Research Article

Anticandidal Activity of Bioplastic Sheets incorporated with Neem Seed Extract

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Received: 17-04-2017; **Revised:** 30-05-2017; **Accepted:** 11-07-2017.

ABSTRACT

This study focuses on the fabrication of anticandidal biopolymer sheets through the incorporation of neem (*Azadirachta indica)* seed extract. *Bacillus pumilus* MTCC 7055 strain was screened to assess its polyhydroxy alkanoates (PHA) accumulating ability through Nile blue and Sudan black staining. The polymer was extracted using sodium hypochlorite digestion followed by chloroform precipitation. Characterization of PHA was done using UV- visible spectrometry (UV-spec), Fourier Transform Infra-Red (FTIR) spectroscopy, and Nuclear Magnetic Resonance (NMR) and Differential Scanning Calorimetry (DSC) analysis. Chloroform extract of neem seeds were incorporated into PHA and bioplastic sheets was fabricated using solvent casting technique. The blended films were explored for its antifungal activity against *Candida albicans*. The results suggested that the anticandidal activity of the fabricated biopolymer sheets could offer promising development in medical packaging and in preventing candidal infections.

Keywords: *Bacillus pumilus*, polyhydroxy alkanoates, Anticandidal activity, *Candida albicans*, Bioplastic.

INTRODUCTION

Increasing environmental consciousness, in the last few decades leads to the development of eco-friendly and biodegradable materials as alternative to synthetic counterparts. The very versatile nature of plastics has made it an inevitable part in every sphere of human life. Due to strong regulation in the disposal of plastics, development of eco-friendly green plastics was considered to be the need of the hour.

Polyhydroxy alkanoates (PHAs) are biocompatible nature biopolymers synthesized and accumulated as intracellular carbon or energy storage material in some bacteria and fungi[1]. Being the polymers of hydroxyl alkanoicacid, they have material properties closely resembling petrochemical plastics.

Candida, a yeast genus causes major fungal thrush on the mucosal region of gastrointestinal, genitourinary tracts and skin of humans. The infection caused by candida called as Candidiasis. These are endo-symbionts of hosts. It may also enter the host if the immune system is not functioning properly. If the pH of the body imbalances, uncontrollable growth of candida occurs cause severe systemic problems especially in moist and warm areas. Mainly women were infected with candida in the urogenital tract called Vulvovaginal candidiasis (VVC).

Development of antimicrobial packaging for food and the medicinal application has increased focuses on the last few decades. Antimicrobial plastics are capable of retarding or inhibiting microbial growth as they are incorporated with active principles like volatile or non-volatile antimicrobial agents[2]. The practice of addition of phytomedicinal components especially herbal formulation into polymers has gained much attention due to potential antimicrobial and antioxidant activity.

The present research was designed to study the production and characterization of PHA from *Bacillus pumilus* MTTC 7055 and its subsequent utilization for fabrication of bioplastic sheets. Incorporation of neem seed extracts into polymer sheets was done to inculcate anticandidal activity to the fabricated biopolymer sheets.

MATERIALS AND METHODS

Microorganism

Bacillus pumilus MTCC 7055 strain was procured from Microbial Type Culture Collection and Gene Bank, Chandigarh, India. Stock culture was grown in nutrient agar medium (containing (g/L), peptone – 5.0, Beef extract-3.0, NaCl-5.0, Agar-20.0).All the media components were of analytical grade and were obtained from Hi Media Laboratories Pvt Ltd, (Mumbai, India) and solvents were purchased from Merck.

Screening for PHA production

Sudan Black Staining

Bacterial test culture was smeared on a clean glass slide and it was heat fixed. A few drops of Sudan black B solution (0.02gm in 100ml ethanol) was added to the smear and left for 10 minutes. Then the slides were washed gently with ethanol and counter stained with safranin and allowed to dry. The slide was observed under oil immersion under optical microscope[3].

