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FATTY ACIDS AS AN ALTERNATIVE CARBON SOURCE FOR MCL-POLYHYDROXYALKANOATE PRODUCTION BY *PSEUDOMONAS FLUORESCENS* SC4

Joao Snoei*[1] ,Luong Dang Nguyen[2],Jukka Seppala[3]
Laboratory of Polymer Technology, Department of Biotechnology and Chemical Technology, Aalto University, School of Chemical Technology, P.O. Box 16100, 00076 Aalto, Finland.
* Corresponding Author: Phone/Fax: +358-40070142; Email: joao.snoei@gmail.com

***Abstract*- Polyhydroxyalkanoates are biodegradable and biocompatible thermoplastics that are produced by bacteria. In this study, the capability of *Pseudomonas fluorescens* SC4 to produce polyhydroxyalkanoates was investigated using different fatty acids as sole carbon sources. High yield production of polyhydroxyalkanoate (PHA) by *Pseudomonas fluorescens* SC4 from alternative carbon sources was achieved. Linoleic acid induced the highest (44.2 wt%) PHA accumulation in bacterial cells into flask fermentation, and (45.2 wt%) in batch Fermentor at 48 h. The biopolymer recovered from fermentation was identified by NMR as medium-chain-length PHA. The strain was also able to produce biopolymer using oleic acid, however at lower concentration (31.9 wt%). No reasonable PHA was recovered from bacterial cells using caprylic acid, capric acid, undecylenic acid and sun flower oil as nutrient sources. Our experiments demonstrated that *Pseudomonas fluorescens* SC4 is a suitable candidate for conversion of fatty acids into value-added products as polyhydroxyalkanoates, as well as fatty acids may be considered an alternative cheap material for the production of mcl-PHA with a high productivity.**

Keywords: Fatty acids, mcl-Polyhydroxyalkanoate, *Pseudomonas fluorescens*, Biopolymers

I. INTRODUCTION

Plastic materials have become an integral part of contemporary life because of many desirable properties including durability and resistance to degradation; they are utilized in almost every manufacturing industry ranging from automobiles to medicine. Plastics are very much advantageous than other materials because as synthetic polymers, their structure can be chemically manipulated to have a wide range of strengths and shapes (Lee 1996).

Recently, the problems concerning the global environment and solid waste management of petroleum based plastics have created much interest in the development of biodegradable plastics, which must still retain the desired physical and chemical properties of conventional synthetic plastics. What makes synthetic plastics undesirable is the difficulty in their disposal. Plastics being xenobiotic are recalcitrant to microbial degradation. Excessive molecular size seems to be mainly responsible for the resistance of these chemicals to biodegradation and their persistence in soil for a long time (Yu 2001). In the recent years, there has been increasing public concern over the harmful effects of petrochemical-derived plastic materials in the environment. Therefore, the development and use of biodegradable plastics is gaining more serious attention.

Among the candidates for biodegradable plastics, polyhydroxyalkanoates (PHAs) have been drawing much attention because of their similar material properties to conventional plastics and complete biodegradability. PHAs are polyesters synthesized by microorganisms as carbon and energy storage materials under conditions of limiting nutrients such as nitrogen, phosphate or oxygen together with an excess of carbon source (Contreras et al. 2013). Under these conditions, several microorganisms are able to divert the usual carbon flux (conversion of Acetyl-CoA in the tricarboxylic acid cycle for creation of energy and metabolites for biomass formation) towards synthesis of PHA.

Over 250 different bacteria, including gram-negative and gram-positive species, have been reported to accumulate various PHAs (Ojumu and Solomon 2004). PHAs can be divided into two broad groups based on the number of carbon atoms in the monomer units; the short chain length polyhydroxyalkanoates (scl-PHAs), which consist of C3-C5 atoms, and medium chain length polyhydroxyalkanoates (mcl-PHAs) consisting of C6-C14 atoms.

