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Polyhydroxyalkanoates (PHAs), bioprocessing using waste oil

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Abstract

Pseudomonas oleovorans NCIMB 6576 and Ralstonia eutropha NCIMB 10442 were used for the production of Polyhydroxyalkanoates (PHA) from industrial waste cooking oils, the bacteria were cultured on tryptone soya broth (TSB) and Tryptone soya agar (TSA). *P. oleovorans* NCIMB6576 gave a better percentage PHB yield (8.2%) with PS oil as carbon source as compared to 6.45% with TS oil. However, a very low yield (0.64%) was recorded when *P. oleovorans* NCIMB6576 was grown on TSB without the oils as carbon source. Ralstonia eutropha NCIMB 10442 gave an appreciable yield of 13.63% and 14.80% with PS and TS oil samples respectively as carbon source with negligible variation in the yields. The results obtained across all experiments were compared with one another. The SEM images from the PHB samples generated from the experiments shows that there is a slight difference in the surface morphologies of the PHB with respect to the oil samples as well as the different bacteria used in the experiment.

Keywords: Polyhydroxyalkanoates; *Pseudomomnas. oleovorans NCIMB6576;* Bioprocessing; Biotechnology; Waste Oil

1. Introduction

Polyhydroxyalkanoates (PHAs) are group of biodegradable polymers of hydroxyalkanoates (HAs) produced by various microorganisms through the accumulation of energy storage materials to overcome stress. The PHAs are synthesized through polymerization of hydroxyacyl-CoA thioesters in a reaction catalysed by PHA synthases. The cost of the carbon source is one of the most common factor that affect PHAs production prices since most microorganisms can metabolize a range of carbon sources. Sugars like glucose and sucrose are common substrates for PHAs production because they are cheap. Plants oils or fatty acids also serve as good carbon sources because they are inexpensive and a better yield coefficient of PHA is obtainable from the fatty acids. Other carbon sources include agricultural and food industrial wastes as well as carbon dioxide which can be used as feedstock by Cyanobacteria and photosynthetic bacteria (Tsuge, 2002).Vegetable oil became a promising resource for polymer production owing to its availability, biodegradability, low toxicity and the double bonds inherent in its fatty acids can serve as a carbon source for polymer production (Xia and Larock, 2010). PHA was also produced from waste frying oil using *Cupriavidus necator* (Known as *R.eutroha*) and 1.2g/L PHB concentration was obtained similar to the concentration if glucose was used as carbon source (Verlinden *et al.,* 2011). *Wautersia eutropha* (*R. eutropha*) produces medium chained length PHAs with hydroxybutyrate (HB), hydroxyvalerate (HV), hydroxydecanoate (HD) and hydroxyoctanoate (HO) serving as monomers when canola oil was used as energy source to yield about 18.27g/l of PHAs culminating into about 90% of the cell dry biomass (Lopez-Cuellar *et al.,* 2011). *P.oleovorans* grown on mineral medium in a batch fermentation condition produced 26.06% PHA (PHB-co-HV) using *Jatropha curcas* oil as a carbon source

The use of waste materials obtained from agriculture and industries as starting materials for the biosynthesis of PHA is an important strategy for the cost effective production of the polymer and provides an alternative means to overcome

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waste disposal problems. Different convertible substrates obtainable from variety of sources that includes waste cooking oil and plants oils provides triacylglycerols or fatty acids and glycerol which can serve as direct carbon source after hydrolysis of the constituent triacylglycerol backbone (Koller *et al*., 2010). Food industry wastes including oils mainly disposed as wastes in the environment can serve as carbon sources for the microbial synthesis of PHAs, an important bioplastic material (Obruca *et al.,* 2010). In this research, *Pseudomonas oleovorans* NCIMB 6576 and *Ralstonia eutropha* NCIMB 10442 were used for the production of PHB from industrial waste cooking oils.

2. Material and methods

2.1. Microorganisms

Pseudomonas oleovorans NCIMB 6576 and *Ralstonia eutropha* NCIMB 10442 were used for the production of PHB from industrial waste cooking oils. These microorganisms were collected from the culture collection of microbiology laboratory, University of Wolverhampton, UK as streaked plates. The microorganisms were refrigerated at 4^{o}C and cultures were maintained by streaking on TSA plates, once after every two weeks.

