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Optimization of Polyhydroxybutyrate Production by Bacteria Isolated from Solid Waste Transfer Station, Teku, Nepal

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ABSTRACT

Current universal usage of plastics is causing adverse environmental impacts, particularly in the fields of natural resources depletion, production emissions and end of life waste management. This has urged the scientific community to search for potential renewable and eco- friendly resources. Bioplastics are the best alternative to this issue as it degrades naturally in environment by microorganisms. Moreover, bioplastics are produced by microorganisms during nutrient deficient conditions.

In this study, three PHB positive bacteria were isolated from solid waste collected from solid waste Transfer station at Teku, Kathmandu, Nepal. Based on their PHB production, the best isolate was selected for optimization and identification. The effect of different time (24, 48 and 72 hours), pH (6.5, 7, 7.5, 8), temperature (25°C, 30°C, 37°C and 42°C), carbon (glucose, dextrose, fructose, maltose and sucrose) and nitrogen (ammonium sulphate, ammonium nitrate, urea and peptone) sources were studied for high PHB production. The optimized culture conditions for selected isolate in terms of pH, temperature and time were found to be in pH 7 at 37°C for 48 hours respectively. The optimized culture condition in presence of 2% dextrose and 0.5% urea enhanced the PHB production to 81%. On the basis of the morphological and biochemical characterization the selected isolate was identified as *Bacillus coagulans*. The extracted PHB was characterized through FTIR and GC analysis.

KEYWORDS: Bacillus, Bioplastic, Polyhydroxybutyrate (PHB), Fourier-Transform infrared spectroscopy (FTIR), Gas chromatography (GC)

INTRODUCTION

Plastics are synthetic polymers which are derived from non-renewable resources like fossil fuels and have versatile properties such as inexpensive, easily manufactured, durable and resistant to water. They have become a fundamental requisite in day to day life as they can be made to almost any application from packaging goods, gadgets, and electric appliances to food items. Despite their flexible functionality, these synthetic materials once disposed

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remain in the environment. The major issue with plastic is that it is non degradable in nature hence, it has ensured risk in the environment creating a serious problem to normal living beings worldwide.

Alternative to synthetic plastics can be offered by bioplastics which has been an emerging topic to many researchers. PHA (polyhydroxyalkanoates) synthesized by microorganisms is the most commonly explored bioplastic. Among the types of PHA, Polyhydroxybutyrate (PHB) is widely studied due to being biologically degradable and having similar properties like polypropylene. In addition, PHB are biocompatible and can be used in biological applications like medical sutures. PHB are macromolecules produced by bacteria which are accumulated as intracellular granules. These granules are stored as a reserve material by bacteria and are synthesized during stress conditions such as excess carbon source and limited nitrogen and phosphate sources (Getachew & Woldesenbet, 2016). The most commonly studied microorganisms for PHB production belongs to the genera *Alcaligenes, Bacillus, Pseudomonas* and *Azotobacter* (Muthezhilan *et al*, 2014).

PHB is produced in an industrial scale using Gram negative bacteria such as *Cupriavidus necator* (Vandamme and Coenye, 2004), *Alcaligenes latus* and recombinant *Escherichia coli* (Choi *et al*, 1998). However, PHB of those organisms contain the outer membrane lipopolysaccharide (LPS) endotoxins that may induce a strong immunogenic reaction and is therefore undesirable for the biomedical application of the PHAs (Chen and Wu, 2005). Possible removal of endotoxin during purification of poly (3 - hydroxybutyrate) from Gram negative bacteria was reported by lee *et al*, 1999. On the other hand, Gram positive bacteria lack LPS, excreting proteins at high concentration and potentially use of cheaper raw materials, therefore considered as better source of PHAs to be used for biomedical applications (Valappil *et al*, 2007; Lopes *et al*, 2009; Singh *et al*, 2009).

Bacillus is a well-known, industrially robust organism. Bacillus can be easily exploited for a wide range of novel biotechnological applications. As a “Generally Recognized as Safe” (GRAS) organism, it is not a cause of concern because its release into the environment would not be a hindrance. In addition to its highly versatile nature, Bacillus represents a better host for genetic engineering because it lacks endotoxins, which are produced by other widely used host organisms, such as *E. coli* (Singh *et al*, 2009). It can grow easily to very high cell densities on inexpensive feed materials. Many species of Bacillus accumulate PHA (Singh *et al*, 2009; Valappil *et al*, 2007).

Furthermore, the genus Bacillus, in common with many other PHA-accumulating Gram positive

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bacteria, accumulates co-polymers of 3- hydroxybutyrate when grown on different substrates (Valappil *et al*, 2007 and 2008). In spite of the advantages of PHAs compared with petroleum-derived plastics, their use is currently limited due to their high production costs (Ojumu *et al*, 2004). In order to reduce the overall cost, it is important to produce PHA with high productivity and high yield.

The main objective of this research was to isolate gram positive PHB producing microorganisms from solid waste soil and identify the potential PHB producing bacteria. The extracted polymer was characterized via FTIR (Fourier transform infrared spectroscopy) and GC (Gas chromatography). Furthermore, the effect of different nutritional requirement and physicochemical parameters were investigated to achieve higher PHB yield.

METHODOLOGY

Sample Collection and Isolation

The soil samples were collected aseptically in sterile sampling bottle stored in ice box from Solid waste Transfer Station, Teku and were brought to the Microbiology laboratory, Kathmandu University, Dhulikhel for analysis.

