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Analysis, Optimization and Molecular Characterization of PHB Positive Bacteria Isolated from Agricultural Soil Sample

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Abstract

Polyhydroxybutyrates are the widely studied biopolymer because of its biodegradability and non-toxic effect in the environment. This work focused on the isolation, screening, characterization and optimization of the PHB producing bacteria from agricultural soil. About eighty percentage of the isolated bacteria were identified as PHB producing bacteria after Sudan Black Blue staining. Among them three colonies were randomly selected and their morphologically and biochemically characterized. The biopolymer was extracted using sodium hypochlorite method. Potent PHB producer (WJSP1) (13.5µg/ml) was identified by crotonic acid assay. The functional groups of the produced PHB were identified by Fourier Transform Infra-Red spectroscopy (FTIR). The presence of functional groups like carbon group, C=O ester group, CH group, carbonyl group, carboxyl group which was corelated with the peak values1454.33cm^{-1}, 1724.36 cm^{-1}, 1274.95 cm^{-1}, 1049.28 cm^{-1}, 1280-1053 cm^{-1} indicates the presence of PHB. The potent producer (WJSP1) was optimized under different conditions to achieve the best condition for the production of PHB. The maximum production was obtained at the temperature - 37˚C, the carbon source - glucose, pH - 7 and at a time period of 72 hours. 16SrRNA sequencing was performed and the organism was identified as Bacillus cereus from the BLST (Basic Local Alignment Search Tool). A phylogenetic tree was constructed and the sequence was submitted in GENBANK (Accession No: OR192860). The isolated bacteria Bacillus cereus is an efficient producer of PHB under laboratory condition in presence of glucose as a chief carbon source. Large scale production of PHB can be done in large scale fermenters in industries. Large scale production of PHB will help the environment to get rid of the petroleum-based bioplastics.

Keywords: *Polyhydroxybutyrate (PHB), Sudan Black Blue staining, crotonic acid assay, Fourier Transform Infra-Red spectroscopy, Bacillus cereus, 16SrRNA sequencing.*

1. Introduction

Microorganisms like bacteria, yeast can produce various types of PolyHydroxyAlkanoates (PHAs) based on the various types of the carbon sources available. The first discovered PHA is PHB and it is the most common PHA to be present in nature (Ansari.S *et al*., 2014). PolyHydroxyButyrates(PHBs) are biodegradable thermoplastics (Kim.B.S 2000). PHB is generally a carbon assimilated product which is often used as an energy storage molecule by the microorganisms (Steinbuchal *et al*., 2002). PHB exist as a crystalline granule in the cytoplasm of bacteria (Prasana *et al*., 2011). PHB is an eco-friendly polymer which occupies about 90% of cells dry weight (Rydz.J *et al*., 2015). The properties like biodegradability and biocompatibility makes PHB ideal for many applications in veterinary, medicine and agriculture (Wang.J *et al*., 2007). The enzyme PHA synthase obtained from the *Bacillus* species

can incorporate both short chain length PHA and medium chain length PHA which indicates that the *Bacillus* species is a novel producer of PHA with different monomeric compositions (Tajima.K *et al*., 2003). Approximately 40 PHA synthase genes are isolated from several microorganisms (Rehm.B.H *et al*., 1999).

The molecular structure of PHB will not vary based on the characteristic features of strain and the mode of carbon source available for the microbes (Volva.T.G *et al*., 2000). Numerous microbes isolated from various environment has the ability to produce PHB, but the microbes isolated from marine rarely produce PHB (Numata *et al*., 2016). The microbes can produce high quantity of PHB in a medium containing excessive carbon and even if there is a depletion of other nutrients like nitrogen, phosphorous and oxygen (Philip.S *et al*., 2007). Most commonly PHB producing organisms are *Bacillus, Alcaligens, Azetobacter, Pseudomonas etc.* (Singh.P *et al*.,2011). Three tones of glucose were needed for the microbes to produce one tone of PHB (Collins 1987). Halobacterium is the first marine bacterium reported for the accumulation of PHB (Kirk *et al*., 1972). Mostly soil bacteria can degrade these PHB molecules easily (Anderson.A.J *et al*., 1990).

