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# Co - Production of Polyhydroxyalkanoates and Biosurfactants using *Pseudomonas luteola*

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## ABSTRACT

Co-production of Poly (β-hydroxyalkanoates) (PHAs) and Biosurfactants (BS) is made possible because they both contain hydroxyalkanoic acids as their main constituents. This study investigated the use of glucose and molasses at varying concentrations as alternative carbon substrates for the co-production of PHAs and BS. Biosynthesis was for 7days at $30^0$C during which the best PHA was produced from 8% glucose (101 mg\L) on the third day and the best BS from 4% molasses (52 mg/L) on the fifth day. The produced PHAs contained hexadecanoic, octadecenoic and octadecanoic acids from molasses and hexadecanoic and octadecenoic acids from glucose. Some fractions from the open column chromatography were positive to both Anthrone and Rhodamine B test reagent and others negative to Ninhydrin test reagent indicating that the presence of a glycolipid; the PHAs on the other hand showed monomers of alkanoic acids.

**Keywords**: Biosurfactant (BS), Glucose, Molasses, Poly (β-hydroxyalkanoic acids) (PHA) and *Pseudomonas luteola.*

## INTRODUCTION

Polyhydroxyalkanoates (PHAs) are water insoluble storage compounds produced by some microorganisms that serve as reserves of carbon and energy (Anderson and Dawes, 1990). Biosurfactants (BS) on the other hand, are surface active amphiphilic compounds with a hydrophobic portion having a dimer of 3-hydroxyalkanoate which is the monomeric unit of PHAs (Siddhartha *et al*., 2009). These biological polymers are gradually replacing those of chemical origin and have attracted biotechnological and environmental interest because of their degradability and bio-compactable nature (Pantazaki *et al*., 2011).

Several species of pathogenic and non-pathogenic *Pseudomonas* are capable of producing Polyhydroxyalkanoates (PHAs) and Biosurfactants (BS) as reported by Marcia *et al*. (2011). *Pseudomonas* (*Chryseomonas*) *luteola* is a gram negative motile rod that is strictly aerobic and grows optimally at $30^0$C on Nutrient, Trypticase Soy or MacConkey Agar (Anzai *et al*., 1997).

The feasibility of co-producing PHAs and BS has been reported by Horia *et al*. (2011) and Marsudi *et al*. (2008) towards reducing the production costs. However, production and marketing is presently limited by the accumulation levels of the bacterial specie used and the high costs of the materials involved in the biosynthesis of these biomolecules as reported by Khanna and Srivastava (2005). New strains and preferably recombinant strains having a high accumulation percentage have to be isolated and less expensive substrates chosen as this accounts for about 50% of the production costs to solve these limitations (Moreno *et al*., 2007; Makkar and Cameotra., 1999). Molasses, Palm oil mill effluents, waste cooking oils, cassava waste water and residues from vegetable oil refineries have been reported to be excellent substrates for PHA and BS co-production (Nitschke and Pastore, 2006; FAO, 2005; Patel and Desai, 1997).

This study report the co-production and characterization of PHA and BS using *Pseudomonas luteola* obtained from a petroleum contaminated site in Ogun State Nigeria.

## MATERIALS AND METHODS

**Sample collection:** Soil sample was obtained from the Nigerian National Petroleum Corporation (NNPC) oil depot in Mosimi Shagamu located in Ogun State, Nigeria (N $06^0$45'17.1" and E $003^0$33'08.5").

**Bacterial Isolation:** One gram of soil sample was serially diluted ($10^6$) and dispensed asceptically into plates containing Mineral Salt Medium (MSM) Agar. The plates were then incubated at 27 ˚C for 14 days. Filter paper (Whatman 1; 11 µ) was impregnated with sterile crude oil and then suspended on the cover of the Petri-dish modified from the report of Balogun and Fagade (2010). The MSM Agar was made up of Basal Salt Medium (BSM) containing (g/L): $K_2HPO_4$, 1.8; $KH_2PO_4$, 1.2; $NH_4Cl$, 4.0; $MgS0_4.7H_2O$, 0.2; NaCl, 0.1; yeast extract, 0.1 and $FeCl_2.4H_20$, 0.05 and the trace elements solution contained: $H_3BO_3$, 0.1; $ZnSO_4.7H_2O$, O.1; $CuSO_4.5H_2O$, 0.05 and $MnSO_4$. $H_2O$, 0.04 and Bonny light crude oil (an internationally accepted form of crude oil from Bonny, Rivers State, Nigeria) was used as the carbon source. Five percent agar was added to solidify the medium. Ten milliliters of the trace element solution was added to the Basal Salt Medium to make up one liter of the MSM.

