International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 8 | Issue 3 | JUL-SEPT | 2018 | 379-387
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

STUDIES ON UTILIZATION OF FRUIT PEEL EXTRACTS FOR THE PRODUCTION OF POLYHYDROXYBUTYRATE USING *BACILLUS SUBTILIS*

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ABSTRACT

The aim of the present study was to utilize the fruit peel waste extracts for the production of polyhydroxybutyrate (PHB) using Bacillus subtilis. Two different fruit peel waste extracts were developed and tested as cheap substrate for the production of PHB. Several experiments were performed to optimize various parameters associated with medium and environmental factors for maximizing the production of PHB using B. subtilis. The result showed that the maximum production of PHB of 3.35 g/L was accumulated during 48 h of incubation at 35°C when initial pH was maintained at 7.0. Among the two different fruit wastes tested, sapota peel extract was found to give maximum production of 2.68 g/L when 50% concentration was supplemented with the production medium. In case of apple peel extracts, maximum PHB production of 2.46 g/L was found when 60% extracts was amended with production medium. The PHB extracted from both fruit wastes were characterised using FTIR studies which confirmed the presence of polyhydroxybutyrate.

KEY WORDS

Polyhydroxybutyrate, Fruit peel wastes, FTIR

INTRODUCTION

The use of different group of chemicals for the production of plastic is found to be extremely toxic which poses a grave hazard to the entire biosphere. The major problem lies in the degradable property of plastic and their relative components in the environment. Generally, these plastics were originated from less expensive petrochemicals constituents which lead to significant problem because of its continual presence in the environment [1]. These components apart from creating huge impact in ecosystem tend to cause serious problems like cancer, Nerve damage, birth defects and also affect immune systems. Due to impending fossil fuel crisis and environmental concern, recently the bio-based synthetic polymers and biodegradable polymers have become the major topics of interest. These polymers are found to be excellent alternative due to their biodegradability and environmentally friendly nature [2,3].

Among the different types of biodegradable polymers, the microbial derived biopolymers, polyhydroxy butyrate (PHB) were found to be 100% biodegradable. Polyhydroxy butyrate are produced by different types of microorganism, especially bacteria as reserve inclusion bodies accumulated when they grow under nutrient limiting stress conditions. The bacterial synthesized PHB possesses similar properties to synthetic polymers such as polypropylene which makes them an excellent replacement for the existing commercial plastics developed using petroleum-based derivatives [4,5].

However, the major hindrance in the production and commercialization of PHB is mainly due to their production costs when compared with petrochemical based plastic materials. The high production cost of

microbial based PHB can be reduced by selecting efficient bacterial strain, optimizing fermentation conditions and extraction process [6,7]. Several researchers have reported that the overall production cost of PHB depends on the carbon substrate source utilized. Hence, researchers are finding an alternative way to utilize cheap raw materials such as waste organic materials, less expensive carbon substrates which may drastically reduce the production cost of PHB [8-10]. Therefore, selection of efficient cheap carbon substrate is a key aspect which should be economically viable and readily available for the growth of microorganisms and PHB production. The present study was aimed to utilize fruit peel waste extract as carbon source for the production of PHB using *Bacillus subtilis*.

MATERIALS AND METHODS

Reagents

In the present study microbiology media, chemicals and reagents used were of analytical grade procured from SRL Pvt. ltd., Mumbai, India and Himedia Pvt. Ltd., India.

Screening for PHB granules

For the present study, the strain *Bacillus subtilis* MTCC 9763 was utilized for the production of PHB. The above test strain was purchased from Microbial Type Culture Collection (MTCC), Chandigarh, India, subcultured in nutrient agar slant and stored at 4°C. For screening of PHB studies, the freshly prepared 48 h culture was subjected to sudan black B staining (Sudan balck B, 0.3 % in 70% ethanol). The bacterial smear was stained with Sudan black B stain for 2 min followed by safranin treatment for 15 seconds [11]. The dried smear was then observed for purple color PHB granules within pink cells under high power objective.