This study focuses on the isolation and optimization of PHB producing bacteria. The microbe was optimized at different conditions like temperature, pH, time period and various carbon sources. The morphological and biochemical characters of the microbe was studied and PHB was extracted by sodium hypochlorite method. FTIR was done to identify the functional group of the extracted PHB. The gene sequence of the organism is identified by 16SrRNA sequencing and submitted in GEN bank.

2. Materials And Methods

Collection of soil sample:

The soil sample used for this study was collected from Mullencherry of Vilavancode Taluk.

Isolation of bacteria:

Serial dilution was done to get discrete number of bacteria. One gm soil was weighed and dissolved in 10 ml of distilled water which is noted as 10^{-1} dilution. 1ml of soil solution from this tube is transferred to another tube to get 10^{-2} dilution. This step is followed until we get 10^{-10} dilution. Nutrient agar media is used for the growth of bacteria at 37˚C for 24 hours.

Screening of PHB producing bacteria using Sudan Black Blue stain:

PHB producing bacteria was detected by using Sudan Black Blue stain (Burdon *et al*., 1942). Sudan Black Blue stain was prepared by dissolving 0.3g of powdered Sudan Black Blue in 100ml 70% ethanol (Christiana Thapa *et al*., 2018). The bacteria grown on the media was screened for PHB production by pouring prepared Sudan Black Blue stain on the plates and the plates are kept undisturbed for 30 minutes. To remove excess stain the plates were washed with decolourizer ethanol. The PHB producing colonies will be remained in bluish black colour.

Microscopic screening of PHB producing bacteria:

Bacterial smear was made in microscopic glass slide and the smear was flooded with Sudan Black Blue stain for 10 minutes and then washed with water and then again flooded with counter stain saffranine for 5 minutes and observed under light microscope (Monika Sharma *et al*., 2015).

Biochemical characterization:

Gram staining, spore staining, catalase test, indole test, methyl red test, Voges Proskauer test, triple sugar ion test, urease test, starch hydrolysis test, gelatin hydrolysis test, citric acid utilization test, hydrogen sulphide test was done to identify the biochemical characteristic of the isolated bacteria.

Extraction of PHB:

The produced PHB was extracted by sodium hypochlorite method (Rawte *et al*., 2002). The incubated culture was centrifuged at 10,000 rpm for 10 minutes. To the pellet 10ml of sodium hypochlorite was added. It was then incubated in a water bath at 50˚C for 1hour. After incubation, it was then centrifuged at 5000 rpm for 15 minutes. To the pellet equal volume of acetone was added and centrifuged at 5000 rpm for 15 minutes. To the pellet equal volume of ethanol was added again and centrifuged at 5000rpm for 15 minutes. To the pellet add 5ml of boiled chloroform and pour it in a sterile petri plate and let it undisturbed overnight to evaporate. The powdered PHB in the plate was scratched with a spatula and stored for later analysis.

Crotonic acid assay:

Crotonic acid test assay was done to perform the quantitative test of PHB. When produced PHB polymer is dissolved in concentrated sulfuric acid, the PHB will be depolymerized and converted into crotonic acid (Godbole 2016). 10 mg of produced PHB is dissolved in 5ml of sulfuric acid and heated in a water bath for 10 minutes. The resultant brown coloured crotonic acid solution was measured for absorbance at 235nm against sulfuric acid blank in UV spectrophotometer (Darshan Marjadi *et al*., 2014). Quantitative test for the produced PHB was done using UV spectrophotometer (Aslim *et al*., 1998). A standard graph was drawn with standard PHB obtained from Sigma. Concentration of the test sample was known from the graph and the amount of PHB produced was estimated by the amount of sample taken.

FT-IR analysis for isolated polymer:

FT-IR spectrophotometer was done to identify the presence of particular functional groups on the polymer at the range of 4000 to 400cm^{-1} (Deepa Aryaraj *et al*., 2021)

Optimization of the isolated bacteria:

The isolated bacteria were optimized at different temperatures (28˚C, 30˚C and 37˚C), at different carbon sources (glucose, sucrose and fructose), at different pH (6.5, 7, 7.5) and at different time period (24hrs,48hrs and 72hrs).