**Screening for Polyhydroxyalkanoate Production:** The presence of PHA as intracellular granules was confirmed by staining the cells with Sudan Black B as reported by Amutha and Sreenivasan (2010). Loop full of isolate was mixed with 20% brine and smeared on a clean glass slide, the smear was then air dried and fixed by immersing in 2% acetic acid for 5 mins. The slide was removed, covered with 0.3% Sudan Black solution and kept at room temperature for 15 mins. Excess stain was drained off and allowed to dry. Xylene was then added, excess xylene was drained off and counter stained with 0.5% aqueous Safranin for 10 sec. Slide was then washed with distilled water, dried and examined under light microscope (at 100X).

**Screening for Biosurfactant Production:** Drop Collapse Test: Bacterial isolate was inoculated into a liquid medium consisting of 20 ml MSM to which 2% glucose solution had been added and 0.1% crude added after 3 days and incubated for 7 days at 180 rpm. This was then centrifuged at 15,000 for 25 min to obtain the supernatant. Mineral oil was sterilized at 160 °C for 2 h and left to acclimatize for 2 days. Drop collapse test was then carried out using the method of Balogun and Fagade (2010).

Emulsification Index Test: Two milliliters' of the sterile mineral oil was added to the same amount of supernatant and vigorously, shaken using a vortex mixer to ensure uniformity for 2 min and left undisturbed for 24 h. The volume of oil that separated after 24 h of standing was measured. The emulsification index was taken as a percentage of the height of emulsified layer divided by the total height of the liquid column (Onwurah and Nwuke, 2004).

**Bacterial characterization:** The biochemical characterization of the screened isolates was done using API 20E Idenification kit (BioMérieux). Among the isolates, *Pseudomonas luteola* was chosen for PHA and BS co-production because it was produced the best result from the various screening techniques used.

**Simultaneous Production of PHAs and BS:** Cells from slant were inoculated in bacteriological peptone and yeast extract for 24 h at 30°C. One ml of this solution was added to flasks containing MSM and glucose

(2%, 4% and 8%) and MSM and molasses (2%, 4% and 8%). Flasks were then kept in a rotary shaker and incubated at $28^0$C for 7 days at a speed of 180 rpm (Saranya *et al*., 2010).

The MSM medium contained BSM and 10 ml of microelements that were sterilized separately before usage. The BSM contained g/L of distilled water: 0.66 g of $(NH_4)_2SO_4$, 2.3 g of $KH_2PO_4$, 7.3 g of $Na_2HPO_4.12H_2O$, 0.25 g of $MgSO_4.7H_2O$, 0.3 g of $NaHCO_3$, 0.1 g of $CaCl_2.2H_2O$. The microelement solution contained 0.58 g of $ZnSO_4.7H_2O$, 3.96 g of $MnCl_2.4H_2O$, 0.6 g of $H_3BO_3$, 5.56 g of $FeSO_4.7H_2O$, 5.62 g of $CoSO_4.7H_2O$, 0.34 g of $CuCl_2.2H_2O$, 0.04 g of $NiCl_2.6H_2O$ and 0.06 g of $NaMoO_4.2H_2O$ per litre of 0.5 N HCl (Choi and Yoon, 1994). Agitation speed was monitored daily and adjusted to control foaming while maintaining aerobic condition. The pH was adjusted to neutral using 0.2 M NaOH and 0.1 M HCl and aliquots of 10 ml was taken at regular intervals for analysis (Horia *et al*., 2011).