Production and estimation of PHB

For the production studies, the freshly prepared overnight test strain, *Bacillus subtilis* MTCC 9763 was inoculated into a sterile mineral salt medium (MSM) (composition: 0.16g Yeast extract, 1g Urea, 4g Na_2HPO_4, 1.52g KH_2PO_4, 0.52g $MgSO_4{\cdot}7H_2O$, 0.02g $CaCl_2$, 40g Glucose, distilled water 1L) supplemented with trace element solution of 0.1 ml (composition g/L: 0.02g $FeSO_4{\cdot}7H_2O$, 0.13g $ZnSO_4{\cdot}7H_2O$, 0.06g H_3BO_3, 0.06g $(NH_4)6MO_7O_{24}$. $4H_2O$). The base medium, glucose and trace element solutions were autoclaved separately and then reconstituted before the inoculation of test strain. The flask was incubated at 37°C for 48 h in a rotary shaker incubator at 120 rpm. At the end of incubation, the cells were digested using sodium hypochlorite (30%) for 20 min and then residues were separated at 5000 rpm for 15 min, washed with distilled water, followed by acetone and ethanol. The residues were then dissolved in 5 ml of boiling chloroform and kept for complete evaporation. The estimation of PHB was determined using Law and Slepecky method [12]; where 5 mL of concentrated sulphuric acid was added to the test tube containing PHB and kept in a boiling water bath for 40 minutes for the formation of crotonic acid which can be quantified by analysing using UV spectrophotometer for measuring absorbance at 235 nm [13].

The dry weight of the test strain utilized for the production of PHB was estimated by centrifuging 100 ml of culture broth at 10,000 rpm for 15 min at 4°C. The obtained pellet was washed with deionised water and allowed to dry at 80°C till constant weight was observed [11].

Optimization studies

Optimization studies such as effect of incubation time, pH, temperature, and carbon source were performed to study maximum production of PHB in the MSM medium. For incubation period, the cells were extracted at different time intervals ranging from 0 h to 96 h, pH of the medium from 6 to 9 and temperature varying from 25 to 50°C. Similarly, to study the effect of different carbon source on PHB production, the glucose present in the MSM medium was replaced with different carbon sources such as, fructose, lactose, sucrose, maltose, galactose and mannitol. At the end of each experiment, the cells were extracted, digested as described earlier and estimated for PHB production using Law and Slepecky method [12].

Utilization of fruit wastes for PHB production

The fruits peel wastes of sapota and apple were collected from local juice shops and processed in the laboratory [14]. The peel wastes were washed thrice in tap water followed by distilled water and then kept for shade drying to remove the presence of moisture. The dried peel wastes were then ground using mixer grinder to make fine powder. For the preparation of fruit peel extracts, peel wastes of sapota and apple of 1 g were separately mixed in 100 mL of sterile water and kept in rotary shaker incubator for 48 h at 37°C. The filtrate was then collected by passing it through Whatman filter paper No.1 and utilized for further studies.

To study the effect of fruit wastes on PHB production studies, the fruit peel extracts such as, sapota and apple

of varying concentration were mixed with MSM medium (10 to 100 %). The medium was sterilized, inoculated with the test strain *B. subtilis* and incubated at 37°C for 48 h in a rotary shaker incubator (120 rpm). At the end of incubation, the cells were digested and estimated for PHB concentration as described earlier.

Characterization of PHB

For characterization studies, PHB was extracted from the bacterial cells grown separately using sapota and apple peel extracts. The PHB was then dissolved in 5 mL of chloroform and kept for drying [15]. The dried PHB was then mixed with KBr to form a pellet and analyzed for FTIR spectra in the region between 4000 – 400 cm^{-1} using Fourier transformed infrared spectroscopy. The FTIR spectrum obtained was then compared with the existing reports to determine the presence of PHB in the extracted samples.

RESULTS AND DISCUSSION

In the present study, the test strain was screened for the presence of PHB granules using sudan black B staining. The result showed the presence of purple colour granules within the pink colour bacterial cells, confirming the synthesis of PHB. Further, the strain was subjected for the PHB production using MSM medium and the cells were digested using sodium hypochlorite method for the extraction of PHB. The amount of PHB was determined by the formation of crotonic acid in the presence of concentrated sulphuric acid using Law and Slepecky method [12].

