudomonas* isolate were recorded at 30°C after 48 h of incubation which was significantly the best temperature for PHB and biomass production (P≤ 0.05) compared with other temperature values. 36.5 % of PHB yield accompanied with lowering of surface tension at 28mN/m after 48 h of incubation at 30° C. Species of *Pseudomonas* grow well at 28–30°C, which is more appropriate for most of the species (Aagot *et al*., 2001). The increase of temperature beyond 35°C has negative impact on PHB production (Figure 10). The decrease in PHB production at high temperature could be due to low PHB polymerase enzyme activity (Korotova *et al*, 2001). 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. The experimental results showed that the highest CDW and PHB production accompanied with decreasing of surface tension.

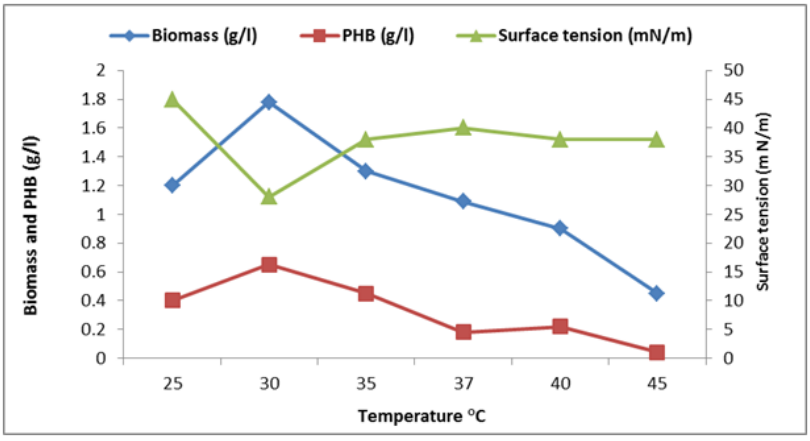

Figure 10: Effect of various temperatures on PHB production by *Pseudomonas* aeruginosa DW7.

Effect of oil source on PHB production

Different carbon types affect PHB production was evaluated using various carbon sources including (olive oil, sunflower oil, sesame oil corn oil and glycerol).The oils were sterilized separately and added to MSM at a final concentration of 1% (v/v), to evaluate their effects on PHB accumulation in bacterial cells. The cultivation was performed at a temperature of 30°C, pH7 and an agitation rate of 150 rpm under aerobic conditions. However, all the carbon sources enhanced PHB synthesis in different ratio; corn oil waste was the best to support PHB synthesis. Compounds of interest in waste oils are foreign food residues, readily available nitrogen compounds, peroxides and short-chain compound formed during heating. Residual carbohydrates, proteins and fats from foods, available nitrogen compounds, peroxides and heat-degradation products could also be metabolized and may have contributed to increased PHB production (Rehm *et al*. 1998). The results from the fatty acid analysis are indicating that saturated fatty acids lead to build-up of more energy-rich PHB in bacteria than with unsaturated fatty acids. Traditionally the PHB synthesis mechanism has been described as a *de novo* route in which fatty acids are transformed into acetyl-CoA by β-oxidation cycles (Rehm *et al*. 1998). It is possible that saturated fatty acids are more easily converted to acetyl-CoA than unsaturated fatty acids. Isolation of new bacterial isolate capable of utilizing the cheap carbon source is essential to reduce the cost is the major concern at industrial level (Sharmila *et al*, 2011).

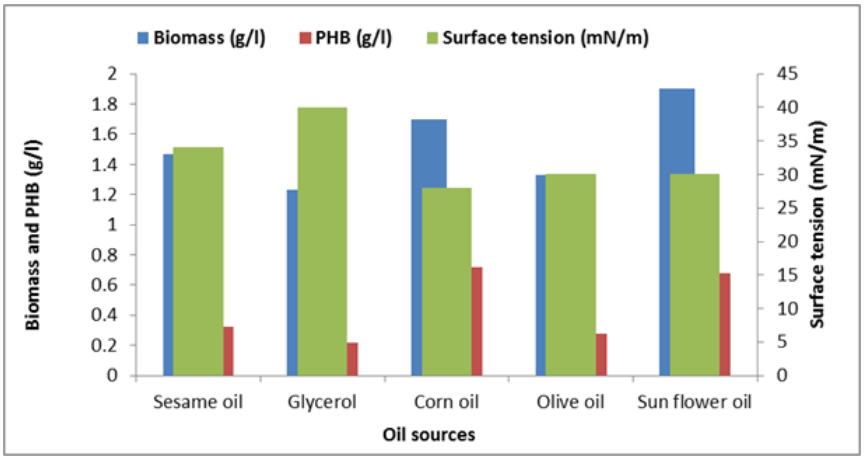

Figure 11: Effects of various oil sources on biomass and on PHB production by *P aeruginosa*Dw7.

