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Research Article

OPTIMIZATION AND CHARACTERIZATION OF BIOPLASTIC PRODUCED BY *BACILLUS CEREUS*

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ABSTRACT

Plastic pollution involves the accumulation of plastic products in the environment that adversely affects wildlife, wildlife habitat, or humans. The bioplastic is an alternative, more ductile and less elastic than other plastics, widely used in the medical industry, and it is also biodegradable. The present study aims the optimization and production of bioplastic by *Bacillus cereus.* Soil samples were collected from different areas of Nagpur city and were used to perform serial dilution and were grown on HiCrome Bacillus Agar media. 11 colonies of *Bacillus cereus* were isolated. Among 11 isolates 6 were gives positive result in screnning process for bioplastic production. Among 6 isolates giving positive results, isolate 4 was selected because it was giving highest production which was 5.8 gl^{-1}. Among all four carbon sources, maltose was found to give highest production i.e., 5.9 gmL^{-1}. Peptone was found to be giving highest production of PHB among all four different nitrogen sources. Production was found to be 6.3 gmL^{-1}. *Bacillus cereus* was found to be giving highest production at pH 7 and found to be 6.2 gmL^{-1}. At 35°C, it was found that organisms were giving highest result and produced 6.3 gmL^{-1}. *Bacillus cereus* can be used for PHB production on large industrial scale, solving by this one of the problems of solid waste management that results from the accumulation of plastics and saving the environment from additional air pollution caused by its recycling.

INTRODUCTION

Plastic is a material consisting of any of a wide range of synthetic or semi-synthetic organic compounds that are malleable and can be molded into solid objects. The world's first fully synthetic plastic was bakelite, invented in New York in 1907 by Leo Baekeland who coined the term 'plastics' (*Edgar David. 2009).* Many chemists contributed to the materials science of plastics, including Nobel laureate Hermann Staudinger who has been called "the father of polymer chemistry" and Herman Mark, known as "the father of polymer physics"(*Teegarden, David M. 2004).* The word *plastic* is derived from the Greek (*plastikos*) meaning "capable of being shaped or molded", from (*plastos*) meaning "molded". Plastics are typically organic polymers of high molecular mass, but they often contain other substances. They are usually synthetic, most commonly derived from petrochemicals, but many are made from renewable materials such as polylactic acid from corn or cellulosics from cotton linters. Plasticity is the general property of all materials that are able to irreversibly deform without breaking, but this occurs to such a degree with this class of moldable polymers that their name is an emphasis on this ability. Due to their relatively low cost, ease of manufacture, versatility, and imperviousness to water, plastics are used in an enormous and expanding range of products, from paper clips to spaceships. They have already displaced many traditional materials, such as wood, stone, horn and bone, leather, paper, metal, glass, and ceramic, in most of their former uses. In developed countries, about a third of plastic is used in packaging and another third in buildings such as piping used in plumbing or vinyl siding. Other uses include automobiles (up to 20% plastic), furniture, and toys. In the developing world, the ratios may be different - for example, reportedly 42% of India's consumption is used in packaging. Plastics have many uses in the medical field as well, to include polymer implants, however the field of plastic surgery is not named for use of plastic material, but rather the more generic meaning of the word plasticity in regard to the reshaping of flesh (Andrady AL 2009).

Plastic pollution involves the accumulation of plastic products in the environment that adversely affects wildlife, wildlife habitat, or humans. Plastics that act as pollutants are categorized into micro-, meso-, or macrodebris, based on size.