At present, approximately 150 different constituents of PHAs have been identified as homopolymers or as copolymers (Ching Lee and Kumar 2007). The main reason for the possible formation of these diverse types of PHAs is due to the extraordinarily broad substrate specificity of PHA synthases as well as because of the effects of the types of carbon sources fed to the microorganisms and the metabolic pathways that are active in the cell.

The PHAs are non-toxic, biocompatible, thermoplastics that can be produced from renewable resources. They have a high degree of polymerization, are highly crystalline, optically active and isotactic (stereochemical regularity in repeating units), peizoelectric and insoluble in water. These features make them highly competitive with polypropylene, the petrochemical-derived plastic, as well as the most characteristic property that distinguishes PHA from petroleum based plastics is their biodegradability. PHAs are

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degraded upon exposure to soil, compost, or marine sediment (Tsuge 2002).

However, besides the remarkable properties of polyhydroxyalkanoates, one of the main problems in developing biodegradable polymers as substitutes for conventional plastics is their high price compared with petrochemical derived plastics. Although the cost of production has decreased due to development of new fermentation techniques and recombinant strains, the price of PHA remains still high. A suitable alternative to reduce the price of production would be the use of inexpensive carbon source.

Fatty acids are desirable feedstock for PHAs production because they are relatively cheap compared to most sugars. Although the production of PHAs using sugars has been optimized to achieve high productivity, the cost of production is still high because sugars contribute to low PHAs yield. In addition, the theoretical yield coefficient of PHA from fatty acid (e.g., 0.65-0.98 kg/kg from butyric acid) is considerably higher than that from glucose (0.32-0.48 kg/kg) (Reddy et al. 2003). However, bacterial fermentation using fatty acids still has some problems. One major problem is the relatively low growth rate of available PHA-producing bacteria. Even if a bacterium, that the growth rate of which on fatty acids is rather high, is employed for PHA production, the PHA content in the dry cells is relatively low (Chan et al. 2006).

In this study, we attempted to produce Polyhydroxyalkanoate (PHA) by a wild-type strain *Pseudomonas fluorescens* SC4 isolated from Finish soil utilizing different fatty acids as sole carbon source cultivated into flasks batch and 3L lab scale bioreactor fermentation. The results obtained during fermentation suggested that PHA production from fatty acids has a potential advantage for reduction on the production cost. Furthermore, based on the presented results, we emphasized the biotechnological importance of bacterial strains inhabiting Nordic soils.

II. MATERIAL AND METHODS

Bacterial Strain

Pseudomonas fluorescens SC4, which was isolated from soil in Finland, was used in this study. The stock culture was maintained on LB agar slants overlaid with 20% (v/v) glycerol and kept at − 20 °C.

Carbon Sources

All chemicals were obtained from Sigma–Aldrich Company Ltd. Linoleic acid, Oleic acid and Sun flower oil were used as sole carbon source at 1.5% (v/v) concentration; Caprylic acid, Capric acid and Undecylenic acid were added to MM minimal medium to final concentrations of 60, 30 and 20 mM, respectively.

Culture Media and Inoculum Preparation

The strain was pre-cultivated overnight into LB broth containing 10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl. For PHA production a mineral salt medium (MM), composed of 2.0 g/L KH_2PO_4, 0.6 g/L Na_2HPO_4, 1.0 g/L $MgSO_4.7H_2O$, 1 g/L $(NH_4)_2SO_4$, 1g/L yeast extract and 1 mL/L of trace element (1.3 g/L $ZnSO_4.7H_2O$, 0.2 g/L $CaCl_2$, 0.2 g/l $FeSO_4.7H_2O$, 0.6 g/L $(NH_4)_6Mo_7O_{24}.4H_2O$ and 0.6 g/L H_3BO_3), was used. Fatty acids were used as carbon sources at the indicated concentrations above. A modified mineral medium was used in the fermentation in the bioreactor containing (g/L): $Na_2HPO_4 \cdot 2H_2O$ 1.2, KH_2PO_4 4, $MgSO_4.7H_2O$ 2, $(NH_4)_2SO_4$ 2, yeast extract 1 and trace element solution 2 ml. The carbon sources were sterilized separately at 121 °C for 20 min and then aseptically added into the flask containing the other components at room temperature. The pH of the final culture medium was adjusted to 7.0 before bacterial inoculation.