Pseudomonas sp. synthesized only MCL-PHA, comprising C6 to C16, from the various waste vegetable oil which is very useful substrate for the production of PHB from bacteria, since it has a similar composition (glycerol, palmitic acid (16:0), oleic acid (18:1), and

linoleic acid (C18:2) to corn oil (Van Dyk *et al*,1995). It has been cleared from the results from the current study that the maximum PHB was produced with cooking corn oil as a carbon source, it was observed that PHB content reached 0.72 g/l with 1.72 g/l of biomass (fig 11). It was found from statistical analysis results that corn oil was significantly P≤0.05 the best carbon source for PHB production with maximum yield 42.4 % of PHB with lowering of surface tension to 28 Mn/m. Vegetable oils have been found to be possible substrates in the production of PHAs (Alias and Tan 2005; Kahar *et al*. 2004). 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 (Reddy et *al*, 2009). Song and colleagues, (2008) reported the utilization of WCO from corn oil to produce medium chain length PHA (mcl- PHA) by *Pseudomonas* sp. strain DR2. *Pseudomonas* sp. strain DR2 is capable of accumulating 37.3% mcl-PHA. Waste streams from oil mills or used oils, which are even cheaper than purified oils can be used too (Fernández *et al*. 2005; Mumtaz *et al*. 2010).

Since fermentations produce more PHB when waste frying oil is used (compared to pure vegetable oil) the compositional changes during the frying process play an important role in the bacterial conversion to PHB. 98-99% of the components of pure oils are fatty acids (Tabee 2008). During frying complex components are formed in the oil and residue of food products are added to the oil. It is known that many chemical processes occur during the prolonged heating of vegetable oils under influence or air and moisture. The dominant process during heat treatment of oils is oxidation of fatty acids under influence of oxygen (Boskou and Elmadfa 1999). The mcl-PHA produced by *Pseudomonas* sp. strain DR2 is gluey and rubbery (Song *et al*., 2008). The ability of various *Pseudomonas sp* to convert oil and fats into mcl-PHA has been tested by Rao *et al.* (2010).

Effect of nitrogen source on PHB production

Various nitrogen sources included: (Ammonium chloride, Ammonium sulphate, Peptone and Yeast extract) were used to evaluate the effect of nitrogen source on cell growth and PHB accumulation. Data presented in figure (12) showed that out of different nitrogen sources tested ammonium sulphate was found to be optimum for maximum PHB production. The statistical results showed that *Ammonium sulphate* was significantly the best nitrogen source in PHB and biomass production at P≤0.05 which was 1.4 and 2.8 g/l respectively yielding of 48.3% of PHB. Conversely, although all nitrogen sources positively affected PHB synthesis,

Ammonium sulphate had the largest effect followed by ammonium chloride. Hence, figure (12) shows that the medium with corn oil and ammonium sulphate as the carbon and nitrogen source, respectively, apparently had a positive effect on PHB production. Peptone was found to be the least supporter of PHB production. It was reported that ammonium sulphate could induce the production of PHB (Nair *et al*, 2007).

These results are in agreement with the results obtained by Khanna and Srivastava, (2005) who also observed the highest PHB production (2.2g/100 ml) by gram negative bacteria on MSM medium supplemented with ammonium sulphate. Raje and Srivastav, (1998) also worked on the accumulation of PHB by bacterial isolates with ammonium sulphate. Beaulieu *et al.* (1995) in a similar study reported that ammonium sulfate was the best nitrogen source for PHB production in synthetic medium containing different carbon sources by bacterial isolates.

Effect of nitrogen source concentration on PHB accumulation

As shown in figure (13) the maximum PHB production of 1.35g/l, and biomass was 2.7 g/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 reported work by Basak *et al*, (2011). The statistical results showed that 1g/l was significantly $P \leq 0.05$ the best concentration to produce PHB with percentage of 51%, while no significant differences appeared between other less concentrations.

