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Research Paper

Semi-scale production of PHAs from waste frying oil by *Pseudomonas fluorescens* S48

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Abstract

The present study aimed at developing a strategy to improve the volumetric production of PHAs by *Pseudomonas fluorescens* S48 using waste frying oil (WFO) as the sole carbon source. For this purpose, several cultivations were set up to steadily improve nutrients supply to attain high cell density and high biopolymer productivity. The production of PHAs was examined in a 14 L bioreactor as one-stage batch, two-stage batch, and high-cell-density fed-batch cultures. The highest value of polymer content in one-stage bioreactor was obtained after 60 h (33.7%). Whereas, the two-stage batch culture increased the polymer content to 50.1% after 54 h. High-cell-density (0.64 g/L) at continuous feeding rate 0.55 mL/l/h of WFO recorded the highest polymer content after 54 h (55.34%). Semi-scale application (10 L working volume) increased the polymer content in one-stage batch, two-stage batch and high cell density fed-batch cultures by about 12.3%, 5.8% and 11.3%, respectively, as compared with that obtained in 2 L fermentation culture. Six different methods for biopolymer extraction were done to investigate their efficiency for optimum polymer recovery. The maximum efficiency of solvent recovery of PHA was attained by chloroform–hypochlorite dispersion extraction. Gas chromatography (GC) analysis of biopolymer produced by *Pseudomonas fluorescens* S48 indicated that it solely composed of 3-hydrobutyric acid (98.7%). A bioplastic film was prepared from the obtained PHB. The isolate studied shares the same identical sequence, which is nearly the complete 16S rRNA gene. The identity of this sequence to the closest pseudomonads strains is about 98-99%. It was probably closely related to support another meaningful parsiomony analysis and construction of a phylogenetic tree. The isolate is so close to Egyptian strain named EG 639838.

Key words: *Pseudomonas fluorescens* S48, biopolymer, PHAs, batch, two-stage batch, high-cell-density fed batch, bioreactor, recovery.

Introduction

Plastic materials have become an integral part of contemporary life because of many desirable properties including durability and resistance to degradation. The non-degradable plastics accumulate in the environment with an increasing rate. Recently, the problems concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics, which must still retain the desired physical and chemical properties of conventional synthetic plastics. One of the biodegradable plastic materials under development includes polyhydroxyalkanoates (PHAs) (Slater *et al.*, 1998). However, one of the problems facing the development of biodegradable polymers as substitutes for conventional plastics is their high price compared with petrochemical derived plastics. Therefore, many efforts have been made to reach low-cost PHA production processes (Verlinden *et al.*, 2007). Different potent strains, which grow to high-cell-density in cheap cultivation media and accumulating high

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PHA content via cost-efficient fermentation process and enabling easy recovery methods as well, were described in numerous investigations and patented processes (Kinoshita *et al.*, 2005). Since one of the main obstacles that hinder an economic feasible production of PHA is the cost of carbon substrate (25% to 28% of total production cost). However, the abundance of inexpensive waste materials from agriculture, industry or agro-industrial such as waste frying oil present them as an alternative cheap substrates for microbial fermentations, in particular for the production of microbial polyesters (Da Silva *et al.*, 2009).

Either fed-batch or continuous cultivation techniques can be used for the production of PHA with high productivity. Fed-batch culture has been the most popular culture system to achieve a high cell density and PHB content (Kim *et al.*, 1994; Lee and Yoo, 1994; Kim *et al.*, 2003; Valappil *et al.*, 2007). In this system, it is essential to maintain optimal concentration of nutrients during fermentation. This can be achieved by using various feeding strategies (Lee *et al.*, 1997; Wang and Lee, 1997; Abdel Hafez *et al.*, 2009; Lopez-Cuellar *et al.*, 2011).

In view of these facts, the current investigation is aimed to study the semi-scale production of PHAs from waste frying oil using different fermentation techniques as batch and two-stage batch and high-cell-density fed-batch cultures in 14 L bioreactor.

Materials and Methods

Microorganism and culture conditions

Pseudomonas fluorescens S48 was used in this investigation for accumulating PHAs from waste frying oil (WFO) (Gamal *et al.*, 2011, 2012). The bacterial culture was maintained by transferring at regular intervals on nutrient agar slants. Slants were kept at 4 °C until used. For PHAs 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, $(NH_4)_6Mo_7O_{24}$ 0.1 and 35% HCl 10 mL) and 10 mL waste frying oil as the sole carbon source. The pH was adjusted to 7.0 before sterilization.

Cultivation was done in a Bioflo 3000 14-liter fermenter (New Brunswick Scientific Co., Inc. Edison, NJ 08818-4005 USA), with a working volume of 10 liter (24 cm inner diameter and 45 cm height) and a d/D value (relation of stirrer diameter to vessel diameter) of 0.286 was used for cultivation. The bioreactor was equipped with three stirrers, each containing six paddles and a Fundafoam mechanical destroyer. In addition, sterilizable probes were inserted into ports to measure dissolved oxygen, pH and temperature. The operation were controlled and recorded. Cultivation was carried out at 30 °C and 350 mM/L/h oxygen transfer rate obtained through powerful fermentation agitation motor and 6-bladed Rushton impellers dissolve 20% air saturation in the medium, which was controlled by agitation at 500 rpm and aeration rate 1 vvm. Unless otherwise stated, the pH of medium was adjusted at 7.0 ± 1 by the controlled addition of NaOH (5 N) or 4 N HCl. The operations were controlled and recorded by a digital control unit (DCU) in combination with the software package. Samples of 10-20 mL were withdrawn from the culture fluid for analytical purposes.

