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# Bioconversion of Agricultural Wastes to Polyhydroxybutyrate by *Azotobacter vinelandii*

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**ABSTRACT**

The aim of the present study was to evaluate the potential of different agricultural wastes like wheat bran, rice polishing and molasses for the synthesis of polyhydroxybutyrate (PHB) by *Azotobacter vinelandii*. The optimization of different carbon sources and various physical parameters was also performed. Highest yield of PHB was obtained by fermentation of 4% (w/v) wheat bran after 48 h of incubation with 4% (v/v) inoculum volume at pH 7.0 and 37°C. Among different nitrogen sources tested, 0.2% peptone gave the better yield (285 mg/100 mL), while yeast extract decreased the amount of PHB. The outcomes of the present data indicate that agricultural wastes can be used for the production of polyhydroxybutyrate that will help in the solid waste management.

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**Authors' Contribution**
**TA, SS and MT designed the study, carried out the experiments and wrote the manuscript. HM, ARA, SF and ASH analyzed the data.**

**Key words**
**Polyhydroxybutyrate, Agricultural wastes, *Azotobactor vinelandii*, Fermentation.**



## INTRODUCTION

Plastic has gained importance in modern life due to its use in many industries for different purposes. It is usually made up of material that is derived from petrochemicals and cannot be degraded. So, these materials are accumulating in environment and are posing serious pollution problems (Hamieh *et al*., 2013). Therefore, it is required to replace such materials with biodegradable polymers, like polyhydroxyalkanoates (PHAs) for manufacturing of plastic (Barunegg *et al*., 2004). The principle biodegradable polyesters belonging to PHAs class are polyhydroxyvalerate, polyhydroxyoctanoate and poly-3-hydroxybutyrate (PHB) (Lakhawat *et al*., 2012).

As physical properties of polyhydroxybutyrate are similar to polypropylene and polyethylene so it has attracted much interest to be used as bioplastic. However, it is biodegradable and nontoxic as compared to synthetic polymers, so its use is environmentally more beneficial (Reddy *et al*., 2003). PHB is mostly used as packaging material in food industry because it is highly waxy and degradable. In pharmaceutics, it can be used as microcapsules in therapy and as material for cell and tablet packaging. It also has numerous applications in other areas such as fertilizers, insecticides, cosmetics, disposable items and also for long term dosage of drugs. Bioplastic also plays a vital role in medical field, including surgical stitches, repair devices and patches, slings, cardiovascular patches, biodegradable pins for fixation of bone and cartilage, stents to treat weak arteries, devices to regenerate tissues, tendon repair devices, bone marrow framework, and wound dressings (Hassan *et al*., 2016).

Commercially, PHB can be synthesized utilizing β-butyrate or β-hydroxybutyric acid as monomers, however this approach is economically unfeasible. Therefore, fermentation production of PHB using specific microorganisms is the most appropriate path by virtue of its simple and mild conditions during production. It is produced by many bacteria in response to physiological stress such as excess of carbon and limitation of nutrients like nitrogen, phosphorus and oxygen (Belal, 2013).

High cost of substrates used and recovery process employed, makes fermentation an expensive strategy for PHB production (Rehman *et al*., 2016). According to research, 3 tons of glucose must be used for each ton of polymer produced (Gouda *et al*., 2001). Thus, commercially cheap PHB can be achieved using substrates such as methanol, beet molasses, starch, whey and cane molasses that are used as a sole carbon sources (Suzuki *et al*., 1986; Kim, 2000; Gouda *et al*., 2001). Pakistan, as an agricultural country, produces a lot of agricultural byproducts annually (Amin *et al*., 2014). These nutrient rich and cheap substrates can be used for the synthesis of PHB through fermentation (Ramadas *et al*., 2009).

The current research work has been focused on production of polyhydroxybutyrate by *Azotobacter vinelandii,* using agro-industrial wastes such as wheat bran, rice polishing and molasses as substrates through

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submerged fermentation.

## MATERIALS AND METHODS

*Microorganism and culture maintenance*

The strain of *Azotobacter vinelandii,* NRRL-14641 was obtained from Agricultural Research Service (ARS) of United States Department of Agriculture (USDA). The organism was cultured and preserved on Burk's Nitrogen free agar medium slants (Saeed *et al*., 2016).

Bacterial culture was inoculated into 250mL of conical flask containing 25mL of Jarman broth and then incubated at 37ºC till OD reached to 0.6 at 660 nm (Saeed *et al*., 2016).

