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Research Article

COMPARITIVE STUDY ON EFFICACY OF ALKALINE AND ACID EXTRACTION FOR PHA RECOVERY FROM *BACILLUS SUBTILIS* NCDC0671

Mridul Umesh[1]* and Mamatha C[2]

[1]Department of Life Sciences, CHRIST (Deemed to be University), Hosur Road, Bengaluru 560029, Karnataka, India
[2]Department of Bioscience, MES College, Marampally, Aluva, Ernakulam 683107, Kerala, India

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ABSTRACT

The increasing global concern regarding the environmental hazards caused by the use and careless discharge of synthetic plastics has led to the increasing focus on bioplastics that are nontoxic and ecofriendly. Polyhydroxyalkanoates are a class of truly biodegradable polymers with material properties closely resembling their synthetic counterparts. Their biodegradability and biocompatibility had made them widely useful for application in medicine and industry. In spite of their potential application the major factor that hinders its commercialization is the production cost contributed significantly by extraction process for PHA recovery. This research work focuses on screening rapid and simple acid and alkali based method for PHA production. Further the effect of incubation temperature, concentration of acid/alkali and time period of treatment on PHA recovery was also assessed. The results obtained from the present study revealed that maximum PHA recovery of 76.53 ± 0.27% was obtained when the bacterial biomass was treated with 0.5 M NaOCl concentration.

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are the most widely studied class of bioplatics that are completely biodegradable. PHA's are polyesters of Hydroxyalkonates (HA's) synthesized by numerous bacteria as an intracellular carbon and energy storage compound. These are the only plastics produced exclusively by microorganisms and hence are completely degraded to benign compounds. Downstream processing has a great significance in determining the overall cost of commercial PHA production process (Jacquel *et al.,* 2008). Extraction of intracellular PHA granules from NPCM is a major technical challenge in PHA recovery as both are in same phase (solid phase). It is an integral component in PHA production process and determines PHA purity, material properties and overall economics of the fermentation process. Extraction and purification of PHA from microbial cells basically works on two strategies – NPCM dissolution and PHA solubilizing. In PHA solubilizing approach, PHA is selectively dissolved in appropriate organic solvent followed by precipitation. In the second approach, NPCM components are subjected to chemical digestion leaving behind pure PHA. Methods of PHA recovery differ among various bacterial strains based on their cell wall structure, composition and fermentation conditions. Often pretreatment methods are a prior requisition for effective PHA recovery as they facilitate effective cell disruption leading to release of PHA granules. Heat pretreatment has a direct impact on the integrity of cell envelope as it destabilizes the outer membrane (Kapritchkoff *et al*., 2006). Alkaline pretreatment method using NaOH involves the release of cellular proteins (Tamer *et al*., 1998). In salt pretreatment water is forced to move out of the cells due to high salt concentration leading to cell shrinkage associated with dehydration (Tamer *et al*., 1998). Pretreatment of microbial biomass through freezing makes them succeptible to digestion by NaOCl and SDS in further extraction steps (Dong and Sun, 2000).

Variety of chemical digestion methods have been evaluated for the recovery of PHA from cellular biomass. The approach is based on the solubilization of non-PHA cellular mass (NPCM) leaving behind PHA. Alkaline treatment method usually employs the use of alkalis like potassium hydroxide, sodium hydroxide and ammonium hydroxide capable of effectively digesting NPCM leaving behind high purity PHA with PHA

Corresponding author:* **Mridul Umesh
Department of Life Sciences, CHRIST (Deemed to be University), Hosur Road, Bengaluru 560029, Karnataka, India

yield greater than 91% (Choi and Lee, 1999). Ammonia extraction has an added advantage as the waste generated could serve as nitrogen source for successive PHA fermentation steps (Page and Cornish, 1993).Inorganic acids like sulfuric acid and hydrochloric acids were evaluated to be effective for PHA recovery (Choi and Lee, 1999). In practice the NPCM including peptidoglycan component making the cell wall is digested by acid treatment whereas the PHA granules resistant to acid attack are released out of the cellular biomass (Yu and Chen, 2006). The cheap mineral acids at appropriate concentrations selectively solubilize the NPCM with little decomposition of the polyesters.