**Polyhydroxyalkanoate Analysis:** Ten ml of the fermentation broth was centrifuged at 3 000 rpm for 20 min, the cells were then washed with 10 ml of saline and re-centrifuged to get pellets which were then suspended in 5 ml of sodium hypochlorite (4% active chlorine) and incubated. The extract was centrifuged and washed with 10 ml of cold diethyl ether, recentrifuged at 8 000 rpm to get purified PHA which was dried to a constant weight at 105°C for 24 h (Ceyhan and Ozdemir, 2011). Characterization of the monomer composition of the accumulated polyhydroxyalkanoates was done using Gas Chromatography-Mass Spectrometry (GC-MS model 6890-5973N Agilent Technologies UK) as previously reported by Braunegg *et al*. (1978).

**Biosurfactant analysis:** To quantify the biosurfactant, 10 ml of the fermentation broth was centrifuged at 3 000 rpm for 50 min using a cold centrifuge at 4°C. The supernatant was precipitated with 3 volumes of cold acetone and left to stand at 4°C for 10 h. The precipitate was re-centrifuged and evaporated to dryness, then re-dissolved in sterile water and dried to constant weight (Abouseoud *et al*., 2007).

Characterization of the biosurfactant was done according to Balogun (2009). Fifty ml of the fermentation broth was centrifuged at 3 500 rpm for 20 min, the pH of the supernatant was adjusted to 2 using drops of concentrated HCL and left to stand at 4°C for 24 h. The solution was then centrifuged twice at 3500 rpm for 20 min, the supernatant removed and the residue dissolved in 50 mM $NaHCO_3$. Extraction was with chloroform: methanol: cell free extract (2:1:3) to give a precipitate. The precipitate was further purified by running it through open column chromatography packed with Sephadex LH 20 as a stationary phase and methanol as the mobile phase. Five ml elluent from the column was collected in clean glass bottles; methanol in the glass bottle was allowed to evaporate leaving the fractionated compound. Elluents were assayed for amino acids, lipids and carbohydrate using Ninhydrin, Rhodamine B and Anthrone reagent respectively. Fractionated biosurfactant (0.04 ml) was dispensed into small test tubes and 0.4 ml of reagents added, tubes were shaken and observed for colour change.

**Statistical Analysis**

Microsoft Excel (2010) and SAS 9.1 (2002-2003, NC USA) were used to analyze data for this research. On the other hand, descriptive statistics was carried-out using Pearson correlation coefficient and analysis of variance (ANOVA) was performed at P=0.05.

## RESULTS AND DISCUSSION

Soil and water are known habitat and source of versatile microorganisms. The organism used for the simultaneous production of PHA and BS was isolated from soil sample contaminated with hydrocarbon suggesting that *P. luteola* can metabolize the carbon present and the residual crude present in the soil and water as reported by Bodour *et al*. (2003).

Screening for organisms that produce biosurfactant has been previously reported by several researchers using the haemolytic activity test (Maneerat and Phetrong, 2007), the drop collapse test (Balogun and Fagade, 2010) and the emulsification activity test (Onwurah and Nwuke, 2004). *Pseudomonas luteola* was selected because it showed the best screening result. The high value of emulsification stability exhibited by *P. luteola* points to the fact that it is an emulsifier and has the ability to reduce surface tension. Emulsification Index (E.I) for *P. luteola* was observed to vary slightly from the E.I reported by Pruthi and Cameotra, (1995) and Lai *et al*. (2009) probably because of the different environments of isolation.

Table 1 illustrates 7 days biosynthesis of PHA and BS produced by *P. luteola* from molasses and glucose. On the first day of fermentation, no significant difference was observed when both substrates were used, however, *P. luteola* produced significantly higher mean values of BS from glucose (16.5) and molasses (19.17) than PHA. Day 2 of the fermentation showed that both substrates did not significantly affect BS production and PHA produced was significantly highest using molasses (12.33) as a substrate. *P. luteola* on day 3 showed the highest mean value for PHA (73.33) and BS (14.33) using glucose as a substrate. Significantly high value was recorded for BS (7.67) and PHA (11.00) production using glucose on the fourth day. Day 5 of the fermentation revealed that both substrates did not significantly affect BS production but the PHA produced was significantly highest using glucose (29.00). On the sixth day of the

Fermentation PHA production was highest when molasses (12.50) was used as a substrate while BS produced showed no significant difference from either glucose or molasses. On the last day of fermentation no significant difference was obtained using any of the substrates to produce BS and the highest value of PHA was produced using molasses (10.50).

