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UTILIZATION OF POMEGRANATE WASTE FOR THE PRODUCTION OF BIOPOLYMER

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ABSTRACT

Plastic is one such material that poses a big threat to the environment because this is non-degradable product it creates pollution. Polyhydroxyalkanoates (PHAs) are natural biodegradable microbial polymers with a promising potential to replace synthetic plastics. PHAs are insoluble storage granules that microorganisms accumulate in stressful conditions, under excess of carbon and deficiency of other essential nutrients. This research is focused on devising a sustainable approach to produce PHAs by *Pseudomonas aeruginosa* and *Bacillus substilis* using pomegranate waste as a carbon source. Pomegranate waste was treated with water and acid hydrolysis for the effective production of PHA and lignin degradation. Acid hydrolysis shows better lignin breakdown when compared with water hydrolysis. This study reveals that *Pseudomonas aeruginosa* (4.9g/l) produce a higher yield than *Bacillus subtilis* (4.4g/l) under optimized temperature, pH, and substrate concentration. The obtained PHA analyzed qualitatively by Sudan Black Test and Nile Blue Test and finally confirmed by FTIR spectroscopy method.

Keyword: Lignocellulosic waste, Polyhydroxyalkanoates, *Pseduomonasaeruginosa*, *Bacillus substilis*, FTIR.

1. INTRODUCTION

Plastics are the pollutants which are categorized into micro (1µm-5mm), meso (5mm-200mm) or macro (>200mm) debris on the size (Rodrigo *et al*., 2022). There are 1.1 to 8.8 million metric tons of plastics waste in the oceans every year, living organism in the marine and also the surroundings gets affected through this rising pollution. Some researchers suggested that by the year of 2050 there will be more plastics while compare to the fish in the oceans. These are known as the "Trash islands" or the "garbage patches". The larger piece of plastics takes atleast 400 years to break into fragments as the micro-plastics. These lead to tiny piece of plastics which accumulate micro-beads in the oceans. By the recent survey they show 15 to 50 trillion pieces in the oceans. The usage is higher now because of their low cost and compatibility, by using these plastics it creates the serious issue to the environment because this is not a degradable product it creates the pollution. Normally the plastics are destroyed by using the burn when the plastics are burn it releases dangerous gas to the environment and it also responsible for the carbondioxide, toxic gas emissions (Ezeoha *et al.,* 2013). The chlorinated plastics release the harmful chemical to the soil which enters into ground water. This is harmful to species that intake those chemical mixed water. For this plastic replacement scientists were discovered to produce the biodegradable plastics which are from the bio-wastes. The use of Biodegradable plastic growers can reduce the amount and cost of disposal Photo-degradable plastics, mulch film and biodegradable plastics have been considered by researchers to replace petroleum based plastics (Halley *et al.,* 2001). Plantation industries also searching for the alternative for the plastics (Kang *et al*., (2013). Biodegradable plastic has been in discussion in number of years mainly because of their degradable capacity (Nonato *et al.,* 2001). It helps in the recovery of material, renewable source utilization and reduction of landfills (Mostafa *et al.,* 2015).

2. POMEGRANATE AS A SUBSTRATE

Punica grantum belongs to the family of Lythraceae, the genus has two common species: Punica granatum and Punica protopunica which are located between Iran and the Himalayas in India (Stover et al., 2007; Soriano et al., 2011). Lignocellulosic materials in the pomegranate are composed of 34–55 wt % cellulose, 11–25 wt % lignin, and 20–40 wt % hemicellulose in addition to extracts of pectin, resins, waxes, ash, and minerals. The Punica granatum have the proximate composition of pectin- 27%, hemicellulose - 10.8%, cellulose - 26.2%, lignin - 5.7%.

Lignin can function as a plasticizer, stabilizer, or bio- compatibilizer in bioplastics, which will produce different properties on bioplastics (Yang et al., 2019).

Food waste is one of the big sources of an inexpensive carbon source, due to its widespread availability and has the potential to produce biopolymer when it is recycled. Based on the carbon sources a variety of microorganisms is helpful for the production of PHA.

PHA producers can also be classified as:

(i) Hydrocarbon degraders as a PHA producer.

(ii) Halophiles as a PHA producer.

