APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Research and Graduate Studies Division,
Khon Kaen University, Thailand

Effect of thyme oil on mechanical and antimicrobial potential of solvent casted poly (3-hydroxybutyrate)

Mahak Mittal[1], Naveen Kumar[1], Anita Yadav[2] and Neeraj K. Aggarwal[1,*]

[1]Laboratory of Fermentation Technology, Department of Microbiology, Kurukshetra, Haryana, India
[2]Department of Biotechnology, Kurukshetra, Haryana, India
[*]Corresponding author: nkumar@kuk.ac.in

Received 14 November 2022
Revised 21 January2023
Accepted 12 February 2023

Abstract

Poly(3-hydroxybutyrate) (PHB) is a biopolymer that substitutes petroleum-based conventional plastics in the packaging sector. Production of PHB is presently limited due to its higher cost of production. Therefore, this work has been designed to synthesize PHB by utilizing orange peel as a cheap carbon source. *Pseudomonas putida* has been used to accumulate PHB in the medium with orange peel hydrolysate. This strain has produced 5.2±0.03 g/L of PHB in orange peel hydrolysate medium under 72 h of incubation. The organism was able to synthesize 55% PHB of the cell biomass. Extracted PHB was confirmed by Nuclear Magnetic Resonance (NMR). Solvent casted films of extracted (neat) PHB and thyme oil (TO) (30%) incorporated PHB were prepared. Both the films were tested mechanically and microbiologically. The incorporation of TO reduces the tensile strength up to 22 % more than that of pure or neat PHB film, but improves the antimicrobial potential. TO incorporated PHB film was resistant against *Bacillus subtilis* (Gram positive), *Candida albicans* (fungal strain) and *Escherichia coli* (Gram negative). These findings suggest that TO incorporated PHB films could be used to develop eco-friendly active packaging products.

Keywords: Antimicrobial activity, Mechanical potential, Packaging film, Polyhydroxybutyrate, Thyme oil

1. Introduction

Petroleum-based plastics are the main reason behind urbanization; their strength and uses in a variety of industries have aid in raising our standard of living [1,2]. However, the piling of plastic garbage has resulted in serious environmental issues and ecosystem devastation. Every year, over 34 million tons of plastic garbage are produced worldwide, with 93 percent ending up in landfills and oceans. Both practices negatively impact Apart from environmental concerns and diminishing fossil fuel reserves, the idea of substituting current conventional plastics with bioplastics is gaining traction among the general population. Polyhydroxyalkanoate belongs to the energy-reserved chemicals accumulated by microbes that are being hailed as viable alternatives to traditional plastics, particularly in the biomedical and packaging industries [4]. These biopolymers are biodegradable, immunologically safe, and have physical qualities comparable to ordinary polymers [5,6]. Among Polyhydroxyalkanoates (PHAs), Poly(3-hydroxybutyrate) (PHB) are the most common and well-studied members, synthesized by many bacteria.

PHB consists of 3- hydroxybutyrate (3HB) as the monomeric unit and exhibits semi-crystalline properties. PHB has been employed in pharmaceutical applications, food sectors, and agricultural films [5,7]. Different bacterial strains synthesize PHB during their metabolism. *Pseudomonas putida* strains, like other PHB - accumulating bacteria, use PHB as a carbon and energy storage to adapt with changing environmental factors in their native habitats [7]. These ubiquitous, aerobic, gram-negative bacteria are popularly known to produce many primary and secondary metabolites [8,9]. PHB is one of the value-added compounds produced by this bacterial species [10]. Different strains of *P. putida* like *P. putida* KT2440, *P. putida* S12, *P. putida* GPo1 etc. have been employed for their potential to synthesize PHB from various substrates [11,12,13].

However, compared to low-cost conventional plastics, PHB is less competitive due to its high production costs. Major factors leading to the relatively high cost of PHB production include lower polymer yield, high cost of substrate used and productivity of the production processes [14,15]. The substrate is estimated to account for nearly half of the total production cost. Therefore, using renewable carbon sources would be a viable technique for lowering manufacturing costs [16]. Furthermore, antimicrobial packaging can restrict the growth of microorganisms by prolonging the initial phase of the growth, thus lowering the microbial counts [17]. Synthetic agents, such as sorbate, benzoate, nitrate, propionate, etc., are employed [18]. On the other hand, natural agents are also present, for example, plant and spice extracts: cinnamon, clove, carom seeds, thyme, and oregano, called as essential oils [19]. These extracts mainly contain terpenes, phenolic and aliphatic alcohols [20,21].