Detection of Polymer Accumulation

Nile Red (Sigma-Aldrich, USA) diluted in dimethylsulfoxide (DMSO) (0.25 mg/mL) was filtered and added to the sterile MM medium. The tested strain was cultivated into agar plates at 30 ºC for 72 hours. The agar plates were exposed to UV light (312 nm) after cultivation to detect PHA granules inside the cells. The cells were subsequently observed under fluorescent microscopy (Labophot Microscope, Nikon Instruments, New York, USA).

Shaking Flasks Experiment

Precultures were prepared from solid agar medium and incubated for overnight at 30 °C and 180 rpm. Studies on PHA production were conducted in sterilized 250 mL Erlenmeyer flasks with 50 mL of mineral culture medium for the others substrates, inoculated with 1 mL of bacteria from a seed culture and incubated at 30 °C with shaking at 180 rpm. The fatty acids were added as sole carbon source in each test flask according to the text above. After a preliminary screening at 48 h to analyze dry biomass and PHA accumulation, a proper timing of fermentation was established at 24, 48, 72 and 96 h; the process was monitored by Nile Red fluorescent dye to check the PHA production. The cells were harvested by centrifugation at 5000 rpm, for 20 min, at 20 °C (Beckman Coulter, avanti J-HC), washed with saline solution at 0.9 % and lyophilized overnight. (Virtis, benchtop 2K). All the assays were done in triplicate.

Batch Culture into Fermentor

Bioreactor BBraun with a working volume of 3000 mL were filled with 950mL of mineral medium adding fatty acids (1% v/v) as carbon source. The bioreactor was sterilized by autoclaving at 121 ^{0}C for 20 min. A pre-inoculum was cultivated in 50 mL LB medium overnight at 30 ^{0}C. The bioreactor temperature was maintained at 30 ^{0}C and pH 7, aerated at 300 rev.min^{-1} with 2 vvm O_2 injected into the system. Every 12 hours during the experiment 10mL of culture was centrifuged at 4.500 rpm, dry mass PHA content and residual biomass were determined. All the assays were done in duplicate.

Dry weight estimation

Cell Dry weight (CDW) was estimated from 5 mL of culture broth. The cell suspension was centrifuged at 5000 rpm for 15 min at 20 °C(Beckman Coulter, avanti J-HC), washed with distilled water several times, transferred to pre-weighed vials and dried in an at 70°C till constant weight.

Determination of Nitrogen Concentration

The nitrogen concentration (as ammonium ion) in the medium was measured using an Ammonium Assay kit (BioAssay Systems, USA).

Isolation of PHA from lyophilized cells

For the extraction of the PHA, the cells were harvested by centrifugation at 5000 rpm for 30 min and then lyophilized. The lyophilized cell mass was subjected to soxhlet extraction for 24 h using chloroform as the solvent. The PHA was then concentrated by vacuum rotary evaporation and precipitated using 10 volumes of ice-cold methanol. The precipitate obtained was centrifuged and air-dried.

Polymer Identification

The identity of individual monomer unit was confirmed by nuclear magnetic resonance (1H-NMR) spectroscopy. ^{1}H-NMR spectra were acquired by dissolving the polymer in deuterochloroform (CDCl3) at a concentration of 10 mg/ml and analyzed and recorded at 300.1 MHz with a spectrum of 32 kB data points. The equipment used was a NMR spectrometer model Mercury 300 MHz (Varian Inc., Palo Alto, CA, USA).

III. RESULTS

Nile red staining was used in the microscopic observation for a possible accumulation of intracellular PHA by the isolated strain. Further confirmation of the presence of accumulated granule was made by fluorescence microscopy (Figure 1).