(iii) Cyanobacteria as a PHA producer.

(iv) Plant Growth Promoting Rhizobacteria (PGPR) as a PHA producer.

(v) Antibiotic Producers as PHA producers.

From this, we took Pseudomonas aeruginosa and Bacillus subtilis because these bacteria have effectual production of biopolymer when compared to all other bacteria which produce PHA enormously.

Pseudomonas aeruginosa

Pseudomonas aeruginosa was gram-negative, rod-shaped encapsulated bacteria, citrate catalyze, and oxidase-positive. It is mostly found in soil, skin flora, water, and vegetation. It grows well in the culture at 37˚C and tolerates temperatures up to 42˚C it withstands environmental stresses. This organism is capable of producing extensive colonization and aggregates into the synthesis of bio-films. It also breakdown tarballs and oil from oil spills. This bacteria is also found on medical equipment by causing cross-infection in hospitals, and catheters in clinics and it also decomposes hydrocarbons.

Bacillus subtilis

Bacillus subtilis is the gram-positive, catalyze-positive bacteria that is found in the soil and gastrointestinal tract of humans and ruminants. This is also called the hay bacillus or grass bacillus.

POLYHYDROXYALKANOATES

The polyhydroxyalkanoates were first described by Lemoigne, a French scientist (Jiun et al., 2010). These are polyesters that are hydrophobic and insoluble in water. Their properties are mostly similar to those of their monomer building blocks. A huge variety of biopolymers can be produced by microbial fermentation. This occurred in the storage polymer in the prokaryotic cells. PHA has the advantage of being produced from renewable resources and thus being independent of crude oil price and its availability. PHAs are classes of natural polyesters that accumulate within different bacteria and archaea as intracellular granules acting as a carbon and energy pool. PHAs are known to be distinctive bioplastics that can be chemically modified or bioengineered to be used as high-value medical biomaterials such as sutures, particulate vaccines, scaffolds in tissue engineering, and drug carriers or to be used as low-value bioplastics.

3. MATERIALS AND METHODS

The lignocellulose waste (Pomegranate peel) was selected as the substrate because of their nutrient values in the pomegranate peels by considering the waste from the wealth it was inoculated with microorganisms for biopolymer production. Pomegranate (Punica granatum) is a polysaccharide source that can break down from polysaccharides to monosaccharides by water treatment and acid hydrolysis methods. Pseudomonas aeruginosa and Bacillus subtilis bacterium was taken specifically because of their PHA synthesis for the production of bio-film by using these microorganisms. This bacterium was inoculated in the pomegranate substrate.

PRETREATMENT

3.2.1 ACID HYDROLYSIS

100g of pomegranate powder was taken and 70 ml of 2N of HCl was mixed with 200 ml distilled and boiled at 100ºC for 45 minutes for the reduction of the polysaccharides to monosaccharides for the substrate preparation (K Preethi et al., 2015). The concentration of sulfuric acid, temperature, and time of hydrolysis was used as dependent variables, whereas hemicellulose, cellulose, lignin contents, weight loss, extractives, total carbohydrates, and reducing sugars, were recorded as responses for PPW composition.

3.2.2 WATER TREATMENT

100g of pomegranate powder was taken and it was boiled at 100ºC for 1 hour in a water bath for the break down of the polysaccharide. The untreated, acid hydrolyzed and water treated pomegranate peel was analyzed for the lignin, hemicelluloses, cellulose concentration, ash, carbon dioxide, protein, energy, and moisture content. Dependent variables such as concentration of temperature and time of hydrolysis are used to detect the concentration, whereas hemicellulose, cellulose, lignin contents, weight loss, extractives, total carbohydrates, and reducing sugars, were recorded as responses for PPW composition.

4 INOCULATION OF MICROORGANISM

Pseudomonas aeruginosa and Bacillus subtilis were inoculated in the pomegranate substrate by the various concentrations of the acid hydrolyzed substrate (6, 8, 9, 10, 12g) Vs pH (5, 6, 7, 8, 9) Vs temperature (37^0C) (Anteneh et al., 2016). The OD and the biomass were analyzed by every 4 hours till 52 hours. By concluding that the effective production of Biomass is on centrifuging for 15 minutes and the pellet was allowed to dry in an oven (Anteneh et al., 2016).

