

Production of Sterilized Medium Chain Length Polyhydroxyalkanoates (Smcl- PHA) as a Biofilm to Tissue Engineering Application

Anteneh Getachew¹, Admas Berhanu¹ and Andualem Birhane²

¹Department of Biotechnology, College of Natural and Computational Sciences, Wolkite University, Wolkite, Ethiopia

²Department of Biology, College of Natural and Computational Sciences Arba Minch University, Arba Minch, Ethiopia

Abstract

The current increase in the utilization of polyhydroxyalkanoates (PHAs) in various industrial and biomedical applications is due to their biodegradability, compatibility, resorbability and piezoelectricity. In the present study, we developed a modified medium chain length PHAs (Mmcl-PHA) by sterilizing surfaces of PHAs extracted from bacterial isolate. FTIR analysis of the neat polymer confirmed the presence of functional groups corresponding to alkyl halide, alkyne, hydroxyl group, and alkane groups. Sterilization of PHA using ethylene oxide as a medium was resulted modification of minor band differences in the absorption spectrum of homopolymer of PHB (scl-PHA) in to the co polymer of P (HB-co-HV) medium chain (mcl-PHA). The variation on to carbonyl (C=O) ester groups modified without significant changes to physico-chemical properties of the polymer were noticed. Sterilization of PHA using ethylene oxide has been evident surface modification properties suited to tissue engineering application of scaffold fabrication.

Keywords: Polyhydroxyalkanoates; Sterilization; Modification

Introduction

Polyhydroxyalkanoates (PHAs) are a category of natural polyesters as energy reserves in several species of microorganisms. They are wholly biodegradable, biocompatible and piezoelectric biopolymers that have attracted much attention recently as the biomaterial of choice for medical applications [1]. Polyhydroxyalkanoates can be considered as the natural biodegradable biomaterials used in different human clinical conditions. These biopolymers, due to their bioactive properties, they tend to have greater biological interaction with the cells, which allow them to have better performance in the biological system [2]. Adequate investigations have now been suggest PHA biopolymers promise to have a significant role in tissue engineering and the development of novel living tissue products for therapeutic applications [2,3]. These pledges of PHAs are due to it characteristics of biocompatible, support cell growth, guide and organize the cells, allow tissue in growth and, ultimately and degrade to non-toxic products [4].

Tissue engineering approaches employs scaffolds surface to be conducive to cell attachment and subsequent tissue growth [5]. This scaffolding composed of natural materials is commonly used as scaffolds to interact with biological systems to accomplish desirable medical outcomes in modern healthcare, providing alternatives to overcome the limitations and restrictions imposed by the use of autograft and allograft tissues [6]. Reactive polymer blending is one of the most economic and versatile way to produce materials combining the desired properties through forming the compatibilizing agents by inducing the chemico-physical interactions between polymer blends [7]. Hence, surface properties of PHAs have to be suited using different sterilization methods without altering its mechanical strength or thermal properties. Expectations for use of these scaffolds are increasing as the knowledge regarding their chemical and biological properties expands, and new biomedical applications are investigated [8].

Numerous surface modification techniques have been discovered and used deliberately for tissue engineering applications. To mention only a few, such modification techniques include gas plasma, Alfa radiation and ethylene oxide as well as the use of different chemicals, acids and bases were investigated [9,10]. The aim of this work has focused on production of PHAs polymer from bacterium isolated from different sample source. The polymer was further modified using ethylene oxide

treatment and, the monomers for the scaffold requirements have been fabricated and subsequently characterized using FTIR spectroscopy.

Methods

Isolation of bacterial strains

Ten different samples were collected from different localities around Arba Minch town (sewage sludge, waste water and river sludge). The samples were used for isolation of bacteria by serially dilution techniques (S^{-1} to S^{-6}) in nutrient agar medium. The isolated bacterial colonies were screened and potential isolates were preserved on nutrient agar slants and glycerol stocks, until further use [11].

Screening of PHAs producing bacterial strains

The relative occurrences of PHAs accumulating bacteria from samples were studied by microscopic screening by Sudan Black B staining and florescent staining of acridine orange. The accumulation of PHAs in the isolates was ranked according to Nandini et al. [9] screening method. The black and yellow stained isolates were ranked in terms of + symbol. The medium stained colonies as ++ symbol, strongly stained colonies as +++ symbol while excellently stained as ++++ symbol.