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(*Hammer, J. et.al., 2012)* The prominence of plastic pollution is correlated with plastics being inexpensive and durable, which lends to high levels of plastics used by humans. (Hester, Ronald E. *et.al.,* 2011) However, it is slow to degrade. Plastic pollution can unfavorably affect lands, waterways and oceans. Living organisms, particularly marine animals, can also be affected through entanglement, direct ingestion of plastic waste, or through exposure to chemicals within plastics that cause interruptions in biological functions (*Lytle, Claire Le Guern 2015)*

Bioplastics are plastics derived from renewable biomass sources, such as vegetable fats and oils, corn starch, or microbiota. Bioplastic can be made from agricultural by-products and also from used plastic bottles and other containers using microorganisms. Common plastics, such as fossil-fuel plastics (also called petrobased polymers), are derived from petroleum or natural gas. Production of such plastics tends to require more fossil fuels and to produce more greenhouse gases than the production of biobased polymers (bioplastics). Some, but not all, bioplastics are designed to biodegrade. Biodegradable bioplastics can break down in either anaerobic or aerobic environments, depending on how they are manufactured. Bioplastics can be composed of starches, cellulose, biopolymers, and a variety of other materials. *Hong Chua 1999)* Thermoplastic starch currently represents the most widely used bioplastic, constituting about 50 percent of the bioplastics market. Bioplastics can be made from proteins from different sources. For example wheat gluten and casein show promising properties as a raw material for different biodegradable polymers. The aliphatic biopolyesters are mainly polyhydroxyalkanoates (PHAs) like the poly-3-hydroxybutyrate (PHB), polyhydroxyvalerate (PHV) and polyhydroxyhexanoate (PHH). PHB production is increasing. Polyhydroxyalkanoates are linear polyesters produced in nature by bacterial fermentation of sugar or lipids. They are produced by the bacteria to store carbon and energy. In industrial production, the polyester is extracted and purified from the bacteria by optimizing the conditions for the fermentation of sugar. More than 150 different monomers can be combined within this family to give materials with extremely different properties. PHA is more ductile and less elastic than other plastics, and it is also biodegradable. These plastics are being widely used in the medical industry (*Song J. H. 2009).*

Polyhydroxybutyrate was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne. (Lemoigne, M. (1926) PHB is produced by microorganisms (such as *Ralstonia eutrophus, Methylobacterium rhodesianum* or *Bacillus megaterium*) apparently in response to conditions of physiological stress; (Ackermann Jörg-uwe. 1995) mainly conditions in which nutrients are limited. The polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA. This latter compound is then used as a monomer to polymerize PHB. (Steinbüchel Alexander (2002) PHAs granules are then recovered by disrupting the cells. (Jacquel N. (2008) Most commercial plastics are synthetic polymers derived from petrochemicals. They tend to resist biodegradation. PHB-derived plastics are attractive because they are compostable and derived from renewables and are bio-degradable. Firmicutes and proteobacteria can degrade PHB. Bacillus, Pseudomonas and Streptomyces species can degrade PHB. *Pseudomonas lemoign*e, *Comamonas* sp. *A cidovorax faecalis, Aspergillus fumigatus* and *Variovorax paradoxus* are soil microbes capable of degradation. *Alcaligenes faecalis, Pseudomonas*, and *Illyobacter delafieldi*, are obtained from anaerobic sludge. *Comamonas testosterone* and *Pseudomonas stutzeri* were obtained from sea water. Few of these are capable of degrading at higher temperatures; notably excepting thermophilic *Streptomyces sp.* and a thermophilic strain of *Aspergillus sp*. (Yutaka Tokiwa *et.al*., 2009)

MATERIAL AND METHODS

Collection of soil sample

Soil samples were collected from various areas such as Butibori, Manewada, Shantinagar, Bharat Nagar and Gandhinagar of Nagpur city. The soil sample was collected from10-15 inches depth of soil in sterilized sealed packet and transported within 24 hrs in laboratory for further process.

Isolation of Bacillus cereus from samples and maintenance of culture

1gm of each soil sample was dissolved in 10ml of autoclaved distilled water. Then it is serially diluted. 0.1ml of this solution is poured in respective plates of HiChrome Bacillus Agar plate and spread it over the media with the help of sterile spreader. Incubate the plates at 37°C for 24 hours. After incubation, different types of isolated colonies were obtained on HiChrome Bacillus Agar plates. Among these dark blue colour colonies were picked and were inoculated on different HiChrome Bacillus Agar plates to obtain pure culture. Then an isolated colony was picked and was inoculated on nutrient agar slants. These Nutrient Agar slants incubated for 24 hours at 37°C. After incubation these subcultured slants stored at 4°C and maintained for its identification on the basis of their morphological, cultural and biochemical characteristics.