Figure 1, 2 and 3 illustrates the quantities of BS and PHA produced from *P. luteola* using glucose and molasses. From the figures it was observed that PHA production from glucose got to its peak of 440 mg/L in day 3 and continued to decline producing only 5 mg/L by the 7$^{th}$ day. Biosurfactants on the other hand showed a non-systematic production curve and had its peak on the 6$^{th}$ day (138 mg/L). 2% glucose was observed to yield a maximum value of PHA (95 mg/L) on the 3$^{rd}$ day and BS produced a maximum value of 9.5 mg/L also on the third day (Figure 1). 4% glucose yielded maximum value of 25 mg/L on the 3$^{rd}$ day while BS produced a maximum value of 54.5 mg/L on the 6$^{th}$ day (Figure 2). 8% glucose yielded a maximum amount of PHA (100 mg/L) on the 3$^{rd}$ day and BS (23.5 mg/L) on the 5$^{th}$ day (Figure 3). PHA production when molasses was used as a carbon source got to the peak on the 3$^{rd}$ day (202 mg/L) and BS production on the 5$^{th}$ day (166 mg/L). 2% molasses yielded maximally PHA (19 mg/L) on the 3$^{rd}$ day and BS (16 mg/L) on the 6$^{th}$ day (Figure 1).

**Table 1**: Biosynthesis of Polyhydroxyalkanoates (PHA) and Biosurfactants (BS) by *P. luteola* from glucose (GLU) and molasses (MOL).

| DAYS | PHA | | BS | |
|---|---|---|---|---|
| | GLUCOSE | MOLASSES | GLUCOSE | MOLASSES |
| 1 | 8.83±2.51[a] | 6.00±1.39[a] | 16.5±4.82[a] | 19.17±5.69[a] |
| 2 | 5.33±1.36[b] | 12.33±2.42[a] | 8.33±1.74[a] | 7.17±1.85[a] |
| 3 | 73.33±15.40[a] | 33.67±7.65[b] | 14.33±4.63[a] | 11.17±2.89[a] |
| 4 | 11.00±4.70[a] | 9.00±2.86[a] | 7.67±1.58[a] | 3.33±1.20[b] |
| 5 | 29.00±3.59[a] | 19.17±4.34[b] | 11.17±3.96[a] | 27.67±7.89[a] |
| 6 | 5.00±1.91[b] | 12.50±2.67[a] | 23.00±15.60[a] | 23.00±3.93[a] |
| 7 | 1.25±0.25[a] | 10.50±0.5[b] | 7.50±3.43[a] | 5.33±2.29[a] |

Values are Mean ± Standard Error.
Means within a column with the same letters are not significantly different at p>0.05.

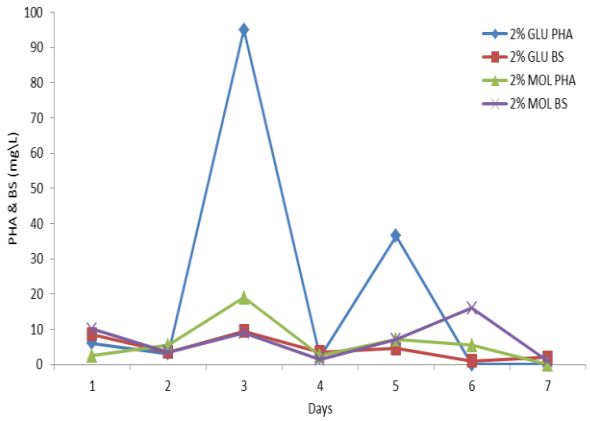


**Figure 1**: Biosynthesis of Polyhydroxyalkanoates (PHA) and Biosurfactant (BS) from *P. luteola* using 2% glucose and molasses.