Quantification of PHAs and selection of one best isolate

For screening of one best isolate, PHAs extracted from each isolate measured and one best isolate was selected. For production of PHAs mineral salt medium (MSM) was used for fermentation process. The medium MSM contained the following ingredients (g/L): Urea (1.0), Yeast extract (0.16), KH_2PO_4 (1.52), Na_2HPO_4 (4.0), $MgSO_4 \cdot 7H_2O$ (0.52), $CaCl_2$ (0.02), Glucose (40), and trace element solution 0.1

***Corresponding author:** Anteneh Getachew Woldemariam, Wolkite University, Department of Biotechnology, P.O. Box -07, Wolkite, Ethiopia, Tel: +251-0913429224; Email: antutensu@gmail.com

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ml. Trace element solution contained (g/L): $ZnSO_4 \cdot 7H_2O$ (0.13), $FeSO_4 \cdot 7H_2O$ (0.02), $(NH_4)_6MO_7O_{24} \cdot 4H_2O$ (0.06) and H_3BO_3 (0.06). Both glucose and trace element solution were autoclaved separately, and reconstituted prior to inoculation. For PHAs production, 100 mL of production medium was inoculated with 1 mL of the organism. All the flasks were incubated on a shaker incubator at 37°C and 150 rpm for 48 hrs and the process was triplicated. Cells were collected by centrifuging at 10,000 rpm for 15 min, samples dried at 55°C to constant weight [12,13]. Quantification of PHAs has been done by crotonic acid assay of standard curve the method described by Burdon [14]. Then concentrated (98%) of 10 ml hot H_2SO_4 was added to the polymer granules. The addition of sulfuric acid converts the polymer into crotonic acid, which is brown colored. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank. By referring to the standard curve, the quantity of PHAs produced was determined and calculated using the following formula.

Residual biomass (g/L) = Dry Cell Weight (g/L) – Dry weight of extracted PHA (g/L)

PHA accumulation (%) = Dry weight of extracted PHA (g/L) / Dry Cell Weight (g/L) × 100%

Characterization of PHAs producing isolate

The positive PHAs producing bacterial isolates were subjected to a set of morphological, physiological and biochemical tests for identification. Biochemical characterization was done according to Bergey's Manual of Determinative Bacteriology.

Preparation of biofilm

PHA film was prepared by homogenizing 370 mg each of a PHA powder with PEG, glycerol in 40 ml of chloroform. The solutions were poured onto glass plate and dried at 30°C to obtain the films [15].

PHA sterilization using ethylene oxide

The biofilm further modified using the concept of the ethylene

oxide treatment technique described by Marois et al., 1999 [10]. Biofilm treated with ethylene oxide for 8 hrs 38°C with 65% humidity and subsequent measurement of changes within functional groups of treated polymer was quantified using FTIR.

Fabrication of PHA scaffolds

For fabrication of PHA, dispersing of a leachable material within a scaffolding material was required. Subsequently, fabricating the required material, shape, and then removing the leachable material to leave a porous scaffold [9].

PHA authentication

FTIR spectroscopy was used to record the PHA spectrum on the range of a scan 400 to 4000 cm at room temperature (27°C) was applied. A 0.01 g of the biofilm sample was prepared in dichloromethane and the mixture was then applied on to the NaCl crystal window and the spectrum was read after the solvent has been dried [16].

Results

Isolation of bacterial strain

A total of 15 representative colonies of bacteria isolated from 3 sampling areas. The isolated colonies were taken from 10^{-4} serial dilution of nutrient agar plate. The colonies were purified by repeated streaking on nutrient agar medium. Each colony of pure culture was individually picked based on distinct morphological characteristics and subjected to screening procedure of PHAs production.