Propanolysis of PHB:

The sample containing the polymer in chloroform as well as the standard PHB and P(HB-*co*-HV) were taken in crimp top vials and evaporated. The polymer was esterified with propanol containing hydrochloric acid (4:1). Trichloroethylene was used as solvent and the reaction was allowed to continue in tightly sealed crimp top vials at 100ºC for 2 hours. After boiling to room temperature, the esterified samples were supplemented with 1ml of water for phase separation.

TLC:

The separated ester (organic) phase and aqueous phase of the sample were spotted onto TLC plate and allowed to dry at room temperature. The TLC run was carried out in 4:1 proportion of benzene and ethyl acetate, and the plate was visualized with iodine vapour and Rf value were calculated.

$$Rf = \frac{DISTANCE\ TRAVELLED\ B\ Y\ SOLUTE}{DISTANCE\ TRAVELLED\ B\ Y\ SOLVENT}$$

Molecular characterization of isolated bacteria:

Genomic DNA was isolated using NucleoSpin Tisue Kit (Macherey-Nagel). Further the DNA was confirmed by agarose gel electrophoresis and PCR was done for the amplification of DNA.

Sequence analysis:

The quality of the sequence was identified using Sequence Scanner Software v1. Sequence alignment and required editing of the sequence was done using Genious Pro v5.1 (Drummond *et al*., 2010). The molecular identification of the bacterial strain was identified by16S rRNA sequencing. (Gurubasappa *et al*., 2015).

16SrRNA analysis:

The obtained sequence was compared with 16S rRNA database of National Centre for Biotechnology Information using the BLAST algorithm (Altschul *et al*., 1997). Phylogenetic tree was constructed and the obtained 16S rRNA sequencing was submitted on the GEN BANK.

3. Results and Discussion

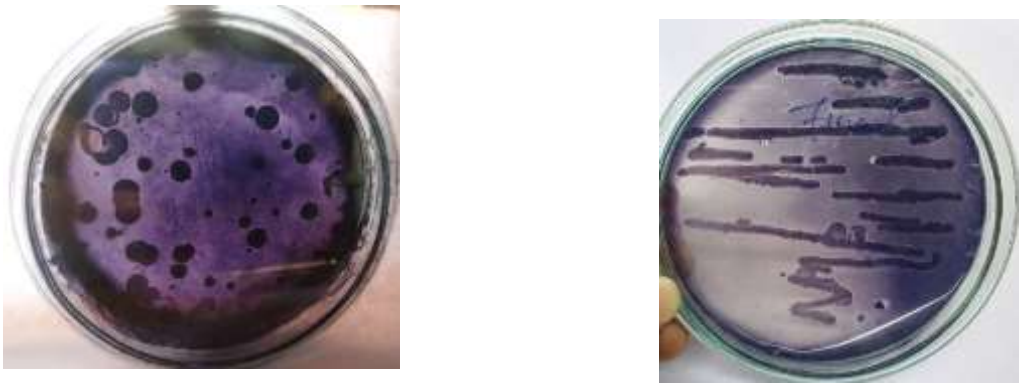


Figure 1. Sudan black blue stained colonies

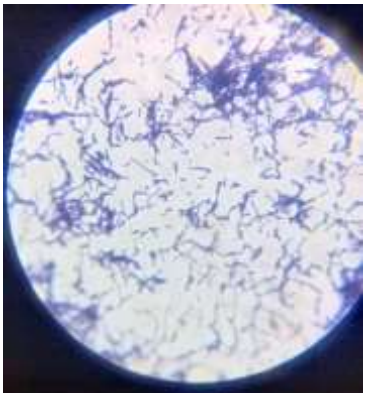


Figure 2: Gram staining

Figure 3: Produced PHB powder

Table:1 Biochemical Characterization of The Isolated Organism

DESCRIPTION	INTERFERENCE		
	WJSP1	WJSP2	WJSP3
Colony morphology	Rod shaped, waxy, flat colonies with irregular edges	Rod shaped, white and slightly pigmented colonies	Rod shaped, creamy white and flat colonies
MICROSCOPIC EXAMINATION			
Gram's staining	+	-	-
Spore staining	+	-	+
BIOCHEMICAL TEST			
Catalase test	+	+	-
Indole test	-	+	-
Methyl red test	-	-	-