Bioreactor as a one-stage batch culture

In this experiment the fermentation vessel (bioreactor) containing 9800 mL productive medium without WFO was autoclaved at 121 °C for 40 min. WFO (1%) was added after sterilization. The fermentation medium was inoculated with 1% standard inoculum of the bacterial strain. The standard inoculum was prepared in a conical flask (250 mL) containing 100 mL of nutrient broth medium inoculated with a loop of *Ps. fluorescens* S48 and incubated at 28-30 °C with shaking (300 rpm) for 24 h prior to inoculation (5×10^8 cfu / mL).

The final working volume was 10 liter. Initial pH was adjusted to 7 ± 0.1 which was not controlled during the fermentation period. Temperature, dissolved O_2 and speed of agitation were kept at 30 °C, 20% of air saturation and 500 rpm, respectively, during cultivation.

During fermentation, samples (10-20 mL) were withdrawn from the culture (fermentation vessel) periodically. The samples were centrifuged at 15000 x*g* for 4 min at 4 °C. The sediment (biomass) was washed twice with distilled water, and then dried at 70 °C to constant weight.

Bioreactor as a two-stage batch culture

The production of PHAs was carried out in two-stage cultivation. In the first stage, two sterile conical flasks (1000 mL) each containing 400 mL nutrient medium was inoculated with 10 mL standard inoculum of the bacterial strain, then incubated at 30 °C for 24 h on rotary shaker (150 rpm) in order to get a luxurious growth. Then the culture fluid was centrifuged at 15000 x*g* for 4 min at 4 °C and the bacterial cells were collected and suspended in additional sterile productive medium to inoculate the bioreactor vessel to give a final working volume of 10 L sterile productive medium. The cultivation conditions and microbiological determinations were done as mentioned before.

Bioreactor as high-cell-density fed-batch culture

This experiment of fed-batch culture was constructed to study the effect of washed high-cell-densities (0.64 g/L) of *Ps. fluorescens* S48 on PHAs production. WFO was fed continuously at 0.55 mL/L/h during the first 18 h of cultivation (according to the obtained results from Gamal *et al.* (2012) for the semi-scale production. Samples (10-20 mL) were taken from the growing culture periodically under

aseptic conditions to determine the cell dry weight, PHAs produced and residual carbon.

In all cultivations, polymer in samples was generally precipitated and determined as dry weight and PHAs content was determined as dry weight percent. The extraction of PHAs was implemented by the chloroform-sodium hypochlorite method (Hahn *et al.*, 1994). Total organic carbon was determined in supernatant according to Walinga *et al.* (1992). Polymer content (%) and productivity (g/L/h) were calculated according to Lee and Choi (1998) and Lee (1996), respectively. The parameters of polymer yield (%), conversion coefficient (%) and carbon utilization efficiency (%) were calculated according to Ramadan *et al.* (1985).

PHAs recovery

Six different methods for bioplastic recovery were performed as the following:

Biopolymer recovery by commercial sodium hypochlorite solution

The culture fluid was centrifuged in polypropylene centrifuge tubes which had been previously washed thoroughly with ethanol and hot chloroform to remove plasticizers. The cell paste was resuspended in a volume of commercial sodium hypochlorite solution (clorox or equivalent) equal to the original volume of medium. After 1 hr at 37 °C the lipid granules were centrifuged, washed with water, and then washed with acetone and alcohol. Finally, the polymer was dissolved by extraction with three small portions of boiling chloroform, the chloroform solution was filtered, and the filtrate was used for biopolymer assay (Law and Slepecky, 1961).

Biopolymer recovery by using dispersions of sodium hypochlorite and chloroform

A 1 g portion of freeze-dried cells was treated with a dispersion containing 50 mL of chloroform and 50 mL of a diluted sodium hypochlorite solution (3% vol/vol). After the cells were treated at 30 °C for 1 h, the mixture was centrifuged at 4000 x*g* for 10 min, which resulted in three separate phases. The upper phase was a hypochlorite solution, the middle phase contained non-PHB cell material and undisrupted cells, and the bottom phase was chloroform containing PHB. The upper phase was removed first with a pipette, and the middle phase was separated by filtration from the chloroform phase. Finally, bottom phase was chloroform containing PHB. PHB was recovered from the chloroform phase by nonsolvent precipitation (mixture of methanol and water 7:3, vol/vol) five times the volume of chloroform and filtration (Hahn *et al.*, 1994).

Biopolymer recovery by using acetone and chloroform

Freeze-dried cell powder was washed with hot acetone for 20 min. After drying, the cell powder was mixed with 50 volumes of chloroform for 48 h at 30 °C. The clear polymer solution was recovered by centrifugation to remove the majority of the non-PHB cell material; this was followed by polishing filtration. Finally, pure biopolymer was obtained by no solvent precipitation (five times the volume of chloroform) and filtration. The nonsolvent used was a mixture of methanol and water 7:3 (vol/vol) (Hahn *et al.*, 1995).

Biopolymer recovery by sodium hypochlorite

A sodium hypochlorite solution was diluted with distilled water to give concentrations 3% (vol/vol). The biomass was mixed with a hypochlorite solution, Biopolymer granules were separated from the aqueous fraction containing cell debris by centrifugation. The Polymer recovered was rinsed with distilled water, centrifuged again, and then rinsed with acetone. 1% (wt/vol) biomass suspension was treated for 1 h at 30 °C (Hahn *et al.*, 1995).

Biopolymer recovery by using chloroform

Lyophilized cell pellets were ground in a mortar and the resulting powder was extracted with chloroform for 4 h at 50 °C. The PHA-containing chloroform phase was concentrated and extracted once with water to remove residual solid particles. The organic phase was evaporated to dryness and the resulting crude extract preserved for further analyses. Purified PHAs were obtained by repeated precipitations in 10 volumes of cold methanol (Simon-Colin *et al.*, 2008).