Screening and characterization of PHAs producing strains

The relative occurrences of PHAs accumulating bacteria from samples were studied. A striking prevalence of PHAs producing bacteria was observed in wastewater sample. Out of the 20 bacterial isolates obtained from 15 samples, three isolates were showed positive response to staining procedures by accumulation of PHAs granule inside the cell (Figure 1). Screening procedures such as Sudan black and fluorescent staining results depicted in

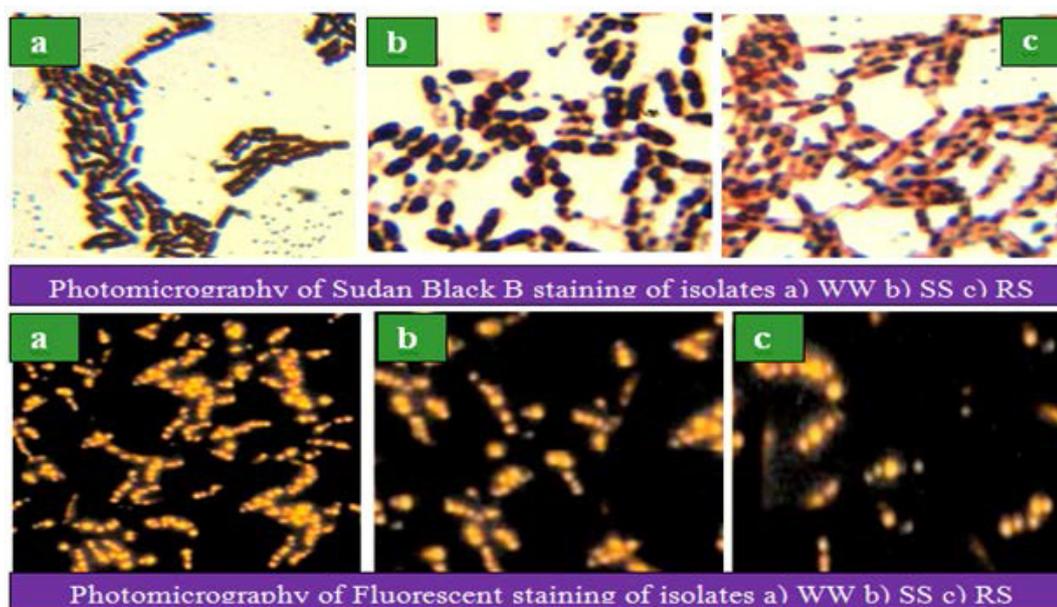


Figure 1: Photomicrography of Sudan Black B and Fluorescent staining of acridine orange of isolates.

Sample source	Designation of Isolates	Biochemical characteristics				PHA Accumulation	
		Endospore	Gram Staining	Catalase Production Utilization	Citrate	Sudan Black B	FIFluorescent staining
Waste water	WW	+Ve	+Ve	-Ve	+Ve	++++	++++
Sewage sludge	SS	-Ve	+Ve	+Ve	+Ve	+++	+++
River sludge	RS	+Ve	+Ve	-Ve	-Ve	++	++

Table 1: Biochemical characteristics and screening of isolates.

Table 1. The strains also subjected to biochemical characterization, from the result all isolates found as *bacillus* species (Table 1).

Selection of one best isolate

The isolates were further compared and screened by the amount of PHAs they produced utilizing glucose as a sole carbon source in their growth medium. The better PHAs producing isolates were identified based on the amount of cell dry weight of extracted PHA (Table 1). Isolate WW was selected for further studies as it produced the highest amount 46.28% cell dry weight of PHA.

Biofilm production and surface modification

PHA powder has been used as a matrix for production of a neat biofilm sheet. The neat biofilm sheet was further modified by using ethylene oxide treatment to suit its surfaces for tissue engineering application. On surface analysis, the blends exhibited higher values of roughness compared with the neat films (Figure 2).

Scaffold fabrication

Surface modified biofilm was subjected to scaffold fabrication. The

scaffolds were fabricated by using waxy components for intended shape and the component subsequently separated. The scaffolds fabricated for different tissue engineering applications are depicted in Figure 3.

PHAs authentication

FTIR spectroscopy was used to record the PHA spectrum on the range of a scan 400 to 4000 cm was applied. Both neat and modified form of polymers resulted in different peaks assigned for functional groups as shown below in Figure 4.

Discussion

Isolation screening and characterization of PHA producing strains

In present study potential PHAs polymer accumulating bacteria were isolated from diverse sources and the efficient strain was selected for further studies (Table 1). Among 15 bacterial isolates, 3 were able to produce PHAs which was confirmed by Sudan black B and fluorescence staining by observing the presence of granule inside the cell. Significant PHAs was produced by isolates from wastewater. The morphological



Figure 2: PHAs Polymer a) powder polymer extracted from bacterial isolate b) The neat biofilm sheet produced by chloroform method c) Modified form of the neat film blends using ethylene oxide.