Thyme, extensively used as a spice, has also been recently explored for its antimicrobialproperties [22]. Thymol, p-cymene, γ-terpinene, and carvacrol are four key active ingredients in thyme oil, which have antibacterial activity against food pathogens and a variety of microbiological species in varying amounts depending on the species employed [23]. Thyme oil was found to suppress the growth of clinical strains of *Enterococcus, Staphylococcus, Pseudomonas,* and *Escherichia coli* [24]. In addition, it had the highest antibacterial activity when tested against L. monocytogenes to preserve minced fish meat than rosemary and cinnamon essential oils [25]. The mechanism of antimicrobial potential of thyme oil against different microorganisms like *Pseudomonas, E. coli* etc., is attributed to the presence of thymol and carvacrol constituents, which enhanced the permeability of the bacterial membranes, causing leakage of amino acids, inorganic ions, and adenosine triphosphate (ATP) [26]. Thereby, damaging bacterial structure and cellular constituents.

With this context, the present work has been planned to exploit discarded orange peel waste as a substrate for the synthesis of PHB by employing *P. putida* MTCC 672 and its subsequent utilization for the preparation of antimicrobial film by incorporating thyme oil (TO) as an antimicrobial agent. The decision to choose TO was based on the literature reported [24, 25, 28, 37]. Furthermore, the microbial strain was also selected from the literature, which can accumulate PHB on a wide range of feedstocks. Overall, the current study deals with comparing mechanical and antimicrobial evaluation of neat PHB and PHB, TO film.

2. Materials and methods

2.1 Chemicals and microorganisms

Pseudomonas putida (MTCC 672), was procured from Microbial Type Culture Collection (MTCC), Chandigarh, in a lyophilized state. As per recommendations of MTCC, the organism was revived in a nutrient broth medium. The stock culture was maintained on slants of Luria Bertani at 4°C and subculture monthly. The PHB-producing capability was confirmed by the method of Sudan Black staining [29]. All the media components, solvents, and thyme oil used were purchased from HiMedia Laboratories Pvt Ltd.

2.2 Substrate pretreatment

Orange peels were obtained from juice corners in Kurukshetra University, Kurukshetra, Haryana, India. These were washed with water and sterilized in an autoclave followed by the addition of distilled water up to 250 ml. The obtained mixture was heated at 70°C for 25 min and filtered through muslin fabric. Next, 1% hydrochloric acid was poured into the resultant filtrate and heated for half an hour at 120°C. Finally, the prepared solution was neutralized using 1 M sodium hydroxide. This resultant peel hydrolysate was utilized for the medium preparation for PHB production.

2.3 Preparation of inoculum

A single loop of *P. putida* (MTCC 672) was inoculated in the nutrient medium [5g peptone (CAS No. 73049-73-7), 5g NaCl (CAS No. 7647-14-5), 3g beef (CAS No. 68990-09-0) in 1000 mL distilled water], preserved under incubating conditions for 24 h, and utilized as the starter culture for the production of PHB.

2.4 Media Preparation and growth parameters

PHB was synthesized in an MMS medium made up of 2.5 g peptone, 2.5 g yeast extract (CAS No. 8013-01-2), 10 g Glucose (CAS No. 50-99-7), 0.10 g Sodium chloride, 0.20 g Magnesium sulfate (CAS No. 10034-99-8) and 0.50 g Potassium dihydrogen phosphate (CAS No. 7778-77-0) in the hydrolysate of orange peel (1000 mL). The medium was set to pH 7, inoculated with the one-day-old culture of *P. putida* MTCC 672, and preserved under incubating conditions at 35°C in shake flask at 120 rpm. Growth and production of PHB were followed up to 120 h and checked at regular intervals for maximum yield. Modified mineral salt (MMS) medium in dist. H_2O (without orange peel hydrolysate) was employed as the comparative standard for the production of PHB.

2.5 PHB extraction and characterization

After culture growth in media with orange peel hydrolysate, the broth was collected and centrifuged at 7500 rpm for 15-16 min. in a cooling centrifuge (Producer: REMI, Cat No: C-24BL) [30]. The supernatant was discarded, the pellet was treated with 10 ml sodium hypochlorite (CAS No.7681-52-9) and incubated at 50°C for 60 min. Next, the mixture was again centrifuged at 12000 rpm for half an hour and washed with distilled water, acetone, and alcohol sequentially. Finally, the PHB was extracted with boiling chloroform and was filtered through pre-wetted Whatman's No 1 filter paper. The filtrate was evaporated at room temperature, followed by the addition of 10 mL of conc. Sulphuric acid was kept at 100°C for 10 min in a water bath. The solution was cooled, and the absorbance was measured at 235 nm against the sulphuric acid blank. The amount of crotonic acid was calculated from a standard curve made from commercially available Poly β-hydroxybutyrate (Sigma, USA, Cat No: 36350-2) [31]. Produced PHB was subjected to analysis of the NMR.