*Optimization of culture conditions for hyper-production of PHB*

The basal medium for fermentation contained different agricultural by-products as carbon source (wheat bran, rice polishing and molasses) in varying concentrations (1-5 g), $MgSO_4$ (0.02 g), NaCl (0.01 g), peptone (0.25 g) and yeast extract (0.25 g) per 100 mL. The flasks were incubated at 30ºC, pH 7.0 for 48 h to select the best carbon source for the production of PHB by *Azotobacter vinelandii* (Shivakumar, 2012). Then incubation time (12, 24, 36, 48, 60 and 72 h) was optimized to obtain the maximum yield of PHB (Khosro *et al*., 2012). The effect of different volumes of inoculum (2, 4, 6, 8 and 10%), pH levels (5, 6, 7, 8 and 9), degrees of temperature (23, 30, 37, 44 and 51ºC) was studied for hyper-production of the bioplastic (Hamieh *et al*., 2013). Different nitrogen sources *i.e.* peptone and yeast extract in varying concentrations (0.15, 0.2, 0.25, 0.3, 0.35%) were used to obtain high amount of the biopolymer (Singh *et al*., 2011). All the experiments were carried out in triplicates in shake flask of 250 mL (Erlenmeyer flask) having 25 mL of the fermentation medium.

*Extraction and determination of PHB yield*

For the extraction of the PHB, 25mL of the media was collected after fermentation and then centrifuged at 8000 rpm for 15 min. The pellet was used for further process and treated with sodium hypochlorite equivalent in volume to culture and incubated at 30°C for 2 h. Then the mixture was centrifuged for 15 min at 10,000 rpm and the pellet was further treated with distilled water, acetone, methanol and diethyl ether, respectively for the extraction. Finally, the polymer was obtained by dissolving the pellet in 5ml of boiling chloroform and filtering the solution through Whatmann Filter Paper No. 1. After evaporation of chloroform to dryness, the powder form of PHB was obtained. The PHB was gravimetrically estimated by weighing the dried precipitates (Singh *et al*., 2012).

To estimate the purity, the granules of biopolymer were dissolved in hot chloroform. Then 10ml hot sulphuric acid was added and finally placed in a water bath at 100°C for 10 min. The addition of sulfuric acid converts polyhydoxybutyrate into crotonic acid. After cooling, the absorbance was read at 235nm against a sulfuric acid blank. By referring to the standard curve, the quantity of poly-ß-hydroxybutyrate produced was estimated (Khosro *et al*., 2012).

*Statistical analysis*

All the experiments were performed in triplicates. The data was analyzed on SPSS 13.0 software, by comparing mean through One-Way ANOVA and multiple comparison was made through LSD and Descriptive analysis (Saeed *et al*., 2016).

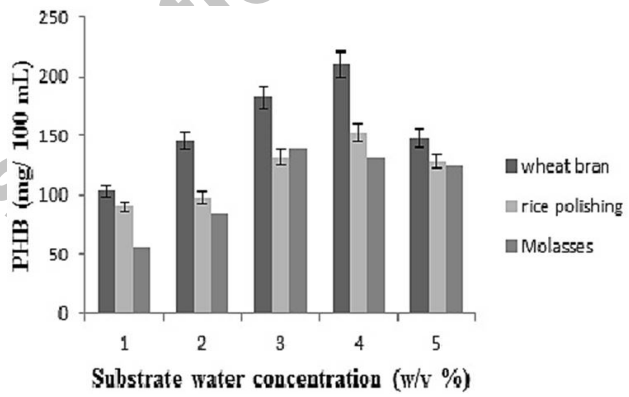


Fig. 1. Effect of different substrates production of PHB by *Azototobacter vinelandii*.

## RESULTS AND DISCUSSION

Pakistan as an agricultural country produces a lot of agricultural waste annually. Production cost of PHB is very high due to utilization of costly substrates and expensive downstream processing. However, use of cheaper substrates and economic recovery method in fermentation process can reduce the production cost. Therefore, the main purpose of this study was to optimize different parameters for maximum production of PHB using cheap agricultural by-products by *Azotobacter vinelandii*.

The significantly ($P<0.05$) highest PHB production (210 mg/100mL) was observed at 4% wheat bran, followed by 4% rice polishing (152 mg/ 100 mL) and 3% molasses produced the least amount (140 mg/ 100mL) after 48 hours of incubation (Fig. 1). Similar results were reported in the study conducted by Chaijamrus and Udpuay (2008), that maximum yield of PHB was observed at 4% molasses

and 4% corn steep liquor from *Bacillus megaterium* (Yield=43%). Aarthi and Ramana (2011) also reported high PHB yield (1.28 g/L) by *Bacillus mycoides*, using wheat starch as the media. Lakhawat *et al*. (2012) observed highest PHB yield (400 mg/100 mL) using 4% dilution of distillery spent wash by mutant *Azotobacter vinelandii* UWD. Sukan *et al*. (2014) reported maximum amount of PHB (2.5 mg/100 mL) using water treated orange peel as the substrate by *Bacillus subtilis* OK2. Gowda and Shivkumar (2014) stated that mango peel yielded high amount of biopolymer (0.403 mg/ 100 mL) by using *B. thuringenesis* IAM 12077. Soya flour was suggested as the best substrate for PHB production (0.089 mg/100 mL) by Shivkumar (2012).