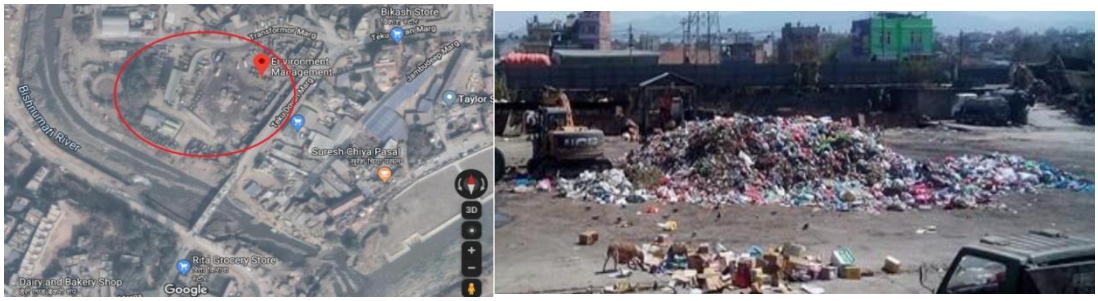

Figure 1. Sample collection site (Solid Waste Transfer Station, Teku, Nepal)

Isolation of PHB Producing Bacteria

One gram of soil sample was serially diluted from 10^{-1} to 10^{-7} dilutions, followed by standard pour plating method. 100 µl of each dilution was taken and poured onto Nutrient Agar (NA) plates. The plates were incubated at 30°C for 24-48 hours. Colonies with different morphology, color, pigmentations etc. were isolated in pure form and maintained on slants and stored at 4°C.

Screening of PHB Producing Bacteria

The isolated colonies were screened for PHB production by Sudan black B staining (Hartman, 1940). The cells appearing blue-black color under microscope were accredited as PHB positive strains.

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Similarly, the isolated colonies were further screened by Nile Blue A staining (Ostle & Holt, 1982). The cells showing bright yellowish-orange color under fluorescence microscope were confirmed as PHB positive isolates.

Morphological and Biochemical Characterization

The morphological characteristics of the colony and the cell were studied based on shape, color, surface, margin, elevation, texture, opacity, Gram's staining and spore staining. Biochemical tests were performed using different kits such as HiCarbo kit [KB009] and HiBacillus identification kit [KB013] from Himedia for identification of isolated bacteria. Likewise, starch hydrolysis test, IMViC test, oxidase test, catalase test and nitrate reduction test were also performed.

Optimization of Cultural Parameters for Maximum PHB Production

Optimization of Physical Parameters

Physical parameters such as time, temperature and pH were evaluated on Nutrient medium for the production of PHB by the selected isolates. The effect of temperature at 25°C, 30°C, 37°C and 42°C and pH variation of 6.5, 7, 7.5, and 8 respectively at different time intervals of 24, 48 and 72 hours were determined using UV spectrophotometer at 640 nm.

Optimization of Nutritional Parameters for PHB Production

Effect of Carbon Source

Thirty five carbohydrate utilization tests were studied using Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose, - Inulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbitol, Mannitol, Adonitol, Arabitol, Erythritol, alpha-Methyl-D-glucoside, Rhamnose, Cellobiose, Melezitose, alpha-Methyl-D-Mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Citrate, Malonate and Sorbose. Based on the positive test results three monosaccharides glucose, fructose and dextrose plus two disaccharides maltose and sucrose were selected for optimization study. The effect of these carbon sources at different concentration as 1%, 2%, 3% and 4% on PHB production was optimized.

Effect of Nitrogen source

The isolate was grown in media containing the best carbon source and the effect of different nitrogen sources such as ammonium sulphate, ammonium nitrate, peptone and urea were evaluated. The best nitrogen source was selected on the basis of their PHB

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yield. Furthermore, the effect of different concentrations of nitrogen source (0.5, 1 and 1.5%) on PHB production was optimized in the presence of the best carbon source detected from the study.

Harvesting of cells and dry cell weight (DCW) determination

After 48hrs incubation at 30°C, 10 mL of homogenous bacterial culture was poured into sterile 15 ml falcon tubes and centrifuged at 8000 rpm for 20 minutes. Culture medium was collected and centrifuged at 8,000 rpm for 20 min. Supernatant was discarded and the cell pellet was dried at 40°C to estimate the dry cell weight (DCW) in units of g/ml

Extraction of poly- -hydroxybutyrate (PHB)

The extraction of PHB was performed by sodium hypochlorite-chloroform dispersion method. The culture was transferred into falcon tubes, followed by centrifugation at 8000 rpm for 20 minutes to collect the pellets. The pellets obtained were treated with 4% (v/v) sodium hypochlorite and chloroform in the ratio 1:1. The mixture was incubated at 37°C for 1 hour. After incubation, the tubes were centrifuged at 8000 rpm for 20 minutes. Three different layers upper phase containing sodium hypochlorite solution, the middle phase containing cell materials and undisrupted cells and bottom phase containing chloroform with PHB were formed in the tubes.. The bottom phase was transferred into new tubes using Whatman No. 1 filter paper. The tubes were then placed at 40°C for evaporation. The percentage of intracellular PHB accumulation was estimated as the percentage composition of PHB present in the dry cell weight. Mixture of methanol and water in the ratio of 7:3 (v/v) was added to chloroform solution for the PHB extraction. The residual mass was defined as total DCW minus PHB weight. PHB (%) was defined as the percentage of the ratio of PHB to DCW.

$$\text{PHB \%} = \frac{\text{Dry weight of extracted PHB}}{\text{DCW}} \times 100$$

PHB film preparation

The chloroform solution containing PHB was poured in a petri dish and the plate was then left overnight for evaporation. After evaporation of the solvent, PHB was obtained as a tough, translucent film which was further analyzed by FTIR spectroscopy and Gas Chromatography.

