Effect of pH and Temperature on Synthesis of Polyhydroxyalkanoates from Dairy Waste Water

YOGESH S¹, NIRMAL KUMAR G¹, SARAVANAKUMAR P¹, DHAYANANTH N² and RAMESH BABU N G²,
Department of Biotechnology, Adhiyamaan College of Engineering, Hosur-635109, India

Abstract

The recent years Polyhydroxyalkanoates (PHA), a kind of bioplastic are renewable and become order of the day. Dairy waste water has high concentration of organic compounds present in the effluent hence it suitable as substrate for the growth of microorganisms. This study aims at screening and identifying potential condition for the production PHAs from dairy industry waste water. The samples had pH varying from 3 to 9 and maintained at varying temperatures of 28°C to 38°C respectively and then incubated for 72 hrs at 150 rpm. The screening of PHA was performed by Sudan Black B staining by identifying the appearance of blue coloured granules in bacteria cells. The accumulated PHA in bacterial cells was extracted by using a solvent. The maximum yield of PHA 70.81% was observed at pH 6 and in case of temperature at 34°C maximum yield of PHA 60.61% was obtained. The analysis for PHA was carried in thin layer chromatography and UV Spectroscopy confirmed the presence of PHA.

Key words: Dairy wastewater, Polyhydroxyalkanoates (PHA), Sudan Black B Stain, Parameters

1. Introduction

Plastics are non-biodegradable causing them to be inert to natural and chemical breakdown and they also emit greenhouse gas during their production and combustion process. In order to overcome these problems, synthetic biology is being used to produce polymer from biomass which do not have any petroleum based origin nor environmental impacts. These plastics are collectively called bioplastics.

Bioplastics are bio-based polymer that can be directly derived from microorganisms or can be synthesized as result of chemical reaction of biological reactants that can compromise the properties of conventional plastics like polypropylene. Bio-based materials such as polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols are potential candidates for substitution of syntheticplastics. Among these, polyhydroxyalkanoate (PHA), which belongs to the group of polyoxoesters has received intensive attention because it possesses biodegradable thermoplastic properties (Jiun-Yee Chee et al., 2010).

PHA is synthesized from acetyl coenzyme A by a sequence of three reactions catalyzed by 3-ketothiolase, acetoacetyl CoA reductase dehydrogenase and PHA synthase and this pathway is regulated by oxygen limitation. Under oxygen depletion condition, the acetyl-CoA will no longer enter the TCA cycle at the same rate, it instead is converted to acetoacetyl-CoA by 3-ketothiolase, the first enzyme of the PHA biosynthetic pathway followed by the synthesis of PHA as final product (Anderson et al., 1990).

Water management in the dairy industry is well documented but effluent production and disposal still remain a problematic issue for the dairy industry. The turbid nature of dairy waste water because of high organic materials was reported by Shaikhparween (2009). The high concentration of organic compounds present in the effluent is suitable for the growth of microorganisms. Cullinson (1982) identified carbohydrate, fats, proteins, minerals, vitamins and water as nutrients in dairy waste water.

The production cost ultimately depends on the substrate used as carbon source and the extraction methodology. The present work involves using dairy industry waste water as a potential source for the production of PHA. Since dairy waste water has high carbon source to support the growth of bacterial cells and also high BOD that regulates the metabolic pathway results in production of PHA. The present study on PHA synthesis was identified by utilization of microorganisms in dairy waste water naturally as the inoculum with the effect on operational parameters such as pH, temperature. Dairy waste water can be used as a cheaper source and an alternative methodology for the production of biopolymers/ bioplastics.
2. MATERIALS AND METHODS

2.1 Sample collection

Dairy industry effluent was collected from Dharmapuri cooperative milk producers unit, Krishnagiri, Tamilnadu. A Sample was taken from the fresh effluent and stored in sterilized polyethylene bottle and maintained at 4°C in a refrigerator. A sample of 500ml each was taken and the pH 3, 4, 5, 6, 7, 8 and 9 was adjusted by adding 0.1N HCL and 1M NaOH accordingly. Similarly, a sample of 500ml each were taken and their temperature was maintained in the range of 28°C, 30°C, 32°C, 34°C, 36°C and 38°C. The samples were kept in shaker incubator for 72hrs at 150 rpm.

2.2 Screening of PHA

The Slide method was followed to identify the PHA granules in the culture by using Sudan black B staining method. The method helps to confirm the synthesis of PHA granules by up taking the stains in the bacteria as intracellular products after incubation for 48hr in the samples.