The present research work describes elaborate methods for sorting out the ideal extraction method for effective recovery of PHA from bacterial biomass by comparing alkaline and acid extraction methods. The effect of temperature and concentration of various agents were assessed to finalize an effective recovery method for enhancing PHA recovery

MATERIALS AND METHODS

Microorganism and chemicals

Bacillus subtilis NCDC0671strain used in this study was obtained from National Dairy Research Institute, Karnal, India. Media components were purchased from HiMedia Laboratories Pvt Ltd, (Mumbai, India). All the media components were of analytical grade and solvents were procured from Merck (Mumbai, India). No experimental animals have been used for the whole study.

Inoculum preparation

Bacillus subtilis NCDC0671 inoculum was prepared in sterile nutrient broth (Himedia, India) that was contained in test tubes. The test tubes were inoculated with 2-3 loops of *Bacillus subtilis* NCDC0671 strain previously subcultured and maintained in nutrient agar slants. The inoculated tubes were incubated at 37°C for 24 h. The inoculum contained approximately 2 x 10^4 CFU/mL of *Bacillus subtilis* NCDC0671.

PHA production in shake flasks

Modified nutrient broth medium comprised of the following; Glucose (10 g), Peptone (5 g), Yeast Extract (3 g), NaCl (5 g) in distilled water (1000 mL) was prepared and used as fermentation medium for PHA production (Radha*et al.,* 2015). The pH of the medium was adjusted to 7 and inoculated with 24 h old culture of *B. subtilis* NCDC0671 (1%)andincubated at 37˚C in a shaking incubator (120 rpm) for 72 h. For each extraction process PHA production was carried out in 100 mL production media contained in 250 mL Erlenmeyer flasks in triplicates. After incubation period the bacterial cells were harvested by centrifugation at 5000 rpm for 10 min and subjected to various extraction methods. The CDW and PHA content were recorded using the method suggested by Chien*et al*., 2007.

PHA recovery using alkaline extraction

Bacterial biomass (100 mg) was suspended in distilled water (5 mL) and pH was adjusted to 11 using NH_4OH, NaOH and KOH at varying concentrations (0.1 M, 0.5 M, 1 M and 5 M). The biomass suspension treated with alkalis was incubated in 50 mL tubes at 200 rpm at 30˚C for 1 h. The suspension was subsequently centrifuged at 10000 g for 10 min at 4°C.The pellet was washed twice with distilled water and dried at 60°C overnight (Jiang *et al.,* 2015). The CDW and PHA content were recorded. The ideal concentration of alkali providing maximum PHA recovery was selected for studying the effect of temperature and on alkaline extraction of PHA.

Effect of treatment temperature on alkaline PHA extraction

The bacterial biomass (100 mg) was suspended in distilled water (5 mL) and pH was adjusted to 11 using NH_4OH, NaOH and KOH (at the ideal concentration fixed in the previous step) and incubated in 50 mL tubes (200 rpm) at varying temperatures (30˚C, 60˚C and 80˚C) for 1 h. The suspension was subsequently centrifuged at 10000 g for 10 min at 4°C.The pellet was washed twice with distilled water and dried at 60°C overnight.The CDW and PHA content were measured. The ideal concentration of alkali and optimum temperature providing maximum PHA recovery was selected for studying the effect of treatment time on alkaline extraction of PHA.

Effect of treatment time on alkaline PHA extraction

The bacterial biomass suspension prepared in the same as described above was incubated in 50 mL tubes at 200 rpm in the ideal temperature range fixed in the previous step for varying time periods (1 h – 24 h). The suspension was subsequently centrifuged at 10000 g for 10 min at 4°C.The pellet was washed twice with distilled water and dried at 60°C overnight. The CDW and PHA content were measured.

PHA recovery using acid extraction

The bacterial biomass (5% w/v) was treated with varying concentration of H_2SO_4 (2.5%, 5% and 10% v/v) at 30°C for 3 h. After the acid treatment, the pH value was set to 10 using a 0.5 N NaOH solution, and the solid was washed with water. Finally the recovered solid fraction was treated with sodium hypochlorite (3% v/v) for 1 h for removing residual proteins. The suspension was subsequently centrifuged at 10000 g for 10 min at 4°C.The pellet was washed twice with distilled water and dried at 60°C overnight (Abelairas *et al.,* 2015).The CDW and PHA content were measured.