Identification of Bacteria

Identification of *Bacillus cereus* was done basis of morphology by performing Gram staining and motility, biochemical by testing sugar fermentation using Glucose, Lactose, Mannitol , Maltose, Sucrose, IMViC Test, Catalase test, Oxidase test, Triple Sugar Iron (TSI) test, Urease test and cultural characteristics by inoculating bacteria on Hichrome Bacillus Agar.

Screening for the production of PHB

Screening by plate assay method with Sudan Black B dye

Nutrient Agar plates were prepared with additional 1% glucose. These plates were inoculated with required culture. These plates were incubated for 72 hrs at 37°C. Then they were stained with Sudan Black B dye and were kept undisturbed ate room temperature for 30min. They were then washed with ethanol to remove excess stain. (Joraleerut P. *et.al.*, 2014)

Screening on slide using Sudan Black B dye

Smear of culture was made on a clean glass slide. It was stained for 10min with Sudan black solution, rinsed with water and counter stained with 0.5% saffranin for 5min and observed at 1000x magnification. (Sharma M. *et.al.*, 2015)

Carbol fuchsin staining was performed to determine the intracellular production of PHB by the isolate. A thin smear of all the isolated were stained with carbol fuchsin stain for 45 secs. (Sharma M. *et.al.*, 2015)

PHB Production

All PHB producing isolates were subjected to fermentation in PPM (PHB Production media contains $MgSO_4.7H_2O$ – 0.1gm/l, K_2HPO_4 – 0.5gm/l, NH_4NO_2 – 0.1gm/l, Glucose – 20gm/l, Malt Extract – 0.5gm/l, Yeast extract – 1gm/l and pH - 7) for PHB production. Inoculum was prepared by inoculating loopful of culture in 5ml of PPM culture media for 24hrs. 2ml of this inoculum was inoculated in 100ml of culture PPM media for 72hrs at 37°C. For optimization different sugars and nitrogen sources are used at different pH. (Sharma P *et.al.*, 2014)

Extraction and Quantification of PHB

PHB extraction from bacterial cells was done with sodium hypochlorite digestion method (Demain and Davis, 1999). After 72h of incubation at 35°C, 10ml of the culture was taken and centrifuged at 5000rpm for 15min. Supernatant was discarded and pellet was suspended in sodium hypochlorite solution for 1 hr at 37°C for complete digestion of cell components except PHB. Samples were centrifuged at 8,000 g for 20 min to collect PHB granules. Precipitated PHB was washed with acetone, water and dissolved in chloroform. Then it was allowed to evaporate overnight and dried pellet obtained was used for PHB estimation. Finally, granules were mixed with 5 ml of concentrated H_2SO_4 and the tubes were capped and heated for 10 min at 100°C in water bath to convert PHB to crotonic acid. Absorbance of the solution was read at 235 nm against sulphuric acid blank. PHB concentration was determined from an established standard curve abd PHB production was estimated spectrophometrically at 235 nm (Law and Slepecky, 1961).

Optimization for PHB production

Different factors affecting PHB production by *B. cereus* were optimized *i.e.*, Carbon source, nitrogen source, pH and temperature.

Carbon sources: Different carbon sources were used for PHB production i.e., Fructose, Glucose, Maltose, Sucrose. Production broth with different sugars were inoculated with culture and were incubated for 72 hrs at 35°C. PHB was extracted and quantified using UV spectrophotometer.

Nitrogen sources: Different nitrogen sources were used in PHB production and they are, Peptone, Ammonium sulphate, Tryptone, Urea. Production broths were inoculated with culture and were incubated for 72 hrs at 35°C. PHB was extracted and quantified using UV spectrophotometer.

pH: Production media was prepared by using carbon source and nitrogen source giving highest production at different pH i.e., 6, 7, 8 and 9. Production broths were inoculated with culture and were incubated for 72 hrs at 35°C. PHB was extracted and quantified using UV spectrophotometer.

