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PRODUCTION OF BIOPLASTIC FROM THE ISOLATED *LACTOBACILLUS*

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ABSTRACT
Plastic has been a vital part of our life. However, disposal of these non-degradable petroleum-derived plastic has threaten our ecosystem. The solution to this problem is bioplastics which are a potential replacement to the synthetic plastic, because has mechanical properties similar to polypropylene and completely biodegradable too. In this work, an attempt was made to isolate potent PHB producing lactic acid bacteria from curd sample. These isolate were then morphologically and biochemically characterized. Sudan Black B was used for primary screening of bacterial isolates for PHB production. The PHB biopolymer was extracted using sodium hypochlorite digestion followed by chloroform precipitation. The PHB granules were confirmed by UV- visible spectrometry analysis and the presence of functional groups in extracted PHB was confirmed by FTIR analysis. The bioplastic film was prepared using PHB powder and biodegradation and solubility study were conducted. In conclusion, study of these lactic acid bacteria may indicate their special role in PHB production and *Lactobacillus* can be used for PHB production and saving the environment from pollution.

KEYWORDS: *Lactobacillus*, Biopolymer, PHB, Sudan Black, Production, FTIR analysis.

INTRODUCTION
Plastics are synthetic or semi-synthetic materials which are typically polymers of high molecular mass obtained from petroleum and natural gas. The phenomenal rise in the usage of plastics is due to their low cost and better properties which include flexibility, rigidness, brittleness and ability to be molded into variety of shapes and lighter (Stevens, 2002). However, the waste generated can be devastating to ecosystems. The major sources of land pollution include plastics, metal and glass containers, food wrapping, worn-out machinery, old furniture, garbage, etc (Modebelu and Edward., 2014). The solution to this problem is bioplastic (Polyhydroxyalkanoates) which are a potential replacement to the synthetic plastic.

Biodegradable polymers play an increasingly significant role in plastic engineering by replacing commodity, non-degradable and nonrenewable petrol-based polymers **(**Park *et al*., 2004 and Stepto., 2006**)**. Polyhydroxybutyrate (PHB) is a biodegradable and biocompatible thermoplastic, there are a class of bacterial polyesters collectively called poly-hydroxyalkanoates (PHAs), accumulated intracellularly as reserve granules by many bacteria in harsh environmental conditions (Kim., 2000).

The present research was designed to isolate PHB producing bacteria and to study the production and characterization of PHB from *Lactobacillus.*

MATERIAL AND METHODS
Sample Collection
The curd sample was collected under aseptic condition in sterile screw cap tube to isolate the bacterium *Lactobacillus*.

Isolation of bacterial strains
Isolation of *Lactobacillus* from curd sample was carried out by serial dilution of the sample in saline solution followed by plating of the samples on Mann Rogosa Sharpe (MRS) agar media. These plates are incubated at 37^{o}C for 2 days.

Screening of isolates for PHB production using Sudan Black B dye
The isolate was screened for PHB production using the lipophilic stain Sudan Black B on agar plate and Sudan black staining.

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 (0.02 gm in 100 ml 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.

Screening for PHB on Solid Agar

The bacterial isolate was qualitatively tested for PHB production following the viable colony method of screening using Sudan Black Dye. For this screening of PHB producers, nutrient agar media supplied with 1% of glucose was autoclaved at 121^{o}C for 20 minutes. This media was poured into sterile petri plates and allowed for solidification. The bacterial isolates were streaked on the plate. These plates were incubated for 24 hours. After the incubation, ethanolic solution of 0.02% Sudan Black B was spread over the petri plates containing colonies and was kept undisturbed for 30 min. They were washed with 96% ethanol to remove excess stain from colonies. Then the results were observed.

Characterization of PHB producing isolates

Microscopic observations of the bacterial isolates were studied using Gram staining, Endospore staining and motility test (Hanging drop Technique). Various biochemical tests such as Indole test, Methyl red, Voges Proskauer, Citrate utilization test, Urease test, TSI test, Nitrate test, Oxidase test, Catalase activity and carbohydrate fermentation (Glucose, Lactose, Sucrose, Maltose, Mannose and Xylose) were carried out for the identification of *Lactobacillus.* Gelatin, Starch and Casein Hydrolysis test were also performed.

Inoculum Development

Inoculum for PHB production was prepared from 48 hours old slant culture of *Lactobacillus* sps incubated at 37°C. One loop of cells was transferred into 100 ml of sterile MRS broth taken in conical flask. The flask was incubated at 250 rpm at 37°C for 24 hours and used as inoculum for PHB production.