Effect of treatment temperature on acid extraction of PHA

The bacterial biomass (5% w/v) was treated with varying concentration of H_2SO_4 (2.5%, 5% and 10% v/v) at varying temperature (30°C, 60°C, 80°C and 100°C) for 3 h. pH adjustment and sodium hypochlorite digestion was performed in the same way as described in previous section. The optimum temperature providing maximum PHA recovery was selected for studying the effect of treatment time on acid extraction of PHA.

Effect of treatment time on acid extraction of PHA

The bacterial biomass (5% w/v) was treated with varying concentration of H_2SO_4 (2.5%, 5% and 10% v/v) at optimized temperature for various time intervals (1 h, 3 h, 6 h and 24 h). pH adjustment and sodium hypochlorite digestion was performed in the same way as described in previous section. The CDW and PHA content were recorded.

Statistical analysis

All the experiments were done in triplicates. Statistical analysis was done using statistical software Minitab 17.1.10 version. The standard error values have been displayed as Y error bars in graphs. The significant difference was determined with 95 % confidence level ($P < 0.05$).

Characterization of PHA

Characterization of extracted polymer was done using FTIR analysis. Extracted granules were dissolved in saline solution (30 kg m^{-3}) and 20 µl of the solution was deposited on KBr disc. The sample was then dried and IR spectra as recorded with a Bruker model FTIR spectrometer coupled to a Bruker IR microscope fitted with an IBM compatible PC running OPUS, Version 2.2 software (Mridul *et al*., 2018).

RESULT AND DISCUSSION

An effective method for isolation and purification of polyhydroxyalkanoates from bacterial biomass is a key step that determines the process profitability in the fermentation system. An ideal method for PHA recovery should lead high recovery and purity level at affordable cost. In this study a comparative analyses of various recovery methods were evaluated for PHA recovery. The effectiveness of the methods employed was assessed based on the percentage PHA recovered and the extent of solubilization of NPCM.

PHA recovery using alkaline extraction

Alkaline method for PHA recovery works by reacting with cellular lipoproteins, solubilizing the cell wall material and releasing the intracellular components. Alkalis dissolve the mucopeptide components in the cell as well as thechoic acids leading to the release of intracellular PHA component (Archibald *et al*., 1969; Cornett *et al*., 1979). In the present study effect of various alkalis like NaOH, NaOCl and NH_4OH on PHA recovery from bacterial biomass was examined under different temperature range and time periods. Maximum PHA recovery of 76.53 ± 0.27% was achieved with 0.5 M NaOCl concentration. Both NaOH and NH_4OH at 0.5 M concentration yielded maximum PHA recovery when compared with the other concentrations studied (Figure 1).

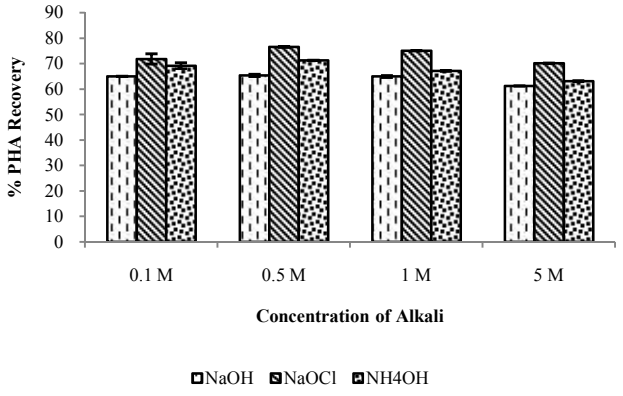

Figure 1 Effect of various concentrations of alkalis on PHA recovery

Each value is mean ± SD (triplicates); each value is significant at $P < 0.05$.