2.3 Slide method

The culture was heat fixed on a slide and immersed in 0.5% (w/w) Sudan Black B staining with ethylene glycol for 5min. Then the slide was air dried and excess amount of stain was destained using xylene several times. Then the counter stain (0.5% w/v saffranin) was added for 5 to 10 seconds. The slide was washed with tap water and dried. The stained cells were observed under oil immersion for the presence of PHA.

2.4 Extraction of PHA

After incubation, 1ml of culture was centrifuged at 4000 rpm for 35 min. The pellet was treated with 1ml of sodium hypochloride and centrifuged at 4000 rpm for 20 min. The pellet was washed by centrifugation at 4000 rpm with distilled water followed by acetone and methanol respectively. The pellet was suspended in 0.5 ml of chloroform and evaporated.

2.5 Quantification of PHA

The bacterial culture was centrifuged at 6000 rpm to obtain the cell pellet and dried to estimate the Cell Dry Weight (CDW) in units of g/L (Du et al., 2001). Residual biomass was estimated as the difference between dry cell weight and dry weight of extracted PHA (Zakariaet al., 2010). This was calculated to determine the cellular weight and accumulation other than PHAs. The percentage of intracellular PHA accumulation is estimated as the percentage composition of PHA present in the dry cell weight.

\[
\text{Residual biomass} \ (\text{g/L}) = \text{CDW} \ (\text{g/L}) - \text{Dry weight of extracted PHA} \ (\text{g/L}) \\
\text{PHA accumulation} \ (%) = \left( \frac{\text{Dry weight of extracted PHA} \ (\text{g/L})}{\text{CDW} \ (\text{g/L})} \right) \times 100\%
\]

2.6 Assay of PHA

2.6.1 Thin Layer Chromatography

About 50 µl of samples was loaded on the TLC plate and allowed to run in the solvent system consisting of ethyl acetate and benzene (1:1) mixture for 40 min. For staining, 50ml of iodine solution was vaporized in water bath at 80-100°C. TLC plate was kept over the beaker containing vaporizing iodine solution for 5-10 min in order for PHA to get saturated with iodine vapor. After 10 min, green-black coloured spots indicated the presence of PHA. The R_f(Retardation factor) value was measured and compared with a standard chart.

2.6.2 UV-Absorption spectrum

UV absorption spectrum of the polymer was analyzed following its conversion to crotonic acid by treating 0.5ml of sample with 4.5 ml of conc. H_2SO_4 and kept in water bath for 10 min. Later the absorbance was measured between 160 to 300 nm with UV spectrophotometer. The confirmation of PHA was determined by observing peak at 230 nm.

3. RESULTS AND DISCUSSION

Bioplastics (Biopolymers) are being widely studied as biodegradable materials for substitution of oil derived polymers. Polyhydroxyalkanoates (PHA) that can be directly derived from microorganisms or can be synthesized as the result of a chemical reaction of a biological reactant. A cost reduction in the biosynthesis process could be obtained either by searching for new cheap substrates, new fermentative strategies, and new recovery and purification steps or by using microorganisms capable of synthesizing and accumulating PHAs to high concentrations.
In the present work, synthesis of PHA is derived from dairy waste water as an inoculum and one of the cheap substrates obtained as waste after the production of various milk products and cleaning of reactor from storage tank. PHA can be produced in almost all bacteria in the form of intracellular inclusions. Another aspect introduced in the present study to identify suitable parameters to be used by a consortium of microorganisms that helps in potential synthesis of microbial environment.

In response to certain deficient growth conditions, biosynthesized PHAs can make up to 90% of the dry cell mass (Lei Pei et al., 2011). The PHA production process was defined through a series of steps, taking into account and optimizing the operative parameters involved. Some microbes such as *Rolstoniaeutropha* (>80%), *Alcaligeneslatu* (>75%), and *Pseudomonasoleovorans* (>60%) in their wild forms can produce PHAs in sufficient quantity (ranging from 50% to 80% of the dry cell mass).

The PHA granules could exactly quantified by increase in their cell mass weight corresponds to the ability of microorganisms. Sudan Black B staining method was used to screen the PHA synthesis from bacteria in the mixed culture. Bluish black colonies indicate the presence of PHA in the culture (Adwitiya pal et al., 2009). Similarly, the appearance of blue coloured granules under fluorescence microscope in 40X magnification in the cytoplasm confirmed the presence of the PHA synthesized in bacteria cells from dairy waste water.

Several extraction methods for PHA recovery have been developed. These involve centrifugation, filtration, extraction with organic solvents (chloroform and methanol), treatment with sodium hypochlorite, and digestion with enzymes. In this study, solvent extraction method was followed for the extraction of PHA from the culture after incubation up to 72 hrs obtained by repeated centrifugation with various solvents. The purified polymer was highly soluble in chloroform, NaOH, moderately soluble in dioxane, pyridine, and toluene, but insoluble in water, sodium hypochlorite, acetone, ethanol, methanol, and diethyl ether (Adwitiya pal et al., 2009). A soluble form of extract in the chloroform was obtained and dried at room temperature [Fig 1].

