Chapter 4:- Standardization of an efficient extraction and recovery process

1. Introduction

Polyhydroxyalkanoates (PHA) are biodegradable, biocompatible, microbial thermoplastics which has potential to replace petroleum-derived thermoplastics (De Koning et al 1997).

However, the use of biologically produced polymers is currently limited because of high production costs. Significant contributors to the cost of production are the productivity of PHAs by the chosen bacterial strain, carbon source and downstream processing. The difficulty of PHB recovery from microorganisms has been the primary obstacle to its commercial exploitation.

Most of the PHA separation methods employ solvent extraction or non-PHA biomass digestion. Digestion of biomass other than PHA (De Koning et al 1997) typically consists of heat treatment, enzymatic solubilization, and surfactant washing. Drawbacks include expense, complexity and binding of materials such as surfactants to the PHA granules. Several enzymatic, centrifugation and washing steps are typically needed to achieve acceptable purity.

Solvent extraction is simpler in terms of the number of steps employed but there are still important choices to be made when designing a process. A PHA extraction process invariably involves three steps. These are biomass pre-treatment, solvent extraction, and polymer purification. The pre-treatment step may incorporate enzymes to degrade proteins and DNA, heating to denature these macromolecules, surfactants to remove...
lipids and/or solvents to remove water and polar lipids. Solvent extraction of PHA most often involves chlorinated hydrocarbons such as chloroform (Ramsay et al 1990).

PHA recovery mainly involves the use of solvents to solubilize the PHA (Baptist 1962, Lafferty et al 1988) or enzymes, surfactants and/or inorganic chemicals to solubilize the non-PHA cellular materials (Holmes, 1985). With solvent extraction, high product purity and recovery efficiency have been achieved with MCL-PHA. Solvents are commonly used in lab scale recovery. Safety concerns and high recycling costs make them less practical on a large scale. In non-solvent methods, surfactants have been used on various bacteria (Choi and Lee 1997, De Koning et al 1997), but they tend to adsorb to the PHA granules and are difficult to eliminate. Enzymes provide mild recovery conditions, but are expensive. For example, De Koning et al. (1997) estimated that the MCL-PHA recovery step was one third of the total production cost, with almost half of the expense being enzymes and chemicals. Although hypochlorite was effective in PHB recovery (Berger et al 1989), there was a molecular weight loss even at optimized digestion conditions (Ramsay et al 1990). KOH and NaOH are easier to handle than solvents, less expensive than enzymes, milder than hypochlorite and have been used in PHB recovery (Choi and Lee, 1997). It was not clear whether it would be efficient in MCL-PHA recovery given the differences in SCL- and MCL-PHA properties. SCL-PHA is highly crystalline (Holmes 1985) and denser than water (1.23 g/cm3), while MCL-PHA has a low degree of crystallinity and a density close to that of water (1.019 g/cm3).
The objective of the present study was to evaluate different extraction and recovery techniques for PHA extraction from the bacterial isolate grown in Jatropha biodiesel byproducts and to standardize the efficient and cost effective PHA extraction and recovery process for PHA production utilizing Jatropha biodiesel byproducts.

2. Materials and Methods

2.1 Microrganism

Isolate SM-P-3M was used for all the recovery and extraction experiments. The isolate SM-P-3M was grown in 20g/L Jatropha biodiesel byproduct containing crude glycerol and 100ml/L Jatropha oil cake hydrolysate as the sole carbon and nitrogen source without the addition of any other additional nutrients and salts in the medium.

2.1 Cell settling by pH variation

100ml of culture broth containing cells was subjected to pH variation from pH 1 to pH 14. The cells suspension was transferred to separating funnel and the cells were allowed to settle at room temperature 32±5°C. The lower phase of settled cells was collected from the separating funnel and the PHA was extracted using the sodium hypochlorite method.

2.1 PHA recovery through solvent extraction

Dried cell powder 1g was taken in two different cellulose thimbles; the cell powder was mixed with 80 ml of chloroform and methylene chloride and was kept for 5 hours in automatic soxhlet apparatus at 90°C. The clear polymer solution was collected followed by filtration. The solvent was recovered by rotavapour. Finally, PHA was recovered from the chloroform and methylene chloride solution by nonsolvent precipitation, by adding dropwise the solution to methanol (methanol: chloroform = 9:1). Finally, the PHA-precipitate was filtered by simple filtration and then dried by evaporation at 60°C.
2.2 PHB recovery using sodium hypochlorite.

30 ml of 5% sodium hypochlorite solution was added to 1-g of dried cell powder and mixed properly by vortexing. After 15–30 min at 37°C, when the solution containing biomass and sodium hypochlorite turns white, it was considered that all the cellular residues are being digested. The PHA granules were collected by centrifugation. Pellet obtained was washed with water, methanol and acetone consecutively for the removal of the residual impurities by centrifugation at 8000 rpm for 20 minutes and then dried by evaporation at 60°C.