Nile blue Staining

The fluorescence plate assay is a preliminary screening method for PHA production[4]. Complex nitrogen limiting agar medium having glycerol as C- source and Nile blue A

as stain were used for the preliminary screening method for identification of PHA accumulating strains. Test cultures were grown in NA slants with 1% NaCl. 16h cultures were patched onto Nile Blue A agar plates and incubated at 37°C for 3-4 days. The appearance of fluorescent colonies in nutrient agar plate incorporated with Nile Blue indicated the ability to produce PHA molecules.

Inoculum preparation

Inoculum for PHA production was prepared from 48 h old slant cultures of *Bacillus pumilus* MTCC 7055 incubated at 30°C. One loop of cells (2×10^3 CFU/mL) was transferred into 100mL of sterile nutrient broth taken in Erlenmeyer flask. The flask was incubated at 250 rpm at 30°C for 24 h and used as inoculums for PHA production.

PHA production and extraction

PHA production was carried out using modified mineral salts medium which contained following ingredients: 0.02% MgSO4, 0.05% KH2PO4, 0.25% peptone, 0.25% yeast extract, 5% glucose, 0.01% NaCl with pH 7[5]. The sterilized medium was inoculated with *Bacillus pumilus* MTTC 7055 (10% v/v) and incubated at 250 rpm and 30°C for 96 h. Extraction of PHA was done using chloroform extraction method[6]. The fermented medium was centrifuged at 10000 rpm for mins. The cell pellet was dried and weighed to calculate the cell dry weight (CDW). The cell pellet was suspended in sodium hypochlorite solution (4%) and incubated at 37°C for 1-2 h for complete digestion of cell components except for PHA. The mixture was centrifuged at 10000 rpm for 10 mins to collect the PHA granules and the sediment was washed using distilled water. PHA granules were washed twice with acetone and diethyl ether mixture (1:1). The resultant PHA granules were dissolved in boiling chloroform, air dried and weight of the polymer was recorded.

Characterization of PHA

UV-vis spectrometric analysis of PHA

The extracted PHA was dissolved in chloroform and subjected to scanning in UV-vis spectrophotometer (Shimadzu) in the range of 800-200 nm against chloroform blank and the spectrum was analyzed[7].

FTIR analysis of PHA

The functional group present in the biopolymer was characterized using Fourier Transform Infra-Red (FTIR) spectroscopy. In the present study, PHA was characterized in the frequency ranges of 4000-500 cm^{-1} (Schimadzu 8400s) to analyze the functional groups[8].

NMR analysis of PHA

The monomeric constituent of the produced biopolymer was characterized using Proton Nuclear Magnetic Resonance (^{1}H NMR analysis). ^{1}H NMR analysis was carried out using extracted polymer sample (5 mg) in deuterated chloroform ($CDCl_3$) and analyzed on Bruker Avance III spectrometer at 400 MHZ using tetramethyl silane as internal standard[9].

DSC analysis of PHA

Differential Scanning Calorimetry (DSC) is a routinely used technique for analysis thermal properties of bacterial biopolymer[10]. DSC analysis of the sample was carried out using Mettler Toledo 822C instrument at the temperature range of -50°C to 400°C (at a rate of 10°C per minute).

Neem seed (*Azadirachta indica*) extract preparation

Neem seeds (*Azadirachta indica*) were collected from Bharathiar University campus, Coimbatore, Tamilnadu, India. The seeds were authenticated as those of *Azadirachta indica* by Department of Botany, Bharathiar University, Coimbatore, and Tamilnadu, India. Neem seed extract preparation was done by soaking the dried seeds in chloroform (1:10v/w) for 8 days at room temperature[11]. The extract was filtered through Whatmann filter paper No.1 into sterile vials. The steps were repeated thrice and the collected extracts were used for anticandidal assay[12].