2.2. Industrial waste oil samples

Two industrial waste oil samples (supplied by Living fuels company, Kings Street, Nottingham) coded PS and TS were utilised individually as carbon sources for PHA production.

The oil samples differ in their fatty acids (FA) compositions with varying range of monounsaturated, polyunsaturated and saturated fatty acids as highlighted by the iodine number of each sample as shown in table 1a and b

Table 1 a The fatty acid composition of the oil samples and 4b, showing the iodine number of the oils used as carbon sources in this research

a	Fatty acids	TS oil (g/100g)	PS oil (g/100g)
	Saturated	11.06	9.72
	Monounsaturated	48.76	59.30
	Polyunsaturated	35.78	26.58

b	Oil sample	Iodine number
	TS	106.2
	PS	98.1

2.3. Growth media

The growth media used in this experiment are tryptone soya broth (TSB) and Tryptone soya agar (TSA). Tryptone soya broth (Lab M, UK) consist of water, 17g/l trptone, 3g/l soy peptone, 5g/l NaCl, 2.5g/l K_2HPO_4 and 2.5 g/l D-glucose. This was prepared by dissolving 30g/l of the powder in 1L of distilled water and stirring with a magnetic stirrer (VELP scientifica). 250ml of the broth was then transferred into 500ml conical flasks and autoclaved at 121^{o}C before use.

TSA (Lab M, UK) contains distilled water, 15g/l tryptone, 5g/l soy peptone, 5g/l NaCl and 12g/l Agar No.2. The agar was prepared by dissolving 37g/l TSA powder in 1L of distilled water (equivalent to 7.4g in 200ml of distilled water in separate bottles). These were autoclaved at 121^{0}C and poured aseptically into Petri dishes and allowed to cool so that it becomes solid.

Ringer solution (Lab M, UK) was used for serial dilution (1/4 ringer) by dissolving one tablet in 500ml distilled water and dispensed into test tubes before autoclaving at 121^{o}C. The solution aids in keeping the cells alive before they are plated on agar.

2.4. Starter preparation

To prepare the starter culture which was used for the fermentation experiment, a single colony of *P.oleovorans* NCIMB 6576 or *R.eutropha* 10442 was inoculated into a conical flask filled with 20ml TSB. This was incubated in a rotatory shaker (Brunswick Scientific, UK) at 30°C and 150rpm for 24 hours.

2.5. Sonication

To create a homogenous mixture of the oil and TSB, the mixture was sonicated to break the oil into fine droplets to enhance its miscibility. 50ml of TSB was transferred into a sterile beaker to which 2.5ml of oil was added (equivalent to 2.5g) and sonicated for 10 minutes at 0.5 active and 0.5 passive intervals with 50% power using a sonicator (Bandelin sonopulz). This was then transferred aseptically back into the 500ml conical flasks to form a broth-oil mixture (with initial oil concentration of 10g/l). This procedure was carried out for each flask since all experiments were carried out in triplicates.

2.6. The fermentation process

Batch fermentation in conical flasks was carried out by preparing 250ml of TSB into each flask (of which 50ml of it was initially sonicated with 2.5ml oil to form a homogenate). The initial oil concentration in the mixture was approximately 10g/l. The oil- broth mixtures in the flasks were inoculated with 100µl of the starter culture. The shake flasks were then incubated in a rotatory incubator at 150rpm, 30°C for 48 hours. However, the fermentation was stopped at intervals to enable sample collection at time (0, 3 and 6, 24, 27, 30 and 48 hours).

2.7. Determination of total cell dry weight and solvent extraction of polymer

The total cell dry weight or biomass was determined by dispensing known quantity of the fermentation medium into 50ml centrifuge tubes after the fermentation was stopped after 48 hours. These tubes were centrifuged (HERMLE Z 300K) for 10 minutes at 3500rpm at 4°C and the resulting supernatant was discarded leaving the bacterial pellets. The pellets were washed by adding distilled water to the centrifuge tubes and centrifuged again at 3500rpm for 10 minutes at 4°C to get rid of any residual oil. The supernatant was discarded again leaving behind the bacterial biomass. This was kept in a -20°C freezer for 24 hours.