Biopolymer recovery by using sodium dodecyl sulfate (SDS)

The cells were harvested and treated with 10% SDS at 100 °C for 20 min. After centrifugation, the pellets were washed, dried and extracted with chloroform at 60 °C for 1 h. The non-PHB cell matter was removed by filtration and the dissolved PHB was separated from chloroform by evaporation, washed twice with methanol, filtered out and dried at 60-70 °C (Jiang *et al.*, 2008).

GC analysis of biopolymer composition

Composition of PHA was determined by GC as described by Mumtaz *et al.* (2009).

Preparation of a biopolymer film

Totally 250 mg of PHB was dissolved in 28 mL chloroform. The solution was evenly distributed into 5 petri dishes. The dishes were maintained at 30 °C to allow complete evaporation of chloroform. The evaporation of solvent resulted in formation of PHB films in the petri dishes. Vacuum drying was further applied to completely remove any possible solvent remained in the films (Kai *et al.*, 2003).

DNA extraction and PCR amplification

The bacteria were grown in nutrient broth on a rotary shaker (120 rpm) at 20 °C for 24 h. Bacterial Genomic

DNA Mini-Prep Kit (Axygen cat. No. V110440-05) was used to isolate DNA as advised by the manufacturer. The universal 16S primers used were: F1 5' AGAGTTT(G/C)ATCCTGGCTCAG 3' R1 5' ACGG(A/C)TACCTTGTTACGACTT 3'

Primers were checked for specificity using the PROBE CHECK function of the Ribosomal Database and the BLAST search facility at the National Center for Biotechnology Information. DNA amplification was conducted on pure 2 to 3 µL DNA sample with about 150 ng of DNA per 1 µL of sample in a perkin Elmer 2400 (Nowalk, CT) thermocycler. The F1 and R1 primers amplifying the PCR reactions were added with a final volume of 100 µL with 0.2 µM of each primer, 2.0 mM $MgCl_2$, 200 µM dNTPs and 2.5 units of Maxima® Hot start Taq DNA Polymerase (Fermentas, www.fermentas.com) mixed in the 1X PCR buffer. DNA was amplified over 35 cycles of denaturation for 1 min at 94 °C, annealing at 55 °C for 1.5 min and extension at 72 °C for 2 min. After the last cycle, DNA was extended at 72 °C for 10 min. Amplification was confirmed by analyzing 5 µL of PCR reaction mixture on 1% agarose gel (Promega). The resulting PCR products sizes were ranged from 1450 to 1500 bp.

DNA sequencing

The PCR-product was purified using QIAquik PCR Purification Kit (Qiagen), and sequenced using automatic ABI 310 DNA Sequencer, Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer. The sequencing was performed in one direction using one of the previously described primers (Lane, 1991; Lane *et al.*, 1985). Sequencing data was analyzed by two different computer alignment programs, DNAStar (DNASTAR, Inc., USA) and Sequence Navigator (Perkin, Corp., USA).

Determination of phylogenetic relationships

The BLAST database (Altschul *et al.*, 1997) of National Center for Biotechnology Information was used to compare resolved sequence of the *Ps. fluorescens* S48 with known 16S rDNA sequences. Determination of phylogenetic relationships was analyzed by the program Phylogenetic Analysis megAlign of DNAstar version 7.0. The robustness of the internal branches of the trees was estimated by bootstrap analyses using 1000 replications in a heuristic search with random stepwise addition (111 replications) (Vinnere *et al.*, 2002). Bootstrap majority-rule (> 50%) consensus trees were obtained.

Statistical analysis

The collected data were statistically analyzed using IBM® SPSS® Statistics software (2011).

Results and discussion

Semi-scale production of PHAs

The successful production of PHAs in both shaken flasks (Gamal *et al.*, 2011) and 3 L laboratory fermentor (Gamal *et al.*, 2012) cultures generate the trials of semi-scale production of PHAs in 14 L laboratory fermentor cultures. Different fermentation techniques were applied in order to increase the PHAs yield using WFO as the sole carbon source.

One-stage batch culture

In batch culture, the cell mass increased gradually leading to record the maximum values of cell dry weight and polymer concentration (2.93 and 0.92 g/L, respectively) after 72 h of cultivation (Table 1). Whereas, the maximum PHAs content (33.7%) was obtained after 60 h

Table 1 - Semi-scale production of PHAs by *Ps. fluorescens* S48 on productive medium containing WFO (waste frying oil) as carbon source throughout 72 h at 30 °C using bioreactor as a batch culture.

Time (h)	Cell dry weight (g/L)	PHAs concentration (g/L)	Residual cells (g/L)	PHAs content (%)	PHAs productivity (g/L/h)	PHAs synthesis rate (g/g/h)	Final pH
0	0.15^{j}	0.00^{i}	0.15^{j}	0.00^{i}	0.000^{g}	0.000^{g}	7.1^{c}
6	0.17^{i}	0.00^{i}	0.17^{i}	0.00i	0.000^{g}	0.000^{g}	6.9^{d}
12	0.25^{h}	0.04^{h}	0.21^{h}	16.00^{h}	0.003^{f}	0.016^{a}	6.8^{e}
24	1.15^{g}	0.19^{g}	0.96^{g}	16.52^{g}	0.008^{e}	0.008^{e}	6.9^{d}
30	1.38^{f}	0.31^{f}	1.07^{f}	22.46^{f}	0.010^{d}	0.010^{c}	7.1^{c}
36	1.56^{e}	0.45^{e}	1.11^{e}	28.48^{e}	0.013^{b}	0.011^{b}	7.2^{b}
48	1.99^{d}	0.59^{d}	1.40^{d}	29.65^{d}	0.012^{c}	0.009^{d}	7.2^{b}
54	2.65^{c}	0.79^{c}	1.86^{b}	29.81^{c}	0.015^{a}	0.008^{e}	7.3^{a}
60	2.74^{b}	0.91^{b}	1.83^{c}	33.70^{a}	0.015^{a}	0.008^{e}	7.2^{b}
72	2.93^{a}	0.92^{a}	2.01^{a}	31.72^{b}	0.013^{b}	0.006^{f}	7.3^{a}