Production of PHB

PHB production was carried out using N_2 deficient medium which contained following ingredients: 1% Glucose, 0.02% $MgSO_4$, 0.01% NaCl, 0.05% KH_2PO_4, 0.25% Peptone and 0.25% Yeast extract with pH-7. The N_2 deficient medium was prepared in 250 ml conical flask and sterilized properly. The sterilized medium was inoculated with *Lactobacillus* sps. The flask was kept in rotatory shaker at 37^{o}C for 48 hours.

Extraction of PHB

After 48 hrs incubation, culture was collected and centrifuged at 10,000 rpm for 15 min and lyophilized. The lyophilized pellet was digested with 4% sodium hypochlorite solution at 37^{o}C for 20 min. Then pellet was collected by centrifugation at 10,000 rpm for 15 min, after that the pellet was washed with water, acetone, ethanol respectively for washing and extraction. Finally polymer was dissolved in chloroform and kept for complete evaporation.

Characterization of PHB

UV-vis spectrometric analysis of PHB

The extracted PHB was dissolved in sulphuric acid and subjected to scanning in UV-vis spectrophotometer in the range of 200-300 nm against sulphuric acid blank and the spectrum was analyzed.

FTIR analysis of PHB

The extracted PHB polymer samples were prepared in KBr pellet and FTIR absorption spectrum was recorded in a range of 400-4500 cm-1.

Preparation of Bioplastic film

0.5 gram of PHB powder was placed in a beaker and 2 ml of distilled water was added and stirred using glass rod, followed by 0.6 ml of 0.1M HCl was added as the solution requires a slightly acidic medium for the formation of plastic. 0.5 ml of glycerol (plasticizer) was added to this mixture. The solution was heated for 30 mins. 0.6 ml of 0.1M NaOH is added to neutralize the acidity of the mixture. The plastic solution was poured on a glass petri plate and the petri plate was placed under the oven at 130^{o}C. This process was continued for about 24 hours and the bioplastic film was obtained.

Solubility studies

Samples of bioplastic were cut into small pieces and were inserted into a test tube containing different solvents (Ammonia, Glacial acetic acid, Chloroform, Acetone, Methanol and Sulphuric acid and water).

Biodegradability studies

400 ml beaker and preweighed piece of bioplastic were taken; the preweighed bioplastic material prepared was placed under the beaker containing soil at a depth of 5 cm from the surface. Some amount of water was sprinkled on the soil so that bacterial enzymatic activities could be enriched. These samples were kept in the beaker for about 10 days and each 3 days of interval the weight of the bioplastic material was measured and results were recorded accordingly.

RESULTS

Sample collection and Isolation of bacterial strains

The curd sample was collected. The sample was serially diluted and followed by plating of the samples on MRS agar media and plates were then incubated at 37^{o}C for 48 hours. After 48 hours of incubation well formed isolated colonies were obtained.

Sudan Black B Staining

The *Lactobacillus* sps upon staining with Sudan black revealed the presence of intracellular dark PHB granules inside pink colored bacterial cells. This represents the PHB producing isolates.

Screening for PHB on Solid Agar

The isolate were found to be positive for PHB production; showing PHB granules which appeared as black colour colonies when stained on plate with the Sudan Black stain. (Figure-1).

Figure- 1: Screening on plate.

Characterization of PHB producing isolates

Bacterial colonies were characterized by Microscopic observations and various biochemical tests. (Table-1).

Table-1: Gram staining & Biochemical results.

Gram staining	Positive – Rod
Endospore staining	Negative
Motility	Non – Motile
Indole test	Negative
Methyl red	Positive
Voges Proskauer	Negative
Citrate utilization test	Negative
Urease test	Negative
TSI test	Negative
Nitrate test	Negative
Oxidase test	Negative
Catalase test	Negative
Glucose	Positive
Lactose	Positive
Sucrose	Positive
Maltose	Positive
Mannose	Positive
Xylose	Negative
Gelatin Hydrolysis test	Negative
Starch Hydrolysis test	Positive
Casein Hydrolysis test	Negative

Production and Extraction of PHB

The N_2 deficient medium induces the PHB production and PHB crystal was collected after extraction process. This biopolymer was used for further studies.

UV-vis spectrometric analysis of PHB

The sample solution was subjected to UV- visible scanning over the range of 200- 300 nm. Sharp absorbance peaks were observed at 220 nm which indicated the presence of PHB molecules. (Figure-2).