The biomass production was analyzed by centrifuging the sample at 5,000 rpm for 10 minutes and the collected pellet was allowed to dry a hot air oven at 30˚C for 30 minutes. The supernatant was checked for biopolymer formation.

5 PHA SYNTHESIS

The PHAs which are synthesized by the microorganism produced by the bacterial fermentation of sugar or lipid which is converted into CO2 by microorganism (Sneha et al., 2017). Polyhydroxyalkanoates are some of the most common reserve substances accumulated by prokaryotes (Tiwari et al., 2012). Bacteria generate energy through channelizing organic matter through the tricarboxylic acid cycle (TCA) cycle under normal environmental conditions . However, under stress conditions arising out of excess carbon rich organic matter and limitations of nutritional elements such as nitrogen, potassium, phosphorus, oxygen, and magnesium, bacteria switch from energy producing to energy conserving pathway (Singh et al., 2019).

6 SCREENING OF PHA

The bacteria screened for the presence of the PHA. Nile Blue staining is done for screening and the conformation of the polyhydroxyalkanoate by Sudan Black B staining. The colonies which was isolated and screened for PHA production by Sudan Black staining and based on the magnitude of the staining it was calculated (Nandini et al., 2011). The Sudan Black was stained a left overnight and the result was observed.

QUANTITATIVE TEST

The anti-oxidant content and potential health benefits associated with consuming pomegranate and pomegranate-containing products has lead to increased consumer demand for this crop resulting in it becoming a high value crop. 50ml of 24 hrs of culture was taken and it was centrifuged at 6000rpm for 20 minutes then the pellet was suspended in 5 ml sterile water and vortex for 8 minutes to this 2ml of cell suspension 2 ml of 2N HCl was added and heated to the boiling temperature for 2hrs in the water bath, then again centrifuged at 6000rpm for 20 minutes and to this 5ml of chloroform was added. Bio-plastic film was prepared by dissolved in chloroform and the spectrum was analyzed for a sharp peak at 240nm (Selvakumar *et al*., 2011).

3.7 QUALITATIVE TEST

3.7.1 SUDAN BLACK SLIDE TEST

The bacterial culture was smeared and it was heat fixed and solution was added few drops and let for 10 minutes, then slide was washed with ethanol gently and counter stain with the safranin and it was left to dry it was then oil immersed to get the clear view under the microscope.

3.7.2 SUDAN BLACK TEST

The Bacteria were plated and the Sudan Black was spread after the growth of the organism. It was left over night in the incubator for the absorption of the Sudan black into the bacteria. The dark blue color indicates the PHA presence in the plate.

3.7.3 NILE BLUE TEST

2 g of PHA sample was taken and heated gently then few drops of nitric acid was added to that sample and cooled and double the volume of sample with distilled water. Then the nitrite solution was added and the appearance of blue color shows the presence of PHA.

3.8 PREPARATION OF BIOPALSTIC FILM

Sample of the bio-plastic film was prepared by dissolving it in 50 mg PHA extract in 10 ml chloroform (Rawia *et al.*, 2013). After adding the chloroform it was kept in the shaker for the PHA formation. The layer which is obtained at the top is our required film.

3.9 FTIR SPECTROPHOTOMETRY ANALYSIS

Fourier transform infrared spectroscopy is used to obtain an infrared spectrum of emission or absorption of a liquid, solid or gas. This term originates from Fourier transform to convert raw data into actual spectrum. 1 mg of extracted sample was dissolved in 5 ml chloroform.

4. RESULT AND DISCUSSION

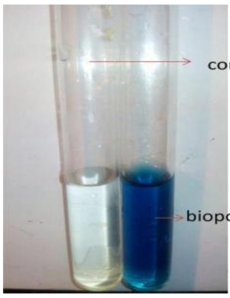
By comparing substrate (6, 8, 9, 10, 12g) Vs pH (5, 6, 7, 8, 9) Vs temperature (37˚C) *Pseudomonas aeruginosa* at pH-7, substrate-80g/l at 37˚C shows 4.9g of biomass and *Bacillus subtilis* at pH-6, substrate-90g/l at 37˚C with 4.7g of biomass reveals the effective biomass while compared to substrate, pH and temperature.