2.3 PHB recovery by using dispersions of sodium hypochlorite and chloroform.

One gram portion of cell powder was treated with a dispersion containing 50 ml of chloroform and 50 ml of 5% sodium hypochlorite solution. The mixture was kept in agitated condition on a shaker. After the cell powder was treated at 37°C for 1 h, the mixture was separated in a separating funnel, which resulted in three separate phases. The upper phase was a hypochlorite solution, the middle phase contained non-PHA cell material and undisrupted cells, and the bottom phase was chloroform containing PHA. The lower phase was collected, and the middle phase was separated by filtration from the chloroform phase, the chloroform was recovered using rotavapour. Finally, PHA was recovered from the chloroform phase by nonsolvent precipitation, by adding dropwise the chloroform solution to methanol (methanol: chloroform = 9:1). Finally, the PHA-precipitate was filtered by simple filtration and then dried by evaporation at 60°C.
2.4 PHA recovery through enzymatic digestion treatments

A cell suspension was prepared by adding 1g cell powder in approx 10 ml water. This suspension was then subjected to heat treatment by autoclaving at 121°C for 1 min prior to enzymatic treatment. The suspension was initially subjected to digestion with 0.3 g protease and 0.08 g SDS at pH 8.5 and a temperature of 55°C for 20 min. This was followed by further treatments with 0.4 g EDTA and 0.005 g lysozyme at pH 7 and a temperature of 30°C for 15 min. Finally the suspension was centrifuged at 4000g for 10 minutes and the pellet collected was rinsed with water and dried at 60°C.

2.5 Chemical digestion of non-PHA cell material by sodium hydroxide and potassium hydroxide

1 gm of dried cells in the total digestion volume of 10ml were treated at 37°C for 1 h with 0.1 N sodium hydroxide- NaOH and 0.1N potassium hydroxide- KOH to digest non-PHA cell material for the recovery of PHA. The cells were mixed properly by vortexing with chemical solution to digest non-PHA cell material; PHA granules were separated from the aqueous fraction containing cell debris by centrifugation at 2500g for 20 min. The PHA granules recovered were gently rinsed with distilled water, recentrifuged, and air-dried.

2.6 Determination of molecular weight by Gel Permeation Chromatography (GPC)

Using the universal calibration method, the molecular weight of PHA sample were determined with gel permeation chromatography (GPC). GPC (Waters 2695 separation module coupled with 2414 R1 detector) equipped with two serially connected column (Waters styragel THF 4E, Waters styragel THF 5). Monodisperse poly-styrene (Mp of 22701, 103000, 419000, 22800, 34, 40,000 Waters. USA) and chloroform were used as a molecular weight standard and mobile phase, respectively.
3. Results and discussion

3.1 Cells settling and coagulation by variation in pH

Ease of recovery of PHA is a very important parameter for economical feasibility of the production process of PHA. An effective method of cell harvesting of the bacterial cells was investigated by variations in the pH of the culture broth using NaOH and HCL solution. The suspended cells rapidly coagulated and initially floated but started settling after 30 to 60 minutes, which allowed simple harvesting of the concentrated cells from the culture broth. The pH for successful coagulation was pH-14 (Figure 1 and 2) and almost all the cells were recovered from the culture broth.

Figure 1. Effect of different pH on cells coagulation and settling
Figure 2. Cells coagulation and settling at pH 14 after 30 to 60 minutes in separating funnel and compared to control in which after 60 minutes there is no visible settling or coagulation of cells observed.

The PHA obtained from the control was 0.491 gm while PHA obtained from the cells settled was 0.397 gm which shows approximately 19.14% decrease in the yield of the PHA obtained from the cells settled at pH 14, which might be due to the degradation of the polymer at higher pH value.

3.2 PHA Recovery by Chloroform Extraction

Chloroform and other chlorinated hydrocarbons dissolve all PHA from culture biomass. The dissolved polymer is separated from the solvent, usually by evaporation or precipitation with alcohol such as methanol. Although more economical ways of recovering PHB have been improved. The recovery of PHA using chloroform and
methylene chloride was done using automated soxhlet apparatus (SOCS PLUS SCS 6, Pelican equipments).

Figure 3. PHA recovery using chloroform and methylene chloride in automated soxhlet apparatus- SOCS PLUS SCS 6, Pelican equipments.

The PHA obtained from chloroform was 0.762 g while that obtained from methylene chloride was 0.685 g from 1g of cell powder, which shows that there was 10.1% decrease
in the recovery of PHA by methylene chloride. The weight average molecular mass of the
PHA recovered by chloroform extraction was 8,99,898. The polydispersity index (PI)
(weight average molecular weight/ average molecular weight) represents the molecular
weight distribution obtained was 4.3. The average molecular weight (Mw) of the PHA
recovered using methylene chloride was 4,38,700 and the polydispersity index obtained
was 2.84.