To investigate the effect of carbon sources on the production of PHA, different carbon sources were provided at 1.5 % (v/v) of oleic acid, sun flower oil and 60, 30, 20 mM (v/v) of caprylic acid, capric acid and undecylenic acid respectively in standard PHA mineral medium. Figure 2a shows the time course of biomass production (CDW) by the isolate with different carbon sources. The microorganism utilized sun flower oil, capric acid and oleic acid for growth, and biomass accumulation giving highest yield with oleic acid as substrate after 96 h of incubation reaching up to 2.1 g/L CDW, followed by the fatty acid groups capric acid with 0.87 g/L and sun flower oil 0.65 g/L, both after 96 h of cultivation. In contrast with the other fatty acids tested, caprylic acid and undecylenic acid were not a good substrate for microbial growth, and no growth was detected after 96 h of cultivation. Although the strain *Pseudomonas fluorescens* SC4 used sun flower oil and capric acid as carbon source in comparison with oleic acid, the selected substrates did not serve for the production of PHA, since no PHA has been detected on sun flower and only 0.13 g/L was produced by capric acid as shown on Figure 2b. Among the 5 tested substrates, the fatty acid oleic oil proved to be the best substrates for PHA accumulation. On 1.5 % oleic acid, the maximum yield of PHA was obtained after 96 h of cultivation, with PHA accumulating up to 0.67 g/L ~ 31.9 wt%.

The ability of strain SC4 to utilize cheap carbon sources for PHA accumulation was investigated in 250 mL Erlenmeyer flasks containing 50 ml of mineral medium in the presence of linoleic acid. Cultures were incubated at 30 ^{0}C and 180 rpm, and samples were taken at different intervals under sterile conditions for analysis. The figure 3a & b showed that the strain was able to utilize the tested carbon source for growth. On 1.5% (v/v) linoleic acid, the maximum yield of PHA was obtained after 144 h of cultivation, with biomass reaching 6.7 g/L CDW and PHA accumulating to 2.96 g/L. The nitrogen source (ammonium sulphate) was completely depleted after approx. 96 h coinciding with the time when the polymer started to be obtained. After 120 h of cultivation there was no significant increase on biomass (CDW 6.7 g/L) as clarified on Figure 3a, however comparing the results obtained for PHA production at this point (2.4 g/L), it was possible to demonstrate an increase on polymer accumulation after this time. A reasonable explanation for the long delay on cell growth would be in the case of the used fatty acid; probably in the beginning of the reaction there was difficult to metabolize the fatty acid due to composition of material. Significant PHA yields are shown in this study when cultivating the tested strain SC4 in platform shaker, being the PHA accumulation of 26.7 wt%, 37.2 wt%, 38.9 wt% and 44.2 wt% recovered after 72, 96, 120 and 144 h of cultivation (Figure 3b).

Pseudomonas fluorescens SC4 was cultivated for 48 h in a modified mineral medium (as described in the section culture media and inoculum preparation), additional 1 g/L of yeast extract had been supplemented into fermentation to improve nutrient content and fatty acid uptake. Samples have been taken for biomass and polymer measurement regularly after beginning of the experiment. To increase the production of PHA, 3-L stirred bioreactor with 1-L working volume was considered using the optimal condition obtained from the Erlenmeyer flask scale (1.5% v/v linoleic acid as sole carbon source). The CDW and PHA production as function of time are shown in Figure 4. The batch system reached a steady state with the CDW (6.75 g/L) and the maximum PHA (3.05 g/L) after 48 h, representing 45.2 wt% of accumulation.

The strain started to accumulate biopolymer after 15 h of fermentation, and increased until the end of the experiment. After the first sampling time (15 h) the polymer content reached 0.41 g/L with CDW 3.01 g/L (13.6 wt%) of CDM from linoleic acid as a carbon source. The second sampling lasted 24 h and the values were quantified as 3.95 g/L with 1.44 g/L, respectively CDW and PHA formation. A total limitation of the nitrogen source was achieved after 18 h of fermentation, extra ammonium sulphate (0.25 g/L) was added into system after 24 h to enhance cell metabolism. After 36 h of cultivation the CDW was quantified as 5.72 g/L with 41 wt% of PHA accumulation. Regarding the nitrogen evolution, the extra addition of nitrogen was completely consumed by the cells after 36 h of cultivation.