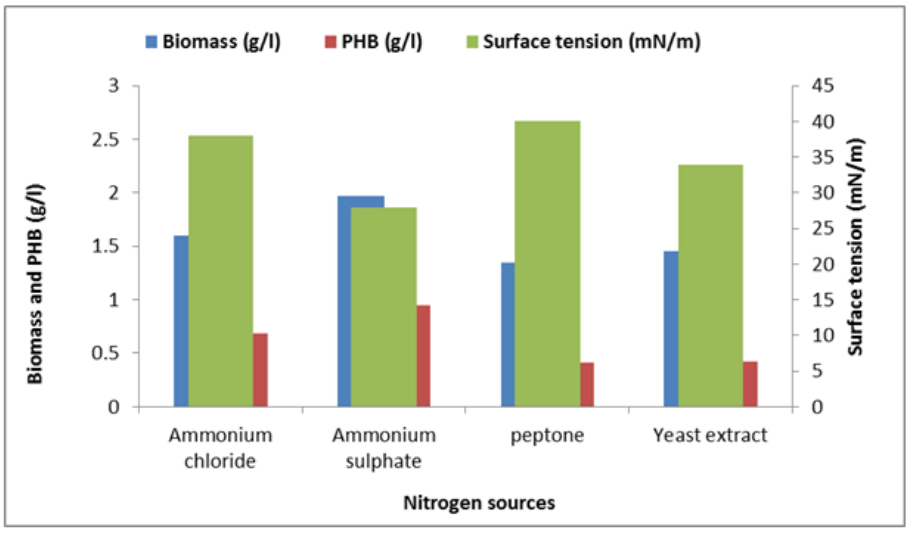

Figure 12: Effect of Nitrogen sources on biomass and PHB Production by *P aeruginosa* DW7.

Koutinas *et al*. (2007) reported that ammonium sulfate is the best nitrogen source at 1 g/l concentration for the PHB production by bacterial isolates.

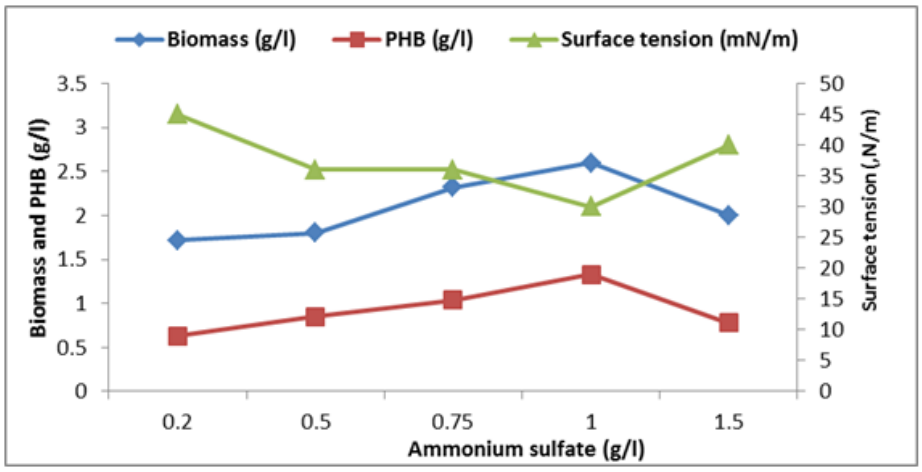

Figure 13: Influence of different concentration of ammonium sulfate on PHB production by *P aeruginosa* DW7.

Effect of oil concentration on PHB accumulation

The results shown that highest biomass and production of PHB were (1.98 and 3.7 g/l) respectively and accumulating 53 % of PHB was observed with corn oil concentration of 3%, resulting decreasing of surface tension to 28 mN/m (fig 14), which was significantly the best concentration for PHB and biomass production at ($P \leq 0.05$) after 48 h of incubation than other concentrations values for *P.aeruginosa* Dw7.

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 Khandpur *et al*, (2012), which referred to that P *aeruginosa* growth with increasing the concentration of oil more than 3% led to the death of microorganism and decreasing of PHB production.

Different strains of *Pseudomonas* have been reported for PHB production using oily substrates. As the number of bacterial cells increased the requirement of carbon source for growth increased and production of biosurfactant increases resulted in access of bacteria to oil carbon source.