3.3 PHA recovery using sodium hypochlorite

The hypochlorite solution was used, based on the fact that it can dissolve nearly all
components of cell except PHB granules. In addition, low sodium hypochlorite
concentration reduces the cost of production. But, high concentration of sodium
hypochlorite can also attack the PHB granules and causes severe degradation of PHB,
rendering the PHB unsuitable for some applications like medical applications, in which
highly pure PHA is used. Since recovery of PHA contributes significantly to the overall
economics, development of a process that allows the simple and efficient extraction of
polymers is desirable.

The PHA recovered using the sodium hypochlorite was 0.756 g from 1 g of the cells. The
average molecular weight (Mw) of the PHA recovered using sodium hypochlorite was
6,52,420 with a polydispersity index of 1.61.

The important features of sodium hypochlorite such as strong oxidizing properties and
non selectivity can be manipulated to digest non PHA cellular mass and facilitate PHA
recovery.
3.4 PHB recovery by using dispersion of sodium hypochlorite and chloroform.

Using the dispersion of sodium hypochlorite and chloroform the degradation of PHA by hypochlorite can be minimized and thus minimize the problems associated with conventional PHB digestion method (Hahn et al 1994). When the dispersion system was used with the isolate SM-P-3M cells grown in Jatropha biodiesel byproducts, however the molecular weight decreased much more than it decreased when the sodium hypochlorite solution was used alone. The PHA recovered using dispersion of sodium hypochlorite and chloroform was 0.728 g from 1 g of the cells and the average molecular weight (Mw) obtained was 2,79,385 with a polydispersity index of 3.9.

3.5 PHA recovery through enzymatic digestion treatments

The environmental implications and human toxicities associated with halogenated compounds, however, have created a need for much safer separation processes. One separation method that is both gentle (100% biological) and selective is through enzymatic digestion. Various studies in recovery processes through enzymatic digestion treatments have been undertaken by many researchers. (Holmes 1985, Eggink and Northolt 1999, Yasotha et al 2006). The PHA recovered by enzymatic digestion was 0.587 g from 1 g of the cells and the average molecular weight (Mw) obtained was 5,49,324 with a polydispersity index of 1.53. The enzyme technique is attractive because of their mild operation conditions. Since enzymes are very specific with respect to the reactions they catalyze, recovery of PHA with good quality could be expected. Nevertheless, the high cost of enzymes and complexity of the recovery process outweigh its advantages.
3.6 Chemical digestion of non PHA cell material using sodium hydroxide and potassium hydroxide.

Simple chemicals like sodium hydroxide and potassium hydroxide were examined for their ability to digest non PHA cell material for the recovery of PHA from the cells. The PHA recovered by NaOH was 0.682 g from 1 g of cells and the average molecular weight (Mw) obtained was 553486 with a polydispersity index of 1.74. The PHA recovered by KOH digestion was 0.6243g from 1 g of cells and the average molecular weight (Mw) was 996155 with a polydispersity index of 1.19. There was a decrease of 8.46% in the recovery of PHA by KOH as compared to the PHA recovery by NaOH. The digestion by simple chemicals like NaOH and KOH can be used for efficient and economical recovery. The cost of such simple chemicals is also very lower than other chemicals used in different recovery methods.

The highest PHA recovered was 0.762 g using chloroform soxhlet extraction but the extraction procedure was time consuming and the cost of solvents can contribute to high cost making the recovery process costly. While the PHA recovered by sodium hypochlorite was 0.756g which was almost near to that recovered by chloroform soxhlet method. Since cost is the major deciding factor in the selection of a suitable method, therefore, the sodium hypochlorite method used, was found to be the most simple and effective method resulting in high yield of polymer in less time and thereby making the PHA production process cost effective. Isolate SM-P-3M was found to be the most promising isolate with PHA/CDW (Cell Dry Weight) content of 75% followed by isolate SM-P-1S with 72% PHA/CDW. Exploitation of these two promising isolates for commercial production of PHA using Jatropha biodiesel byproducts as raw material and
the recovery process utilizing the low concentration of sodium hypochlorite for PHA recovery will result in economic and efficient PHA production process.

The final intended application for the PHA will determine the degree of purity of the PHA. For example, in medical applications it is absolutely necessary that the PHA should be free from bacterial endotoxins and other contaminating chemicals and solvents. On the other hand, if the PHA is intended for applications such as films or garbage bags, a lower degree of purity may be acceptable.

A very efficient and cost effective harvesting method to harvest the concentrated cell by variation in pH of the broth is reported in this work which is the easiest way to harvest the cells and can avoid the additional costs for centrifugation and thereby making the whole recovery process for PHA production using *Jatropha* biodiesel byproducts a cost effective process. The success of PHA as a viable alternative to petrochemical-derived polymers, however, will depend upon the design and implementation of efficient and selective means of PHA production and recovery.