Every bacterial strain has its own optimum conditions where the production of PHB is at maximum. Parameters optimization studies were considered as an essential part of the metabolite production process where several factors such as pH, temperature, incubation period, different carbon and nitrogen sources, etc., have an influence on final product. In the present study, the effect of incubation time, incubation temperatures, initial pH of the production medium and different carbon sources were investigated.

The production of PHB depends on the efficient growth of bacterial cells in the production medium. The effect of incubation period on PHB production was studied by regularly extracting the PHB at different time intervals varying from 0 to 96 h. The biomass yield and PHB production was determined at different time intervals of incubation to find out the optimum time when the PHB production reaches its maximum. The results showed that the PHB production was very less during the initial stage of production (0 to 24 h), followed by gradual increase from 24 h to 36 h and reaches the maximum PHB production of 2.99 g/L at 48 h. This was followed by slight decrease in the PHB production till 72 h, and then there was a steady decrease afterwards (Fig 1). This rapid decrease in the PHB production was due the bacterial cells in the decline phase of growth, which lacked required nutrients. Studies also reported that the microbes may also utilize the PHB when there is severe lack of nutrients in the decline phase, which may also result in the reduction of PHB in the medium [16,8]. Mayeli et al. [17] recorded a maximum yield of PHB of about 66 % when the incubation period was at 48 h. Similarly, Getachew and Woldesenbet investigated the accumulation of PHB from ten bacterial strains using MSM medium. They also found that maximum PHB was produced by *Bacillus* sp. when the incubation period was at 48 h which also supports our studies [13].

The effect of pH on PHB production was studied by varying the initial pH of production medium from 6 to 9 (6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0). Maximum PHB accumulation of 3.21 g/L was observed when the initial pH of the medium was maintained at 7.0. The PHB production was affected when the initial pH was increased from 8 to 9 (Fig 2). The hydrogen ion concentration was found to be major factor to influence the production of PHB in environment as it may affect the metabolic process of the bacterial strain and it is very important to be under control. Wei and co- workers reported that an initial pH of 7.0 accumulated the highest PHB content of 43.04% among the different pH tested [18]. Similarly, Sindhu and co-workers also concluded that PHB production was high when the pH was maintained between pH 7 to 7.5 using *Bacillus sphaericus* NII 0838 which agreed with our experimental results [19].