COMPOSITION OF POMEGRANATE

By comparing the untreated powder, normal water and Hydrochloric acid, Hydrochloric acid has slightly higher cellulose with 16.5 % whereas untreated powder have 14.6% , water treated with 15.5%. By comparing the lignin in the Hydrochloric acid shows 15.4 % while compare to the untreated powder with 18.3% and water treated with 16.4%. With these properties HCl treated powdered sample was taken as the substrate. Less lignin content in the hydrochloric acid treated powder helps for easy utilizing of the substrate and growth of microorganisms.

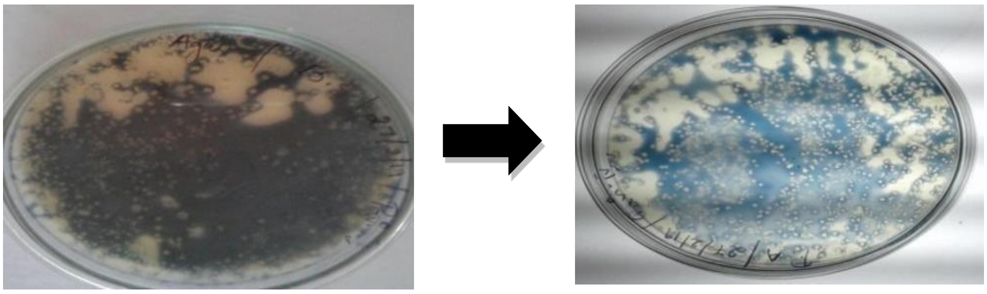
4.2 QUALITATIVE TEST

4.2.1 NILE BLUE TEST



Blue color formation in the supernatant at 52 hours culture confirms the presence of PHA. The blue color formation is due to the breakage of polymerto monomer. The break polymers are efficiently reacts with the chloroform to transform into blue color.

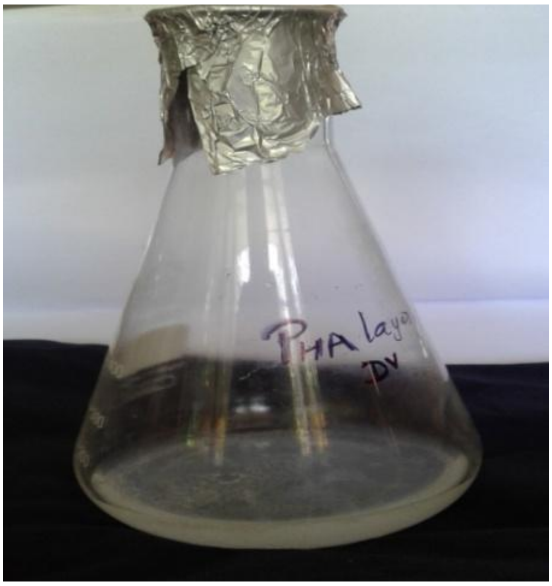
4.2.2 SUDAN BLACK TEST



The culture was stained by Sudan black overnight the plate shows the blue color formation because the microorganisms which have the capability of producing PHA shows blue color formation. The bacteria which produced the PHA will intake the Sudan black strain and reflect blue in color.

4.3 BIOPOLYMER

The culture was centrifuged and the pellet was vortex with sterile water and cell suspension was mixed with 2N HCl and heated in water bath for 1 hour and centrifuged again, the supernatant was heated and mixed with chloroform, it was kept in incubator overnight for the PHA layer formation.