Voges Proskauer test	+	-	-
TSI test	Yellow fully	Pink fully	Yellowish pink
Urease test	+	-	+
Starch hydrolysis	-	+	+
Gelatin hydrolysis	-	+	-
Citric acid test	+	+	-
Hydrogen sulphide test	-	+	-
Carbohydrate fermentation test			
Glucose fermentation	+	-	+
Dextrose fermentation	+	-	+
Sucrose fermentation	+	-	+

Figure 4: Crotonic acid assay

Table 2: Crotonic acid assay

SL.NO	PHB standard concentration (µg/ml)	OD values at 235nm
1	2	0.22
2	4	0.41
3	6	0.5
4	8	0.89
5	10	1.25
6	12	1.41
7	14	1.63
8	16	1.83

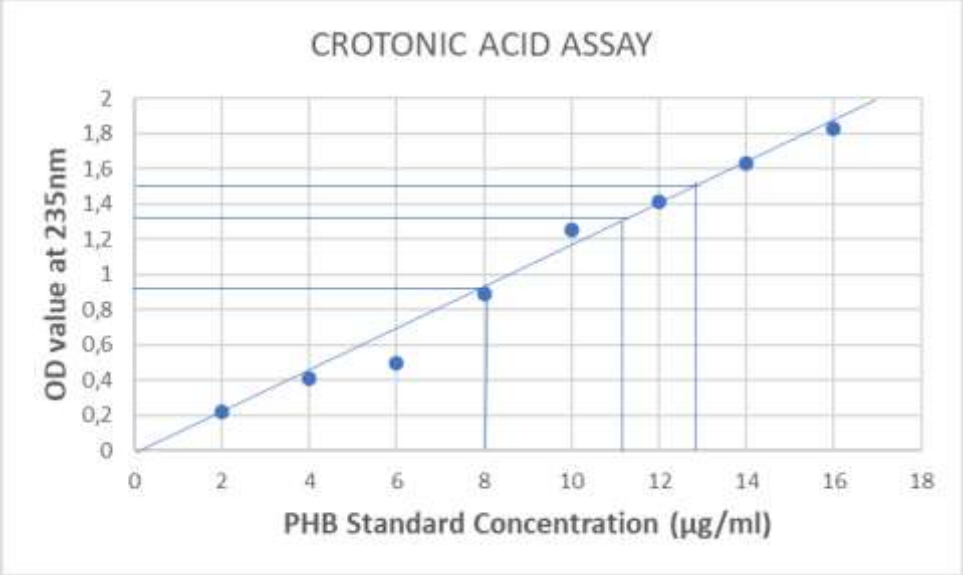


Figure 5: Crotonic acid assay

Figure: 6 Optimization of incubation period

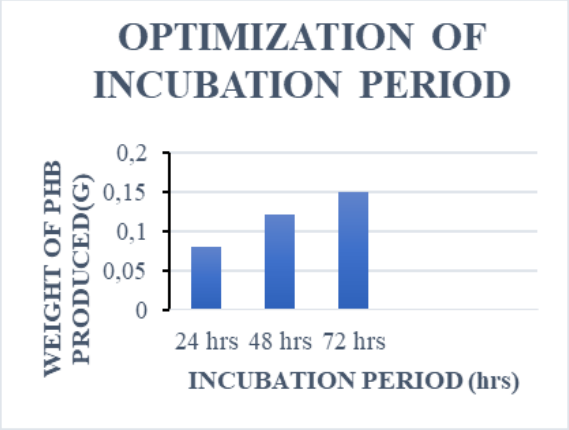


Figure 7: Optimization of Temperature

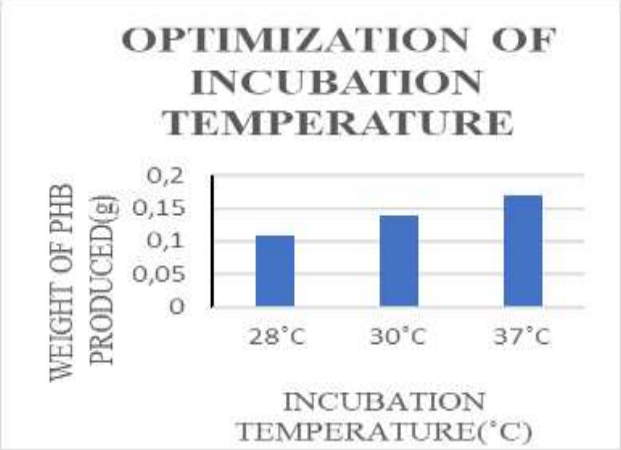


Figure 8: Optimization of carbon sources

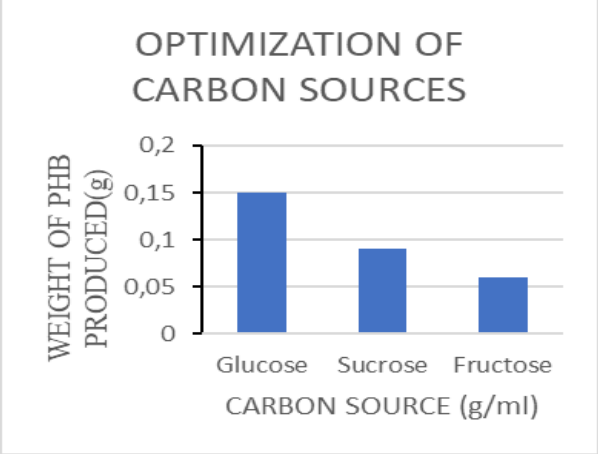
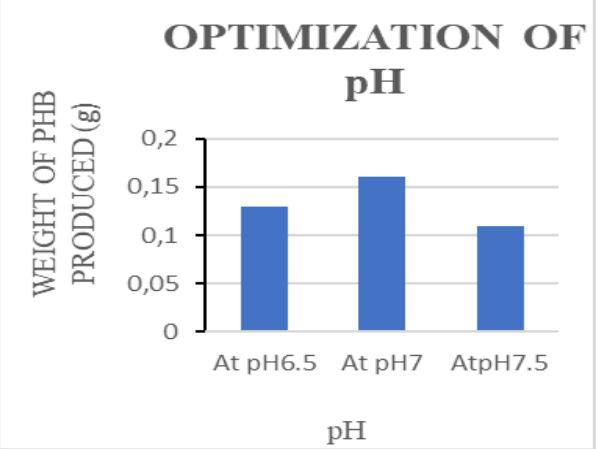