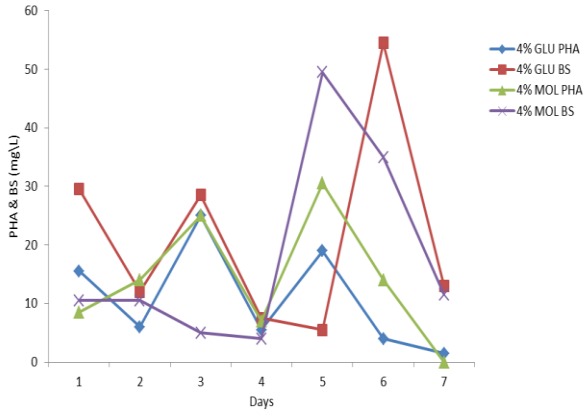


**Figure 2**: Biosynthesis of Polyhydroxyalkanoates (PHA) and Biosurfactant (BS) from *P. luteola* using 4% glucose
and molasses

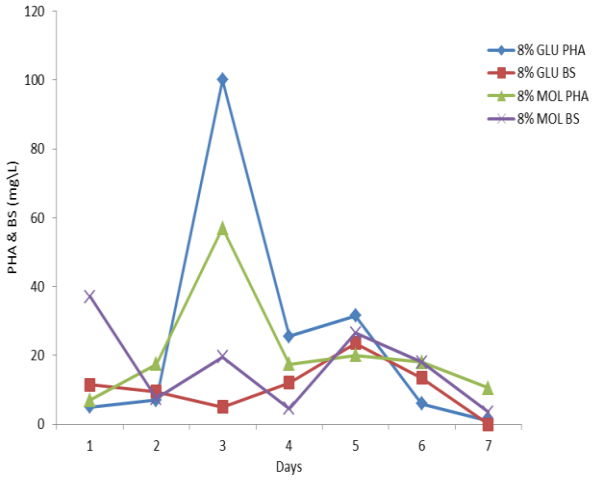


**Figure 3**: Biosynthesis of Polyhydroxyalkanoates (PHA) and Biosurfactant (BS) from *P. luteola* using 8% glucose and molasses.

4% molasses produced maximally PHA (30.5 mg/L) on the 5th day and BS (49.5 mg/L) also on the 5th day (Figure 2). 8% molasses maximally produced PHA (20 mg/L) on the 5th day and BS (26.5 mg/L) also on the 5th day (Figure 3).

After the fourth day, cell growth was observed to reduce drastically probably due to the exhaustion of nitrogen and the Poly(β-hydroxyalkanoic acids) production got to a maximum when all the carbon sources were exhausted. Biosurfactants biosynthesis on the other hand continued even after exhaustion of the carbon source. These tendencies were consistent with previous study by Hori *et al.*, (2002). The GC-MS experiment conducted on the PHA revealed the monomeric β-hydroxyalkanoic acid as being hexadecanoic acid from glucose and both hexadecanoic and octadecanoic acid from molasses. Hexadecanoic acid showed a molecular ion of m/z at 256 having a structural formular of $CH_3(CH_2)_{14}COOH$ and octadecanoic acid had a molecular ion of m/z at 284 having a structural formular of $CH_3(CH_2)_{16}COOH$.

The qualitative test for characterization of the biosurfactant was achieved using open chromatogram. Most of the fractionated biosurfactant tested positive to Rhodamine B and Anthrone reagent but Ninhydrin was not reactive with any of the fractionated crude biosurfactant. The positive reaction of most fractions to Rhodamine B indicates that they are of lipid origin. Okpokwashili and Ibiene (2006) reported similar findings from *Pseudomonas* sp. Also positive reaction of elluents to Anthrone indicates that they consist of carbohydrates as reported by Gunther *et al.* (2005) and Carillo *et al*. (1996). However, Ninhydrin showed no reaction. This suggests that the fractionated biosurfactant was a glycolipid.