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Molecular identification

Isolation of Genomic DNA and 16S rDNA sequence amplification

1mL of 24 hour bacterial culture was transferred into eppendorf tube and centrifuged. The extraction of genomic DNA was done using Invitrogen Purelink Genomic DNA mini kit. From the extracted genomic DNA, 16S rDNA sequence were amplified by PCR using universal bacterial 16S rDNA primers Bact8F forward primer (5'-AGA GTT TGA TCC TGG CTCAG-3') and Bact1492R reverse primer (5' – GGT TACC TTG TTACGA CTT-3'). PCR was performed with 25 µl reaction mixture containing 10 µl of DNA extract as the template, 0.5 µl of 0.2 mM of dNTPs, 1µl of 100nm of each primers, 0.2 µl of 1U taq polymerase with 2.5 µl of 10X buffer, 2 µl M gCl_2. Amplification was carried in MJ minicycler with the following temperature profile: Initial 95°C denaturation for 3 minutes, followed by 30 cycles of 95°C for 3 seconds, annealing at 55°C for 1 minute, extension 72°C for 2 minutes and final extension at 72°C for 6 minutes. Amplified products were separated by agarose gel electrophoresis on 1% (w/v) agarose gel in 1X TAE buffer with ethidium bromide (0.1 µg/mL). The purified 16S rDNA was sent to Macrogen, South Korea for sequencing and identification.

Characterization of Extracted PHB

Fourier-transform Infrared Spectroscopy (FTIR) Analysis

The PHB extracted was analyzed by FTIR spectrophotometer (RAY LEIGH WQF-520A FT-IR Spectrometer) with spectral range of 4000 to 600 cm^{-1}. About 0.1 to 10 mg of PHB sample was measured and mixed homogenously with 100 mg KBr as blank (control). The mixture was compressed by the help of hydraulic press to form a thin film and was fixed in FTIR device for scanning. The FTIR spectra of the bacterial isolate was compared with that of the standard PHB (Santa Crutz Biotechnology, Inc. USA).

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Gas Chromatography (GC) Analysis

The Gas chromatographic analysis was performed on Shimadzu GC-2014 AOC-20i Auto Injector where 2µL Sample was injected in split less mode. Capillary column Rtx®-1 from RESTEK was used with internal diameter of 0.25mm and 30m length with Nitrogen as the carrier gas at a flow rate of 40mL/minute. About 4 mg of PHB sample was taken to a test tube. 2 mL acidified methanol (7% H_2SO_4) and 4 mL chloroform were added into the test tube containing the sample. The test tube containing the sample mixture was closed tightly with Teflon-lined caps and heated for 1 hour at 100°C water bath. 2 mL distilled water was added to the test tube after cooling to room temperature and shaken vigorously. After Centrifugation the chloroform phase containing PHB methyl ester was transferred to GC vial. Standard PHB from Santa Cruz was prepared for positive control and blank was prepared without PHB. 2 µl samples were injected into the chamber. The analysis started at 80°C for 2 minutes and the temperature was increased to 195°C at the rate of 8°C/ minute. After reaching 195°C, the temperature was maintained for 20 minutes prior to the analysis termination ((Braunegg *et al*, 1978), with FID (Flame Ionization Detector).

RESULTS AND DISCUSSION

Isolation and screening of PHB producing bacteria

Twenty-nine strains of bacteria were isolated from soil sample and were screened for PHB production. Out of twenty-nine bacterial isolates, three gram positive bacteria were found to be PHB positive in Sudan black B staining and Nile Blue A staining. Isolate S3 was selected for further studies based on distinct colony morphology and better PHB yield.

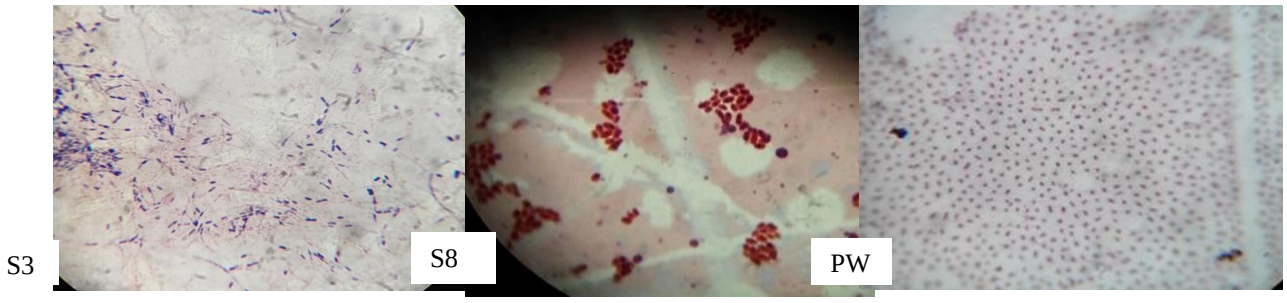

Figure 2. Sudan Black staining of PHB positive isolates

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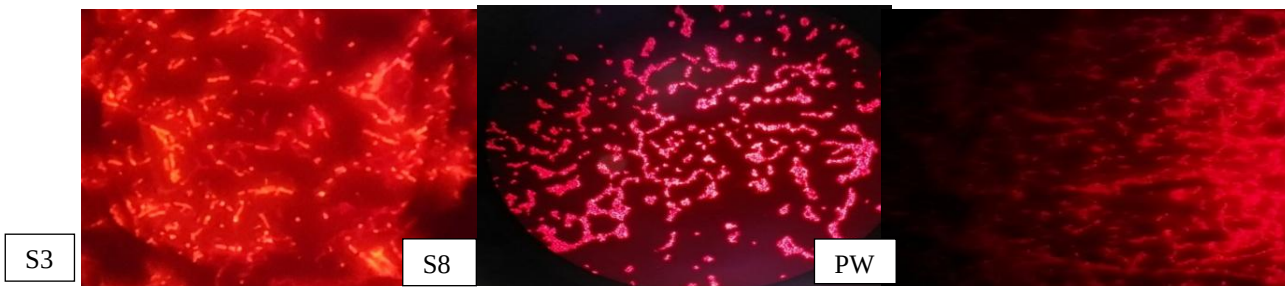