Phytochemical analysis of neem seed extract

The phytochemical constituents in the neem seed extracts were analyzed using standard procedure[13]. The extract was assessed for the presence of alkaloids, saponins, tannins, terpenoids, flavonoids, glycoside, volatile oil and reducing sugar using standard protocols.

Anti-fungal and phytochemical activity of neem seed extract against *Candida albicans*

Anti-fungal activity was assessed using well diffusion method. 20mL of sterilized Potato Dextrose Agar (PDA) medium was dispensed into petriplates and allowed to solidify for 20 mins. Wells were punctured in the solidified media for the addition of seed extracts. The plates were inoculated with overnight lawn culture of *Candida albicans* in a uniform manner. Different concentrations of seed extracts were loaded into the wells. The plates were incubated at 25°C for 24-48 h. the diameter of zone of inhibition was measured after incubation. Fluconazole is used as the commercial control.

Formulation of anticandidal bioplastic sheets

About 5g of PHA powder was dissolved in 95mL of chloroform under the stirring condition at 70°C for 8 mins for complete dissolution of PHA. 10% (w/w) polyethylene glycol (PEG) was added to the mixture and stirred well. 10mL of methanol extract of *Azadirachta indica* was added to the PHA solution and stirred for 8 mins at room temperature to ensure complete dissolution. 20mL of the contents were poured into the sterile petriplates and the solvents were allowed to evaporate completely. The bioplastic sheets were carefully removed and aging was done for 2 weeks and assessed for anticandidal activity. PHA sheets without neem seed extracts were prepared

using the same methodology and used as control sheets to assess anti-fungal activity[14].

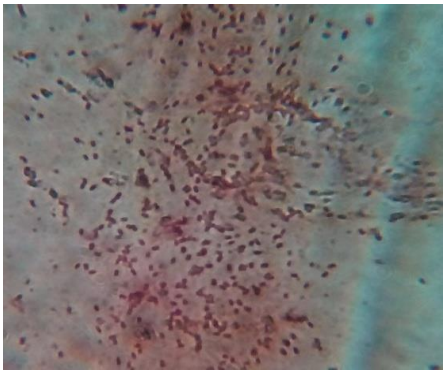
Anticandidal activity of bioplastic sheets

Anticandidal activity of neem seed extract incorporated bioplastic sheet against *Candida albicans* were assessed using Halo test. A Bioplastic sheet (1×1 cm) was placed on the center of PDA plates containing lawn culture of *Candida albicans*. The agar plates are incubated at 25°C for 24-48 h and the zone of growth inhibition around the seeds were recorded after the incubation period.

RESULT AND DISCUSSION

Screening for PHA production

Bacillus pumilus MTCC 7055 upon staining with Sudan black revealed the presence of intracellular dark PHA granules inside pink colored bacterial cells (Figure 1 a). The lipophilic stain Sudan black is routinely used to specifically stain the intracellular PHA granules and is used as a rapid method for screening of PHA producing organism[15].



(a)

(b)

Figure 1: Screening for PHA production (a) Sudan Black Staining (b) Nile Blue Staining

The intensity of fluorescence was evaluated as a method for screening PHA producers through Nile blue staining. *Bacillus pumilus* MTCC 7055 strain produced bright fluorescent when exposed to UV (Figure 1 b). The intensity of fluorescence produced by the culture is directly proportional to the amount of PHA accumulated and thus strain with greater intensity of fluorescence were selected as potential PHA producers.

PHA production and extraction

The amount of PHA produced by *Bacillus pumilus* MTCC 7055 was monitored for 96 h and the results were tabulated in Table.1.

Table 1: PHA production by *Bacillus pumilus* MTCC 7055 at various time intervals

Incubation Period	CDW (g/L)	PHA (g/L)	% PHA
24 h	4.63 ± 0.02	1.92 ± 0.05	41.468
48 h	4.83 ± 0.12	2.54 ± 0.17	52.587
72 h	5.03 ± 0.07	2.84 ± 0.23	56.461
96 h	4.92 ± 0.01	2.67 ± 0.03	54.268

The highest PHA production was observed during 72 h of the incubation period. Further incubation resulted in the decline of PHA productivity probably due to consumption of accumulated PHA for bacterial metabolism due to decreased nutrient content in the fermentation medium.