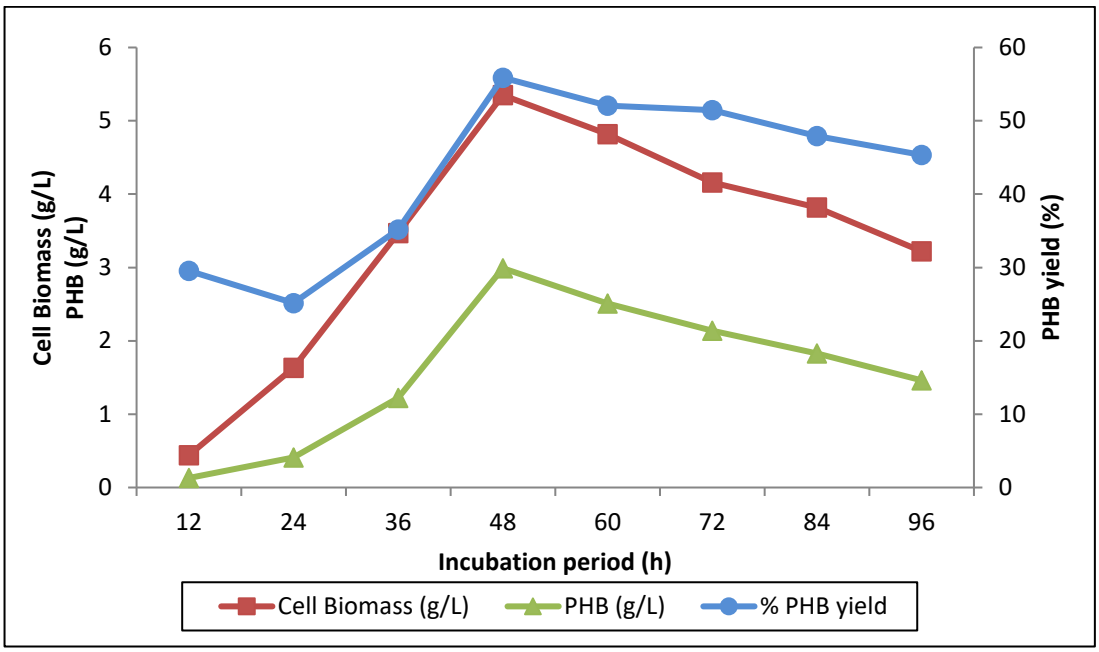

Figure 1: Effect of incubation time on PHB production

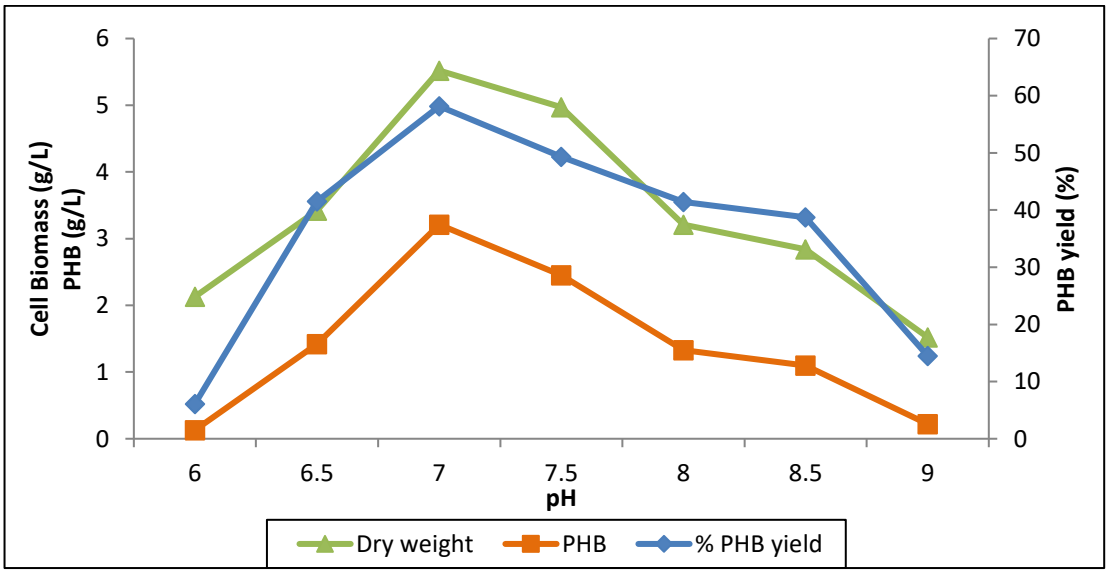

Figure 2: Effect of initial pH on PHB production

Among the different factors, temperature was found to be a critical parameter which varies among different strains and also has profound effect on cellular growth which in turn affects the PHB production process [18]. In the present study, incubation temperature varying from 25 to 50°C (25, 30, 35, 40, 45 and 50°C) was maintained during the production of PHB. Among the different temperature tested maximum PHB of 3.35 g/L was produced when the incubation temperature was maintained at 35°C, followed by 30°C (Fig 3). Incubation temperature beyond 40°C shows a negative impact on the production of PHB, which may be due to fatal effect on bacterial growth which may reduce the PHB production. Similar studies were also in accordance with our study where maximum PHB production was observed when incubation temperature was between 30 to 40°C [20-22]. Researchers also noted that the temperature may also affect the enzyme activity which has major role in the production of PHB [23,24].