Figure 3. Nile Blue B staining of PHB positive isolates

Morphological and Biochemical Characteristics for Identification

The results of morphological and Biochemical characteristics of isolate S3 are tabulated in Table 1and 2. In terms of colony morphology (color, shape, size, elevation, margin, consistency, opacity), basic microscopic observations (gram stain, spore stain, cell size), biochemical tests with reference to the Bergey's manual of Systematic Bacteriology, Determinative Bacteriology and probabilistic identification matrix, the selected bacterial isolate S3 was found closely related to *Bacillus coaglans* (ID score= 0.86) (Sneath *et al*, 1986).

Table 1. Biochemical Characteristics of Isolate S3

Test	**Result**	**Test**	**Result**	**Test**	**Result**	**Test**	**Result**
MacConkey Agar	-	Lysine decarboxylase	-	Melibiose	-	Inulin	+
Indole Test	-	Arginine	+	Arabinose	-	Sodium gluconate	-
Methyl Red Test	+	Ornithine decarboxylase	-	Trehalose	+	Glycerol	-
Voges Proskauer Test	+	Rhamnose	-	Melibiose	-	Salicin	+
Citrate Utilization	-	Cellobiose	-	Xylose	-	Dulcitol	-
Gas from Glucose	+	Melezitose	-	Maltose	+	Inositol	-
H2S Production	-	Rhamnose	-	Fructose	+	Sorbitol	-
Casein Hydrolysis	-	ONPG Test	-	Dextrose	+	Mannitol	-
Esculin Hydrolysis	+	Nitrate reduction	+	Galactose	-	Adonitol	-
Oxidase Test	+	Lactose	-	Raffinose	-	Arabitol	-

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Starch Hydrolysis	+	Xylitol	-	Sucrose	+	Erythritol	+
Urea Hydrolysis	-	α-Methyl-D-mannoside	-	Malonate utilization	-	α-Methyl-D-glucoside	+
Catalase Test	+	Mannose	-	Sorbose	-	Phenylalanine	-

16S rDNA Sequence Analysis

16S rDNA sequence analysis indicated that the isolate belongs to the genus *Bacillus* with nearest species *Bacillus coagulans* with maximum identity of 99% and 98 hits for *Weizmannia coagulans* (*Bacillus coagulans*).

Table 2. Morphological Characteristics of isolate S3

No.	Morphology characteristics	Results
1.	Shape	Circular
2.	Margin	irregular
3.	Elevation	Flat
4.	Texture	Sticky/slimy
5.	Color	White
6.	Opacity	Opaque
7.	Surface	Shiny
8.	Gram's Staining	Positive
9.	Spore staining	Positive

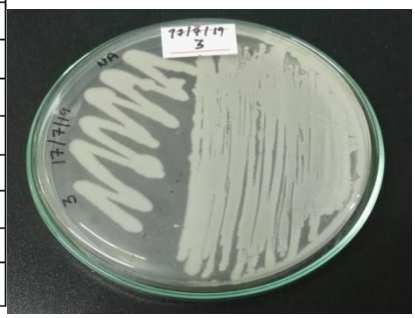

Figure 4. Growth on Nutrient Agar

Optimization of culture conditions of isolate S3

The isolate showed optimum growth at pH 7, 37°C and 48 hrs of incubation respectively Figure 5. Studies conducted by Bonartseva *et al*, 2002 are consistence with the results wherein the maximal PHB production was observed in 48 hours.

Similar results were obtained in other *Bacillus species* which showed that 37°C was a favorable temperature for PHB production (Yu-Hong et al, 2011; Grothe et al., 1999; Tamdogan & Sidal, 2011) and PHB production decreased at temperature extremes due to low enzyme activity at such temperatures. Similarly, Grothe *et al*, 1999; Aslim *et al*, 2002 reported that pH 6.0 to 7.5 as the preferred pH range for PHB production.

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Sangkharak, & Prasertsan, 2008 indicated that PHB was a growth associated product and its accumulation significantly increased when all cultures reached the exponential phase (after 18 hours) till stationary phase. Moreover, it has been reported that cell biomass and PHB production were concomitant with growth conditions and PHB production of a particular strain is related to its biomass. As biomass increases, the bacteria accumulate PHB to the maximum level and the accumulated PHB decreases after the peak biomass production. This might be due to nutrient depletion, which forces the bacteria to use the accumulated PHB as energy source (Getachew & Woldesenbet, 2016).