Characterization of PHA

UV - visible spectrometric analysis of PHA

The extracted PHA was dissolved in chloroform and subjected to UV- visible scanning over the range of 200-800 nm. Sharp absorbance peaks were observed at 240 nm (Figure2) which indicated the presence of PHA molecules[16].

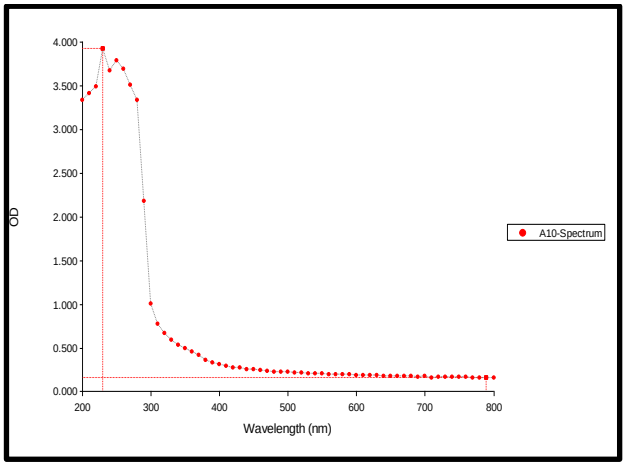


Figure 2: UV Spectra of PHA

Fourier Transform Infra- Red analysis of PHA

The FTIR spectrum confirms the product is, in fact, a bioplastic according to standard IR spectra (Figure 3). The peak at 3628.10 cm^{-1} indicate a strong bond of H stretching originated by terminal OH group, the spectra match similar results cited in literature[17]. The peaks at 2927.94 cm^{-1} corresponds to aliphatic C-H stretching originated from the alkane groups. The peaks at 1631.78 cm^{-1} indicate a weak C=O stretching for the conjugated carbonyl or amide group. The peaks at 1436.97 cm^{-1} correspond to C-H bond contributed by alkanes. The peaks at 970.19 cm^{-1} and 771.53 cm^{-1} correspond to alkyl halides[18].

The other peaks and slight shifts in the peak are owing to the presence of impurities. Thus the FTIR confirms that the product is PHA.

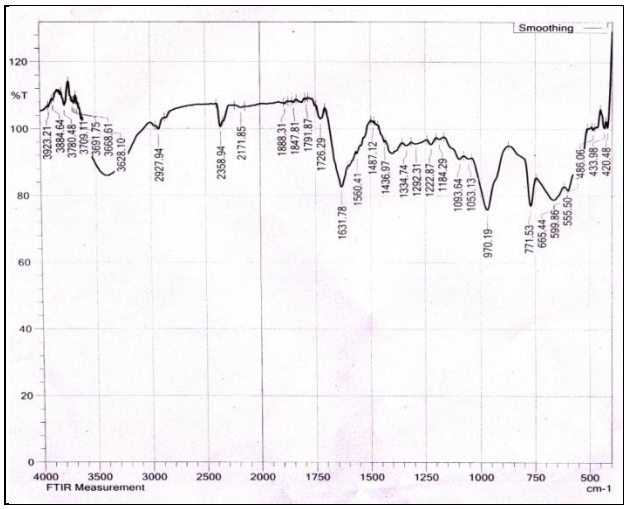


Figure 3: FTIR spectra of PHA

^{1}H NMR

NMR spectroscopic analysis is used to deduce the monomer structure and composition of PHA. Characterization of different types of functional groups – OH, = CH, CH2 and CH3 present in the PHA was done through ^{1}H NMR analysis. The proton NMR spectrum of PHA extracted from *Bacillus pumilus* MTCC 7055 showed the presence of a doublet at 1.2 ppm, which is due to methyl a doublet of quadruplet at about 2.5 ppm due to methylene group and multiplet at 5.2 ppm which is a characteristic of methylene group (Figure 4). This confirms the presence of PHA in the polymer[19].