Nuclear magnetic resonance analyses of the biopolymer produced in mineral medium supplemented with Linoleic acid showed a qualitative evaluation of structural composition. NMR results showed typical signals of PHA. After chloroform extraction, PHA was separated from biomass with hot ethanol (95%) and analyzed by nuclear magnetic resonance (NMR). The results showed a spectrum with the presence of three groups of signals characteristic of the medium chain length Polyhydroxyalkanoate (mcl-PHA): at 2.5 ppm attributed to the methylene group, at 5.2 ppm for the methine group adjacent to an asymmetric carbon atom and at 1.6 ppm characteristic of the methylene protons adjacent to the b-carbon in the side-chains group (Figure 5).

IV. DISCUSSION

The genus *Pseudomonas* is well known for being able to synthetize PHA, many species in this family assimilate several different carbon sources that can be obtained from waste residues or by-products and has the potential to be used

as inexpensive raw materials for PHA production (Aravind et al. 2013). According to (Jiang et al. 2008), *Pseudomonas fluorescens* has been already reported to produce polyhydroxyalkanoates from inexpensive carbon sources, and currently alternatives ways for improving PHA production are being investigated by other researchers with representatives of genus *Pseudomonas* by scaling-up fermentation.

One of the main problems preventing the commercial expansion of microbial polymers is its high cost, even with all the news achievements the price of PHA is still considered much higher than that of synthetic plastic. One considered strategy to reduce its price would be the use of more inexpensive carbon sources (Quillaguaman et al. 2005). Since PHA production from pure glucose, fructose or sucrose has already been well studied and optimized, an option would be the development of new technology and the use of alternative cheaper carbon sources as a key factor in further reducing the PHA production cost. Fatty acids are good material for PHA production because they are inexpensive renewable carbon sources; in addition, the theoretical yield coefficient of PHA from fatty acid is considerably higher than that from glucose (Park et al, 2005).

In the present study, 5 distinct fatty acids have been tested as substitute of glucose on PHA and biomass accumulation in batch cultivation. Oleic and Capric acid were metabolized by our tested strain responsible for 31.9 wt% and 18.5 wt% of biopolymer production, however biomass accumulation was still considered very low in comparison with the experiment using pure glucose as energy source. Tsuge (2002), reported that bacterial fermentation using plant oils or fatty acids has some problems, been one of the major problem the relatively low growth rate quantified on CDW per g of fatty acid. Further experiments are still needed to improve the biomass formation on fatty acids.

Batch fermentation in Erlenmeyer flaks was carried out in this study in order to prove the ability of microorganism in converting a specific fatty acid into biopolymer for further characterization. The strain SC4 was able to accumulate 44.2 wt% of PHA in the presence of higher volume of pure substrate (1.5% linoleic acid), however it was necessary a long time of cultivation, probably due to difficult on metabolizing the substrate by the microbial cells. The value can be considered promising, if compared to other bacteria described in recent works (Alicata et al, 2005; Solaiman et al, 2005). According to Song et al. (2008), *Pseudomonas sp.* strain DR2 accumulated mcl PHA from corn oil and waste vegetable oil containing linoleic acid, and produced mcl PHA up to 37.34 wt% and 23.52 wt% respectively. The use of fatty acids by the tested microorganism showed in this paper may be considered as a good achievement for solving the problem of cost-production by using renewable substrates during PHA synthesis. The polymer started to be accumulated when the nitrogen source was limited. Nitrogen limitation is proved by literature as the initiator of PHA production because the formation of proteins ceases and the flux of carbon are redirected to polymer synthesis (Koller et al. 2007).