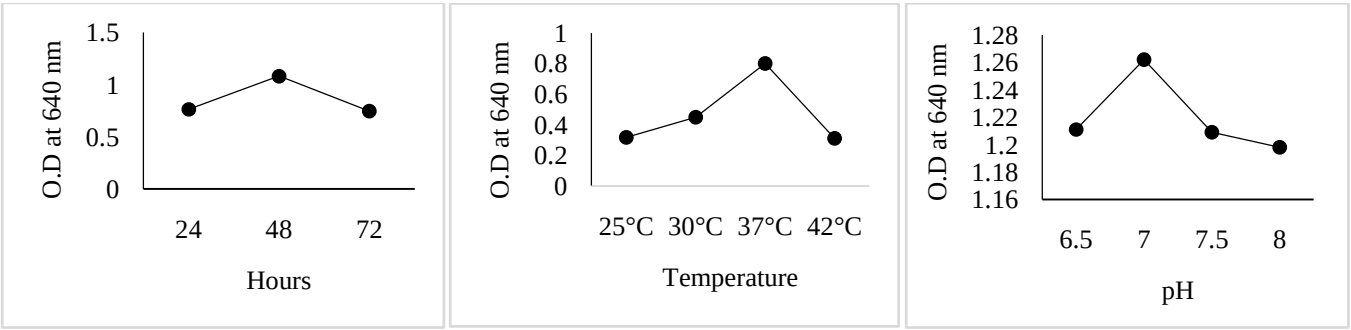

Figure 5. Effect of different incubation period, temperature and pH on growth of isolate S3

Effect of Carbon source

Among the five carbon sources, the highest PHB production was observed on media containing dextrose i.e 2.8 g/L PHB in 5.4 g/L DCW which results to 51.8% PHB yield, followed by sucrose 2.6 g/L PHB in 5.8 g/L DCW. Although the DCW in sucrose was high compared to dextrose however, PHB yield in sucrose was comparatively less i.e 44.8%. Media containing fructose gave 2 g/L PHB in 4.8 g/L DCW thus PHB yield was 41.6%. The least PHB yield was observed in glucose (1 g/L PHB in 3.4 g/L DCW) and maltose (0.8 g/L PHB in 2.4 g/L DCW) with its PHB yield 29.4% and 33.3% respectively (Figure 6). Furthermore, the results on different concentration of dextrose showed that media containing 2% dextrose was the best for PHB production which gave 4.2 g/L PHB in 7 g/L DCW and its PHB yield was 60%. The amount of PHB decreased in media containing more than 2% dextrose as shown in Figure 7.

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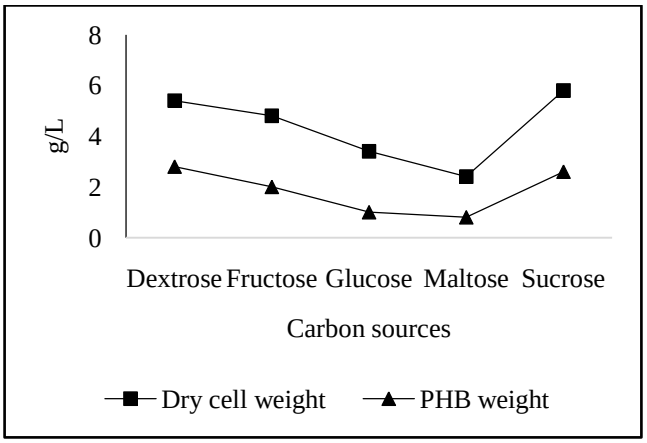

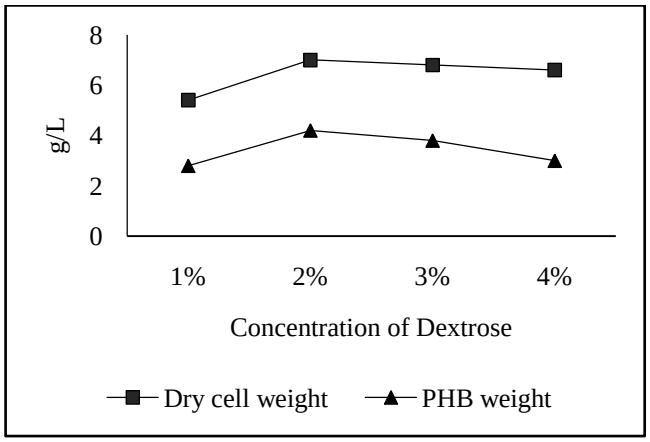

Figure 6. Effect of carbon sources on PHB production **Figure 7. Effect of dextrose concentration on PHB production**

According to Khanna & Srivastava (2005), monosaccharides such as glucose and fructose were readily utilized by bacteria and hence support growth and PHB production, however, the complex molecules like starch and lactose were not utilized for effective PHB production. Aslim et al, 2002 reported the maximum PHB yield of 48% by *Bacillus megaterium*. Similarly, Yelmaz *et al*, 2005 found the maximum PHB yield of 42% by *Bacillus brevis* while *Bacillus coagulans* produced only 24%.

Effect of Nitrogen source

PHB production was enhanced on media amended with urea. The PHB obtained was 2.8 g/L PHB in 4 g/L DCW which gave 70% PHB yield. Besides urea, media containing peptone gave 1.2 g/L PHB in 2.6 g/L DCW with PHB yield 46.15%. The least PHB production was observed in media containing ammonium sulphate (0.4 g/L PHB in 1.6 g/L DCW) and ammonium nitrate (0.2 g/L PHB in 1 g/L DCW) with PHB yield of 25% and 20% respectively (Figure 8).