Figure 9: Optimization of pH



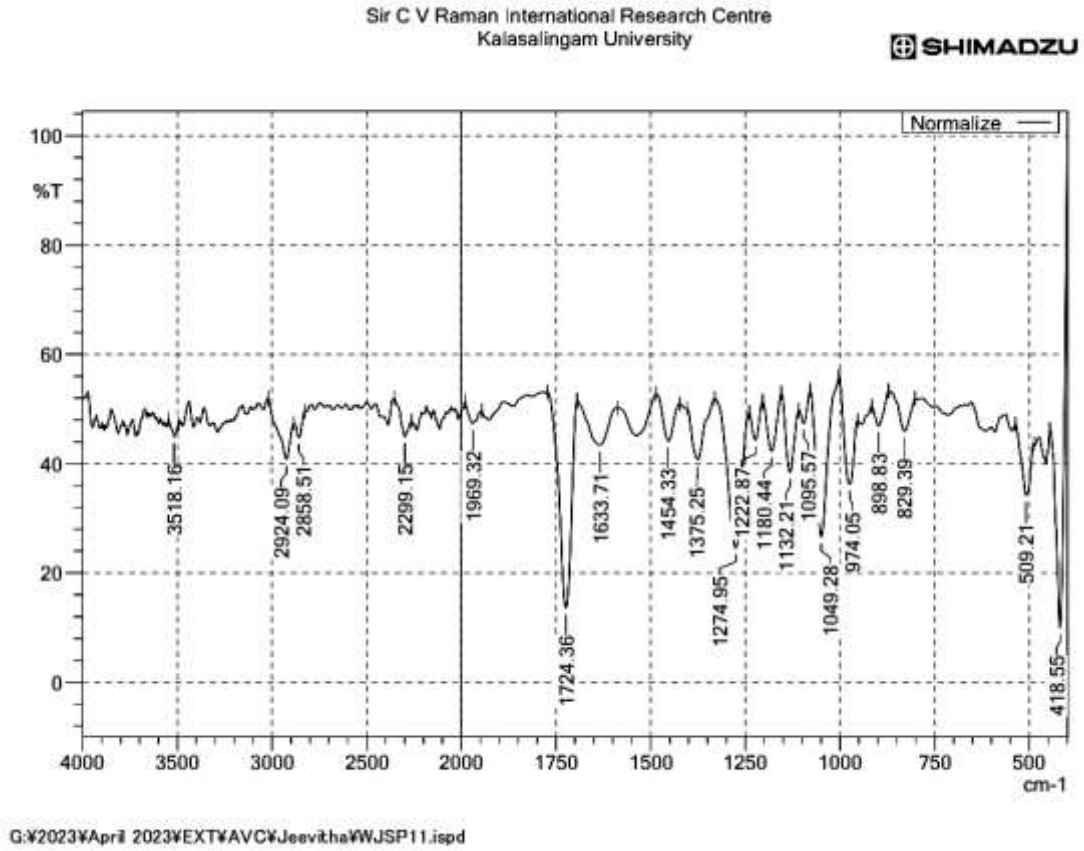
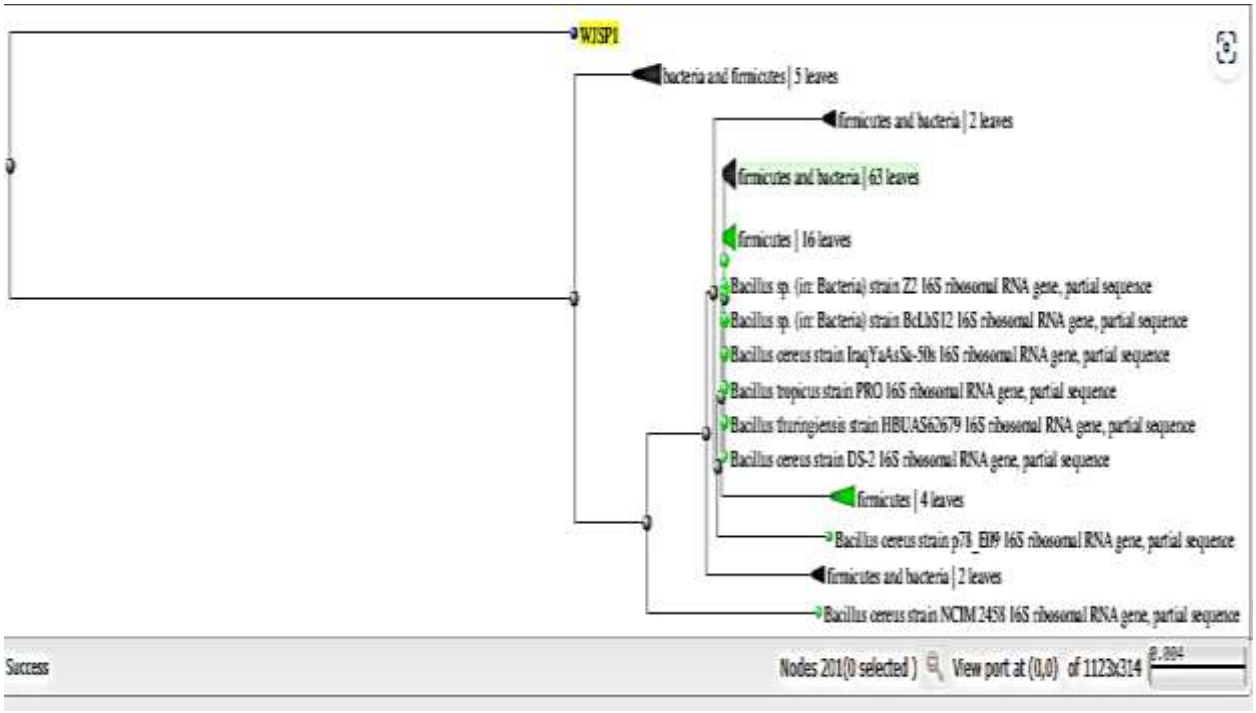


Figure 10: FT-IR Spectrum of PHB extracted from WJSP1

Gene sequencing

>OR192860.1 Bacillus cereus strain WJSP1 16S ribosomal RNA gene, partial sequence
TTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCC
GCGTCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGT
GATCGGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAG
GGAAGAACAA
GTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTA
CGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCA
GGTGGTTTCTT
AAATCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCA

Figure 11: Phylogenetic tree



Soil bacteria plays an enormous role in our daily life and they help in the largescale production of organic and organic products. Several bacterial species like *Bacillus, Pseudomonas, Azatobacter* have been employed as the potential producer of PHB which were isolated from various environments like oil contaminated soil, activated sludge (Shodganga 2011).