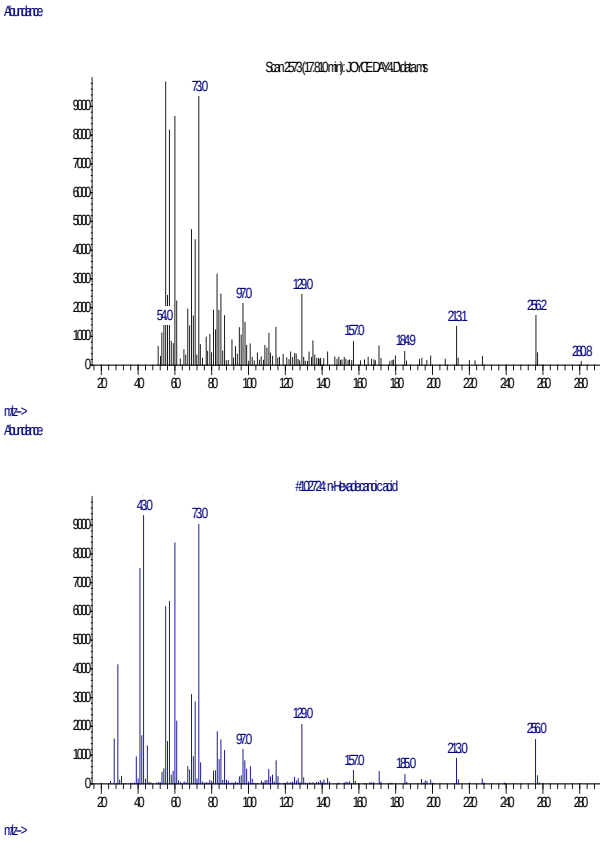


**Figure 4**: Spectrum of Gas Chromatograph showing n- Hexadecanoic acid

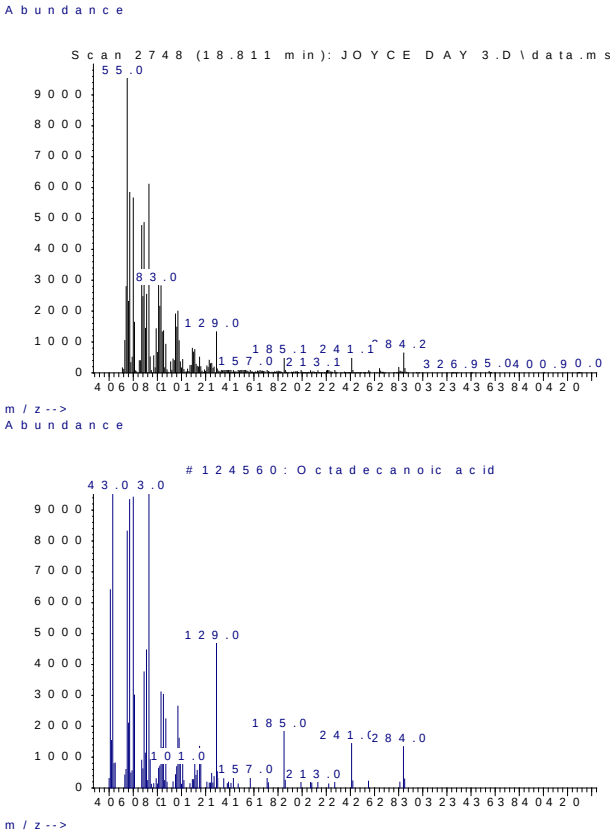


**Figure 5**: Spectrum of Gas Chromatograph showing Octadecanoic acid

## CONCLUSION

The results from this research showed that PHAs and biosurfactants can be produced via microbial fermentation from *P. luteola.* The production of Poly(β-hydroxyalkanoic acids) as a potential substitute material to some conventional plastics has drawn much attention due to their biodegradability and biocompatible properties. Nevertheless, the production cost of PHA has been a major drawback. On the other hand, most surface active compounds used by industries are chemically synthesized and their replacement with biosurfactants can provide good advantages as a result of their unique properties, lower environmental toxicity and higher biodegradability but also as with PHAs large scale production is also a drawback. This study had overcome the challenges of economics of scale by producing both biopolymers from agro-wastes.

It is recommended that optimization studies on the best pH, inoculum load, speed and temperature be done to ascertain the best conditions for production of both PHAs and biosurfactants as their use/application cannot be overemphasized.

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