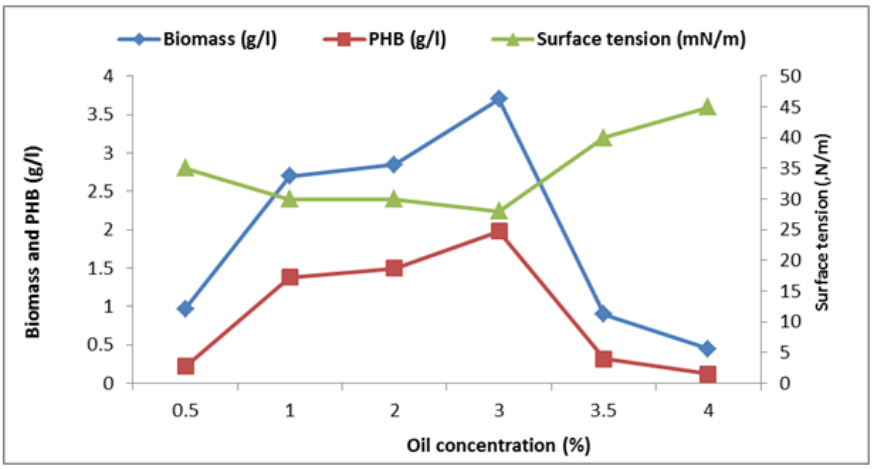

Figure 14: Influence of different concentration of corn oil on PHB yield by *Pseudomonas aeruginosa* Dw7.

Effect of C: N ratio on PHB production

In living organisms, carbon (C) requirements are generally larger than nitrogen (N) requirements. The balance of these elements (C/N) determines how bacteria use an organic material (Chanprateep *et al*, 2008). To enhance PHB production, the C/N ratios of (10:1, 20:1, 25:1, 30:1and 35:1) in MSM were compared to determine the optimal ratio. Figure (15) shows that a C/N ratio of 30:1 obtained the highest values for CDW (4.34 g/L), PHB production (2.30 g/L), and PHB content (54%) of PHB yield. These results suggest that a C/N ratio of 30:1 is optimal for the accumulation of PHB by *p.aerugiosa Dw7*. However, at ratios above 10:1, PHB production consistently increased up to a ratio of 35:1, at which point the C/N ratio decreased. The PHB production and content by *p aerugiosa* Dw7 were decreased to 1.24 g/L and 40% when C/N ratio increased to 35/1 (fig 15). In addition, PHB production by *p .aerugiosa* Dw7 also decreased at C/N ratios higher or lower than 30:1.

These results are attributable to high concentration of carbon source that involves the substrate, and *p .aerugiosa* Dw7 is inhibited, reducing CDW, PHB content and PHB production. Additionally, high C/N and low C/N ratios might affect the physiological conditions of the microorganisms, including cell proliferation and polymerization of PHB (Chanprateep *et al*, 2008). Therefore, the optimal C/N ratio of 30: 1 was used in subsequent experiments.

As shown in (figure 15), 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 Rao *et al.* (2010), which also indicated the ability of various *Pseudomonas sp* to convert oil and fats into *mcl*-PHA. All experiments were performed in duplicate to check the reproducibility of PHB.