Temperature: Production media was prepared by using carbon source, nitrogen source and pH giving highest yield and were inoculated with culture and were incubated at different temperatures i.e., 30°C, 35°C, 40°C and 45°C. Production media was incubated for 72 hrs. PHB was extracted and quantified using UV spectrophotometer.

Thin Layer Chromatography

To detect the presence of PHB by TLC method, the solvent ethyl acetate: benzene (1:1) was used. The Retention factor (R_f) of the compound was Calculated using the formula (Marjadi and Dharaiya, 2014).

$$Rf = \frac{\text{Distance Travelled by solute}}{\text{Distance Travelled by solvenfront}}$$

Confirmation studies using FTIR

Fourier Transform Infrared Spectroscopy (FTIR) was performed to identify the various functional groups present in the extracted PHB. 10mg of the PHB sample was dissolved in chloroform and subjected to FTIR in a spectral range of 4000-400 cm^{-1}.

RESULTS AND DISCUSSION

Isolation of Bacillus cereus

The main goal of the current research was to isolate colonies of *Bacillus cereus* from soil samples of different areas of Nagpur city, and isolate PHB producing bacteria and optimizing and characterizing the produced bioplastic. Soil samples were collected from different areas of Nagpur city and were used to perform serial dilution and were grown on HiCrome Bacillus Agar media. 11 colonies of *Bacillus cereus* were isolated. They were stored on Nutrient Agar media slants for further process. Morphological, Biochemical and cultural tests were performed in order to confirm the species.

Isolates obtained were further tested for PHB production by screening of the isolates by three methods. Among 11 isolates only 6 were found to be giving positive results for PHB production. Those isolates were preserved for further use. Those 6 isolates were used for PHB production by using PHB production media (PPM). Among them one isolate was giving highest production was used for further optimization process. Optimization process was done by using different carbon sources, different nitrogen sources, different pH and different temperature. There was high diversity of bacteria in the soil samples from different area of Nagpur city. Isolates grew well producing different colonies on HiCrome Bacillus Agar plates. Colonical morphology description and Gram stain reaction of the isolates were the initial identification criteria used. Micro-morphological observation of the isolates revealed the organisms had typical characteristics of *Bacillus cereus.*

Table 1 Morphological characteristic of *Bacillus cereus*

Sr.	Test	Result
1.	Gram staining	Gram positive
2.	Endospore staining	Spore forming
3.	Motility	Motile

Table 2 Biochemical test of *Bacillus cereus*

Sr. No.	IMViC Test				TSI (H_2S)	Urea	Enzymatic Action		Sugar Fermentation Test			
1.	Indole	MR	VP	Citrate			Catalase	Oxidase	Glucose	Sucrose	Lactose	Mannitol
	-	-	+	+	-	-	-	+	+	+	-	-