Even though PHAs are been considered as good candidates for replacing petroleum based plastic, the high production cost has limited their use in a wide range of applications. Several fermentation processes are employed in wild strains and recombinant species to develop and improve for more economical production of PHA (Koller et al. 2007). The productivity of Polyhydroxybutyrate cultivated in bioreactors has been reported to reach up to 3 g of PHB/liter/h by employing *A. eutrophus* and recombinant *E. coli* (Lee et al. 1999).

In our study, *Pseudomonas fluorescens* SC4 was cultivated in a stirring tank bioreactor with linoleic acid as sole carbon source at 30 °C and pH 7. The cultivation was carried out under limited nitrogen and excess carbon sources, though extra additional nitrogen was supplied during the fermentation. By optimizing fermentation (Huijbert et al. 1996) could increase the PHA content to 88% of cell dry weight and PHA productivity to 4.94 g/L/h. Significant improvement on biomass and polymer production has been observed in our experiment, raising CDW yield to 6.75 g/L and maximizing the PHA accumulation (3.05 g/L) after 48 h. By using a fed-batch culture with *P. putida* strain using oleic acid as carbon source (Lee et al. 1999) were able to enhance cell concentration, PHA concentration and PHA content obtained in 34 h, resulting in the PHA productivity of 1.79 g/L/h.

Although (Suzuki et al. 1986) observed that the maximum PHA accumulation has been achieved in cells that were not supplied with nitrogen; we attempted to improve the PHA content of cells and the yield of biomass in the fermentation by adding extra nitrogen source. Yeast extract was supplied in the beginning of the experiment to the stirred tank bioreactor. In all experiments after 18 hours of cultivation all the nitrogen has been consumed. 24 hours after inoculation of the bioreactor with *Pseudomonas fluorescens* SC4, nitrogen was fed again into culture to improve the uptake of linoleic acid and biomass growth. The strategy is in accordance with Goff *et al.*, 2007, which described the difference in the quantity of PHA produced by *Pseudomonas putida* CA-3 supplied with nitrogen compared to those not supplied with nitrogen, the highest percentage of PHA accumulated by the tested strain (43 wt% of CDW) was registered with nitrogen feed into cultivation.

The functional groups of the extracted PHA granules were identified by nuclear magnetic resonance (NMR). The main bands and peaks of the 1H NMR spectra correspond to PHA, according to those found in the literature (Sanchez et al, 2003). Furthermore, the chemical signals obtained in the present work agree with those obtained by (Tan et al. 1997; Ashby et al. 2000) for a mcl PHA produced by fermentation. These results showed that strain SC4 was able to accumulate medium chain length PHA using linoleic acid as a substrate.

V. CONCLUSIONS

In order to find out a cheaper microbial production of biomasses and PHAs we have investigated the capability of *Pseudomonas fluorescens* SC4 to convert substrates formed by fatty acids. The bacterial strain showed successfully capability for utilizing linoleic acid and the studied polymer was identified as mcl-PHA, however the same good results were not observed in the other tested fatty acids at the same conditions. It has been proved its potential to produce mcl-PHA in a high content (45.2 wt%) when linoleic acid medium was used as a sole carbon source and giving maximum CDW yield of 6.75 g/L and a PHA productivity of 3.05 g/L after 48 h fermentation in bioreactor. Further study

will be fulfilled on the possibility of *Pseudomonas fluorecens* SC4 to enhance its growth conditions to use more efficiently linoleic acid and oleic acid as sole carbon sources for production of PHAs by using media optimization technique via other fermentations of fed-batch, repeated batch, and repeated fed batch processes to increase the economic feasibility of biopolymer production from renewable materials.