The optimization of incubation period was carried out using 4% wheat bran as the carbon source. The significantly ($P<0.05$) maximum amount of PHB (210 mg/ 100 mL) was produced at 48 h of incubation (Fig. 2A). Similar results were reported in the study conducted by Pandian *et al*. (2009) as highest PHB (0.013 mg/ 100 mL) was reported at 48 h by *Brevibacterium casei* SRKP2 using dairy waste while *Bacillus mycoides* produced increased amount of PHB (1.28g/L) by using wheat starch medium at the end of 48 h of incubation (Aarthi and Ramana, 2011). Khosro *et al*. (2012) also stated 48 h as optimum incubation period for PHB synthesis (34.9 mg/mL) by *Azotobacter vinelandii*. In contrast, Chaijamrus and Udpuay (2008) reported that maximum yield of PHB was observed after 45 hours of incubation (Yield=43%) from *Bacillus megaterium.* Singh *et al*. (2011) observed maximum yield of PHB (0.531 mg/ 100 mL) at 30°C after 72 h of incubation from *Bacillus* sp. *Bacillus thuringiensis* produced maximum amount of PHB after 24 h of incubation in fermentation medium containing carbon sources and nutrient salts (Shivakumar, 2012).

The effect of volume of inoculum was also observed in this study. The significantly ($P<0.05$) maximum yield of PHB (236mg/100mL,) was recorded at 4% of inoculum by *Azotobacter vinelandii* (Fig. 2B). In contrast, Hamieh *et al*. (2013) reported optimum volume of 3mL for higher PHB production from *Lactobacillus acidophilus* and *Bacillus thuringiensis*. Shivakumar (2012) reported maximum PHB production at 200% of inoculum density (250mg/100mL).

Maximum PHB yield (236mg/100mL,) was observed significantly ($P<0.05$) at pH 7 in wheat bran medium from *Azotobacter vinelandii,* at 48 hours of incubation (Fig. 2C). The results were in lined with Maheswari and Dhandayuthapani (2013) as they reported that highest yield of PHB (2g/L) by *Azotobacter vinelanii* KDP was found at pH 7.0. In contrast, Shivakumar (2012) reported 7.5 optimum pH for the PHB production by using carbon sources of pure glucose and agricultural wastes. Hamieh *et al*. (2013) found maximum PHB production at pH 5.5 by *Lactobacillus acidophilus* and *Bacillus thuringiensis*.



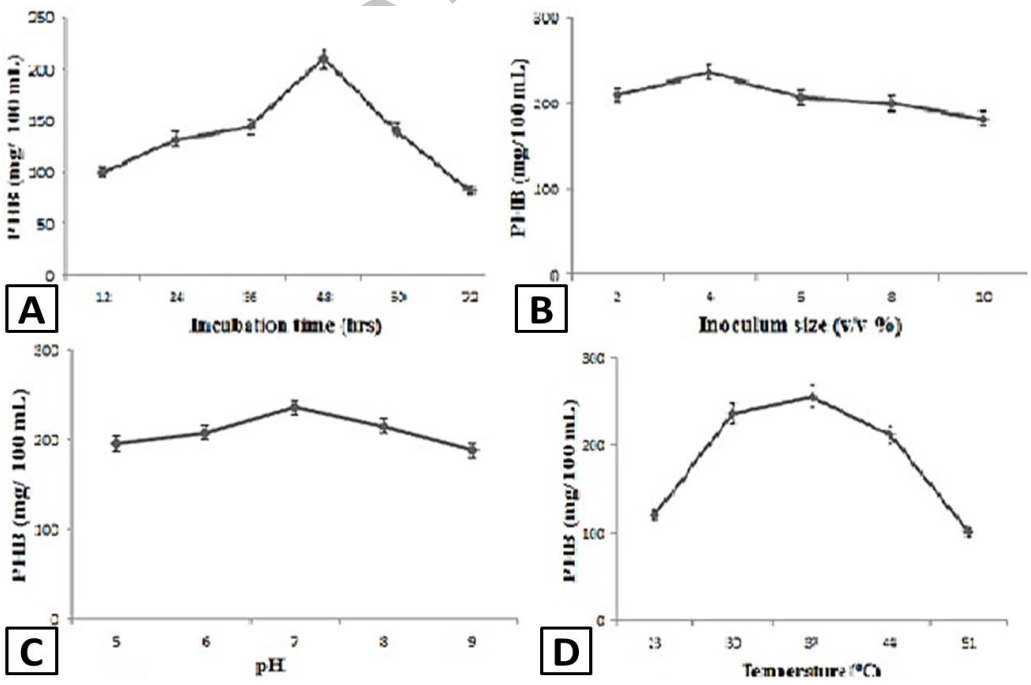


Fig. 2. Optimization of parameters for PHB production by *Azototobacter vinelandii*. A, incubation time; B, inoculum; C, pH; D, temperature.