2.6 Nuclear magnetic resonance (NMR)

The molecular structure of extracted PHB was studied by NMR Jeol 400 MHz. 1H-NMR (400 MHz) was recorded in deuterated chloroform as solvent and chemical shifts were quoted in parts per million concerning internal solvent signal 7.261 for 1H.

2.7 PHB films generation

For film preparation, the conventional solvent casting method was used [32]. 10-15 mL of chloroform (CAS No. 67-66-3) was boiled at 55°C, and 1% w/v i.e., 0.2 g PHB was mixed with the chloroform (20 mL). The solution was agitated continuously to achieve a homogenous mixture. PHB was made completely solubilized in the solvent under the refluxing condition to avoid evaporation of chloroform. Afterward, the resultant PHB solution was poured onto a glass petri plate. Chloroform was evaporated at room temperature for 12 h. Figure 1 shows the process of developing PHB film. Another film was generated by adding thyme oil following the same (solvent casting) method. 30 wt. % TO (percentage weight of total PHB content) was incorporated into the mixture of chloroform, PHB and stirred for 15 mins. The resultant solution was filtered to remove impurities and poured onto a glass petri plate the same as neat PHB was cast. After drying of 12 h, both the films were peeled off carefully from the petri plates and subjected to further mechanical and antimicrobial evaluation analysis.

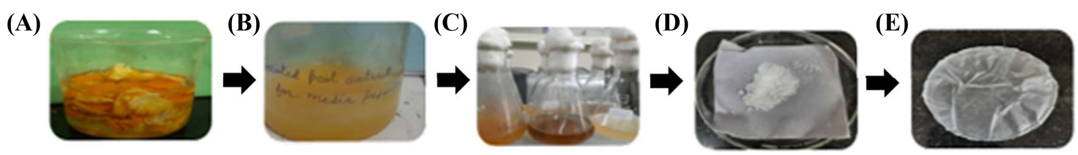

Figure 1 Schematic representation of PHB film generation: (A) orange peel for PHB production, (B) peel hydrolysate, (C) production media for PHB accumulation, (D) extracted PHB power, and (E) solvent castd PHB film.

2.8 Scanning electron microscopy (SEM)

The qualitative analysis of prepared film samples (PHB and PHB/TO30) was examined for their distinctive smooth and fractured surfaces by using SEM JSM 6100 (JEOL). Film samples were coated with gold to take resolved images. Pictures were taken at an accelerated voltage of 5kV.

2.9 Mechanical evaluation

Mechanical properties of solvent-casted films of extracted PHB and thyme oil incorporated PHB (PHB/TO30) were determined by Animatex universal tensile machine (UTM) using the parameters described by ASTM D882-10. Tensile testing for all samples occurred at room temperature without humidity control. The specimens 0.015 mm thick, 10 mm wide and having gauge length of 50 mm were loaded for tensile testing with a cross head speed of 5 mm/min. Every composition was tested in triplicates, and the results were further analyzed statistically by one-way analysis of variance (ANOVA).

2.10 Antimicrobial activity

Agar disk diffusion assay was carried out to evaluate both films' antimicrobial activity. *E. coli* (Gram negative bacteria), fungi *C. albicans* and *B. subtilis* (Gram positive bacteria) were considered for analysis. Experiment was performed by spreading 0.1 mL of the suspension of bacterial cells or fungal spores on the agar plates of nutrient medium. Films were cut into small pieces (approximately 1×1 cm) and placed on the agar plates. To compare, Ciprofloxacin (a standard drug) was used as a control. Plates were incubated overnight at 37°C and 25°C for bacterial and fungal cultures respectively. Zone of inhibition was measured to note the antimicrobial activity of the films [33].