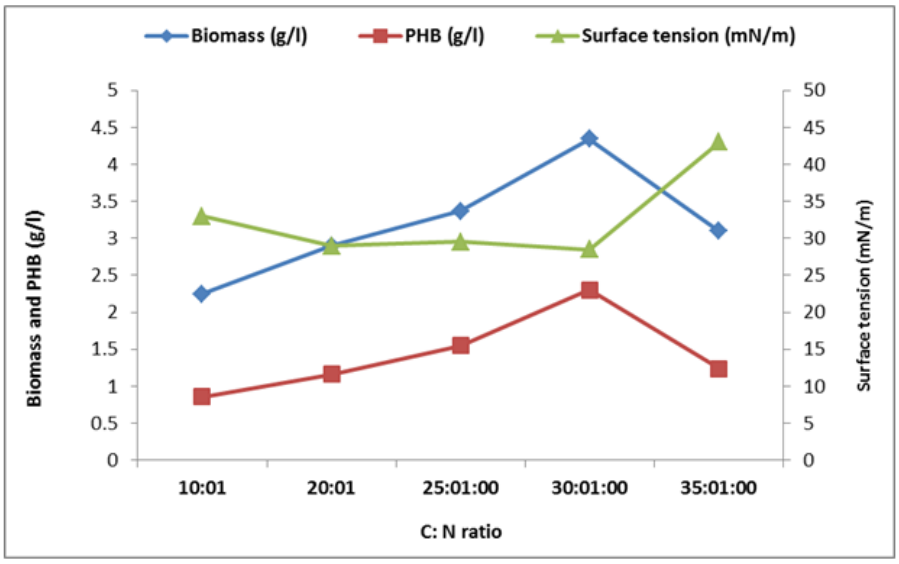

Figure 15: Influence of different C: N ratio on PHB production by *P. aeruginosa* Dw7.

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 aeruginosa* Dw7can 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 53.2% 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.

REFERENCES

1. Aagot, N., O. Nybroe, P. Nielsen, and K. Johnsen. An altered Pseudomonas diversity is recovered from soil by using nutrient-poor Pseudomonas-selective soil extract media. Appl. Environ. Microbiol, 2001; 67: 5233–5239.
2. Alias Z, Tan IKP. Isolation of palm oil-utilising, polyhydroxyalkanoate (PHA)-producing bacteria by an enrichment technique. Bioresour Technol, 2005; 96: 1229–1234.

3. Arnold L., J.Demain, and Davis, Polyhydroxyalkanoates. In: Manual of Microbiology and Biotechnology. Washington, American Society of Microbiology, 1999; 2: 616-627. ISBN-13: 9781555811280.
4. Ayub.ND, Pettinari MJ, Ruiz JA, Lopez NI. A Polyhydroxybutyrate-producing *Pseudomonas* sp. isolated from Antarctic environments with high stress resistance. Curr. Microbiol, 2004; 49: 170–174.
5. Bänziger, S., Tobler, N. The formation of reserve polymers in *Bacillus megaterium*. Microb. Ecology course, 2009; 1-4.
6. Basak B, Ince O, Artan N, Effect of nitrogen limitation on enrichment of activated sludge for PHA production. Bioprocess Biosyst Eng, 2011; 34: 1007–1016.
7. Beaulieu M, Beaulieu Y, Melinard J, Pandian S, Goulet J. Influence of ammonium salts and cane molasses on growth of *Alcaligenes eutrophus* and production of polyhydroxybutyrate. *Appl Environ Microbiol*, 1995; 611: 165.
8. Belma Aslim, Zehra Nur Yukesekdag, Yavuz Beyatli. Determination of PHB growth quantities of certain Bacillus species isolated from soil, 2002.
9. Bonartseva, G.A., Myshkina, V.L., Zagreba, E.D. Poly-bhydroxybutyrate content in cells of various Rhizobium species during growth with different carbon and nitrogen sources. Microbiol, 1994; 63(1): 45-48.
10. Boskou D and Elmadfa I. Frying of Food: Oxidation, Nutrient and Non-Nutrient Antioxidants, Biologically Active Compounds and High Temperatures. CRC Press, New York, 1999.
11. Burdon KL, Fatty materials in bacteria and fungi revealed by staining dried, fixed slide preparations. J. Bacteriol, 1946; 52: 665–678.
12. Chanprateep S, Abe N, Shimizu H, Yamane T, Shioya S. Characterization of new isolated *Ralstonia eutropha* strain A-04 and kinetic study of biodegradable copolyester poly (3-hydroxybutyrate-co-4-hydroxybutyrate) production. Journal of Industrial Microbiology & Biotechnology, 2008; 35(11): 1205–1215.
13. Chen, G.Q. and Wu, Q. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials, 2005a; 26: 6565–6578.
14. Doi, Y., Kawaguchi Y., Koyama N., Nakamura S., Hiramitsu M., Yoshida Y., Kimura H. Synthesis and degradation of polyhydroxyalkanoates in Alcaligenes eutrophus. FEMS Microbiol. Rev, 1992; 103: 103-108.