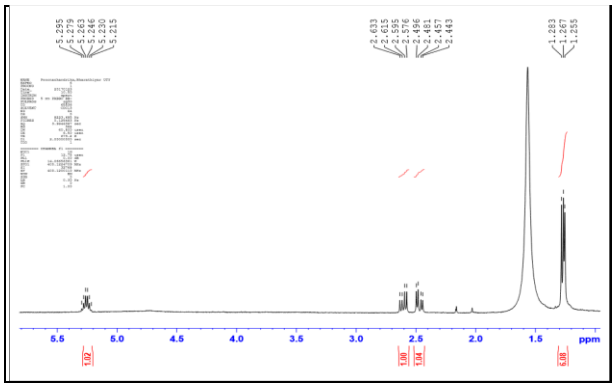


Figure 4: ^{1}H NMR analysis of extracted PHA

Differential Scattering Calorimetry

DSC is a thermo analytical technique used to determine the melting temperature of the polymer. It measures the heat flow between the samples as it undergoes physical transformation such as phase transition. DSC spectrum of extracted polymer from *Bacillus pumilus* MTCC 7055 is shown in Figure 5. The melting temperature of the extracted PHA using sodium hypochlorite method was found to be 117.46°C which is lower compared to what is reported in literal between 173-180°C. The result of the present study was in agreement with the DSC spectrum of PHA produced by *Bacillus substilis*NGO5 using sugar industry waste water[10]. The decrease in melting temperature may be due to the effect of sodium hypochlorite which lowers the molecular weight of the polymer[20].

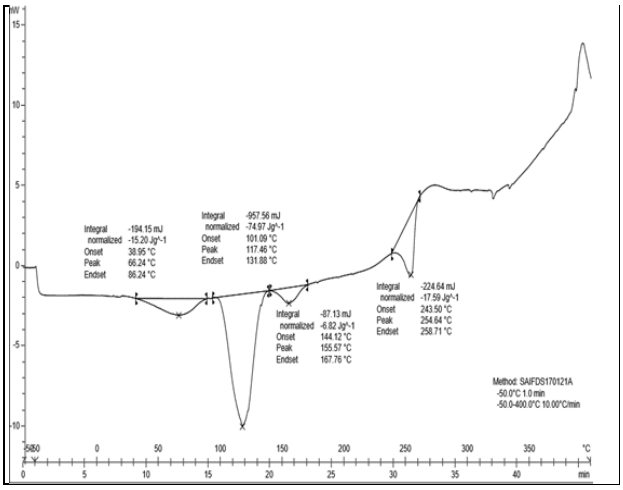


Figure 5: DSC analysis of extracted PHA

Phytochemical analysis of Neem seed extract

The phytochemical constituents present in the neem seed extracts were analyzed using standard procedures and tabulated in Table 3.The results were in correlation with earlier literature[21].

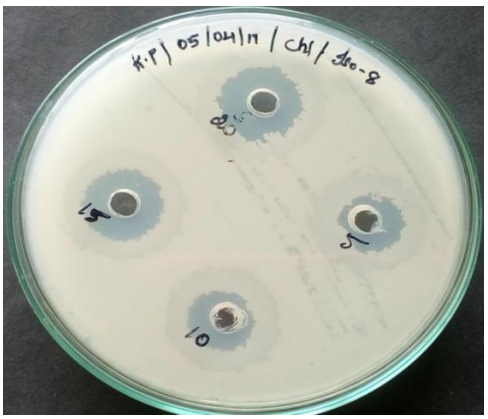
Table 3: Phytochemical composition of neem seeds