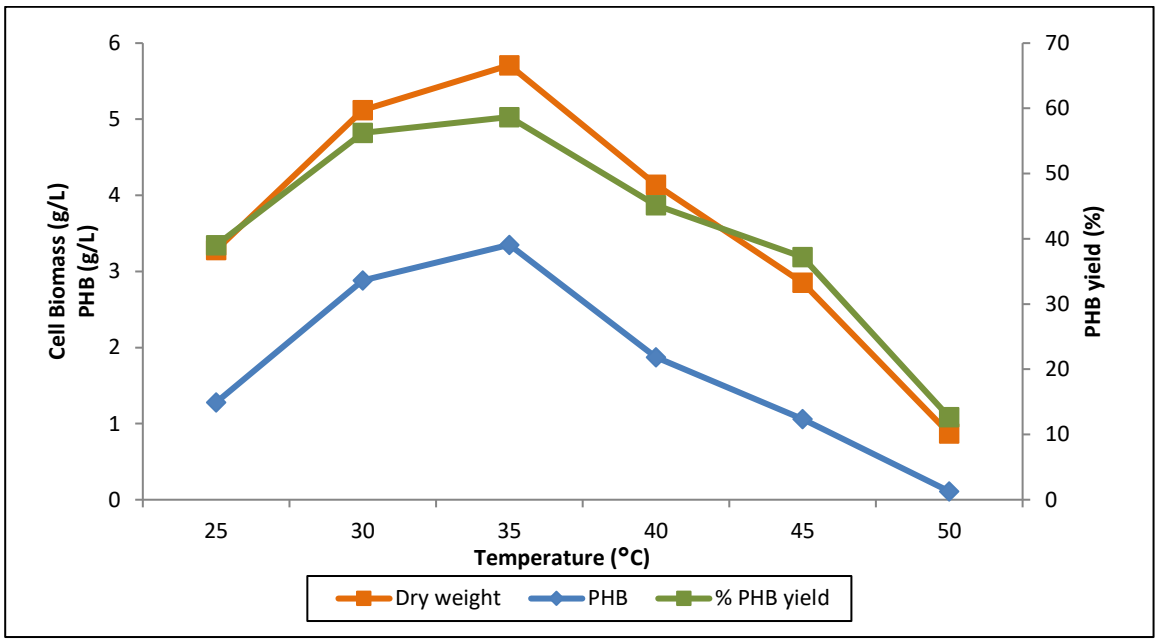

Figure 3: Effect of incubation temperature on PHB production

The effect of interaction of various carbon sources in the production medium for the production of PHB was studied. Different carbon sources such as glucose, fructose, lactose, sucrose, maltose, galactose and mannitol were amended separately and determined for the PHB production. The maximum accumulation of PHB of 3.42 g/L was obtained when glucose was amended in the production medium followed by fructose (2.78 g/L) and sucrose (2.11 g/L) (Fig 4). Similar reports were observed by Gomez and co-workers who confirmed high yield of PHB was found when the medium was amended with glucose as a carbon source [25]. Among the different carbon sources, glucose was found to be easily assimilated with the production medium which may enhance the microorganisms to synthesize increased amount of PHB [8,26]. Comparable results were also reported by Gowda and Shivakumar who have utilized glucose as carbon sources for the maximum accumulation of PHB in the production medium [27].

The major constraint in the commercialization of bio-based plastic is mainly due to their expensive production cost. The utilization of cheap wastes and carbon sources may support the reduction of production cost of PHB which also depends on their ready availability. The utilization of different cheap agro-industrial residues as a carbon sources for the production of PHB using different bacterial strains were reported [28,29]. In the present study, the fruit peel wastes of sapota and apple were collected, processed and the extracts were developed. These extracts were then supplemented with the MSM production medium at varying concentration from 10 to 100% in order to study the effect of cheap substrates for the accumulation of PHB. Among the two, the production medium amended with sapota peel extracts found to accumulate more amount of PHB when compared to the apple peel extracts supplemented medium (Fig 5 & 6). Maximum yield of 55.03 % of PHB (2.68 g/L) was found when the 50% of sapota extract was amended in the production. Similarly, in case of apple extracts, 60% supplemented in the production medium resulted in maximum production of PHB of 2.46 g/L. Several other researchers have also investigated the utilization of various cheap carbon substrates for the synthesis of PHB using bacterial strain. Sharma and Bajaj have reported that maximum yield of PHB was found when molasses was utilized as a sole carbon source than the glucose based medium [30]. Similar results were reported by various researchers who have investigated the utilization of bagasse hydrolytes for the production of PHB from *Cupriavidus necator* and *Pseudomonas aeruginosa* [31,27].