Likewise, nutrient media containing 0.5% urea was found to be best in PHB production with PHB yield of 81%. The PHB obtained was 3.4 g/L in 4.2 g/L DCW. Media containing 1% and 1.5% urea produced 2.8 g/L PHB in 4 g/L DCW and 1.6 g/L PHB in 2.8 g/ L DCW respectively (Figure 9). According to researchers, limited supply of nitrogen source favors the production of PHB. In the study, 0.5% urea was found best for PHB production. This might be due to relatively low nitrogen content of urea that resulted in increased C/N ratio which in turn favored higher PHB accumulation (Basavaraj *et al*, 2013). According to Thammasittirong *et al*, 2017 reported that media containing organic nitrogen sources except with urea were observed high cell mass and PHB production while low cell mass and PHB

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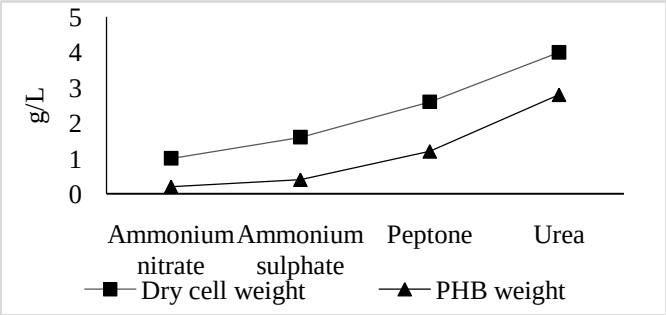
production were observed when using inorganic nitrogen sources. However, Tripathi *et al*, 2012 reported that *Pseudomonas aeruginosa* produced high PHB in media containing urea, ammonium sulfate and ammonium nitrate as nitrogen source. Similarly, Khanna & Srivastava, 2005 investigated the effect of different nitrogen sources on PHB production which showed that urea, ammonium sulfate, ammonium chloride and ammonium nitrate were the best nitrogen sources respectively.



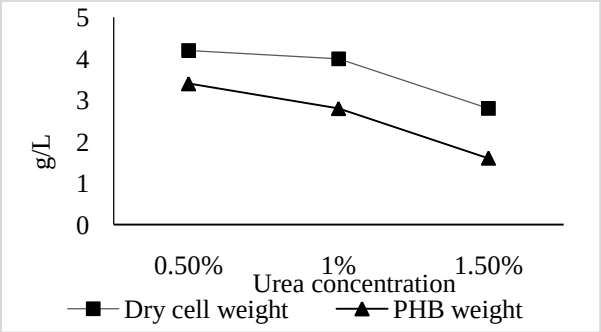


Figure 8. Effect of different nitrogen source on PHB production **Figure 9. Effect of urea concentrations on PHB production**

PHB film

After the culture conditions was optimized in terms of time, temperature, pH, carbon source and nitrogen source, the isolate was cultured in optimized culture condition and PHB film was obtained Figure 10 A, B.

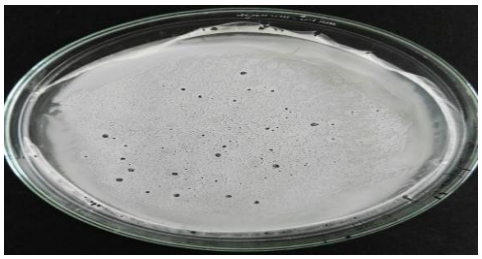
A   B

Figure10. PHB film in Tube A and petri dish B

Characterization of PHB

FTIR analysis:

The presence of prominent functional groups such as C=O, C−O, CH indicates the presence of

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PHB. Figure 10 and 11 illustrates FTIR analysis of extracted PHB showing peaks at 1725 cm^{-1}, 1450 cm^{-1}, 1150 cm^{-1}, 1125 cm^{-1}, 1100 cm^{-1}. When the results of extracted PHB (Figure 12) were compared with the FTIR analysis of standard PHB (Figure 11), similar peaks were observed. The confirmation of PHB is determined by the presence or absence of functional groups on the extracted sample. A strong peak was observed at 1725 cm^{-1} which corresponds to C=O stretch of the ester group present in the molecular chain of highly ordered crystalline structure. The band at 1450 cm^{-1} indicates the presence of –CH2 or methylene group. The band at about 1,200 cm^{-1}, 1000 cm^{-1}characterizes valance vibration of the carboxyl group (Irsath *et al*, 2015; Biradar *et al*, 2017).

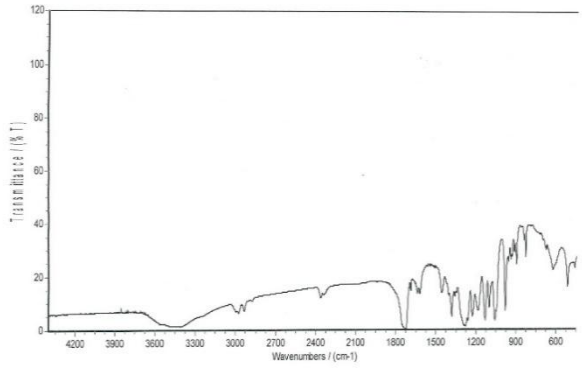

Figure 11. FTIR analysis of standard PHB

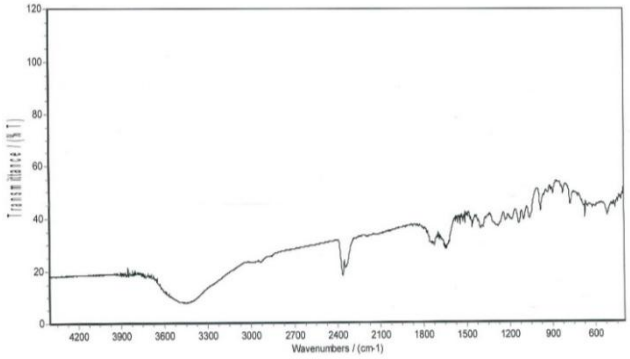

Figure 12. FTIR analysis of extracted PHB

Gas chromatography (GC) analysis:

The extracted PHB was characterized by GC analysis and PHB from Santa Cruz biotechnology was used as standard and Chloroform was used as blank for qualitative analysis. The GC analysis showed a strong peak of chloroform at 2.5 minutes the standard PHB illustrated a second peak at retention time of 4.24 minutes (Figure 13). Similarly, the extracted PHB was compared with standard PHB which showed comparable results (Figure 14). The peak area of standard PHB was observed at 983669 and the peak area observed on extracted PHB was 696098.