PHAs content (%) = Polymer concentration (g/L) / cell dry weight (g/L) x100.
PHAs productivity (g/L/h) = Polymer concentration (g/L) / fermentation time (h) (Lee, 1996).
PHAs synthesis rate (g/g/h) = Polymer concentration (g/L) / residual cells (g/L) / fermentation time (h) (Ramadan *et al.*, 1985).
Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5% level.

fermentation period. The corresponding figures of polymer synthesis rate and productivity were 0.008 g/g/h and 0.015 g/L/h, respectively. A slight decrease was observed on pH values during the first 12 h of growth (7.1 to 6.8), and then increased to 7.3 at the end of fermentation period. Data recorded in Table 2 revealed, that the highest values of utilized carbon (UC), PHAs yield and carbon utilization efficiency (CUE) being 6.12 g/L, 12.11% and 80.53%, respectively, were obtained after 72 h. Whereas, the highest conversion coefficient and specific production rate of PHAs were recorded after 60 and 24 h fermentation period (15.22% and 0.13 h^{-1}, respectively).

Two-stage batch culture

Data presented in Tables 3 and 4 indicated that the cell dry weight of *Ps. fluorescens* S48 and PHAs concentration were increased gradually during the second stage of cultivation (production stage) to record the maximum values (5.42 and 2.60 g/L) after 72 h in productive medium containing 1% WFO as the sole carbon source. The highest values of PHAs content (50.10%) was obtained after 54 h, whereas, the highest values of polymer synthesis rate and productivity were obtained after 36 h (0.025 g/g/h and 0.048 g/L, respectively). During fermentation period, a slight increase in pH value from 7.0 to 7.3 was recorded

Table 2 - Impact of time course on the PHA's parameters (CUE, yield, μ_p and CC) produced by *Ps. fluorescens* S48 on productive medium containing WFO as carbon source throughout 72 h at 30 °C using bioreactor as a batch culture.

Time (h)	Cell dry weight (g/L)	PHAs concentration (g/L)	Carbon concentration (g/L)	Utilized carbon (g/L)	Carbon utilization efficiency (CUE) (%)	Yield (Y) (%)	Specific production rate (μ_p) (h^{-1})	Conversion coefficient (CC) (%)
0	0.15^{j}	0.00^{i}	7.60^{a}	0.00^{j}	0.00^{j}	0.00^{i}	0.000^{h}	0.00^{i}
6	0.17^{i}	0.00^{i}	7.04^{b}	0.56^{i}	7.37^{i}	0.00^{i}	0.000^{h}	0.00^{i}
12	0.25^{h}	0.04^{h}	6.08^{c}	1.52^{h}	20.00^{h}	0.53^{h}	0.000^{h}	2.63^{h}
24	1.15^{g}	0.19^{g}	4.47^{d}	3.13^{g}	41.18^{g}	2.50^{g}	0.130^{a}	6.07^{g}
30	1.38^{f}	0.31^{f}	3.62^{e}	3.98^{f}	52.37^{f}	4.08^{f}	0.114^{b}	7.79^{f}
36	1.56^{e}	0.45^{e}	3.15^{f}	4.45^{e}	58.55^{e}	5.92^{e}	0.104^{c}	10.11^{e}
48	1.99^{d}	0.59^{d}	2.62^{g}	4.98^{d}	65.53^{d}	7.76^{d}	0.075^{d}	11.85^{d}
54	2.65^{c}	0.79^{c}	2.26^{h}	5.34^{c}	70.26^{c}	10.39^{c}	0.071^{e}	14.79^{c}
60	2.74^{b}	0.91^{b}	1.62^{i}	5.98^{b}	78.68^{b}	11.97^{b}	0.065^{f}	15.22^{a}
72	2.93^{a}	0.92^{a}	1.48^{j}	6.12^{a}	80.53^{a}	12.11^{a}	0.052^{g}	15.03^{b}

Carbon utilization efficiency (%) = Utilized carbon (g/L) / initial carbon (g/L) X 100 (Ramadan *et al.*, 1985).
Yield = PHAs (g/L) / initial carbon (g/L) x 100 (Ramadan *et al.*, 1985).
Productivity (g/L/h) = Polymer concentration (g/L)/ fermentation time (h) (Lee, 1996).
Conversion coefficient (%) = Polymer concentration (g/L) / utilized carbon (g/L) X 100 (Ramadan *et al.*, 1985).
Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5% level.

Table 3 - Semi-scale production of PHAs by *Ps. fluorescens* S48 on productive medium containing WFO as carbon source throughout 72 h at 30 °C using bioreactor as a two-stage batch culture.

Time (h)	Cell dry weight (g/L)	PHAs concentration (g/L)	Residual cells (g/L)	PHAs content (%)	PHAs productivity (g/L/h)	PHAs synthesis rate (g/g/h)	Final pH
0	0.92^{j}	0.00^{i}	0.92^{g}	0.00^{i}	0.000^{h}	0.000^{g}	7.0^{d}
6	1.32^{i}	0.00^{i}	1.32^{i}	0.00^{i}	0.000^{h}	0.000^{g}	7.1^{c}
12	1.99^{h}	0.23^{h}	1.76^{h}	11.56^{h}	0.019^{g}	0.011^{f}	7.2^{b}
24	2.94^{g}	0.92^{g}	2.02^{e}	31.29^{g}	0.038^{e}	0.019^{c}	7.3^{a}
30	3.12^{f}	1.32^{f}	1.80^{g}	42.31^{f}	0.044^{c}	0.024^{b}	7.2^{b}
36	3.64^{e}	1.72^{e}	1.92^{f}	47.25^{d}	0.048^{a}	0.025^{a}	7.1^{c}
48	4.82^{d}	2.13^{d}	2.69^{b}	44.19^{e}	0.044^{c}	0.016^{d}	7.1^{c}
54	4.97^{c}	2.49^{c}	2.48^{d}	50.10^{a}	0.046^{b}	0.019^{c}	6.9^{e}
60	5.21^{b}	2.57^{b}	2.64^{bc}	49.33^{b}	0.043^{d}	0.016^{d}	6.8^{f}
72	5.42^{a}	2.60^{a}	2.82^{a}	47.97^{c}	0.036^{f}	0.013^{e}	6.6^{g}

