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Exploitation of agro industrial residues as a substrate for the biodegradable polymer production using polyhydroxybutyrate accumulating bacteria

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Abstract
Agro industrial residues considered as agro-wastes, generated during the processing of crops are produced in large quantities every year worldwide. Major quantities of these on-farm agricultural residues are burnt in the field itself. Polyhydroxybutyrate (PHB), well known macromolecule naturally produced by many species of microorganisms, is being considered as a replacement for conventional plastics; however, the major drawback in PHB commercialization falls due to its high production cost. In the present study agro residues such as wheat bran and rice bran were studied as a cost effective substrate for the replacement of carbon source for the production of PHB using *B. subtilis*. The agro residues were initially hydrolyzed; different concentrations were amended in the minimal salt medium and investigated for PHB production. Several parameters such as incubation time, pH, temperature were optimized prior to the addition of agro residues for the production of PHB. The PHB produced were partially purified and further characterized using FTIR analysis. The results showed that the agro residues are found to be potential suitable substrate for the replacement of carbon source during the production of PHB.

Keywords: Biopolymer, *B. subtilis*, PHB, Rice Bran, Wheat Bran

1. Introduction
The applications of various plastic and plastic based materials developed from petrochemicals possess serious environmental issues due to their non degradability nature. The major advantages of these petrochemical polymers were found to be less expensive however their impact on environment is severe [1]. Due to increase in petroleum prices, adverse environmental impact and forthcoming fossil fuel crisis, the industries and researchers are in search of alternative products such as biodegradable polymers to combat the essential need of the world population [2]. Bio based polymers or biodegradable polymers are found to be better solution in terms of protecting the environment from petroleum based plastic products which are potentially toxic.

Several types of biodegradable plastics with different degrees of biodegradability are available, among them polyhydroxybutyrate (PHB) is found to be 100% biodegradable. Polyhydroxybutyrate are macromolecules produced majorly by bacteria and they are found as inclusion bodies accumulated as reserve material during stress condition in the growth medium [3]. These polymers possess properties which are similar to various synthetic based polymers like polypropylene which makes a good alternative for petroleum based products which can also be produced commercially [4].

However the major drawback is their production costs compared with the existing petroleum based plastic products. Recently various researchers have started investigating different strategies to reduce the production cost of PHB such as, developing bacterial strain with efficient production ability, optimizing the production medium for PHB synthesis, low cost downstream processing methods and utilizing various agro based residues as nutrient sources [5, 6]. This helps in reducing the cost of the PHB production as the production of PHB is majorly depended up on the production medium cost, especially carbon sources [7]. This leads to investigation of various cheap and alternative substrates as a carbon source in the fermentation medium that leads to good availability for the bacterial growth resulting in high PHB yield [8, 9]. Therefore the present study was aimed at producing the PHB using different agro industrial residues as a alternative cheap carbon source in the production medium using *B. subtilis*.

2. Materials & Methods

2.1 Reagents

The chemicals and reagents used in the present study were off analytical grade purchased from SRL Pvt ltd., Mumbai, India. The microbiological media were purchased from Himedia Pvt Ltd., India.

2.2 Strain selection and screening of PHB

The test strain *Bacillus subtilis* MTCC 9763 was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India and stored in the nutrient agar slant at 4°C. The 48 h freshly prepared bacterial suspension was screened for presence of PHB granules using sudan black staining method. Briefly, few drops of the sudan black stain (0.3 g of Sudan black B powder added with 100 ml 70% ethanol) was added to the heat fixed smear of the bacterial suspension and allowed to stand for 2 min. The smear was washed with distilled water and then few drops of safranin was added; kept for 10 – 15 seconds. The smear was washed with distilled water, dried and observed under high power objective for the presence of PHB granules which appears purple in color while bacterial cells appears pink in colour [9].