In this current study the agricultural soil sample was collected and serial diluted and grown on the nutrient agar medium. Sudan Black Blue staining is an efficient method for the staining of PHB producing bacteria, because of the dye's capacity to soluble the fat as it is a fat-soluble dye (Mahitha *et al.*, 2023). More than 80% of the grown bacteria was capable of producing PHB (figure-1). The PHB producing colonies stained with Sudan black blue indicates that these organisms are lipoidal in nature (Williamson and Willan son 1958). Microscopic examination revealed the presence of PHB in the cytoplasm of bacteria.

Colony morphology of the isolated strains were studied. Biochemical characterization test for the bacterial strains were done and tabulated in (table-1). WJSP1 showed positive result in Gram's staining (figure-2) and showed positive results in spore staining, catalase test, Voges Proskauer, urease, glucose, citric acid, dextrose and sucrose fermentation. WJSP2 showed negative results in indole, methyl red starch hydrolysis, gelatin hydrolysis, hydrogen sulphide test. These results indicate that the strain WJSP1 is a gram-positive bacterium which has a similar characteristics of *Bacillus* species.

WJSP2 showed negative results in Gram's staining and spore staining. It showed positive results in catalase test, indole test, starch hydrolysis, gelatin hydrolysis, citric acid hydrogen sulphide and negative results in methyl red, Voges Proskauer, urease, glucose, dextrose and sucrose fermentation test. WJSP3 showed negative result in Gram's staining and positive result in spore staining, starch hydrolysis, urease test, glucose, dextrose, sucrose fermentation and negative results in catalase, indole, methyl red, Voges Proskauer, gelatin hydrolysis, citric acid, hydrogen sulphide test.

The PHB was produced by the isolated bacteria in the selective media. The composition of medium contains glucose, urea, yeast extract, potassium hydrogen phosphate, disodium hydrogen phosphate, magnesium sulphate, calcium chloride, zinc sulfate, ferrous sulfate, ammonium molybdate, boric acid. The three strains were subjected to PHB production. (Mohapatra *et al.*, 2020; Getachew *et al.*, 2016) showed that the production of PHB was maximum when there is excess of glucose and shortage of nitrogen and phosphorous. In this study MSM media was used while (Sujatha *et al.*, 2005) described that Luria Bertani broth gives more yield due to 2% of glucose in that media.

Sodium hypochlorite method is a solvent extraction method used for the extraction of produced PHB (figure-3). (Swathi *et al.*, 2015) described that the treatment of production media by solvent yields produced PHB in the form of pellet. Crotonic acid assay was used to identify the amount of PHB produced during the production process (figure-4). This assay can be used to estimate the amount of PHB produced at different conditions (Slepecky and Law 1960). The amount of PHB produced were

13.5µg/ml, 8.3µg/ml, 11.32µg/ml for the strains WJSP1, WJSP2 and WJSP3 (figure-5). The results interpreted that PHB production was maximum in the strain WJSP1 and hence used for further studies.

Optimization of PHB was done to ensure the maximum production of PHB at different conditions. The following conditions like incubation period, temperature, pH and carbon sources were optimized. At 24 hrs, the production was 0.08g/ 100ml, at 48 hrs the production was 0.12g/100ml and at 72 hrs the production was 0.15g/100ml (figure-6). At 28˚C the production was 0.07g/ 100ml, at 30˚C the production was 0.11g/100ml and at 37˚C the production was 0.14g/100ml (figure-7). In glucose the production was 0.15g/ 100ml, in sucrose the production was 0.09g/100ml and in fructose the production was 0.06g/100ml (figure-8). At pH6.5 the production was 0.13g/ 100ml, at pH 7 the production was 0.16g/100ml and at pH 7.5 the production was 0.11g/100ml (figure-9). These results show that the maximum production was obtained in the carbon source glucose, at temperature 37˚C, at pH 7 and at a time period of 72 hrs and corelated with Mahitha *et al.,* (2023).