PHAs content (%) = Polymer concentration (g/L) / cell dry weight (g/L) x100.
Productivity (g/L/h) = Polymer concentration (g/L) / fermentation time (h) (Lee, 1996).
PHAs synthesis rate (g/g/h) = Polymer concentration (g/L) / residual cells (g/L) / fermentation time (h) (Ramadan *et al.*, 1985).
Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5% level.

Table 4 - Impact of time course on the PHAs parameters (CUE, yield, μ_p and CC) produced by *Ps. fluorescens* S48 on productive medium containing WFO as carbon source throughout 72 h at 30 °C using bioreactor as a two-stage batch culture.

Time (h)	Cell dry weight (g/L)	PHAs concentration (g/L)	Carbon concentration (g/L)	Utilized carbon(UC)(g/L)	Carbon utilization efficiency (CUE) (%)	Yield (Y) (%)	Specific production rate (μ_p) (h^{-1})	Conversion coefficient (CC) (%)
0	0.92^{j}	0.00^{i}	7.60^{a}	0.00^{j}	0.00^{j}	0.00^{i}	0.000^{h}	0.00^{i}
6	1.32^{i}	0.00^{i}	7.07^{b}	0.53^{i}	6.97^{i}	0.00^{i}	0.000^{h}	0.00^{i}
12	1.99^{h}	0.23^{h}	5.78^{c}	1.82^{h}	23.95^{h}	3.03^{h}	0.000^{h}	12.64^{h}
24	2.94^{g}	0.92^{g}	5.40^{d}	2.20^{g}	28.95^{g}	12.11^{g}	0.116^{a}	41.82^{a}
30	3.12^{f}	1.32^{f}	2.48^{e}	5.12^{f}	67.37^{f}	17.37^{f}	0.097^{b}	25.78^{g}
36	3.64^{e}	1.72^{e}	1.68^{f}	5.92^{e}	77.89^{e}	22.63^{e}	0.084^{c}	29.05^{f}
48	4.82^{d}	2.13^{d}	1.29^{g}	6.31^{d}	83.03^{d}	28.03^{d}	0.062^{d}	33.76^{e}
54	4.97^{c}	2.49^{c}	0.66^{h}	6.94^{c}	91.32^{c}	32.76^{c}	0.057^{e}	35.88^{b}
60	5.21^{b}	2.57^{b}	0.28^{i}	7.32^{b}	96.32^{b}	33.82^{b}	0.050^{f}	35.11^{c}
72	5.42^{a}	2.60^{a}	0.07^{g}	7.53^{a}	99.08^{a}	34.21^{a}	0.040^{g}	34.53^{d}

Carbon utilization efficiency (%) = Utilized carbon (g/L) / initial carbon (g/L) X 100 (Ramadan *et al.*, 1985).
Yield= PHAs (g/L) / initial carbon (g/L) x 100 (Ramadan *et al.*, 1985).
Conversion coefficient (%): Polymer concentration (g/L) / utilized carbon (g/L) X 100 (Ramadan *et al.*, 1985).
Specific production rate (μ_p) (h^{-1}) = Ln polymer concentration (g/L) at (T) - Ln polymer concentration (g/L) at (T_0) / T - T_0 (Ramadan *et al.*, 1985).
Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5% level.

during the first 24 h of fermentation and then gradually decreased to reach 6.6 after 72 h. The highest values of UC, CUE and polymer yield were recorded at the end of fermentation period (7.53 g/L, 99.08% and 34.21%, respectively). The corresponding figures of conversion coefficient of WFO carbon was 34.53%.

High cell density fed-batch culture

Using the previous improved results, further optimizations were done using the same fed-batch technique with continuous WFO feeding at 0.55 mL/L/h but with scaled up the volume of the bioreactor to 14 L. Aiming to reach a higher cell density and polymer productivity. Data given in Tables 5 and 6 show that the maximum values of cell dry weight of *Ps. fluorescens* S48, PHAs concentration, content, yield and conversion coefficient were obtained after 54 h (4.21 g/L, 2.33 g/L, 55.34%, 29.91% and 33.87%, respectively), with initial cell dry weight of 0.64 g/L and WFO continuous feeding rate at 0.55 mL/L/h. At this time, the polymer productivity was 0.043 g/L/h. The highest specific production rate of PHAs was obtained during the first 12 h of fermentation period being 0.224 h^{-1}, whereas, the maximum UC and CUE were noticed after 72 h (7.55 g/L and 96.92%, respectively).

Table 5 - Semi-scale production of PHAs by *Ps. fluorescens* S48 on productive medium containing WFO as carbon source throughout 72 h at 30 °C using bioreactor as high-cell-density fed-batch culture with 0.64 g/L cell density and continuous WFO feeding at 0.55 mL/L/h.