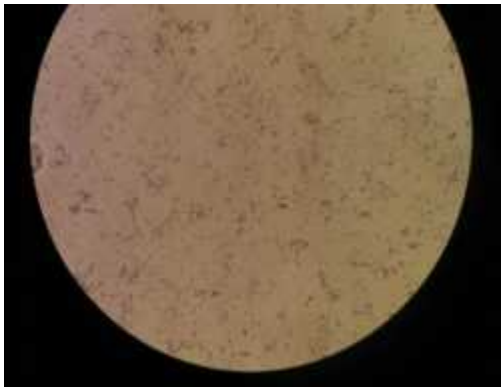

Figure 1 Gram positive staining of *Bacillus cereus* observed under microscope

Figure 2 Biochemical test of *Bacillus cereus*

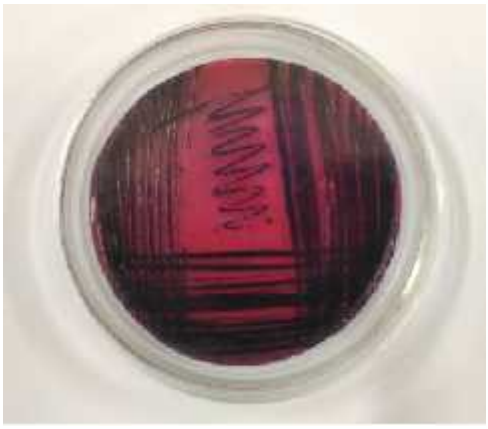

Figure 3 Growth of *Bacillus cereus* on HiCrome Bacillus Agar

Screening of PHB Producing Bacteria

Total 11 isolates were selected for study, among which only 6 were giving positive results for PHB production. There were three different methods used for the screening purpose. Initially Luria Bertini Agar media was prepared with extra 1% glucose was added. In this plate method bacterial, colonies producing PHB took black colour. Next was slide method using Sudan Black B. In this process as well, colonies producing PHB were shown to take black colour observed under microscope. Another slide test was performed with carbon fushin, in which PHB producing bacteria were seen to have taken pink colour. All the isolates were screened and highest PHB producing isolate was used for further PHB production.

Among 6 isolates giving positive result, isolate from sample 4 was found to give highest production.

Figure 4 Bacterial isolate on Nutrient Agar with 1% extra glucose stained with Sudan Black B

After the confirmation by screening of the isolates, they were used for the production process. PHB production medium was used for the production process. After production medium was subjected to centrifugation and extraction. The extracted sample precipitate, boiling chloroform was added and let it to evaporate. The product obtained was in the form of powder and it appeared in the form of sheet. Among 6 isolates, isolate 4 was selected because it was gives highest production which was 5.8 gl^{-1}.

Figure 5 Obtained PHB in powdered form

Bacillus cereus seems to be very versatile in terms of metabolic potential as it has the ability to consume variety of carbon sources. Selected isolate was used for optimization of PHB production. PHB production was optimized for different carbon sources, nitrogen sources, pH and temperatures. Results are as follows:

Effect of carbon sources on PHB production: Among all four carbon sources, maltose was found to give highest production i.e., 5.9 gmL^{-1}. When Fructose was used in the production medium, organisms were found to be giving least production of PHB.

Table no. 3 Optimization Results of PHB production

Optimization factor		Productiongm L^{-1}	Absorbance (235 nm)
Carbon Sources	Fructose	3.2	0.864
	Glucose	3.8	1.216
	Maltose	**5.9**	**2.219**
	Sucrose	4.6	1.561
Nitrogen Sources	Peptone	**6.3**	**2.965**
	Ammonium sulphate	4.9	1.592
	Tryptone	5.2	1.869
	Urea	3.5	0.981
pH	6	4.8	1.584
	7	**6.2**	**2.739**
	8	5.6	2.046
	9	3.5	0.985
Temperature	30°C	4.4	1.364
	35°C	**6.3**	**2.892**
	40°C	5.2	1.854
	45°C	3.8	1.049

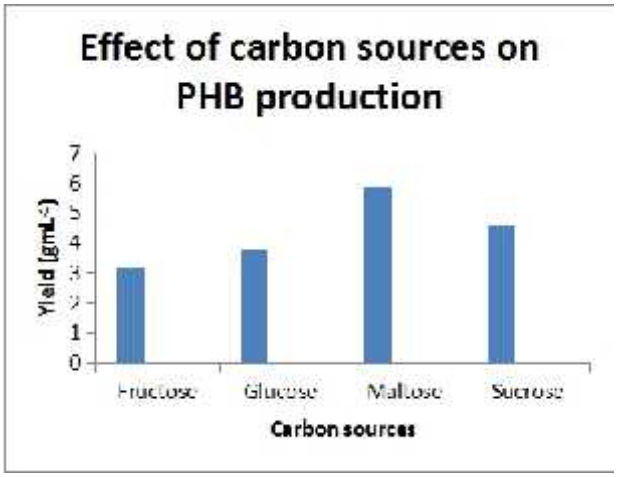

Figure 6a Optimization of culture parameters for PHB production for different carbon sources

Effect of Nitrogen sources on PHB production: Peptone was found to be giving highest production of PHB among all four different nitrogen sources. Production was found to be 6.3gmL^{-1}. Least production was obtained when urea was used as nitrogen source.