3. Results and discussion

3.1 PHB production

Pseudomonas putida was able to produce the maximum amount of PHB in orange peel hydrolysate medium after incubation at 35°C for 72 h under shaking conditions. Accumulation of polymer and organism growth was checked at regular intervals from 24 h to 120 h. Maximum amount of cell dry weight (CDW) and PHB was observed at 72 h in both of the mediums (peel hydrolysate and MMS medium). At this point, 9.31±0.11 g/L CDW, 5.2±0.03 g/L of PHB in orange peel hydrolysate, and 7.53±0.26 g/L CDW, 4.11±0.08 g/L of PHB was recorded in case of MMS medium (Figure 2). The amount of PHB started to decrease after 72 h of incubation in both of the mediums. This culture has produced approximately 55% PHB of CDW in orange peel hydrolysate. However high degree of variation was seen in PHB production by 24 different isolates of *P. putida* [34].

In this study, the biochemical, nutritional, and genetic versatility of 24 isolates of *P. putida* was studied. Synthetic media was employed to cultivate and produce PHB. 14.35±0.45 g/L of PHB was the highest among all the isolates. However, some isolates have produced in the range of 6-8 g/L PHB. In another study, engineered *P. putida* KT2440 produced 31.5% content of mcl-PHA by using minimal mineral medium with acetate as a sole carbon source [35]. These findings suggest that, *P. putida* (MTCC 672) produces a comparatively higher amount of PHB. Hence, this strain is competitive for high production with low-cost media.

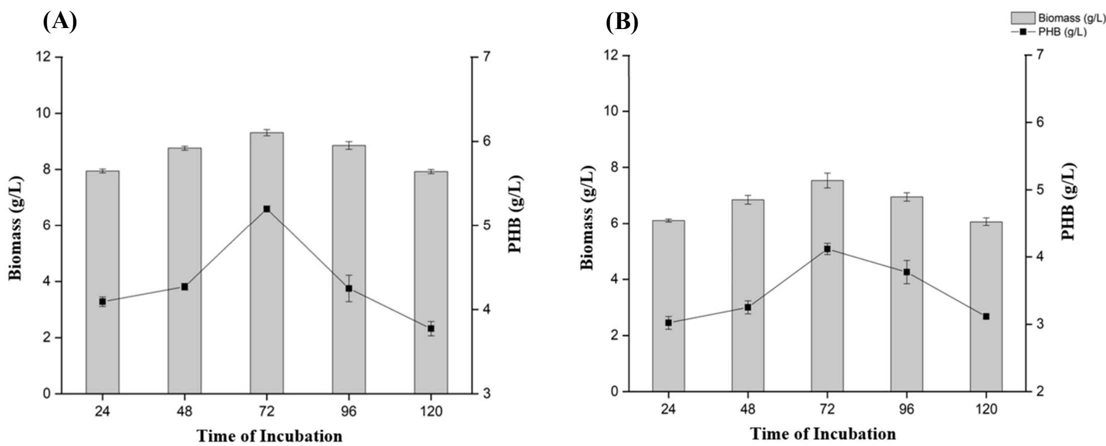

Figure 2 PHB production (A) growth in orange peel hydrolysate medium, (B) growth in modified mineral salt medium.

3.2 Nuclear magnetic resonance (NMR)

The structural details of PHB produced by *P. putida* (MTCC 672) were analyzed by 1H NMR. Three signals such as 5.29, 2.59, and 1.28 mg/L, characteristic of the polyhydroxy butyrate, corresponding to the methane, methylene and methyl group respectively (Figure 3). Likewise, Mohandas et al. [36] found 3 peaks viz., 5.22, 2.56, & 1.21 mg/L of 1H NMR spectrum corresponding to the methane, methylene, and methyl of PHB produced from *Vibrio harveyi.* Similarly, in other studies,, three peaks at 5.2, 2.4, and 1.6 mg/L were found, which correspond to the methane, methylene, and methyl groups of PHB, respectively derived from *P. putida* [13]. These reports of previous literature confirm the structure of PHB.

Furthermore, both PHB film and TO incorporated PHB film was prepared as described in section 2.7. so that mechanical and antimicrobial studies can be made.

3.3 Scanning electron microscopy

Figure 4 shows the morphology of prepared PHB and thyme oil incorporated PHB film. Pure PHB film appeared to have a rough surface, and tiny pores were also observed. These might be due to the evaporation of solvent during film drying. A similar pattern and structures appeared in a study in which PHB film was developed by solvent casting method [37]. However, after introducing 30% TO in the polymeric film, micro structures.