Time (h)	Added WFO (mL/L)	Added carbon (g/L)	Cell dry weight (g/L)	PHAs concentration (g/L)	Residual cells (g/L)	PHAs content (%)	PHAs productivity (g/L/h)	PHAs synthesis rate (g/g/h)	Final pH
0	0.00^{d}	0.00^{d}	0.64^{j}	0.00^{j}	0.64^{j}	0.00^{j}	0.000^{h}	0.000^{i}	7.0^{d}
6	3.30^{c}	2.57^{c}	1.01^{i}	0.06i	0.95^{i}	5.94^{i}	0.010^{g}	0.011^{g}	7.2^{b}
12	6.60^{b}	5.15^{b}	1.30^{h}	0.23^{h}	1.07^{h}	17.69^{h}	0.019^{f}	0.018^{e}	7.2^{b}
24	9.99^{a}	7.79^{a}	1.98^{g}	0.58^{g}	1.40^{g}	29.29^{g}	0.024^{e}	0.017^{f}	7.3^{a}
30	9.99^{a}	7.79^{a}	2.13^{f}	0.71^{f}	1.42^{f}	33.33^{f}	0.024^{e}	0.017^{f}	7.2^{b}
36	9.99^{a}	7.79^{a}	2.42^{e}	0.97^{e}	1.45^{e}	39.98^{e}	0.027^{d}	0.019^{d}	7.3^{a}
48	9.99^{a}	7.79^{a}	3.98^{c}	2.17^{c}	1.81^{d}	54.52^{c}	0.045^{a}	0.025^{a}	7.1^{c}
54	9.99^{a}	7.79^{a}	4.21^{a}	2.33^{a}	1.88^{b}	55.34^{a}	0.043^{b}	0.023^{b}	6.9^{e}
60	9.99^{a}	7.79^{a}	4.11^{b}	2.26^{b}	1.85^{c}	54.99^{b}	0.038^{c}	0.020^{c}	6.8^{f}
72	9.99^{a}	7.79^{a}	3.32^{d}	1.39^{d}	1.93^{a}	41.87^{d}	0.019^{f}	0.010^{h}	6.6^{g}

PHAs content (%) = Polymer concentration (g/L) / Cell dry weight (g/L) x100.
PHAs productivity (g/L/h) = Polymer concentration (g/L) / Fermentation time (h) (Lee, 1996).
PHAs synthesis rate (g/g/h) = Polymer concentration (g/L) / Residual cells (g/L) / Fermentation time (h) (Ramadan *et al.*, 1985).
Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5% level.

Table 6 - Impact of time course on the PHAs parameters (CUE, yield, μ_p and CC) produced by *Ps. fluorescens* S48 on productive medium containing WFO as carbon source throughout 72 h at 30 °C using bioreactor as high-cell-density fed-batch culture with 0.64 g/L cell density and continuous WFO feeding at 0.55 mL/L/h.

Time (h)	Added WFO (mL/L)	Added carbon (g/L)	Cell dry weight (g/L)	PHAs concentration (g/L)	Carbon concentration (g/L)	Utilized carbon (UC) (g/L)	Carbon utilization efficiency (CUE) (%)	Yield (Y) (%)	Specific production rate (μ_p) (h^{-1})	Conversion coefficient (CC) (%)
0	0.00^{d}	0.00^{d}	0.64^{j}	0.00^{j}	0.00^{j}	0.00^{j}	0.00^{j}	0.00^{j}	0.000^{i}	0.00^{j}
6	3.30^{c}	2.57^{c}	1.01^{i}	0.06^{i}	1.01^{f}	1.56^{i}	60.07^{h}	2.33^{i}	0.000^{i}	3.85^{i}
12	6.60^{b}	5.15^{b}	1.30^{h}	0.23^{h}	1.07^{e}	4.08^{h}	79.22^{e}	4.47^{h}	0.224^{a}	5.64^{h}
24	9.99^{a}	7.79^{a}	1.98^{g}	0.58^{g}	3.21^{a}	4.58^{g}	58.79^{i}	7.45^{g}	0.126^{b}	12.66^{g}
30	9.99^{a}	7.79^{a}	2.13^{f}	0.71^{f}	2.48^{b}	5.31^{f}	68.16^{g}	9.11^{f}	0.103^{c}	13.37^{f}
36	9.99^{a}	7.79^{a}	2.42^{e}	0.97^{e}	1.68^{c}	6.11^{e}	78.43^{f}	12.45^{e}	0.093^{d}	15.88^{e}
48	9.99^{a}	7.79^{a}	3.98^{c}	2.17^{c}	1.29^{d}	6.50^{d}	83.44^{d}	27.86^{c}	0.085^{e}	33.38^{b}
54	9.99^{a}	7.79^{a}	4.21^{a}	2.33^{b}	0.91^{g}	6.88^{c}	88.32^{c}	29.91^{a}	0.076^{f}	33.87^{a}
60	9.99^{a}	7.79^{a}	4.11^{b}	2.26^{a}	0.62^{h}	7.17^{b}	92.04^{b}	29.01^{b}	0.067^{g}	31.52^{c}
72	9.99^{a}	7.79^{a}	3.32^{d}	1.39^{d}	0.24^{i}	7.55^{a}	96.92^{a}	17.84^{d}	0.048^{h}	18.41^{d}

Carbon utilization efficiency (%) = Utilized carbon (g/L) / initial carbon (g/L) X 100 (Ramadan *et al.*, 1985).
Yield = PHAs (g/L) / initial carbon (g/L) x 100 (Ramadan *et al.*, 1985).
Productivity (g/L/h) = Polymer concentration (g/L)/ fermentation time (h) (Lee, 1996).
Conversion coefficient (%) = Polymer concentration (g/L) / utilized carbon (g/L) X 100 (Ramadan *et al.*, 1985).
Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5% level.