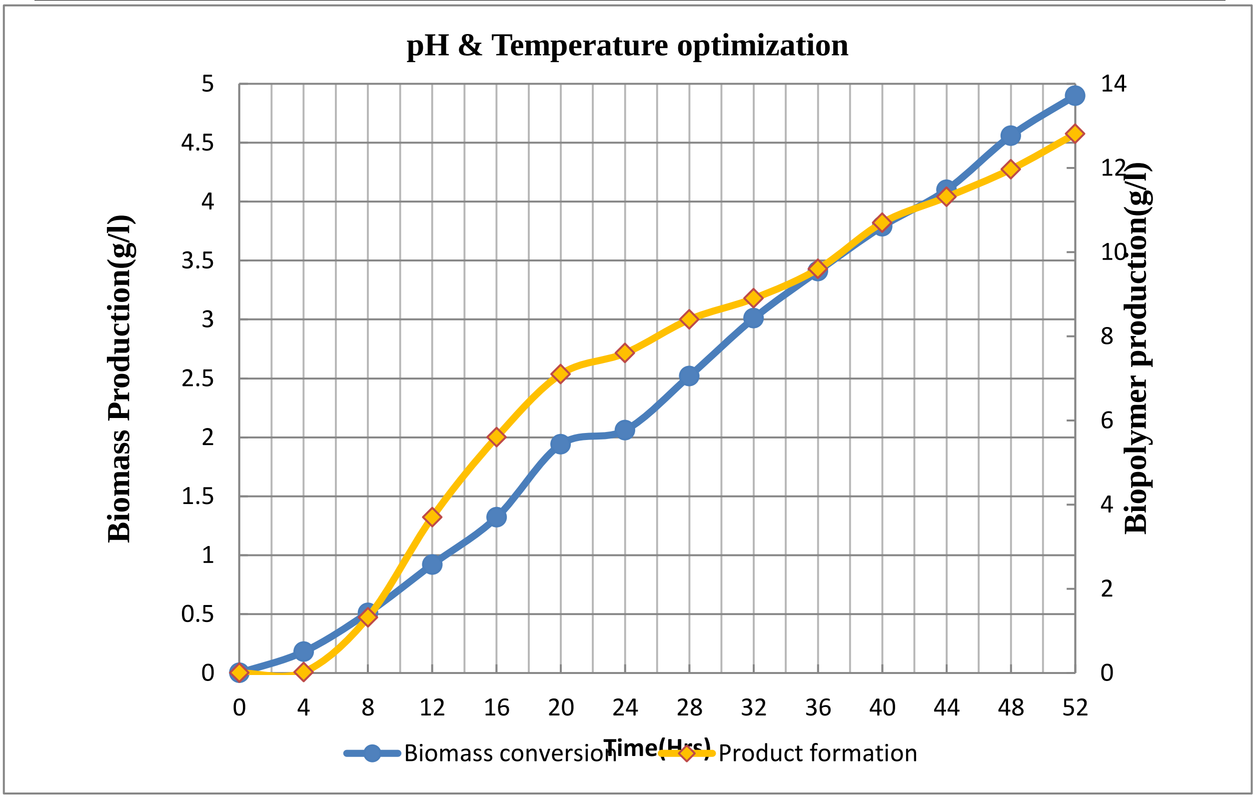


4.4 QUANTITATIVE TEST

By using the substrate as (6, 8, 9, 10, 12g) Vs pH (5, 6, 7, 8, 9) Vs temperature (37^{0}C). In these inoculated *Pseudomonas aeruginosa* at pH-7, substrate-80g/l at 37˚C with 4.9g of biomass and *Bacillus subtilis* at pH-6, substrate-90g/l at 37˚C with 4.7g of biomass shows the effective biomass while compared.