After freezing at -20°C, the pellets were lyophilized by freeze drying using a freeze dryer (Edwards freeze dryer, Modulyo) for 72 Hours. The total cell dry weight in grams is then estimated by weighing in an extraction thimble using a weighing balance, after grinding the freeze dried pellets to fine granular particles using a mortar and pestle. The weight was recorded and the extraction thimble was covered using cotton wool ready for solvent extraction.

2.8. Isolation and Purification of the PHAs

Since the PHAs are accumulated within the cell inside inclusions; a procedure for the recovery or isolation and purification of the PHAs was used. This involves the use of hot chloroform to extract the polymer in an extraction device called the Soxhlet extractor (Thermo scientific) in a fume cupboard for three hours. Approximately 200ml of chloroform HPLC grade (Fischer Scientific) was used. This is followed by precipitation of the solubilized polymer (PHB) using cold n-hexane (Sigma Aldrich) in a 500ml beaker by slowly stirring using a magnetic stirrer. The precipitate forms around the stirrer and settle as a visible precipitate at the bottom of the beaker.

The precipitated polymer is separated from the mixture of n-hexane-chloroform using a forceps to remove large clumps while small particles are filtered off from the mixture using a Watman's filter paper and a funnel. A dropping pipette was used to also purify the polymer by washing it down the filter paper so that it settles at the bottom. The resulting polymer is dried in air and weighed using a weighing balance. The polymer extracted from the bacteria can be used to calculate the percentage yield of the polymer relative to the amount of biomass in grams accumulated as shown below;

$$\% \text{ yield (PHB)} = \text{cell dry weight (g)} / \text{ weight of polymer extracted} \times 100$$

2.9. Statistical analysis

Comparison of the production capacities between *P.oleovorans* NCIMB 6576 with and without the oils as well as *R.eutropha* NCMBI 14402 with the oil samples used in the experiment was also carried out.

2.10. Percentage PHB yield

The dried PHB samples extracted from the biomass after freeze drying was weighed and the percentage yield of PHB was determined from each experiment relative to the amount of biomass generated as can be seen in fig14 below. From the results shown, it can be seen that *P. oleovorans* NCIMB6576 gave a better percentage PHB yield (8.2%) with PS oil as carbon source as compared to 6.45% with TS oil. However, a very low yield (0.64%) was recorded when *P. oleovorans* NCIMB6576 was grown on TSB without the oils as carbon source. *Ralstonia eutropha* NCIMB 10442 gave an appreciable yield of 13.63% and 14.80% with PS and TS oil samples respectively as carbon source with negligible variation in the yields. This is further supported by the bar chart below which shows the distribution of the yield according to the experiments conducted.

Figure 1 Flask containing the fermentation culture (mixture of oil, broth and bacteria)

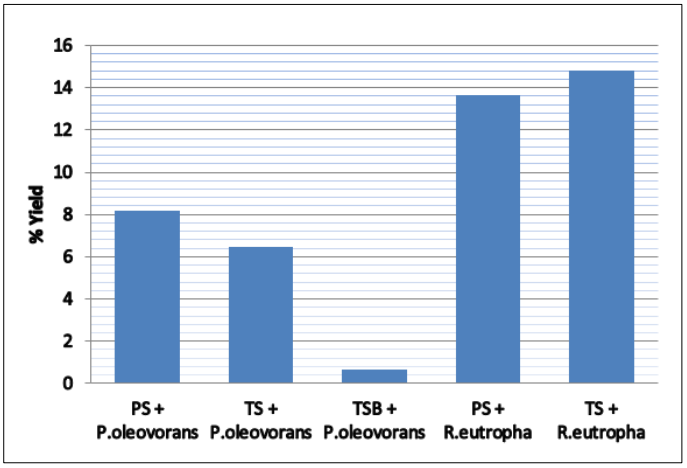

Figure 2 Yield (%) of PHB in the experiments with *P. oleovorans* NCIMB6576 and *R. eutropha* NCIMB 10442 using TSB or TSB + oils (PS and TS)

3. Discussion

3.1. Fermentation

This research aims at production of PHA using *P. oleovorans* NCIMB 6576 with different industrial wastes oil samples in TSB as the growth medium. *P. oleovorans* NCIMB 6576 was grown in 250ml medium at 30°C and 150rpm for the period of 48 hours in the presence of 10g/l of oil samples as carbon source under controlled temperature and agitation.