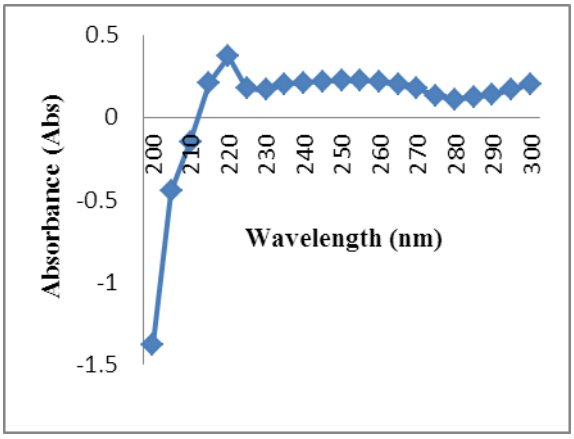

Figure- 2: UV Spectra of PHB.

FT-IR analysis of PHB

The FT-IR spectrum was recorded in the range of 400-4500 cm-1 (Figure-3). The carbonyl group (C=O) gave a strong band in the range of 1658.78 cm-1. In this study, the functional groups of the polymer PHB was confirmed as C=O groups by FT-IR spectroscopy. The methane (CH) group gave a strong band in range of 1411.89 and 2924.09. The (C-O) group showed strong band in the range of 1072.42 and 1242.16. These all prominent absorption bands confirm that the polymer extracted from sample were polyhydroxybutyrate.

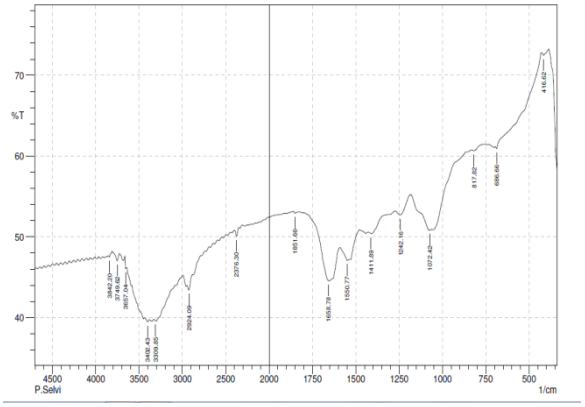

Figure- 3: FT-IR analysis of PHB.

Preparation of Bioplastic film

The biodegradable plastic film was prepared from PHB powder. (Figure-4).

Figure- 4: Bioplastic film.

Solubility studies

Solubility studies were conducted by using various solvents and the results were tabulated in Table-2.

Table -2: Solubility Test for Bioplastic.

Solvents used	Insoluble	Partially soluble	Completely soluble
Ammonia	Yes	--	--
Glacial acetic acid	Yes	--	--
Chloroform	--	Yes	--
Acetone	Yes	--	--
Methanol	Yes	--	--
Sulphuric acid	--	--	Yes
Water	Yes	--	--

Biodegradability studies

The bioplastic kept in the beaker were biodegradable (Table-3), as per our results there were reduced in the weights of bioplastic.

Table -3: Biodegradability of bioplastic.

Sample	Initial quantity taken in grams	after 3 days	after 6 days	after 9 days
Bio plastic	0.16	0.12	0.10	0.7

DISCUSSION

The *Lactobacillus* strain was isolated and cultured in N_2 deficient medium. The N_2 deficient medium induces the PHB production in excess. The cells are then lysed and PHB content was extracted.

In our present study the functional group of PHB granules were identified as C=O, CH and C-O. Previously reported that the FTIR spectrum of the compound showed characteristic bands for the groups C=O, CH and C-O (Sujatha *et al*., 2005). The PHB production was determined after the convention into crotonic acid and absorbance spectra was determined as 220 nm (Hong and Janarthanan 2007). In our present study crotonic acid was formed by acid digestion and absorbance spectra was determined as 220 nm.

The PHB biopolymer blended with the glycerol could help in the formation of plastic film and to perform solubility and biodegradability study.

Solubility is the main properties to check whether the synthesized bioplastic material is sustainable or not. Insoluble property of the bioplastic material in water medium is promising for the synthesis of economically viable product development. Hence the synthesized bioplastic material has got all the substantial properties like biodegradability and insolubility in water medium makes it worthy biomaterial for commercial possibility.

The bioplastic material was degraded by soil bacteria. The degradation studies (soil burial test) conducted is helpful in preparation of environmental friendly product and they can be reused in bio compost preparation.

These PHB have potential candidate for some application in packaging and biomedical material production due to their purity, non-toxic behavior, biocompatibility, biodegradability.

CONCLUSION

Biodegradable plastics can replace petroleum based plastics primarily due to its biodegradability and insoluble property in water. It is more convenient to use bioplastic as they do not lead to the pollution of the environment and can be used widely to decrease the level of pollution.

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