2.3 PHB Production and quantification

The PHB production was carried out in modified mineral salt medium (MSM), {[composition (g/L): Glucose (40), Yeast extract (0.16), Na_2HPO_4 (4.0), KH_2PO_4 (1.52), $CaCl_2$ (0.02), $MgSO_4 \cdot 7H_2O$ (0.52)] and trace element solution of 0.1 ml; [composition (g/L): $ZnSO_4 \cdot 7H_2O$ (0.13), $(NH_4)_6MO_7O_{24}$. $4H_2O$ (0.06), $FeSO_4 \cdot 7H_2O$ (0.02) and H_3BO_3 (0.06)]}. The glucose base medium and the trace element solution was autoclaved separately and reconstituted prior to the inoculation [8].

Freshly prepared overnight suspension of *B. subtilis* was inoculated into the sterile modified MSM medium and incubated at 37°C for 48 h. At the end of incubation, the bacterial pellet was obtained by centrifuging at 10000 rpm for 5 minutes and treated with 30% sodium hypochlorite solution for 20 min at room temperature. The digested cells were again centrifuged at 8000 rpm for 10 min and the pellet was further washed with water, acetone and ethanol. The pellet was then dissolved in chloroform and kept for evaporation at 30°C for 5 min. Five mL of concentrated H_2SO_4 was added and the preparation was incubated at 100°C for 40 minutes in a boiling water bath. The concentration of PHB extracted was analyzed by UV spectrophotometer at 235 nm [10].

2.4 Optimization studies

Optimization studies were carried out using different parameters such as incubation time, pH and temperature for the maximum production of PHB using shake flask culture method. Different incubation time varying between 0 to 96 h, with 6 h interval; initial pH of the production medium (6.0, 6.5, 7.0, 7.5, 8.0) and incubation temperature (30, 35, 40, 45 and 50°C) on PHB production medium was investigated using modified MSM medium at 120 rpm [8]. The pH of the medium was adjusted using 0.1 N NaOH and 0.1 N HCl. At the end of each experiment, the bacterial pellet was digested, extracted, acidified and the concentration of PHB was estimated spectrophotometrically as described earlier.

2.5 PHB production using Agro industrial residues

In the present study, the agro industrial residues such as wheat bran and rice bran were initially subjected to pre-treatment by acid hydrolyses using sulphuric acid [11]. Both the substrates were dried, powdered and then hydrolyzed with 0.5 – 5 % v/v sulphuric acid followed by autoclaving at 121°C for 30 min. The hydrolyzed samples were then filtered and the supernatants were further neutralized using 6N sodium hydroxide and analyzed for their reducing sugar content by DNSA method [12]. The prepared hydrolysate of different concentration (1 to 10 %, v/v) was added to the production medium by replacing the carbon source glucose. The medium was autoclaved, inoculated with the *B. subtilis*, incubated for 48 h and PHB production was estimated spectrophotometrically as described earlier.

2.6 Characterization of PHB

The PHB was extracted from the 48 h grown cells was further washed with acetone and utilized for FTIR analysis. The PHB was mixed with KBr to form a pellet which was then analysed using FTIR (Bruker) absorption spectrum in the range of 4000–600cm^{-1}. The absorbance spectrum obtained was compared with the available literature and the sample was confirmed for the presence of PHB.

3. Results and Discussion

In the present study, the strain *Bacillus subtilis* MTCC 9763 was screened for the presence of PHB granules using sudan black staining. The bacterial smear showed the presence of PHB granules of purple colour within pink colour vegetative cells when 48 h of cultures were subjected to sudan black staining (Fig. 1).

The strain was further utilized for the production of PHB using modified MSM medium. Different parameters such as incubation time, initial pH of the production medium and incubation temperature were optimized. Further, acidified hyrolysate of agro industrial residues such as rice bran and wheat bran were investigated for PHB production by amending it to the modified MSM medium replacing the carbon source (glucose) under optimized condition.

Fig 1: Screening of PHB using Sudan black staining

The effect of incubation time on PHB production was studied by determining the concentration of PHB at every 6 h intervals from 0 to 96 h. The study showed that PHB production was very low till 24 h of incubation, and then there was gradual increase in PHB production from 24 to 48 followed by steady decrease after 54 h. The maximum PHB production was found at 48 h (298 µg/mL) and hence it is considered as optimum incubation time (Fig. 2).