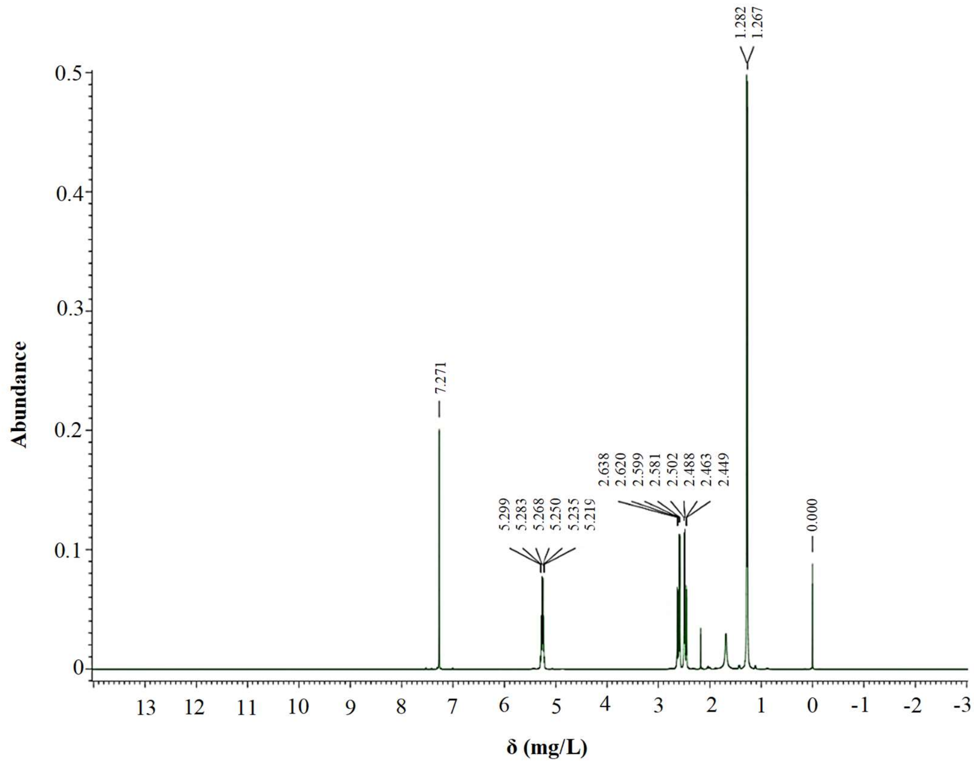

Figure 3 1H NMR spectrum of PHB derived from *Pseudomonas putida* (MTCC 672).

appeared at the film's cross-section area. These indicate the homogenous dispersal of oil in the polymeric matrix. These findingss coincide with the literature where thyme oil incorporated P(3HB-co-4HB) packaging film was developed [38].

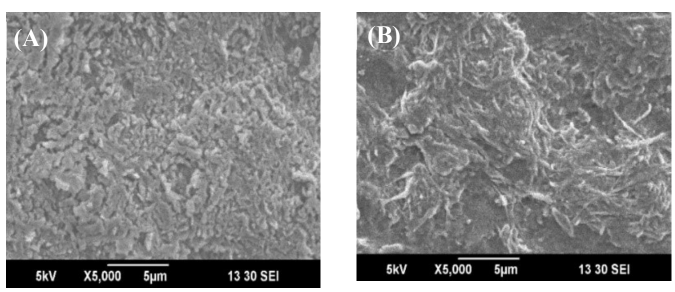

Figure 4 SEM images of prepared film samples. (A) PHB film, (B) PHB/TO30 film.

3.4 Mechanical evaluation

Previous studies elucidated the synthesis of PHB films with varying concentrations of TO i.e., 10%, 20% and 30% (wt. % based on PHB). Their work evidenced the plasticizer effect of thyme oil that was confirmed by reduction in tensile strength and crystallinity [38]. TO 30% was observed to be an ideal film to carry forward for further studies. The tensile strength of PHB expresses the maximum strength of 9.6 ± 0.02 MPa (Table 2). When TO was incorporated into the PHB film, less mechanical resistance in PHB/TO film observed. PHB/TO film exhibited a significant decrease of 22% i.e., 7.4 ± 0.01 MPa compared to pure PHB film (Figure 5). With the decrease in tensile strength, 17% increase in young's modulus has also been evidenced. Findings of the mechanical analysis have coincided with the reports published. The increase in young's modulus was also observed with the incorporation of murtha leaf oil into LDPE films [21]. Similar results were found in a study in which thyme oil incorporated chitosan films were prepared for wound healing applications. The introduction of TO in polymeric film greatly reduced the mechanical properties [39]. Another study where the effect of essential oil and surfactants on wheat and starch films was studied. The reduction in tensile properties was observed upon adding lemon essential oil in the films [40]. Table 1 shows that the statistically significant difference between mean tensile data from one composition to the second is verified by probability factor value ($p < 0.05$).