To evaluate the effect of treatment temperature on accelerating the level of PHA recovery using alkalis, NaOH, NaOCl and NH_4OH at 0.5 M concentration was mixed with bacterial biomass and allowed for alkaline digestion for 1 h at various incubation temperatures. The maximum PHA yield was obtained with 0.5 MNaOCl as depicted in Figure 2. Effect of treatment time on PHA recovery was carried out using 0.5 M alkali solution at 30°C. The result of this study revealed that maximum PHA was extracted after 3 h of alkali treatment and the highest PHA content (73.76 ± 0.05%) was recovered with 0.5 M NaOCl. Further increase in treatment time resulted in decrease in PHA yield probably due to degradation of PHA along with dissolution of NPCM (Figure 3). Choi and Lee (1999) reported that direct treatment of recombinant *E.coli* by 0.2 M NaOH solution resulted in 91% recovery. Hypochlorite is a strong oxidizing agent is effective in killing microorganisms by solubilizing 30% of the cell mass (Peng*et al*., 2002; Koning and Witholt *et al*., 1997). Similar results were observed in the study of Anis *et al.*(2013) with *C. nectar* as production strain. The result of this study is in agreement with recovery of PHA from *P.acidivarans* using alkali under various physiological conditions (Jiang *et al*., 2015). PHA recovered through chemical treatment often fails to meet the required standards for thermoplastic applications due to significant thermal stability deterioration. Alkaline treatment avoids the uses of toxic chemicals thereby reducing process economics and environmental pollution associated with chemical discharge.

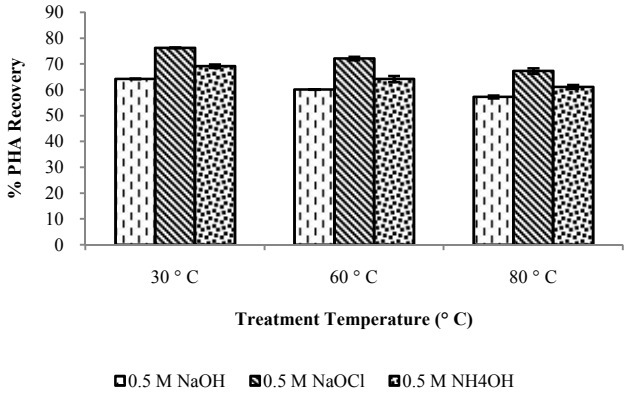

Figure 2 Effect of treatment temperature on alkaline PHA extraction

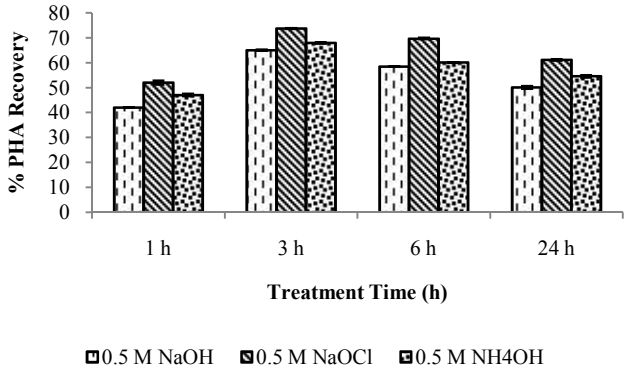

Figure 3 Effect of treatment time on alkaline PHA extraction

Each value is mean ± SD (triplicates); each value is significant at $P < 0.05$.

PHA recovery using acid extraction

The use of acidic treatment for PHA recovery has not been explored much especially with gram positive bacteria. Bacterial cell wall components including peptidoglycan are highly susceptible to acid attack releasing proteins and macromolecules in to the aqueous environment. PHA being highly resistant to acid attack can be thus effectively recovered. The effect of various concentration of H_2SO_4 on PHA recovery at different temperatures from *B.subtilis* cells were depicted in Figure 4. The highest PHA recovery (69.56 ± 1.98%) was observed when the bacterial cells was treated with 5% H_2SO_4(v/v) at 80°C (Figure 4).

Assessment of effect of treatment time on PHA recovery suggested that maximum PHA recovery was observed when the cells were treated for 3 h at 80°C using 5% H_2SO_4 (v/v) (Figure 5). Selective dissolution of PHA through acid treatment for polymer purification from *R.eutropha* was explored by Yu and Chen (2006). These results were in contrast to the observations of Choi and Lee (1999) who reported the inefficacy of H_2SO_4 for PHA recovery from recombinant *E.coli* cells.