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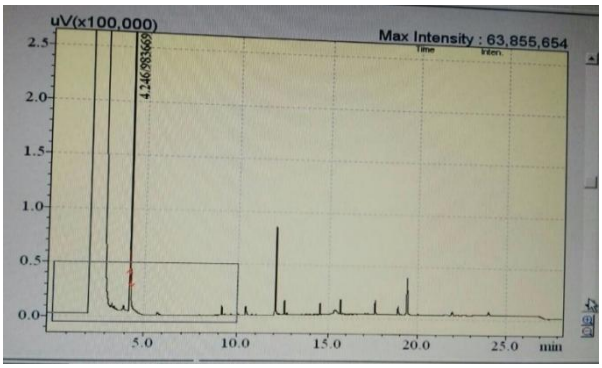

Figure 13. Gas chromatography analysis of standard PHB

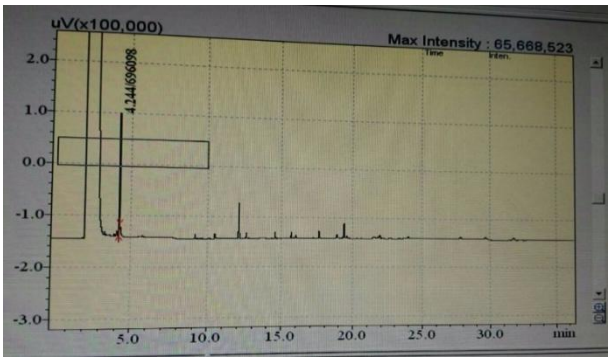

Figure 14. Gas chromatography analysis of extracted PHB

CONCLUSION

The study revealed that large numbers of microorganisms are present in dumping site and PHB producers can also be found in the site. The PHB yield optimization study strongly showed that medium composition with its optimum culture conditions greatly influence PHB production. The *Bacillus spp* isolated from solid waste transfer station, Teku, Nepal can be a potential renewable resource for the production of ecofriendly bioplastic as an alternative to synthetic plastic therefore strongly recommend for pilot scale research.

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REFERENCES

1. Aslim, B., Zehra, N., Beta, Y. (2002). Determination Of PHB Growth Quantities Of Certain *Bacillus Species* Isolated from Soil. *Turkish Electronic Journal of Biotechnology*, Special Issue, 24-30.
2. Basavaraj H, Shyama PP, Mohammed S. (2013). Production Of Polyhydroxybutyrates from *Paenibacillus Durus* BV-1 Isolated From Oil Mill Soil. J Microb Biochem Technol., 5:1948–5948
3. Biradar, G., Shivasharana, C., & Kaliwal, B. (2017). Characterization of Polyhydroxybutyrate (PHB) Produced by Novel Bacterium *Lysinibacillus Sphaericus* BBKGBS6 Isolated from Soil. *Journal of Polymers and The Environment*, *26*(4), 1685-1701. Doi: 10.1007/S10924-017-1054-X

4. Bonartseva, G. A., Myshkina, V. L., Nikolaeva, D. A., Rebrov, A. V., Gerasin, V. A., & Makhina, T. K. (2002). The Biodegradation of Poly-B-Hydroxybutyrate (PHB) by a Model Soil Community: The Effect of Cultivation Conditions on the Degradation Rate and the Physicochemical Characteristics of PHB. *Microbiology*. 71:221 Doi: 10.1023/A: 1015162608031
5. Braunegg, G., Sonnleitner, B., & Lafferty, R. M. (1978). A Rapid Gas Chromatographic Method for the Determination of Poly-B-Hydroxybutyric Acid in Microbial Biomass. *European Journal of Applied Microbiology and Biotechnology*, *6*(1), 29–37. Doi: 10.1007/Bf00500854
6. Chen, G.Q., Wu, Q., (2005). Microbial Production and Applications of Chiral Hydroxyalkanoates. *Appl Microbiol Biotechnol*., 67:592–599.
7. Choi JI, Lee S.Y., Han K. (1998). Cloning of the *Alcaligenes Latus* Polyhydroxyalkanoate Biosynthesis Gene and Use of these Genes for Enhanced Production of Poly (3-Hydroxybutyrate) in *Escherichia Coli*. *Appl. Environ. Microbiol.* 64: 4897-4903.
8. Getachew, A., & Woldesenbet, F. (2016). Production of Biodegradable Plastic by Polyhydroxybutyrate (PHB) Accumulating Bacteria Using Low Cost Agricultural Waste Material. *BMC Research Notes*, *9*(1). Doi: 10.1186/S13104-016-2321-Y
9. Grothe, E., Moo-Young, M., and Chisti, Y. (1999). Fermentation Optimization for the Production of Poly-B- Hydroxybutyric Acid Microbial Thermoplastic. *Enzym. Microbial. Tech*., 25, 132-141.
10. Hartman, T. L. (1940). The Use of Sudan Black B as A Bacterial Fat Stain. *Stain Technology*, *15*(1), 23–28. Doi: 10.3109/10520294009110328
11. Irsath, H., Santhosh, S., Hemalatha, V., Vikramathithan, M., Dhanasekar, R., & Dhandapani, R. (2015). Production and Optimization of Polyhydroxybutyrate using *Bacillus Subtilis* BP1 Isolated from Sewage Sample. *Int. J. Pure App. Biosci.*, *3*(1), 158-166.
12. Khanna, S., & Srivastava, A. K. (2005). Recent Advances in Microbial Polyhydroxyalkanoates. *Process Biochemistry*, *40*(2), 607–619. Doi: 10.1016/J.Procbio.2004.01.053
13. Lee, S. Y., Choi, J. And Wong, H. H. (1999). Recent Advances in Polyhydroxyalkanoate Production by Bacterial Fermentation: Mini-Review. *Int J Biol Macromol* 25, 31-6.
14. Lopes, M.S., Roch, R.C.S., Zanotto, S.P., Gomez, G.C., De Silva, L.F. (2009). Screening of Bacteria to Produce Polyhydroxyalkanoates from Xylose. *World J. Microbiol. Biotechnol,* 25:1751–1756.
15. Muthezhilan, R., Kaarthikeyan1, C. P., Jayaprakashvel, M., & Hussain, A. J. (2014). Isolation, Optimization and Production of Biopolymer (Polyhydroxy Butyrate) From Marine Bacteria. *Pak J. Biochem*, *11*(2), 59–64.