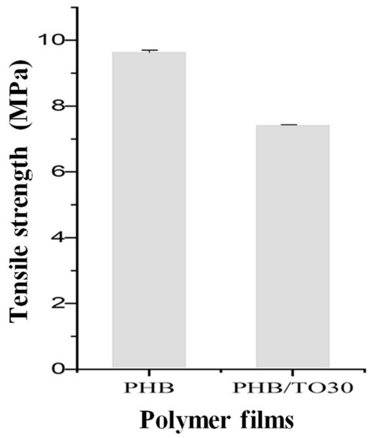

Figure 5 Comparative plot for tensile strength of PHB and PHB/TO30.

Table 1 One-way ANOVA on tensile strength data.

	DF	Sum of squares	Mean square	F value	*p* value
Model	1	7.3926	7.3926	6522.88235	1.40873E-7
Error	4	0.00453	0.00113		
Total	5	7.39713			

Table 2 Tensile strength of prepared film samples.

Sample code	Tensile strength (MPa)
PHB	9.6 ± 0.02
PHB/TO30	7.4 ± 0.01

3.5 Antimicrobial activity

Neat/pure PHB film didn't affect the growth of any microorganism tested. PHB/TO30 was found to be active against all three organisms tested, i.e., *B. subtilis, E. coli*, and *C. albicans.* (Figure 6). A significant zone of inhibition was seen on plates around PHB/TO30 film if we compare with the control used (Table 3.). Plate with standard drugs such as Ciprofloxacin had an 18 mm zone of inhibition. Various reports have been published based on the antimicrobial nature of thyme essential oil. A study has been done to investigate the antimicrobial potential of thyme oil against various multidrug-resistant strains like *Staphylococcus, Pseudomonas, Escherichia, Enterococcus* genus. Thyme oil strongly inhibited all the bacterial strains tested [24]. Antibacterial effect of thyme oil has also been studied against food-borne pathogens. Experiments were carried out with organisms such as *E. coli, B. cereus, Staphylococcus aureus,* etc. Thyme oil inhibited the growth of all organisms tested.

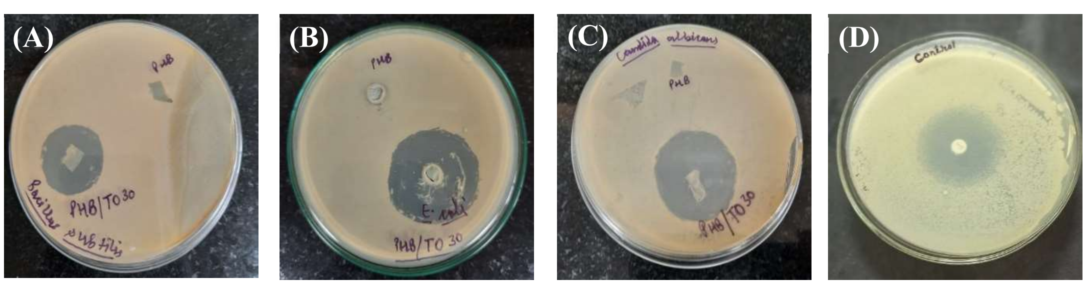

Figure 6 Antimicrobial activity of pure PHB film and PHB/TO30 against different microorganisms. (A) *Bacillus subtilis* (Gram positive bacterium) (B) *Escherichia coli* (Gram-negative bacterium) (C) *Candida albicans* (Fungal strain) (D) Control (Ciprofloxacin).

Table 3 Antimicrobial activity of PHB films.

Microorganism tested	Zone of inhibition (mm)	
	Pure PHB film	PHB/TO30 film
B. subtilis	NA	13 ± 0.5
E. coli	NA	16 ± 0.7
C. albicans	NA	15 ± 0.3

NA: Not Active.

4. Conclusion

In conclusion, we demonstrated the ability of *Pseudomonas putida* (MTCC 672) to produce PHB in a low-cost nutrient medium prepared from orange peel hydrolysate. This strain successfully utilized medium made up of orange peels and produced 5.2±0.03 g/L of PHB under 72 h of incubation. Organism was able to synthesize 55% PHB of the cell biomass. This aids in lower down the production cost of PHB, since an inexpensive substrate has been used. Polyhydroxybutyrate film and PHB/thyme film were also developed through solvent casting. Though, this incorporation reduces the mechanical strength up to 22 % than of pure or neat PHB film but improves the antimicrobial potential. PHB (TO30) was active against three strains *B. subtilis, E. coli, C. albicans.* Considering this, we conclude that thyme oil incorporated PHB film could be a potent active packaging material. However, it can change the sensory properties of the packaged item. More research is required to study further aspects of sensory analysis.