***Pseudomonas aeruginosa* at pH-7, substrate-80g/l at 37˚C**

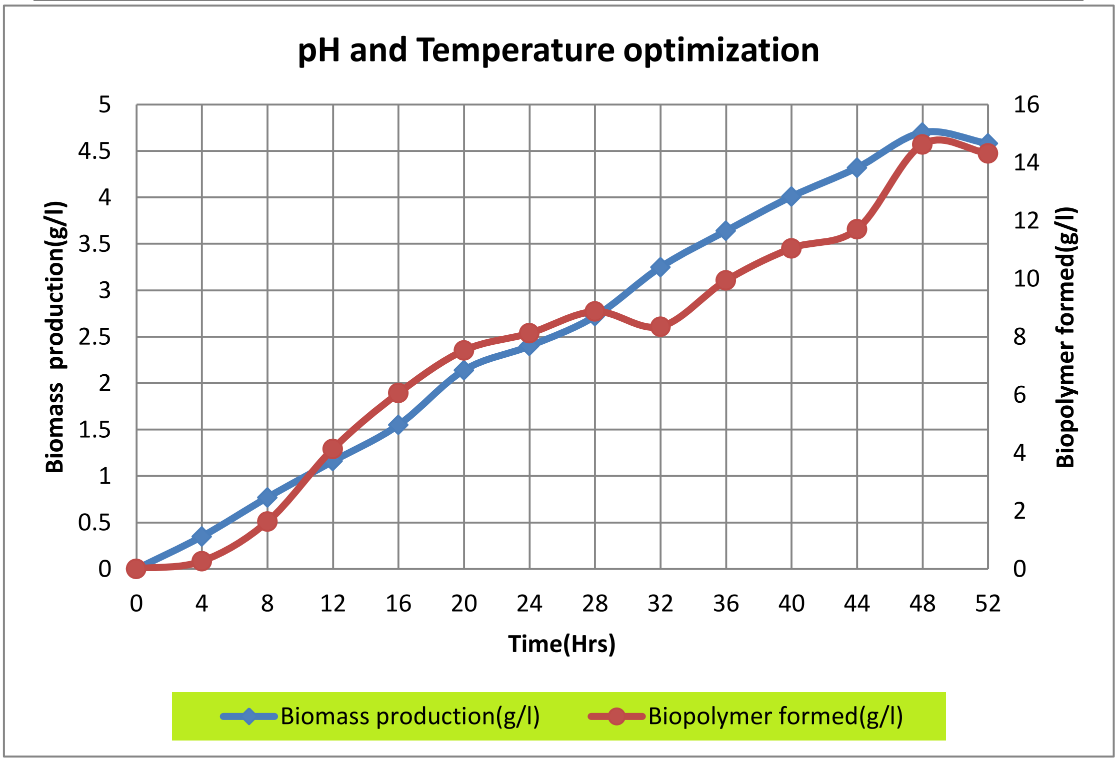
Time (Hrs)	Biomass production (g/l)	Biopolymer formed (g/l)
0	0	0
4	0.44	0.31
8	0.82	1.92
12	1.21	5.01
16	1.59	6.45
20	2.31	7.98
24	2.55	8.3
28	2.89	8.89
32	3.32	9.49
36	3.78	10.9
40	4.11	11.73
44	4.67	13.42
48	4.9	14.31
52	4.72	13.01



***Bacillus subtilis* at pH-6, substrate-90g/l at 37˚C**

Time (Hrs)	Biomass production (g/l)	Biopolymer formed (g/l)
0	0	0
4	0.35	0.27
8	0.77	1.63

12	1.16	4.14
16	1.55	6.06
20	2.14	7.53
24	2.4	8.12
28	2.72	8.87
32	3.25	8.35
36	3.64	9.94
40	4.01	11.05
44	4.32	11.71
48	4.7	14.63
52	4.58	14.32



4.4.1 FTIR ANALYSIS

In this FT/IR 4600 type A model at the standard light source at 45^0 inclined angle, 32 accumulation, TGS detector, speed of 2mm/sec, 7.1mm aperture, cosine apodization, resolution of 4 cm^{-1}**,** at 3000 Hz filter the samples was analyzed.

5. CONCLUSION

The usage of the plastics was increased nowadays for alternating that the bioplastics are produced from the renewable sources it is associated in depending on the biomass. It provoke the global consensus about the climate protection by reducing the green house gas emissions on particularly the carbondioxide and produces PHA which is biodegradable, low cost because it is produced from waste biomass, easily portable, controls pollution. This review reveals the synthesize of the biopolymer from the selected bacteria *Pseudomonas aeruginosa* and *Bacillus subtilis*, qualitative and quantitative measure of the PHA produced from the microorganisms. At pH-7, substrate-10, 37˚C *Pseudomonas aeruginosa* show 4.9 g/l of biomass and at pH-6, substrate-9, 37˚C *Bacillus subtilis* shows 4.57 g /l of biomass. Due to the lack of lipopolysaccharide layer in *Pseudomonas aeruginosa* a gram negative bacteria shows a better yield compared to *Bacillus subtilis* a gram positive bacteria. Biopolymer extracted from the microbial culture was polyhydroxyalkanoates. PHA obtained was examined by Fourier Transform Infra-Red (FTIR) spectroscopy. In future the biofilm were going to test for their degradation capacity and the time required for degradation process.

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