Assay analysis of the extracted samples for the confirmation of PHA was performed with the help of Thin Layer Chromatography. Green-black color band was observed in the TLC sheet that indicates the presence of PHA. According to Rawate and Mavinkruve (2002), the retardation factor was calculated and found to be 0.71 which was compared with the standard chart that confirms the presence of PHA in the chloroform extract.

![Figure 1: Chloroform dried extract of PHA](image-url)

The PHA granules content in the cell was demonstrated by UV spectrophotometer method by crotonic acid conversion (Kato et al., 1992). The polymer granule was dissolved in concentrated sulphuric acid (1mg/ml) and heated at 100°C for 10 min to convert PHA into crotonic acid, which was brown coloured. The solution was cooled and the absorbance was read at different wavelengths between 160-300 nm against a concentrated sulphuric acid as blank in the spectrophotometer.

![Figure 2: UV absorbance spectrum of PHA extracted from samples of dairy waste water](image-url)
From the UV spectrum, the absorbance read for all the pH and temperature varied samples was found to have maximum peaks at 230nm as shown in figure 2. This confirms the presence of PHA in the chloroform extracted samples and then compared with the standard spectrum of the crotonic acid which was found to be at 230nm.

Typically, metabolic processes are highly susceptible to mild changes in pH. The cell dry weights for each parameter were calculated using the formula corresponding to measure the accumulation of PHA from the biomass. The effect of pH on the accumulation of the PHA and their yield for corresponding pH was shown in Table 1. At pH 7 and 8 there was maximum amount of cell dry weight, but PHA accumulation was comparably low in those samples. In case of pH 3, the cell dry weight was very low but the percentage of PHA yield was 59.41%. This shows the evidence of the influence of the pH in the synthesis of PHA. The comparison of PHA synthesis from the Table 1 found the economical yield in the pH 6 and cell dry weight 1.85 g/L which have major role in the scale up the process.

Table 1: Effect of pH on the PHA accumulation

<table>
<thead>
<tr>
<th>S.No</th>
<th>pH</th>
<th>CDW (g/l)</th>
<th>PHA (g/l)</th>
<th>PHA yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>2.24</td>
<td>1.01</td>
<td>45.06</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.79</td>
<td>0.47</td>
<td>59.41</td>
</tr>
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<td>3</td>
<td>4</td>
<td>1.86</td>
<td>0.71</td>
<td>39.35</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1.87</td>
<td>0.95</td>
<td>48.07</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1.85</td>
<td>1.31</td>
<td>70.81</td>
</tr>
<tr>
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<td>7</td>
<td>2.16</td>
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<td>58.26</td>
</tr>
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<td>8</td>
<td>2.28</td>
<td>1.08</td>
<td>47.31</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>0.87</td>
<td>0.23</td>
<td>26.37</td>
</tr>
</tbody>
</table>

The accumulations of PHA granules in the bacterial cells were observed corresponding to their cells dry weight calculated in pH and temperature incubated samples. The sample incubated at pH 6 found to have maximum percentage of yield 70.81% comparatively as shown in table 1. Similarly in temperature varied samples, maximum percentage of yield 60.67% PHA accumulation was observed at 34°C as shown in table 2. These observations help to analyze setting optimum temperature and pH which play a vital role in the growth and accumulation of product in cultures.

4. CONCLUSION

Synthesized of microbial PHAs have emerged as a complementary material for petrochemical based plastics and have drawn much attention due to the biodegradable and biocompatible properties. The use of carbon and other nutrient-rich dairy waste water would be a cheaper substrate in synthesis of PHA by microorganisms that occur naturally in dairy waste water. The microbial environments are highly susceptible to mild changes in pH, temperature and also nutrition in sufficient or insufficient as controlling parameter to alter metabolic pathways. The PHA synthesis in dairy waste water found to have economical yield as optimum condition at pH 6 and at temperature 34°C which will have major effect in the scale up of the process. Owing to enormous amounts of dairy waste water availability, utilization of such a resource be economical and would help in reducing the cost of production of biopolymers.
5. Future work

Dairy waste water can be utilized as a cheap carbon source in harnessing PHA. Further optimization on nutritional requirement, microbial selection via scaling up will lead to increase significant levels in the production of PHAs. Focus on commercialization of production of PHA will bring down the cost and make it available so that it can be used for domestic purposes.

6. References