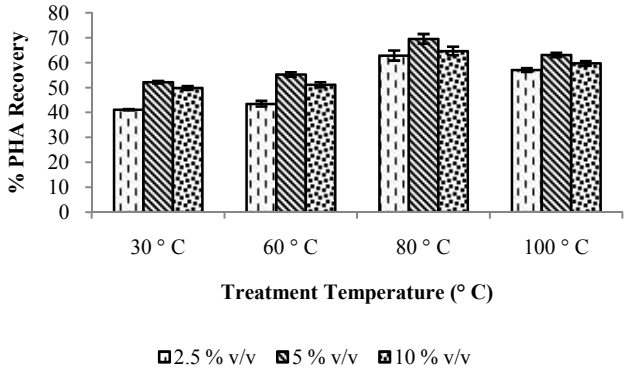

Figure 4 Effect of temperature on acid extraction for PHA recovery

Each value is mean ± SD (triplicates); each value is significant at $P < 0.05$.

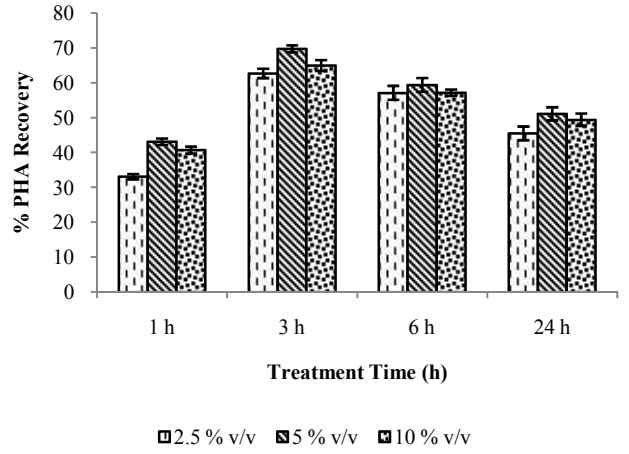

Figure 5 Effect of treatment time on PHA recovery by acids

Each value is mean ± SD (triplicates); each value is significant at $P < 0.05$.

Characterization of PHA

The ability of every chemical compound in the sample to contribute distinct peaks as a characteristic feature of the absorption spectrum serves as the basis of Fourier Transforms Infrared Spectroscopy. It is a most commonly used method to assess the molecular profile of a chemical compound. In the present study FTIR spectroscopy was performed between frequency ranges of 4,000 – 600 cm^{-1} (Figure 6). Intense absorption bands at 1724 cm^{-1} and 1276 cm^{-1} , corresponding to C=O and C-O stretching vibrations of the polymer respectively. The absorption peaks at 972 cm^{-1} and 771 cm^{-1} are due to C-H stretching contributed by methyl, methylene group. These significant absorption bands conforms the structure of PHA (Umesh *et al*., 2017).

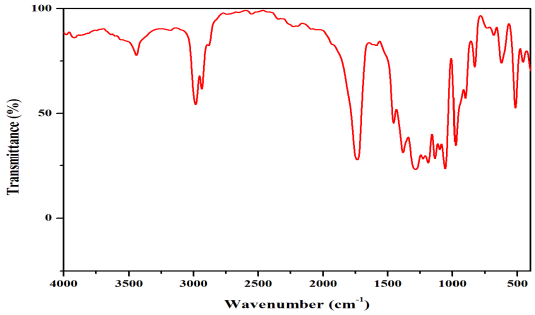

Figure 6 FTIR spectrum of extracted PHA

CONCLUSION

The present chapter outlines the effect of various extraction methods for enhancing PHA recovery from *B.subtilis* NCDC0671 cells grown in modified nutrient broth medium. In this study the comparitive effect of alkali and acid based extraction on PHA recovery was assessed on different incubation temperature and time periods. The presnt study reveals that highest PHA recovery was obtained using alkali extractio employing sodium hypochlorite treatment. Further optimization studies in future including statistical methods can open new arena in efficient recovery of PHA from biomass for its commercial application.

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Compliance with ethical standards

Conflict of interest

The authors declare that there was no conflict of interest.

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