15. Du G, Yu J, Chen J, Lun S. Continuous production of poly-3- hydroxybutyrate by *Ralstonia eutropha* in a two stage culture system. J Biotechnol 88:59–65 biosynthesis pathways as a successful example. Macromol Bioscien, 2001; 1: 1–24.
16. Fernandez D, Rodriguez E, Bassas M, Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa* NCIB 40045: effect of culture conditions. Biochem Eng J, 2005; 26: 159–167.
17. Grothe E, Moo-Young M, Chisti Y, Fermentation optimization for the production of poly (β-hydroxybutyric acid) microbial thermoplastic. Enzyme and Microbial Technology, 1999; 25(1-2): 132–141.
18. Haba E, Vidal Mas J, Bassas M, Poly 3-(hydroxyalkanoates) produced from oily substrates by *Pseudomonas aeruginosa* 47T2 (NCBIM 40044): effect of nutrients and incubation temperature on polymer composition. Biochem Eng J, 2007; 35: 99–106.
19. Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams. Bergey's manual of determinative bacteriology, 9th ed. Lippincott Williams & Wilkins, Baltimore, MD, 1994.
20. Hori K, Marsudi S, Unno H. Simultaneous production of polyhydroxyalkanoates and rhamnolipids by *Pseudomonas aeruginosa*. Biotechnol Bioeng, 2002; 78: 699–707.
21. Huisman GW, Leeuw O de, Eggink G, Witholt B. Synthesis of poly (3-hydroxyalkanoates) is a common feature of fluorescent *pseudomonads.* Appl Environ Microbiol, 1989; 55: 1949–1954.
22. John, H., Ralph, A.(1961). Assay of poly-β-hydroxybutyric acid. J. Bacteriol., 2002; 82: 33–36.
23. Kahar P, Tsuge T, Taguchi K, Doi Y. High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. Polym Degrad Stab, 2004; 83: 79–86.
24. Khandpur P, Jabeen ET, Rohini KVL, Varaprasad Y. Study on production, extraction and analysis of polyhydroxyalkanoate (PHA) from bacterial 4. isolates. IOSR J Pharm Biol Sci IOSRJBPS, 2012; 1: 31–38.
25. Khanna S, Srivastava AK. Recent advances in microbial polyhydroxyalkanoates. Process Biochem, 2005; 40: 607–619.
26. Kim DY, Kim YB & Rhee YH, Evaluation of various carbon substrates for the biosynthesis of polyhydroxyalkanoates bearing functional groups by Pseudomonas putida. Int. J. Biol. Macromol, 2000; 28: 23-29.

27. Korotkova N, Lidstrom ME. Connection between poly-betahydroxybutyrate biosynthesis and growth on C_1 and C_2 compounds in the methylotroph *Methylobacterium extorquens* AM1. J Bacteriol, 2001; 183: 1038–1046.
28. Koutinas AA, Wang R, Webb C. Polyhydroxybutyrate production from a novel feedstock derived from a wheat-based biorefinery. Enzym Microbial Technol, 2007; 40: 1035–1044. doi: 10.1016/j.enzmictec.2006.08.002.
29. Kuniko, M., Y. Nakamura and Y. Doi, New bacterial coployestras produced in Alcaligenes eutrophus from organic acids. Polymer Commun, 1988; 29: 174-176.
30. Law J. H. and R. A. Slepeky. Assay of poly-B-hydroxybutyric acid. J. Bacteriol, 1961; 82: 32-36.
31. Lopez NI, Pettinari MJ, Stackebrandt E., *Pseudomonas extremaustralis* sp. nova, a poly (3-hydroxybutyrate) producer isolated from an Antarctic environment. Curr Microbiol, 2009; 59: 514–519.
32. Muheim, A. and Lerch, K. Towards a high-yield bioconversion of ferulic acid to vanillin. Appl Microbiol Biotechnol, 1999; 51: 456–461.
33. Mumtaz T, Yahaya NA, Abd-Aziz S, Rahman NA, Yee PL, Shirai Y, Hassan MA, J Cleaner Prod, 2010; 1393–1402.
34. Nair LS, Laurencin CT. Biodegradable polymers as biomaterials. Prog Polym Sci, 2007; 32: 762–798.
35. Ostle A G and Holt J G. Nile blue A as a fluorescent stain for polyhydroxybutyric acid. Appl.Environ Microbiol, 1982; 44: 23841.
36. Pawar SN, Edgar KJ, Microbial production of poly-3-hydroxybutyric acid from soybean oil by *Bacillus subtilis*. Biomaterials, 2012; 33: 3279.
37. Poirier Y, Nawrath C, Somerville C, Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. *Biotechnology*, 1995; 13: 142-150.
38. Raje P, Srivastava AK Updated mathematical model fed-batch strategies for poly-β-hydroxybutyrate (PHB) ptoduction by *Alkaligenes etruphus*. Bioresour Technol, 1998; 64: 185–192.
39. Ramachandran H, Abdullah AA. Isolation of PHA-producing bacteria from Malaysian Environment. Proceedings of the 7th IMT-GT UNINET and The3rd International PSUUNS Conferences on Bioscience, 2010; 178–17.