3.2. Percentage yield of PHB

P. oleovorans NCIMB 6576 grown on TSB with the oils as carbon source was able to synthesize PHB in both cases. However, better PHB yield of 0.276g/l (8.2%) was realised with *P.oleovorans* NCIMB 6576 grown on TSB with PS oil as carbon source compared to 0.55g/l (6.45%) when grown in the presence of TS oil. The PHB yield was found to be much lower in the negative control without the oil as carbon source with a yield of 0.02g/l (0.64%). This shows that, although a better yield was obtained with *P.oleovorans* NCIMB 6576 grown with PS oil, the biomass concentration was higher (8.52g/l) in the case of *P.oleovorans* NCIMB 6576 with TS oil relative to the PHB yield compared to 3.36g/l with *P. oleovorans* NCIMB 6576 and PS oil sample. The better PHB yield with PS oil compared to TS oil can be ascribed to the differences in the oil compositions as shown by the iodine number. PS oil has a lower iodine value (more monounsaturated fatty acids) compared to TS oil which might have influenced the PHB yield. Evidence has shown that, PHB synthesis is enhanced by saturated fatty acids in bacteria (Verlinden *et al.,* 2011).

Studies have shown that appreciable yields of mcl-PHAs with diverse compositions are produced by *Psuedomonas* species including *P. putida, P. aeruginosa and P. oleovorans* from plants oils including waste vegetable oils and their fatty acids components (Nitschke *et al.,* 2011, Song *et al.,* 2008).However, it was mentioned that *P. oleovorans* produces PHAs under limited supply of nutrients (Fernandez *et al.,* 2005). Therefore, the growth medium utilised for the production of PHA in this research is TSB which contains some appreciable amount of nutrients which is likely to affect the production potential of the bacteria.

Reported PHA yields using *Pseudomonas sp.* strain DR2 when grown in a medium with low phosphate and nitrogen supplementation after 72 hours using waste vegetable and corn oil yielded 23.52% and 37.34% PHAs (Song *et al.,* 2008). A PHA production yield of 26.06% was realised when *P. oleovorans* ATCC 29347 was grown in mineral medium with *Jatropha* oil by batch fermentation after the oil was first saponified. The saponification became necessary owing to the inability of *P.oleovorans* ATCC 29347 to produce the lipases needed for the breakdown of triacyglycerols (Allen *et al.,* 2010). The percentage yields of PHB recorded for *P.oleovorans* NCIMB 6576 in this research was 8.2% and 6.45% with PS and TS oil respectively, which is much lower than the reported yields cited. Therefore, it is probable that the growth medium utilised for the bacterial growth is not suitable for efficient synthesis of the PHA by the bacteria. The incubation time during the fermentation in this research with TSB was 48 hours which may not be sufficient to ensure efficient yield similar to the output as cited in the literatures above. Another factor that is likely to negate the production of PHB from the oil samples maybe due to impaired metabolism of the constituent triacylglycerols in the oil samples as a result of the inability of the bacteria to produce the lipases needed for the breakdown of the triacylglycerol constituents.

The highest PHB yield was observed in the *R. eutropha* NCIMB 10442 control experiments with the oil samples PS and TS. A better PHB yield of 1.118g/l (13.63%) and 1.113g/l (14.80%) was realised with PS and TS oil as carbon sources respectively compared to *P. oleovorans* NCIMB 6576 with and without the oil samples. Shake flasks experiments with *Cupriavidus necator* (*R.eutropha*) using 20g/l waste frying oil as carbon source yielded 1.2g/l PHB after a period of 72 hours in TBS as a growth medium (Verlinden *et al.,* (2011). *R.eutropha* grown in a nutrient-rich medium supplemented with 20g soybean oil per gram $(NH_4)_2SO_4$ produced up to 0.45g PHA/g of soybean oil equivalent to 7.4g/l in concentration after 72 hours (Park *et al*., 2011). As can be seen, no much difference in the yield exist for the result obtained in this research compared to that obtained by Verlinden *et al.,* (2011) using the same TSB medium even though the period of cultivation is higher in the cited result (72 hours). However, the yield obtained by Park *et al.,* (2011), is higher compared to the yield recorded in this investigation. The carbon source (soybean oil) and time of experiment (72 hours) is different as well. *R. eutropha* was also shown to produce PHAs when cultivated in a medium of low nitrogen when different wastes oils and fats like soybean and rapeseed oil, palm oil and lard as well as tallow were used, yielding 3.5g/l, 5.7g/l and 5.8g/l of PHAs respectively after 72 hours because it was shown to degrade and assimilate different oils and fats for PHA synthesis (Taniguchi *et al.,* 2003). The PHA yields in these reported cases maybe due to the fact that *R.eutropha* NCIMB 10442 can synthesize PHAs even in conditions where nitrogen is not limited in supply in the medium since it has been documented that the bacteria can produce appreciable amount of PHB and P (3HB-co-3HV) and the PHA yield is not influenced by nitrogen limitations (Park *et al.,* 2011). Since the cultivation time in this research is 48 hours, perhaps an increase in the cultivation time can be an added advantage to enhance the yield of the polymer. As can be seen from the cited yields above the fermentation lasted up to 72 hours.