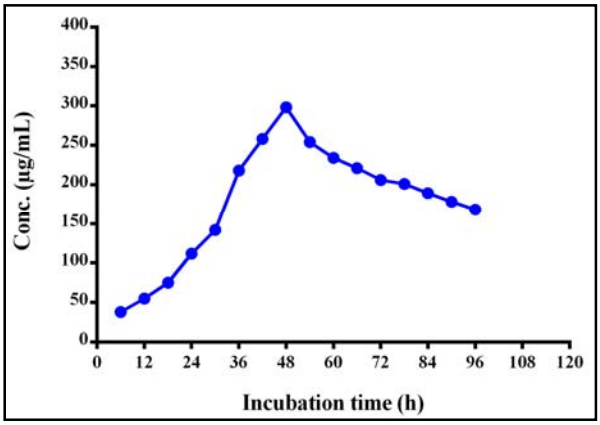

Fig 2: Effect of different incubation period on PHB production

The effect of initial pH of the production medium on PHB production was studied by inoculating *B. subtilis* into the modified production medium with varying pH from 6.0 to 8.0. The maximum PHB production was found when initial pH of the medium was maintained at 7.0 (312 µg/mL) under optimized incubation time (Fig. 3). The change in the initial pH of the production medium will strongly influence the PHB production. Ramadas and co workers have investigated the effect of initial pH on PHB production and they found that a pH of 7.5 influence the maximum production of PHB up to 25 % [12].

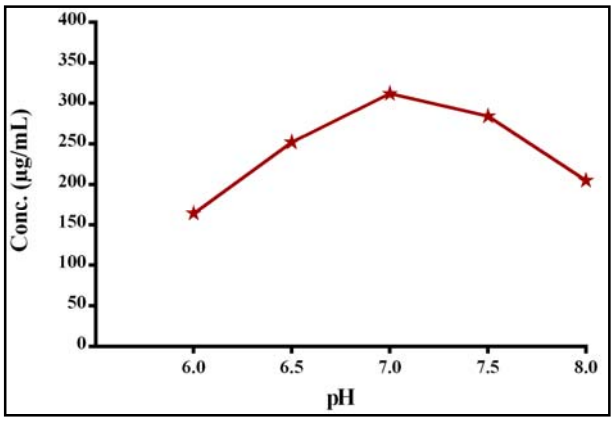

Fig 3: Effect of different pH of production medium on PHB production

The optimum incubation temperature for PHB production was determined by incubating the modified MSM at different temperature such as 30, 35, 40, 45 and 50°C for 48 h. The temperature plays a major role as it directly affects the microorganisms during their growth phase. In the present study maximum PHB production was found when the bacterial strain was incubated at 35°C for 48 h (328 µg/mL). Interestingly, increasing the temperature from 40 to 50°C results in decrease in PHB production as it may affect the growth of bacteria resulting in decrease in the production of PHB in the medium (Fig. 4). Similar studies were performed by Getachew and Woldesenbet who have produced PHB using various low cost agricultural waste materials by *Bacillus* sp [8]. They found that the maximum production was achieved when pH was maintained between 7 to 7.5. Effect of incubation temperature was also studied and the maximum PHB production was supported by an optimum incubation temperature of 37 °C which also corroborate to our study.