5. Acknowledgments

One of the authors, Ms. Mahak Mittal, acknowledged the Rashtriya Uchchatar Shiksha Abhiyan (RUSA) 2.0 for providing financial support and Kurukshetra University, Kurukshetra.

6. References

[1] Kasuya KI, Inoue Y, Tanaka T, Akehata T, Iwata T, Fukui T, et al. Biochemical and molecular characterization of the polyhydroxybutyrate depolymerase of Comamonas acidovorans YM1609, isolated from freshwater. Appl Environ Microbiol. 1997;63(12):4844-4852.
[2] Dusselier M, Van Wouwe P, Dewaele A, Makshina E, & Sels BF. Lactic acid as a platform chemical in the biobased economy: the role of chemocatalysis. Energy Environ Sci. 2013;6(5):1415-1442.
[3] Pathak S, Sneha CLR, Mathew BB. Bioplastics: its timeline based scenario & challenges. J Polym Biopolym Phys Chem. 2014;2(4):84-90.
[4] Mittal M, Mittal D, Aggarwal NK. Plastic accumulation during COVID-19: call for another pandemic; bioplastic a step towards this challenge?. Environ Sci Pollut Res. 2022;29(8):11039-11053.
[5] Keshavarz T, Roy I. Polyhydroxyalkanoates: bioplastics with a green agenda. Curr Opin Microbiol. 2010;13(3):321-326.
[6] Leja K, Lewandowicz G. Polymer biodegradation and biodegradable polymers-a review. Polish Journal of Environmental Studies. 2010;19(2):255-266.
[7] Borrero-de Acuña JM, Bielecka A, Häussler S, Schobert M, Jahn M, Wittmann C, et al. Production of medium chain length polyhydroxyalkanoate in metabolic flux optimized Pseudomonas putida. Microbial Cell Factories. 2014;13(1):1-15.
[8] Dos Santos VM, Heim S, Moore ERB, Strätz M, Timmis KN. Insights into the genomic basis of niche specificity of Pseudomonas putida KT2440. Environ Microbiol. 2004;6(12):1264-1286.
[9] Fravel DR. Commercialization and implementation of biocontrol. Annu Rev Phytopathol. 2005;43:337-359.
[10] Hoffmann N, Rehm BH. Regulation of polyhydroxyalkanoate biosynthesis in Pseudomonas putida and Pseudomonas aeruginosa. FEMS Microbiol Lett. 2004;237(1):1-7.
[11] Kim DY, Kim HW, Chung MG, Rhee YH. Biosynthesis, modification, and biodegradation of bacterial medium-chain-length polyhydroxyalkanoates. J Microbiol. 2007;45(2):87-97.
[12] Meijnen JP, de Winde JH, Ruijssenaars HJ. Engineering Pseudomonas putida S12 for efficient utilization of D-xylose and L-arabinose. Applied and environmental microbiology. 2008;74(16):5031-5037.
[13] Hartmann R, Hany R, Geiger T, Egli T, Witholt B, Zinn M. Tailored Biosynthesis of Olefinic Medium-Chain-Length Poly [(R)-3-hydroxyalkanoates] in Pseudomonas putida GPo1 with Improved Thermal Properties. Macromolecules. 2004;37(18):6780-6785.
[14] Lee GN, Na J. Future of microbial polyesters. Microbial Cell Factories. 2013;12(1):1-4.
[15] Wang Y, Yin J, Chen GQ. Polyhydroxyalkanoates, challenges and opportunities. Curr Opin Biotechnol. 2014;30:59-65.