Figure 3: Examples of PHAs scaffolds fabricated for tissue engineering applications, a) Meshes b) Woven c) Fibers.

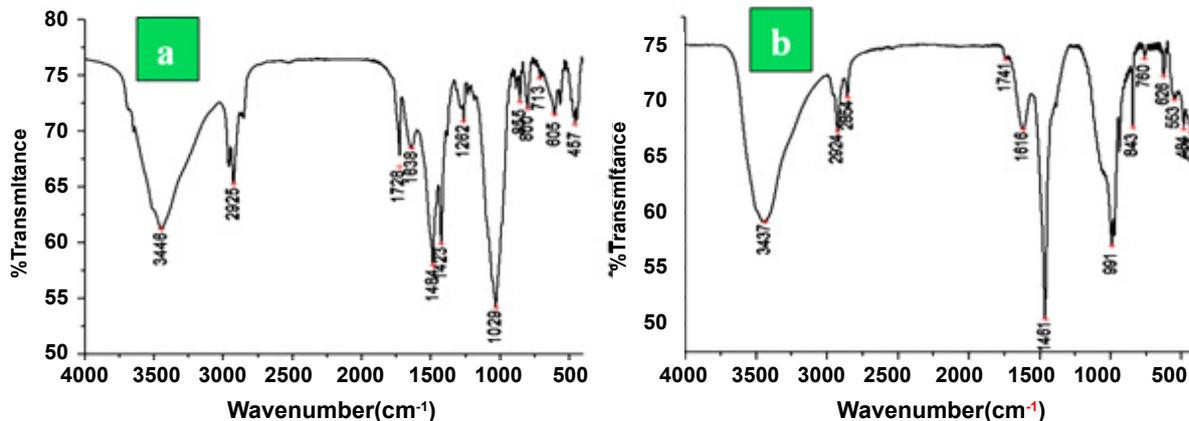


Figure 4: FTIR analysis (a) scl- PHA (P3HB) extracted from isolate WW (b) modified mcl-PHA P(HB-co-HV) using ethylene oxide as medium.

Isolates	Growth at 600nm	PHA (g/l)	Dry weight (g/l)	Residual Biomass (g/l)	PHA % (w/w)
WW	1.159	5.0 ± 0.44	12.0 ± 0.08	7.0 ± 0.05	46.28
SS	1.125	3.9 ± 0.03	11.0 ± 0.00	7.1 ± 0.13	35.45
RS	0.991	3.2 ± 0.07	9.4 ± 0.001	6.2 ± 0.39	34.04

Data represent the mean of 3 different replicates ± standard deviation.

Table 2: PHAs accumulated by the 3 selected isolates after 48 hrs of incubation at 37°C and pH 7.0 using shake flasks as a batch culture.

and biochemical characterization of isolates and their probable genera are depicted in Table 1. Most isolates found were *Bacillus* species. As such, similar results were reported *bacillus* species as an ideal PHA producer [17,18]. One best isolate was selected based on the amount of PHAs accumulation inside the cell. Hence, isolate WW was found to be a promising bacterium which produced (46.28% CDW) of PHAs and selected for further experiments (Table 2).

Surface modification scaffold production and PHAs authentication

Surface properties of any medical device are exceedingly important to interact with the host. As such, tissue engineering approaches employs cell seeded scaffolds that are essential and having surfaces conducive to cell attachment and subsequent tissue growth. Useful surface modification includes changes in chemical group functionality, surface charge, hydrophobicity, hydrophilicity and wettability [19]. In present study, PHAs biofilm produced from bacterial isolate was further modified using ethylene oxide treatment. Result obtained from the experiment was evident, surface properties of PHAs have been suited without altering other properties of the scaffold. Change in functional groups of the polymer blends quantified using FTIR spectroscopy and depicted in figure 4. The fabrication of a suitable scaffold using leachable material has been prepared and depicted in (Figure 2). Significant amount of work has been focused on identifying suitable scaffold configurations for tissue engineering applications. Study reported by Lorena et al. [19] evident, PHA polymer surface was modified by using the solvent-casting method. Using this method the resultant copolymers such as poly (3-hydroxybutyrate), P(3HB), and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(3HB-co-3HHx)) have been explored for use in nerve regeneration. Another study reported by Ralf et al. [8] was noted a scaffold for a trileaflet heart valve using a thermoplastic polyester modified by a salt leaching technique. In this study, a heart valve scaffold was constructed from a thermoplastic

elastomer known as PHAs. The PHAs extracted through fermentation was modified by a salt leaching technique to create a porous, three-dimensional structure, suitable for tissue engineering has been noticed.