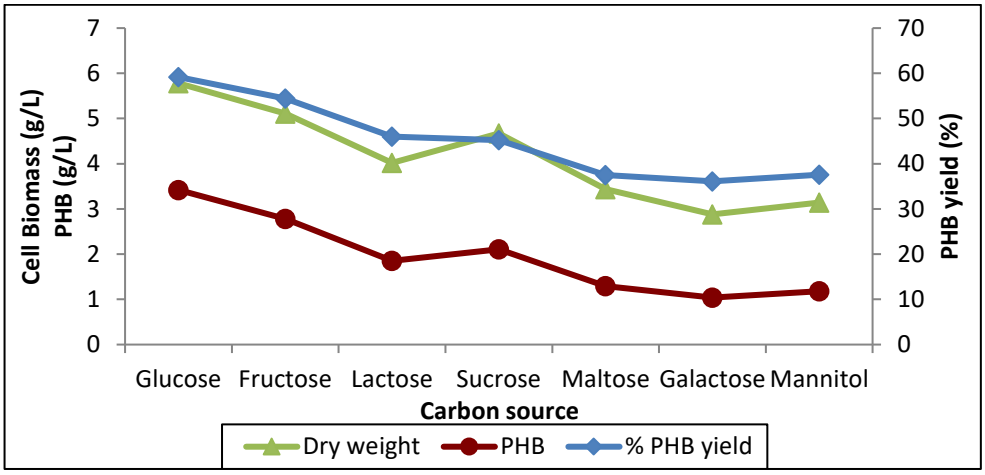

Figure 4: Effect of different carbon sources on PHB production

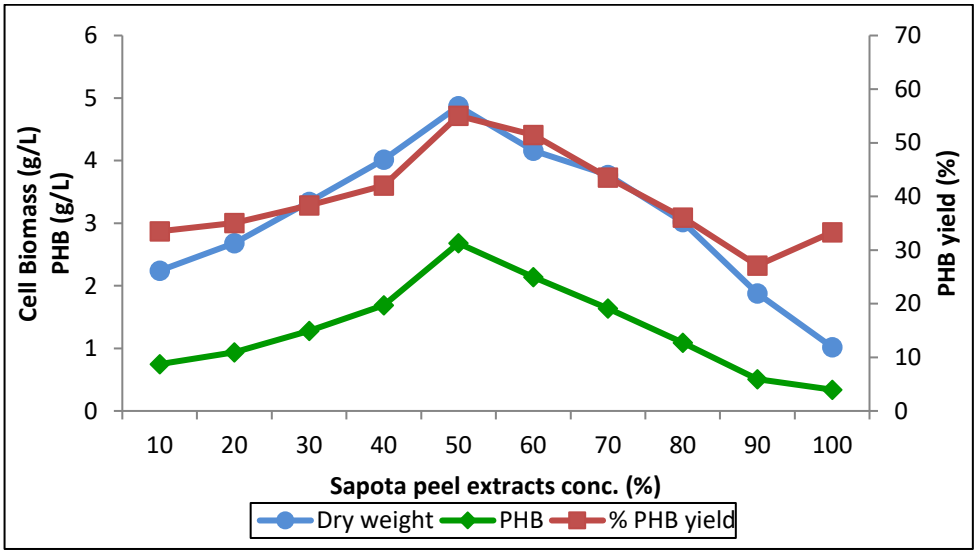

Figure 5: Effect of Sapota peel extracts on PHB production

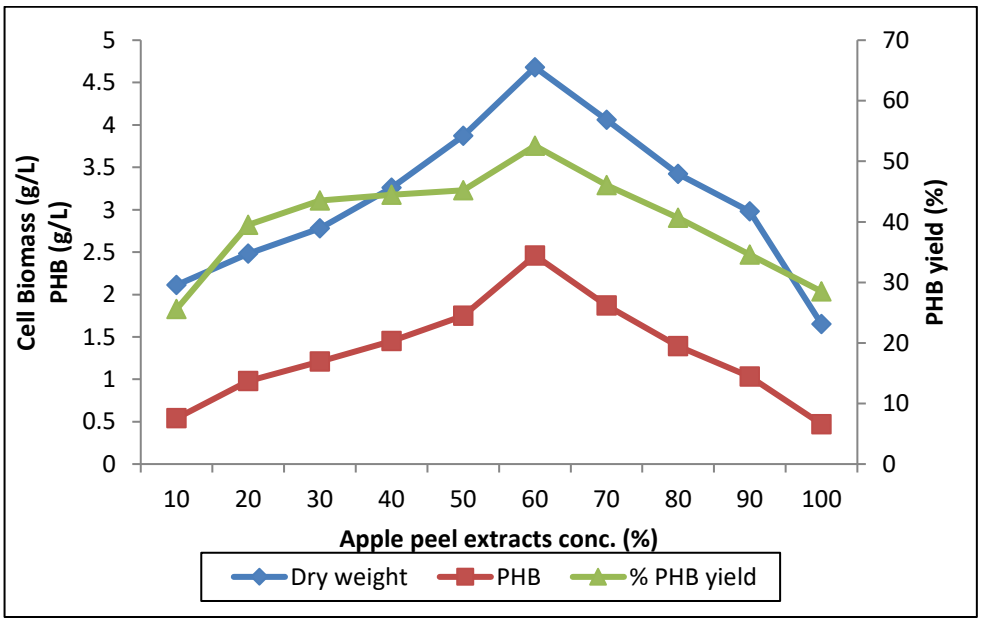

Figure 6: Effect of apple peel extracts on PHB production

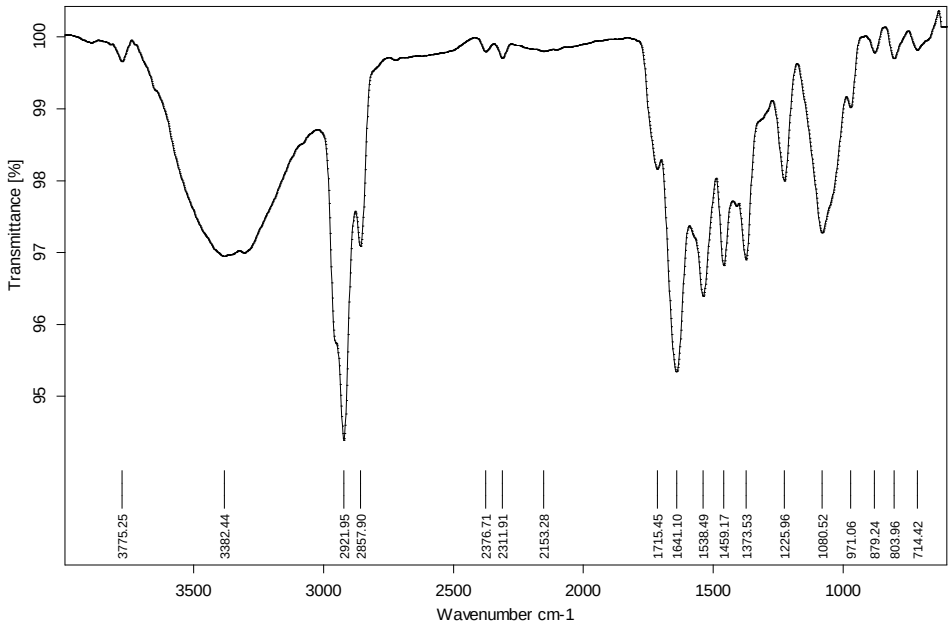

Figure 7a: FTIR spectral analysis of PHB extracted from sapota peel wastes

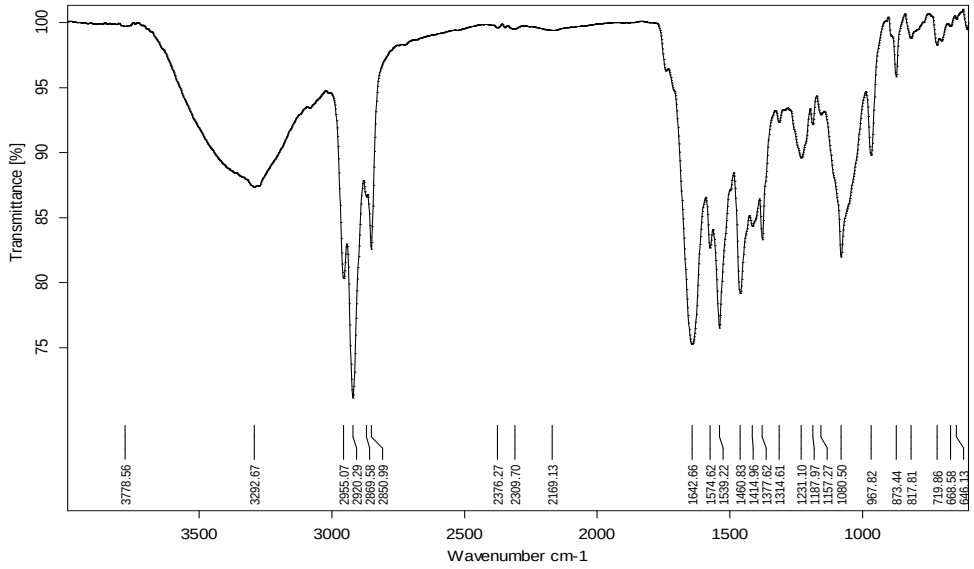

Figure 7b: FTIR spectral analysis of PHB extracted from apple peel wastes

The PHB synthesized form different wastes extracts was characterized using Fourier transform infrared (FTIR) spectrum. The FTIR spectroscopy is a technique used to study different functional groups present in the given sample by its distinct peak in the spectrum which helps in the determination of molecular structure. The degree of spectrum peaks is directly related the concentrations of component present in the given sample [32].