VI. ACKNOWLEDGMENTS

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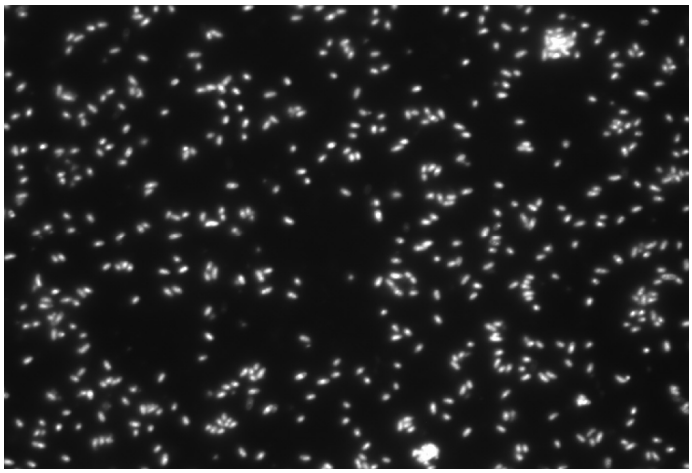
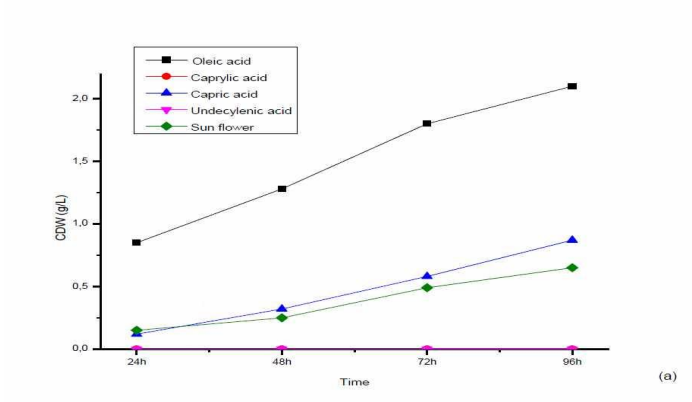


Figure1. Fluorescence of PHA granules using Nile Red staining



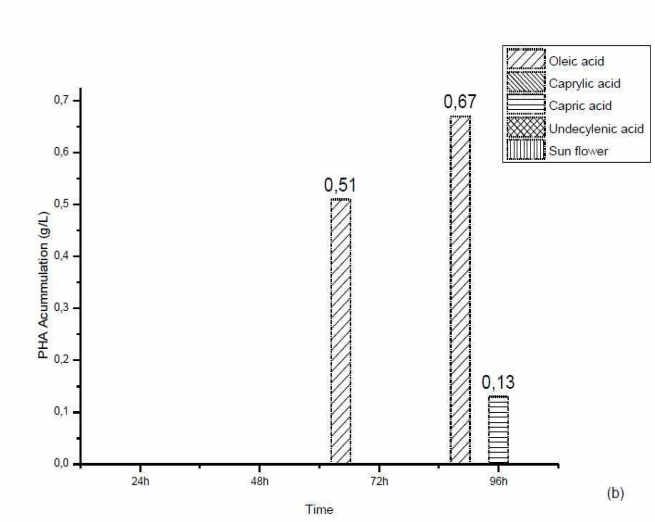


Figure 2. (A) Biomass production (CDW g/L) by *Pseudomonas fluorescens* SC4 with different fatty acids as carbon sources. (B) PHA accumulation (g/L) based on different substrates analyzed each 24 h.