Temperature has an important effect on the growth and metabolism of microorganism. A significantly ($P<0.05$) higher production of PHB (256mg/100mL) was observed at 37ºC (Fig. 2D). These results are in agreement with Hamieh *et al*. (2013) as they reported the same temperature to be optimum for PHB production by *Lactobacillus acidophilus* (412g/ 50 mL) and *Bacillus thuringienesis* (0.367g/50 mL). Aly *et al*. (2013) also observed that maximum yield of PHB (55% in terms of DCW) was found at 37°C by *Bacillus cereus* MM7. In contrast, Tamodgan and Sidal (2011) stated that the temperatures higher and lower than 30ºC decreased the synthesis of PHB by *Bacillus subtilis* ATCC 6633.

Varying concentrations of both peptone and yeast extract were tested to obtain maximum PHB production. The significantly ($P<0.05$) highest yield of biopolymer was observed at 0.2% peptone (298mg/100mL), while yeast extract decreased the production (Fig. 3). These results were in line with Singh *et al*. (2011) and Yuksekdeg *et al*. (2004) who found the peptone the best nitrogen source toward PHB production. In contrast, yeast extract (2.5 g/L) provided maximum production of PHB *i.e.* 50% (9.2 g/L) on the basis of DCW among all organic nitrogen sources tested by *Bacillus cereus* NRRL-B-3711 (Rehman *et al*., 2016).

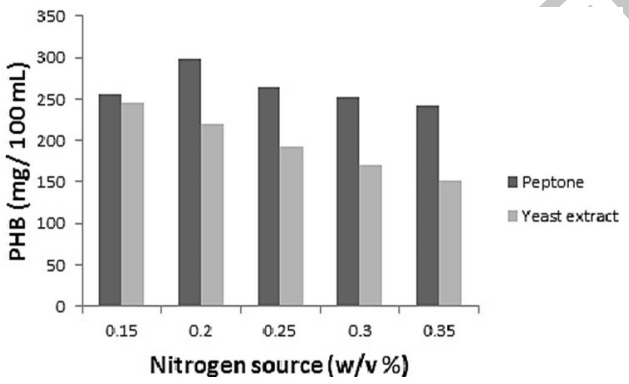


Fig. 3. Effect of nitrogen source (%) on PHB production by *Azototobacter vinelandii.*

The PHB film obtained after extraction (Fig. 4A) was further subjected to spectrophotometric analysis. The bioplastic produced was found to be 97.5% pure in comparison to the standard of Sigma Aldrich with the help of standard graph (Fig. 4B).

## CONCLUSION

It is concluded that the cheap method have been developed for PHB production that will be helpful to reduce the pollution caused by synthetic plastics.

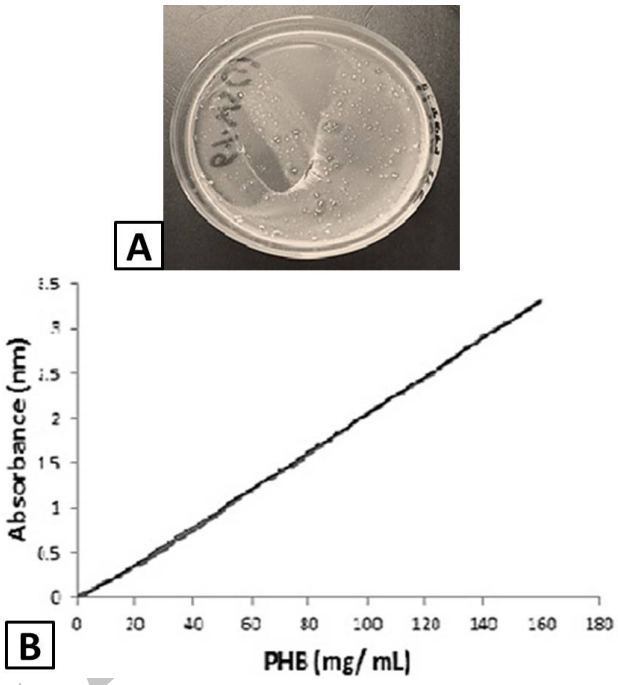


Fig. 4. Polyhydroxybutyrate film (A) after extraction standard graph (B).

*Statement of conflict of interest*

The authors declare no conflict of interest.

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