The FTIR spectroscopy was performed between frequency ranging from 4000- 400 cm^{-1} to evaluate the functional groups present in the extracted PHB. The PHB extracted from both fruit waste extracts namely, sapota peel (Fig 7a) and apple peel extracts (Fig 7b) showed the strong absorption at 3382 and 3292 cm^{-1} representing the O-H bending group. Both the spectrum of PHB shows intense peaks at 2921 and 2955 cm^{-1} for C-H stretch, also marked peaks at 1641 and 1642 confirming the presence of ester carbonyl stretch (C=O) which is well in comparison with standard PHB recorded by Hong and co-workers [15]. In addition, spectrum of PHB extracted from the fruit wastes also shows absorption bands of $–CH_3$ stretch at 1459 and 1460 cm^{-1} which were

similar to the previous results observed by several researchers who characterized PHB using FTIR spectroscopy [33,34].

CONCLUSION

To deal with increased plastic pollution in the current scenario, it is necessary to find an alternative source of synthesizing bio-plastic. Among different methods, synthesizing biopolymers using bacterial sources is found to be a better alternative. In the present study, the strain *Bacillus subtilis* was utilized for the production of PHB under laboratory conditions. Optimum conditions for PHB production were found when pH was 7.0, temperature of 35°C with incubation period of 48h. To reduce the production cost of PHB, two different fruit wastes, such as sapota peel and apple peel wastes extracts were developed and tested for their effect on PHB production by supplementing it into production medium. Both extracts supported the production of PHB, however maximum PHB was found to get accumulated with sapota peel extracts supplemented medium when compared to apple peel extracts. The PHB extracted from media supplemented with both the wastes was subjected to characterization using FTIR. In conclusion, the study explored the role of fruit wastes extracts in the production of PHB thus reducing the production cost. However, further studies such as extraction, operating cost, quality of the PHB are required to substantiate the application of these wastes which may be functionally feasible.

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Received:04.05.18, Accepted: 07.06.18, Published:01.07.2018

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