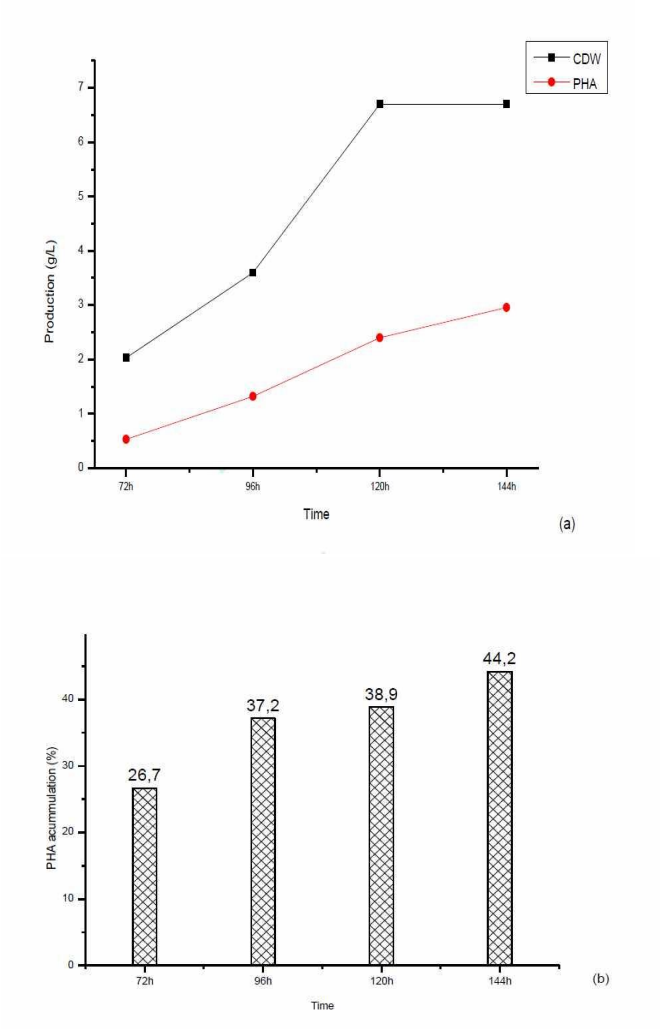


Figure 3. Time curves of fermentation in Erlenmeyer with *Pseudomonas fluorescens* SC4 using linoleic acid at 1.5% (v/v) concentrations: (A) dry biomass (CDW). (B) Polymer (PHA) accumulation.

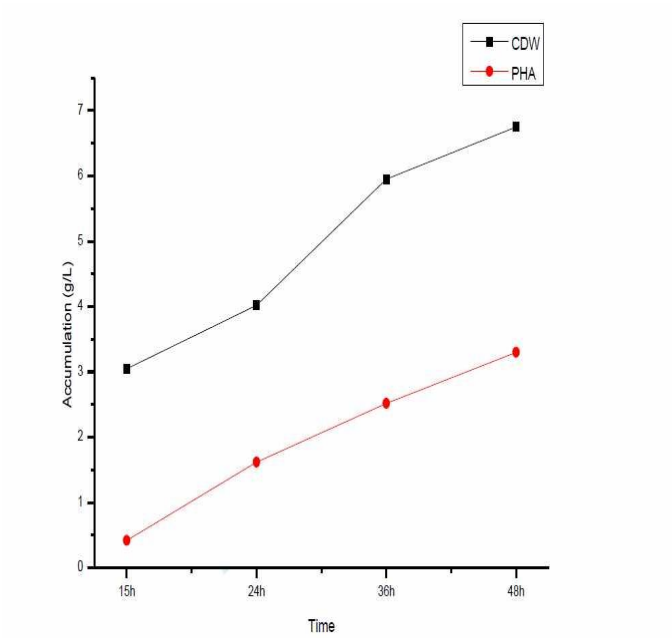


Figure 4. Time curves of fermentation with *Pseudomonas fluorescens* SC4 using 1.5% (v/v) linoleic acid as carbon source into stirring tank bioreactor.

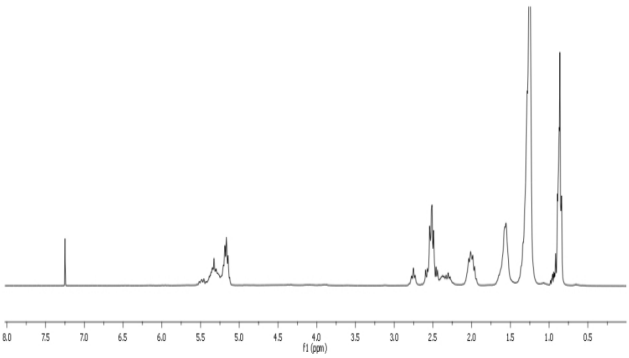


Figure 5. NMR (^{1}H) spectrum of the mcl-PHA extracted from *Pseudomonas fluorescens* SC4 cultivated on linoleic acid as carbon source.