Phytochemicals	Alkaloids	Reducing Sugar	Flavanoids	Saponins	Tanins	Volatile Oil	Glycosides	Terpenoids
Presence/Absence	-	+	-	-	-	-	+	+

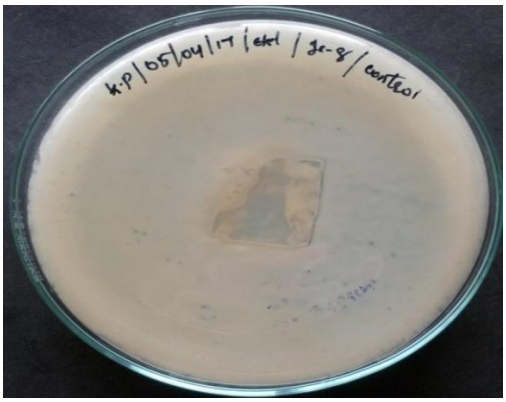
Analysis of anticandidal activity

Assessment of anti-fungal activity of the neem seed extract against *Candida albicans* was performed on PDA plates. The zone of inhibition was measured to evaluate the anti-fungal activity. The highest antifungal activity was observed at the concentration of 5 mg/mL the anti-fungal activity of the neem seed extracts was comparatively lesser as compared to the standard fluconazole. The antifungal activity could be attributed to the phytochemical present in the neem extract. Some of the

previous literature reveals that neem has anti-dermatophytic activity[22].



(a)



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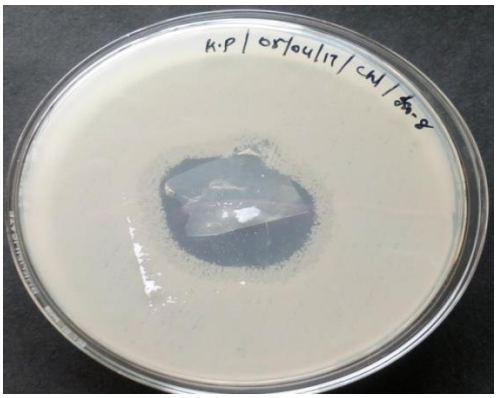

(c)

Figure 6: Anticandidal activity of (a) Neem extract (b) Biopolymer Sheet (c) Control Sheet

Incorporation of phytomedicines into active packaging materials was considered as a prime step to improve their biological activity. The PHA polymer sheets incorporated with chloroform extracts of neem seeds were screened for anticandidal activity. The results of the study revealed that the active principles present in the neem seeds produced a strong antagonistic activity against *Candida albicans* producing a zone of inhibition around the control films is contributed by the antimicrobial activity of chloroform used for film preparation.

CONCLUSION

The use of traditional phytomedicinal concepts in tune with modern material science in emerging as a successful domain especially for the development of wound healing biomaterials and active packaging ingredients. Incorporation of herbal combinations in biomaterials is the first step to improve their antimicrobial and antioxidant activities. Applications of PHA- based polymer sheets in medicine and food industry paves way for tackling the issues associated with synthetic plastic usage and degradation. PHAs due to their fully degradable nature, easy mold ability and similar material properties to their synthetic counterparts have emerged as the best alternative for petroleum- based plastic sheets.

The present study revealed that PHAs could be produced in the minimal nutrient medium using *Bacillus pumilus* MTCC 7055 strain. Detergent based cell digestion method and conventional chloroform precipitation method has proved to be ideally suitable for PHA extraction and precipitation. The active principles in the neem seeds as described in traditional medicinal formulation suggested its incorporation into the biomaterials to improve their biological activity. The promising anticandidal activity illustrated in well diffusion and Halo test suggested the anti-fungal potential of neem seed extracts. The improved anti-fungal biopolymer sheets could offer promising development in medicinal fabrics and wound healing applications.

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Source of Support: Nil, **Conflict of Interest:** None.