FTIR is one of the rapid and powerful tools to obtain information on polymer structure. Because every chemical compound in the sample makes its own distinct contribution to the absorbance/transmittance spectrum [15]. The method is particularly suitable for screening a large number of bacterial cells and it has been demonstrated that PHA present within the cells can be rapidly detected by this technique [15,19]. In the present study, the observed infrared absorption at 3446 and 3437 cm^{-1} was assigned to the hydroxyl group of the polymer chain of both purified and modified polymers respectively [20]. The absorption band at 2925 and 2924 cm^{-1} was assigned to asymmetric C-H stretching vibrations of methyl groups of both polymers. A shift in CH_2 of the lateral monomeric chains was assigned to the stretching vibration at 2854 cm^{-1} has not appeared on the scl- PHA (Figure 4a). Although the absorption bands of 1728 and 1741 cm^{-1} has been reported to be a PHA marker band assigned to carbonyl (C=O) stretch of the ester groups present in the molecular chain of highly ordered crystalline structures according to Randriamahefa et al. [21]. These peaks were comparable with the standard peaks 1728 and 1740 cm^{-1} to scl-PHA and mcl-PHA respectively [22]. Short-chain length PHA represents (scl-PHA; 3 to 5 carbon atoms) and medium-chain length PHA (mcl-PHA; 6 to 14 carbon atoms) monomer units [23]. This kind of PHA is bearing different functional groups that can be modified by chemical reaction to obtain useful polymer and the monomer usually used for medical application of tissue engineering [18]. Minor band differences in the absorption spectrum of homopolymer of PHB and Co polymers of P(HB-co-HV) in ethylene oxide as a medium were noticed. Hence, the variation on to carbonyl (C=O) ester groups evident sterilization of biofilm using ethylene oxide has been modified without significant changes to physico-chemical properties of the polymer. The monomers of this kind of PHA could be given lower melting point and crystallinity to polymer and conducive to cell attachment and subsequent tissue growth (Figure 4b).

P3HB homopolymer had characteristic absorption bands of 1638 and 1484 cm^{-1} , while copolymer of P(HB-co-HV) showed a shift in N-H amid protein in 1616 and 1431 cm^{-1} . Absorption at 1423 cm^{-1} is owed to a terminal CH_3 group of PHB [24,25], while that was not appearing at copolymer P(HB-co-HV). The peak at 1262 cm^{-1} owes asymmetric C-O-C stretches vibration present on PHB. Moreover, series of absorption bands at 1029 cm^{-1} to 457 cm^{-1} were assigned

to C–O and C–C stretches of amorphous phase [26,27]. These series absorptions showed shift to 991 cm^{-1} to 433 cm^{-1} at copolymer P (PHB-co-HV).

Conclusion

The present study illustrated extraction and sterilization of PHAs produced by a bacterial isolate. Analysis of functional group using FTIR evident, absorption bands of 1728 and 1741 cm^{-1} of PHA marker bands assigned to carbonyl (C=O) were comparable with the standard peaks 1728 and 1740 cm^{-1} to scl-PHA and mcl-PHA respectively. Ethylene oxide was used as a potential chemical for sterilization and subsequent modification of PHA for fabrication of tissue scaffolds. In modification treatment the absorption band of homopolymer of PHB shifted to co polymers of P (HB-co-HV) in ethylene oxide as a medium. Other shifts were also observed absorptions assigned to CH_2 , C-O, C-C and N-H amid protein. Additionally, shift in CH_2 of the lateral monomeric chains were assigned to the stretching vibration at 2854 cm^{-1} was not appearing on modified PHA. Therefore, this modification experiment of PHA evident surface properties to suit the intended application often without altering other properties of the scaffold. The functional groups registered on modified PHA such as (alkyl halide, alkyne, hydroxyl group, and alkane groups) were very suitable for attachments and growth of new proliferative cells.

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Conflict of interest

The authors have not declared any conflict of interest.

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