In the previous study fed-batch fermentation (bioreactor 3 L) Gamal *et al.* (2012), a relatively high cell dry weight and high polymer content were achieved after a short incubation time (48 h). Both were higher by a factor of 2.4 and 4.14, respectively, than those recorded in shake flask experiments after 72 h (Gamal *et al.*, 2011). Also, it could be noticed that the application of high cell density fed-batch culture in 10 L fermentation medium (bioreactor 14 L) increased the PHAs content by about 84% and 16.82% than that obtained by batch and two-stage batch cultures (bioreactor 3 L) Gamal *et al.* (2012), respectively, as well as shortened the fermentation period by about 6 h comparing with batch culture. Also it could be stated that using the 10 L fermentation culture led to increase the PHAs content of one-stage batch, two-stage batch and high cell density fed batch cultures about 12.3%, 5.8% and 11.3%, respectively, than that obtained by 3 L fermentation culture (Table 7). These results are in line with those obtained by Valappil *et al.* (2007), they stated that a simple glucose feeding strategy in 20 L batch fermentation increased the polymer yield by 31% compared to the batch culture. Additionally, Riesenberg and Guthke (1999) stated that high cell density cultivations represented an about 10-20 fold increase in growth in comparison to normal cell density growth. Problems encountered by high cell density cultivation are numerous, such as PO_2 deficiency, byproduct formation, and/or metabolic heat production. As a result of the growing industrial interest in high cell density, many attempts have been made to improve high cell density fermentations, such as improving potent strain, and/or applying different types of bioreactors and cultivation strategies.

PHAs recovery

One of the most important prerequisites for an industrial strain for PHA production is how easy PHA can be recovered from non-PHA cell matter (Suriyamongkol *et al.*, 2007). Although several new downstream processes for the extraction of PHAs have been reported as economically effective, such as the application of surfactants and the dispersions of hypochlorite solution and chloroform, solvent extraction methods are still regarded as an adequate way to gain intact polymer with high purity and recovery yield (Ramsay *et al.*, 1990). The organic solvents were investigated to determine their efficiency to recover PHAs and how easy the separation of them from cells debris after extraction could be. Data illustrated in Figure 1 show that the maximum efficiency of solvent recovery of PHA was attained by chloroform–hypochlorite dispersion extraction (method 2, 55%) followed by that extracted with chloroform 60 °C for 1 h after pretreatment the cells with 10% SDS at 100 °C for 20 min (method 6, 53%). However, polymer recovery by hot acetone and chloroform (method 3) or sodium hypochlorite alone (method 4) gave the lowest PHA content (40% and 39%, respectively). The corresponding figures of PHA concentration were 2.3, 2.0, 1.5 and 1.42 g/L, respectively. There is still a need to develop and improve these extraction methods further to make the entire processes much simpler and cheaper.

Analysis of PHA by gas chromatography

Gas chromatography (GC) analysis revealed that polyhydroxyalkanoates (PHAs) produced from *Ps. fluorescens* S48 was solely composed of 3-hydroxybutyric

Table 7 - PHAs production from some Egyptian raw materials by *Ps. fluorescens* S48 via different fermentation strategies.

Cultivation vessel, fermentation strategy	Media used	Cultivation time (h)	Cell dry weight (g/L)	PHAs concentration (g/L)	PHAs content (%) (wt/wt)	PHAs productivity (g/L/h)	Yield (Y) (%)	Conversion coefficient (CC) (%)
Batch shaker (250 mL) (Gamal *et al*., 2011)	Kim *et al.* (2003) medium (synthetic)	72	1.74	0.402	23.1	0.0056	4.02	-
	Modified Kim *et al.* (2003) medium (synthetic)	48	1.24	0.39	31.45	0.005	2.4	-
	Two-stage (washed cells)	48	1.92	0.72	37.5	0.015	4.5	-
	Rice straw	72	0.8	0.16	20.0	0.0022	1.6	-
	Glucose syrup	72	1.56	0.3	19.23	0.004	3.0	-
	Corn stalks	72	0.1	0.13	18.75	0.008	1.3	-
	Productive medium containing corn oil (2%)	72	1.90	1.03	54.0	0.014	5.1	-
	Productive medium containing soybean (1%)	72	3.47	1.8	52.0	0.03	26.09	-
	Productive medium containing WFO (1%)	72	1.44	0.26	12.0	0.036	2.6	-
One-stage bioreactor (3 L) (Gamal *et al*., 2012)	Productive medium containing corn oil (2%) extracted from meal	60	3.47	1.8	52.0	0.03	26.09	-
	Productive medium containing soybean (1%) extracted from meal	60	3.41	2.61	76.8	0.044	36.71	-
	Productive medium containing WFO (1%)	60	2.3	0.69	30.0	0.012	9.14	15.68
Two-stage bioreactor (3 L) (Gamal *et al*., 2012)	Productive medium containing corn oil (2%) extracted from meal	48	2.75	68.7	68.7	0.057	39.9	74.9
	Productive medium containing soybean (1%) extracted from meal	48	4.08	3.19	78.2	0.066	45.7	67
	Productive medium containing WFO (1%)	60	3.8	1.8	47.37	0.03	22.5	35.29
Fed-batch bioreactor, fed with WFO (3 L) (Gamal *et al*., 2012)	Pulsed at specific addition rate of 0.023 mL/L/h	72	1.23	0.33	26.82	0.005	4.10	4.84
	Continuous rate at 0.55 mL/h	72	1.71	0.49	41.88	0.007	6.13	8.75
	High cell density (0.64 g/L) at continuous rate of 0.55 mL/L/h	48	3.46	1.72	49.71	0.036	21.5	29.00
One-stage bioreactor (14 L)	Productive medium containing WFO (1%)	60	0.91	0.91	33.70	0.015	11.97	15.22
Two-stage bioreactor (14 L)	Productive medium containing WFO (1%)	54	2.49	2.49	50.10	0.046	32.76	35.88
High-cell-density fed-batch culture in bioreactor (14 L) with 0.64 g/L cell density and continuous WFO feeding at 0.55 mL/L/h	Productive medium containing WFO (1%)	54	4.21	2.33	55.34	0.043	29.91	33.87

PHAs content (%) = Polymer concentration (g/L) /cell dry weight (g/L) x 100.
PHAs productivity (g/L/h) = Polymer concentration (g/L)/ fermentation time (h) (Lee, 1996).
Yield= PHAs (g/L) / initial carbon (g/L) x 100 (Ramadan *et al.*, 1985).
Conversion coefficient (%) = Polymer concentration (g/L) / utilized carbon (g/L) X 100 (Ramadan *et al.*, 1985).