The nature of PHB production with varied temperature was explained by (Arshad *et al.,* 2007) and he described that the production was maximum at the temperature 28 to 37˚C. (Jini Joseph *et al.,* 2021) described that the maximum production of PHB was obtained at pH 7 and the production declines when the pH is increased. (Grothe *et al.,* 1999) reported that the pH 6-7.5 is optimum for organism *Alcaligens latus.* The production of PHB was maximum at 48 hrs was reported by (Adwitiya *et al*.,2009; Gomez *et al.,* 2020*;* Gouda *et al.,* 2001). (Evangeline *et al.,* 2019) reported that the PHB production was maximum in Bacillus species when glucose is given as a carbon source. (Hungund *et al.,* 2013) described that the role of the carbon sources in the production of PHB was important as they are responsible for three different functions in the bacteria, biomass production, upholding of cell and polymerization of PHB.

Thin layer chromatography was performed to ensure the presence of PHB by the formation of brown spots which indicates the presence of lipid PHB (Subashri Mani *et al.,* 2022). Similar work was done and same observations were made by (Brigham *et al.,* 2010). The rf value of aqueous phase is found to be 0.44, The rf value of organic phase is found to be 0.46 confirming the presence of PHB

FTIR analysis was done, in order to know the functional group present in bacterial extract. About 1mg extracted sample of PHB was dissolved in 5 ml of chloroform. Chloroform was allowed to evaporate to get PHB polymer film and was subjected to FTIR analysis by using FTIR spectrophotometer. Spectra were recorded at 4000 cm^{-1} to 400cm^{-1} range. FTIR spectra of the extracted polymer showing peaks at 1454.33 cm^{-1}corresponds to specific rotations around carbon atoms specific to certain functional groups (Sindhu Raveendran *et al.,*2011). The peak at 1724.36 cm^{-1} corresponds to C=O stretch of the ester group present in highly ordered crystalline structure (Bhagowati *et al.,* 2015). The peak at 1274.95 cm^{-1} corresponds to –CH group (Rohini *et al.,*2006). Fourier-transform infrared (FTIR) absorption band at about 1049.28 cm-1 is a characteristic of the carbonyl group and that a band at about 1,280–1,053 cm^{-1}characterizes valance vibration of the carboxyl group (Mostafa *et al.,* 2020) (figure-10). The functional groups like CH2, CH, C=O, -OH indicates the presence of PHB and its co polymer, similar results were obtained by (Pradhan Shreema *et al.,* 2014). FTIR Spectroscopy helped in elucidating the functional groups of PHB and the presence of PHB in the sample. However, many unidentified peaks were also found along with the functional groups. This indicates that the sample contained many impurities of chemicals along with PHB. (Liu *et al.,* 2014 Dhangdharia *et al.,* 2015) described in his studies that the FTIR spectrum range for PHB is similar for *Bacillus shackletonii, Bacillus megaterium* and commercially available PHB.

To identify the bacteria, WJSP1 DNA isolation, Agrarose gel electrophoresis, 16S r RNA gene sequencing was performed. The sequence was run under BLAST tool and the organism was found to be *Bacillus cereus*. The phylogenetic tree was viewed and it also interpreted that the organism belongs to *Bacillus species* (figure-11). The obtained gene sequence was submitted in the GEN bank and the accession number for the submitted organism is OR192860. (Pati *et al.,* 2020) studied the PHB production of Bacillus species by submerged and solid-state production process and downstream process for the extraction of PHB.

4. Conclusion

By this study it is proved that the Bacillus cereus produce maximum PHB at the temperature 37˚C, carbon source glucose, pH 7, and at a time period of 72 hrs. It is proved that the agricultural land soil will be a good sample source for the production of PHB as it contains about 80% of PHB producing bacteria during screening of bacteria. In the upcoming research, more optimization studies could be done, large amount of PHB can be produced by large scale fermentation techniques and PHB can be produced cheaply by using natural carbon source.

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