4. Conclusion

Results from the experiment revealed that *P. oleovorans* NCIMB 6576 was able to synthesize PHB using the industrial waste oil samples as carbon sources. However, low percentage yield of the polymer was recorded with *P. oleovorans* NCIMB 6576 *with* both oil samples even though the yield was shown to vary with the type of oil sample used in the experiment. More PHB yield was realised with *P. oleovorans* NCIMB 6576 and PS oil as carbon source than with TS oil.

The composition of the oil may be a reason for this finding since PS oil was shown to have a low iodine value (signifying that it has a higher amount of monounsaturated fatty acids) compared to TS oil. It was reported that increase in bacterial PHB synthesis is enhanced by saturated fatty acids compared to unsaturated ones.

The low PHB yield observed with P*. oleovorans* NCIMB 6576 using the oil samples maybe due to the fact that *P. oleovorans* NCIMB 6576 is unsuitable for high PHB (short-chained length) synthesis with oil but for synthesis of mCl-PHAs. It was reported that good yields of mCL-PHAs are realised using *Pseudomonas sp.* including *P. oleovorans* from plants oils and waste

The experiments with *R. eutropha* NCIMB 10442 with the two oil samples yielded more PHB compared to *P. oleovorans* NCIMB 6576. The PHB yield obtained is consistent with that obtained by Verlinden *et al.*, (2011) where 1.2g/l of PHB was recorded using waste frying oil in TSB, although, the period of cultivation was longer in this case. More PHB yield were realised with *R. eutropha* grown in a nutrient-rich medium in the presence of soybean oil as carbon source after 72 hours.

It was also proven that the carbon sources (the oil samples) have a positive influence on the PHB yield from *P. oleovorans* NCIMB 6576 since experiments with the oil samples all gave a higher PHB yield when compared to the negative control without the oils.

The FTIR analysis conducted on all PHB samples produced in this research revealed characteristic peaks that are consistent with that of PHB as reported by Valappil *et al.,* (2007). SEM images of the polymer showed homogeneous spherical granular particles with varying sizes depending on the carbon source and the bacteria.

TGA analysis has shown that the PHB samples produced have varying thermal stabilities since they start to degrade at different temperatures (T_o) which vary according to the carbon source and the bacteria. This varies from 255^{o}C and 248^{o}C with *P. oleovorans* NCIMB 6576 with the oil samples compared to 229^{o}C and 239^{o}C with *R. eutropha* NCIMB 10442.

X-ray diffraction analysis conducted on the PHB samples showed that, all the polymer produce irrespective of the carbon source from *P. oleovorans* NCIMB 6576 and *R. eutropha* NCIMB 10442 are crystalline in nature since they exhibited similar trends in the patterns of the peaks consistent with results obtained as reported by Oliviera *et al.,* (2007) and Santos *et al.,* (2009)

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest in the work, however, traces of constructive criticism was observed at the cost of data collection but eventually, mutual agreement was mate at end and result submitted is a product of duely accepted process by all members of the team.

Statement of ethical approval

All protocols involving ethical standard has been followed duely. Permission for data collection and approval for risks instances were strictly taken into consideration before any practical work was observed. Experience laboratory attendant were made available and strict were followed accordingly.

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