16. Ojumu, T.V., Yu, J., Solomon, B.O. (2004). Production of Polyhydroxyalkanoates, a Bacterial Biodegradable Polymer. African J Biotechnol, 3: 18-24.
17. Ostle, A, & Holt, J. G. (1982). Nile Blue A As A Fluorescent Stain For Poly-3Hydroxybutyrate. *AppliedAnd Environmental Microbiology*, *44*(1), 238–241.
18. Sangkharak, K. Prasertsan, P. (2008). Nutrient Optimization for Production of Polyhydroxybutyrate from Halotolerant Photosynthetic Bacteria Cultivated Under Aerobic-Dark Condition. *Ejournal of Biotechnology*, 11: 3-8.
19. Singh, M., Patell, S.K.S., Kalia, V.C. (2009). *Bacillus Subtilis* as Potential Producer for Polyhydroxyalkanoates. *Microbial Cell Factories,* 8:38.
20. Sneath, P.H.A. 1986 Endospore-Forming Gram-Positive Rods and Cocci, In Bergey's Manual of Systematic Bacteriology. Eds. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G., 2, 1104–1139. Baltimore: Williams and Wilkins. ISBN 0-68307-893-3
21. Tamdoğan, N. & Sidal, Ugur. (2011). Investigation of poly-β-hydroxybutyrate (PHB) production by *Bacillus subtilis* ATCC 6633 under different conditions. Kafkas Universitesi Veteriner Fakultesi Dergisi. 17. S173-S176.
22. Thammasittirong, A., Saechow, S., & Thammasittirong, S. N.-R. (2017). Efficient Polyhydroxybutyrate Production From *Bacillus Thuringiensis* Using Sugarcane Juice Substrate. *Turkish Journal of Biology*, *41*, 992–1002. Doi: 10.3906/Biy-1704-13
23. Tripathi, A. D., Yadav, A., Jha, A., & Srivastava, S. K. (2012). Utilizing Of Sugar Refinery Waste (Cane Molasses) For Production of Bio-Plastic under Submerged Fermentation Process. *Journal of Polymers and The Environment*, *20*(2), 446–453. Doi: 10.1007/S10924-011-0394-1
24. Valappil SP, Misra SK, Boccaccini AR, Keshavarz T, Bucke C, Roy I., (2007). Large-Scale Production and Efficient Recovery of PHB with Desirable Material Properties, From The Newly Characterized *Bacillus Cereus* SPV. *J Biotechnol*. 132:251–258.
25. Valappil, S.P., Boccaccini, A.R., Bucke, C., Roy, I. (2007a). Polyhydroxyalkanoates in Gram-Positive Bacteria: Insights from The Genera *Bacillus* And *Streptomyces. Antonie Van Leeuwenhoek*, 91:1–17.
26. Valappil, S.P., Rai, R., Bucke, C., Roy, I. (2008). Polyhydroxyalkanoate Biosynthesis In *Bacillus Cereus* SPV Under Varied Limiting Conditions And An Insight Into The Biosynthetic Genes Involved. *J Appl Microbiol*, 104:1624–1635.
27. Vandamme, P., Coenye, T. (2004). Taxonomy of The Genus *Cupriavidus*: A Tale Of Lost And Found. *Int. J. Syst. Evol. Microbiol*. 54: 2285-2289.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 5308 - 5324
Received 05 March 2021; Accepted 01 April 2021.

28. Yelmaz, M., Soran, H., Beyatli, Y., (2005).Vdetermination Of Poly-B-Hydroxybutyrate (PHB) Production by Some *Bacillus Spp. World J. of Microbiol. & Biotech.* 21:565–566. DOI 10.1007/S11274-004-3274-1

29. Yu-Hong, W., Wei-Chuan, C., Ho-Shing, W., and Ommurugan, J. (2011). Biodegradable and Biocompatible Biomaterial, Polyhydroxybutyrate, Produced by an Indigenous *Vibrio Sp*. BM-1 Isolated from Marine Environment. *Mar. Drugs.*, 9, 615-624.

30. Zaki, N. (2018). Biodegradable Plastic Production by *Bacillus Spp.* Isolated from Agricultural Wastes and Genetic Analysis Of PHA Synthesis. *Al-Mustansiriyah Journal of Science*, *29*(1), 67. Doi: 10.23851/Mjs.V29i1.115.