[16] Agnew DE, Pfleger BF. Synthetic biology strategies for synthesizing polyhydroxyalkanoates from unrelated carbon sources. Chem Eng Sci. 2013;103:58-67.
[17] Malhotra B, Keshwani A, Kharkwal H. Antimicrobial food packaging: Potential and pitfalls. Front Microbiol. 2015;6:611.
[18] Jideani VA, Vogt K. Antimicrobial packaging for extending the shelf life of bread-A review. Crit RevFood Sci Nutr. 2016;56(8):1313-1324.
[19] Shaaban HA. Essential oil as antimicrobial agents: Efficacy, stability, and safety issues for food application. In: Oliveira de MS, Costa da WA, Silva SG, editors. Essential Oils-Bioactive Compounds, New Perspectives and Applications. London,UK: ntechOpen; 2020. p. 1-33.
[20] Vergis J, Gokulakrishnan P, Agarwal RK, Kumar A. Essential oils as natural food antimicrobial agents: a review. Crit Rev Food Sci Nutr. 2015;55(10):1320-1323.
[21] Lucera A, Costa C, Conte A, Del Nobile MA. Food applications of natural antimicrobial compounds. Front Microbiol. 2012;3:287.
[22] Liu Q, Meng X, Li Y, Zhao CN, Tang GY, Li HB. Antibacterial and antifungal activities of spices. Int J Molec Sci. 2017;18(6):1283.
[23] Borugă O, Jianu C, Mişcă C, Goleţ I, Gruia AT, Horhat FG. Thymus vulgaris essential oil: chemical composition and antimicrobial activity. J Med Life. 2014;7(3):56.
[24] Sienkiewicz M, Łysakowska M, Denys P, Kowalczyk E. The antimicrobial activity of thyme essential oil against multidrug resistant clinical bacterial strains. Microbial Drug Resist. 2012;18(2):137-148.
[25] Abdollahzadeh E, Rezaei M, Hosseini H. Antibacterial activity of plant essential oils and extracts: The role of thyme essential oil, nisin, and their combination to control Listeria monocytogenes inoculated in minced fish meat. Food Control. 2014;35(1):177-183.
[26] Ryu V, McClements DJ, Corradini MG, & McLandsborough L. Effect of ripening inhibitor type on formation, stability, and antimicrobial activity of thyme oil nanoemulsion. Food Chem. 2018;245:104-111.
[27] Liu Q, Meng X, Li Y, Zhao CN, Tang GY, Li HB. Antibacterial and antifungal activities of spices. Int J Molec Sci. 2017;18(6):1283.
[28] Issa A, Ibrahim SA, Tahergorabi R. Impact of sweet potato starch-based nanocomposite films activated with thyme essential oil on the shelf-life of baby spinach leaves. Foods. 2017;6(6):43.
[29] Liu M, González JE, Willis LB, Walker GC. A novel screening method for isolating exopolysaccharide-deficient mutants. Appl Environ Microbiol. 1998;64(11):4600-4602.
[30] Law JH, Slepecky RA. Assay of poly-β-hydroxybutyric acid. J Bacteriol.1961;82(1):33-36.
[31] Law J, Slepecky RA. Assay of poly-b-hydroxybutyric acid. J Bacteriol. 1969;82:52-55.
[32] Stanley A, Murthy PS, Vijayendra SVN. Characterization of polyhydroxyalkanoate produced by Halomonas venusta KT832796. J Polym Environ. 2020;28(3):973-983.
[33] Biemer JJ. Antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method. Ann Clin Lab Sci.1973;3(2):135-140.
[34] Agrawal T, Kotasthane AS, Kushwah R. Genotypic and phenotypic diversity of polyhydroxybutyrate (PHB) producing Pseudomonas putida isolates of Chhattisgarh region and assessment of its phosphate solubilizing ability. 3 Biotech. 2015;5(1):45-60.
[35] Yang S, Li S, Jia X. Production of medium chain length polyhydroxyalkanoate from acetate by engineered Pseudomonas putida KT2440. J Indust Microbiol Biotechnol. 2019;46(6):793-800.
[36] Mohandas SP, Balan L, Lekshmi N, Cubelio SS, Philip R, Bright Singh IS. Production and characterization of polyhydroxybutyrate from Vibrio harveyi MCCB 284 utilizing glycerol as carbon source. J Appl Microbiol. 2017;122(3):698-707.
[37] Anbukarasu P, Sauvageau D, & Elias A. Tuning the properties of polyhydroxybutyrate films using acetic acid via solvent casting. Sci Rep. 2015;5:1-14.
[38] Sharma P, Ahuja A, Izrayeel AMD, Samyn P, & Rastogi VK. Physicochemical and thermal characterization of poly (3-hydroxybutyrate-co-4-hydroxybutyrate) films incorporating thyme essential oil for active packaging of white bread. Food Control. 2022;133:108688.
[39] Altiok D, Altiok E, & Tihminlioglu F. Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications. J Mater Sci Mater Med. 2010;21(7):2227-2236.
[40] Song X, Zuo G, & Chen F. Effect of essential oil and surfactant on the physical and antimicrobial properties of corn and wheat starch films. Int J Biol Macromol. 2018;107:1302-1309.