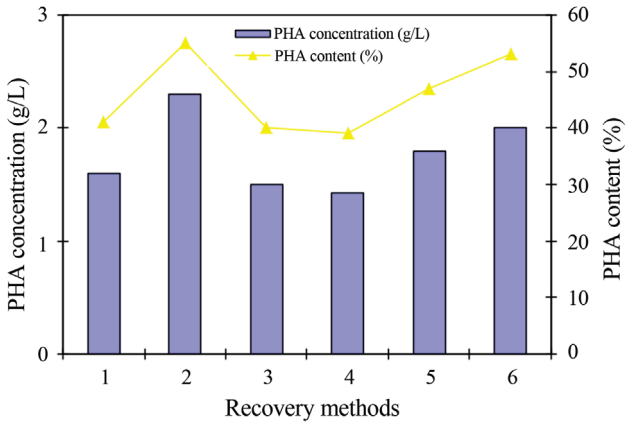

Figure 1 - Efficacy of different recovery methods (1-6) on PHAs extraction from *Ps. fluorescens* S48.

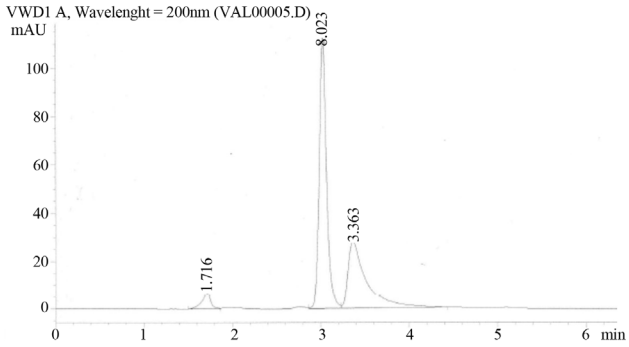

Figure 2 - Gas chromatography (GC) of 3HB-methyl ester indicating that the biopolymer produced by *Ps. fluorescens* S48 was solely composed of 3-hydroxybutyric acid.

Figure 3 - Plastic film made of PHB produced by *Ps. fluorescens* S48.

acid (98.7%) (Figure 2). Therefore, this feature made them highly competitive with polyethylene and petrochemical-derived plastics. The biodegradable biopolymer (PHB) are often preferred materials not only for environmental considerations, but also in medical application such as developing therapeutic devices, for tissue engineering and for slow release drug delivery systems (Nair and Laurencin, 2007). The preparation of PHA polymer films which solely composed of 3-hydroxybutyric acid (98.7%) was implemented according to Kai *et al.* (2003). A film made of PHB was shown in Figure 3.

Genotypic characterization

In case of genus *Pseudomonas*, nearly complete sequences have been determined for the PCR amplified 16S rRNA genes of about 21 species (Moore *et al.*, 1999). In this report, we have further investigated the taxonomic position of the bacterial isolate *Ps. fluorescens* S48, which was proposed here to represent a species, upon sequencing most of the 16S rRNA gene, it was discovered that the isolate *Ps. fluorescens* S48 shares the same identical sequence, which is nearly the complete 16S rRNA gene. The identity of this sequence to the closest Pseudomonads strains is about 98-99%. Concerning the phylogenetic tree constructed in this study it was interesting to note that the isolate *Ps. fluorescens* S48 is so close to Egyptian strain named EG 639838 (Figure 4). Based on phylogenetic study, the analysis of the 16S rRNA gene sequences data for the isolate *Ps. fluorescens* share the same sequence, but

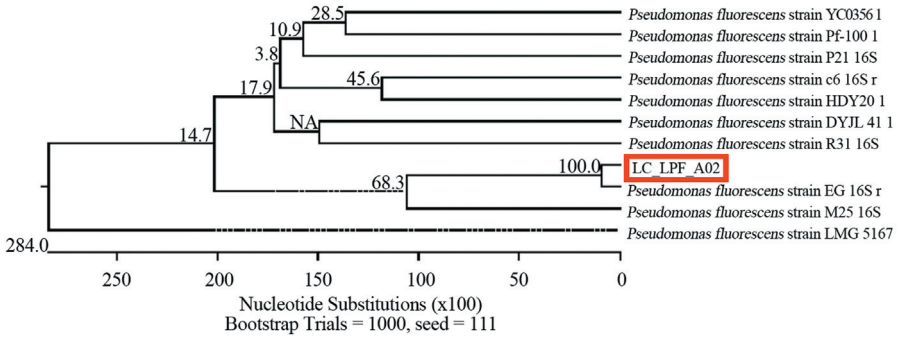

Figure 4 - Neighbor-joining tree showing the estimated phylogenetic relationships of the isolated strain (shown in red) and other closely-related strains of the genus *Pseudomonas* based on comparative analysis of 16 S RNA sequences.

slightly differ from another *Pseudomonas* species. The strain studied was probably closely related to support another meaningful parsimony analysis and construction of a phylogenetic tree. The genetic relationships between the *Ps. fluorescens* S48 and known members of other species of *Pseudomonas* genus were estimated by parsimony analysis (Swofford, 1993) using haustoric search with TBR branch swapping (100 replicates). The bootstrap analyses were run with TBR MULPARS and 1000 replicates.

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