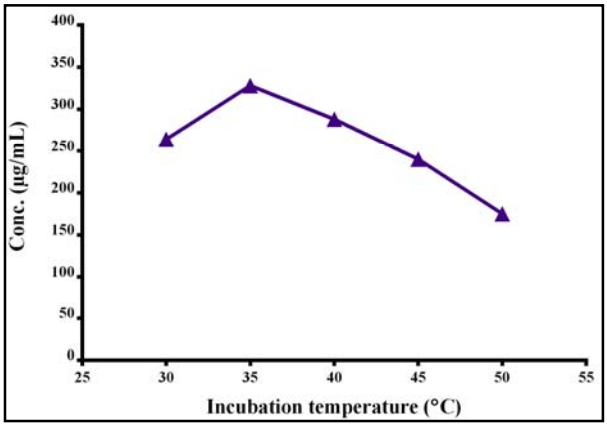

Fig 4: Effect of different incubation temperature on PHB production

Due to increase in the production cost of PHB, there is a huge interest in investigating various cheap sources as nutrient sources for the production of PHB which may contribute in reducing the production cost. In the present study two different agro industrial residues such as rice bran and wheat bran were investigated for the production of PHB by replacing it in the production medium for carbon source. For this, the acidified hydrolysate of rice bran and wheat bran was prepared, diluted and amended in the production medium at varying concentration from 1 to 10% (v/v) in the modified MSM production medium by replacing it with glucose. The results showed increasing the concentration of both rice bran and wheat bran shows a steady increase in the PHB production from 1 to 6% in the production medium. The maximum PHB production was found when rice bran and wheat bran was maintained at 7 % and 6 % respectively in the production medium. However increasing the concentration of rice bran and wheat bran beyond 7 % and 6 % respectively showed a steady decrease in PHB production (Fig. 5).

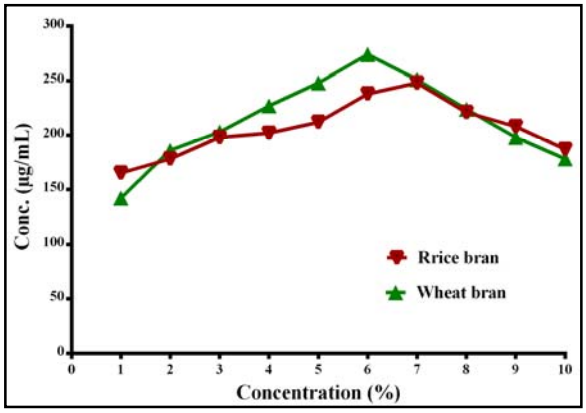

Fig 5: Effect of different concentration of agro industrial residues on PHB production.

Several bacteria can produce PHB using different cheap raw materials such as whey, [13, 14], starch [15], sugar industry waste water [16], sugarcane molasses [17], bagasse [18], wheat bran hydrolysate [19]. Fukui and Doi have investigated the supplementation of plant oils such as olive oil, corn oil and palm as alternative carbon substrates for the production of PHB using *R. eutropha* [20]. Similar studies were also reported by Rusendi and Sheppard who have utilized waste potato starch hydrolyzate as a cheap source for the production of PHB [21].

The PHB produced were characterized using FTIR analysis. The FTIR analysis shows the presence of various bands, especially at 1278 cm^{-1} representing CH stretch which is characteristic peak of PHB. The spectrum also shows strong absorption peak such as 1722 cm^{-1}, 1378 cm^{-1}, 1455 cm^{-1}, 2930 cm^{-1} and 3758 cm^{-1} corresponding to C=O, - CH3, -CH2, -CH and O-H groups respectively which were found to be characteristics peak for the PHB compound. Similar results were reported by various researchers who have also characterized PHB compound using FTIR analysis [22, 23]. Kumalaningsih and their co-workers have found characteristics peak at 2925.81 cm^{-1} who have used *Alcaligenes latus* for PHB production [24]. In another study, Bhuwal and co-workers reported PHB production using various industrial wastes such as pulp, paper, and cardboard wastes. They also characterized PHB using FTIR and found a characteristic peak of 2932 cm^{-1} corresponding to C–H stretch which also correlates with our study [25].

4. Conclusion

The present study provides preliminary investigation of utilizing the different agro industrial residues such as, rice bran and wheat bran as potential carbon source for the production of PHB. The study also confirms that amending the acid hydrolysate of rice bran and wheat bran in the production medium by replacing glucose as a carbon source results in good PHB yield. Thus the present investigation confirms that replacing the carbon source in the production medium using cheap agro industrial residues provides a viable strategy in the production of PHB with low cost and also aids in the solid waste management of agro industrial residues resulting in less pollution and also recycling process.

5. Acknowledgement

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