40. Rau.U, "Biosynthesis and biocompatibility of poly(3-hydroxybutyrate-co-4-hydroxybutyrat e) produced by *Cupriavidus necator* from spent palm oil", Biochemical Engineering Journal, Elsevier, Amsterdam, NL, 2010; 49(1,15): 13-20.
41. Reddy, S.V., Thirumala, M. and Mahmood, S.K. Production of PHB and P (3HB-co-3HV) biopolymers by *Bacillus megaterium* strain OU303A isolated from municipal sewage sludge. World J Microbiol Biotechnol, 2009; 25: 391–397.
42. Reema Aslam1, Faiza Saleem1 and Yasar Saleem. Biotechnological production of polyhydroxybutyrate (PHB) from *Enterobacter aerogenes.* Global journal of multidisciplinary and applied sciences, 2013; 1.1/1-8.
43. Rehm BHA, Kruger N, Steinbüchel A. New Metabolic Link between Fatty Acid de Novo Synthesis and Polyhydroxyalkanoic Acid Synthesis. J Biol Chem, 1998; 273: 24044–24051.
44. Rehm, B.H.A. Steinbüchel A Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis Int J Biol Macromol, 1999; 25: 3-19.
45. Sharmila T, Screening and characterisation of polyhydroxybutyrate producing bacteria from sugar industry effluents. World Journal of Science and Technology, 2011; 1(9): 22–7.
46. Singh G, Mittal A, Kumari A, Goel V, Aggarwal NK, Yadav A. Optimization of poly-β-hydroxybutyrate production from *Bacillus species*. Eur J Biol Scien, 2011; 3(4): 112–116.
47. Singh M, Patel SKS, Kalia VC, *Bacillus subtilis* as potential producer for polyhydroxyalkanoates. Microb Cell Fact, 2009; 8: 38.
48. Slepecky RA, Law JH, Synthesis and degradation of poly-3-hydroxybutyric acid in connection with sporulation of *Bacillus megaterium*. J Bacteriol, 1961; 82: 37–42.
49. Song JH, Jeon CO, Choi MH, Polyhydroxyalkanoate (PHA) production using waste vegetable oil by *Pseudomonas* sp. strain DR2. J Microbiol Biotechnol, 2008; 18: 1408–1415.
50. Spiekermann P, Rehm BH, Kalscheuer R, Baumeister D, Steinbüchel A. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch Microbiol, 1999; 171(2): 73–80.
51. Steinbüchel A. Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxyalkanoats, 2001.

52. Sujatha, K. and Shenbagarathai, R. A study on medium chain length-polyhydroxyalkanoate accumulation in *Escherichia coli* harbouring phaC1 gene of indigenous *Pseudomonas* sp. *LDC-5*. *Lett Appl Microbiol*, 2006; 43: 607–614.
53. Tabandeh, F. and Vasheghani, V. Biosynthesis of Poly (3-hydroxy butytyrate) as a biodegradable polymer, *Iranian J. Pol*, 2003; 12: 37.
54. Tabee E. Vegetable Oils and Fried Potato Products. PhD thesis. 14. Swedish University of Agricultural Sciences, Uppsala; Lipid and Phytosterol Oxidation, 2008.
55. Vedaraman.N Venkatesh. N. Production of surfactin by *bacillus subtilis* mtcc 2423 from waste frying oils.Braz. J. Chem. Eng, 2011; 28(2).
56. Van Dyk "Synergistic Induction of the Heat Shock Response in *Echerichia coli* by Simultaneous Treatment with Chemical Inducers," J. Bacteriol, 1995; 177: 6001-6004.
57. Yuksedag ZN, Aslim B, Beyadliti X, Mercan N. Effect of carbon and nitrogen sources and incubation time on poly-β-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12. African Journal of Biotechnology, 2004; 3(1): 63-66.