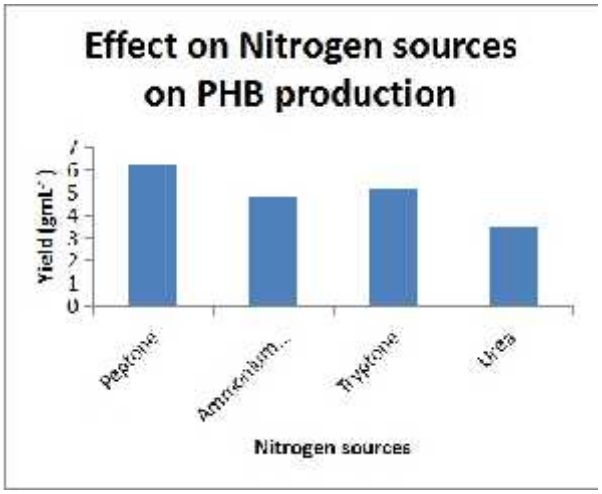

Figure 6b Optimization of culture parameters for PHB production for different nitrogen sources

Effect of pH on PHB production: Organisms were found to be giving highest production at pH 7 and found to be 6.2 gmL^{-1}. At pH - 9, lowest production was obtained.

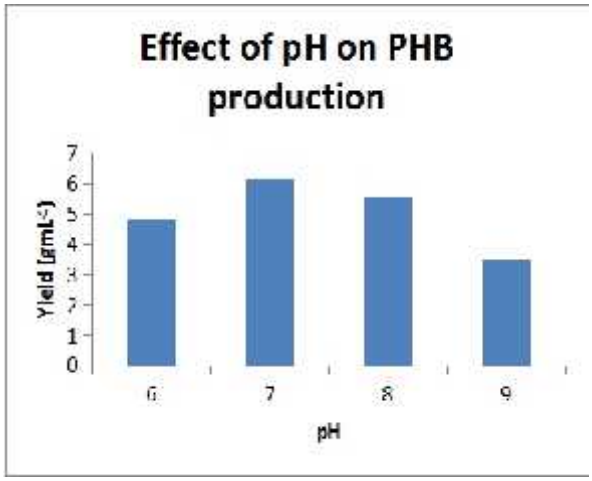

Figure 6c Optimization of culture parameters for PHB production for different pH

Effect of Temperature on PHB production: At 35°C, it was found that organisms were giving highest result. It was found to be 6.3gmL^{-1}. Lowest production was obtained at temperature 45°C.

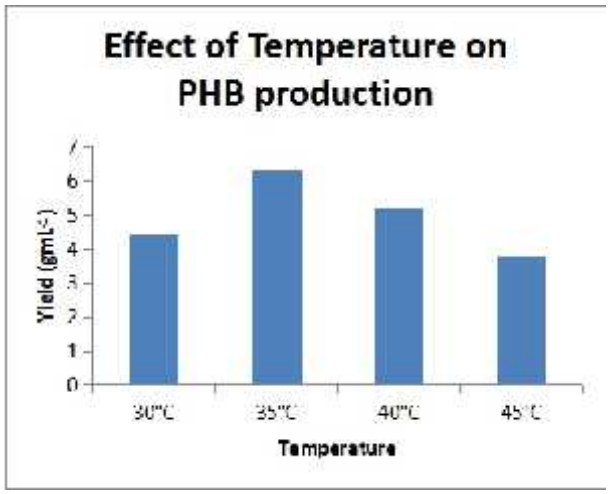

Figure 6d Optimization of culture parameters for PHB production for different temperatures

Characterization of PHB

Thin Layer Chromatography

Table no. 4 Result of Thin Layer Chromatography

Sr. No.	Distance travelled by the compound	Distance travelled by the solvent front	R_f value
1.	3.7 cm	8 cm	0.46

For analysis of PHB, obtained product was used to perform Thin Layer Chromatography. R_f value was found to be 0.46 by calculating values of Distance travelled by the compound and distance travelled by the solvent front from the formula,

The FTIR analysis of recovered polymer revealed absorption bands at 3745.36/cm, 3618.83/cm and 3444.38/cm corresponds to-OH groups.

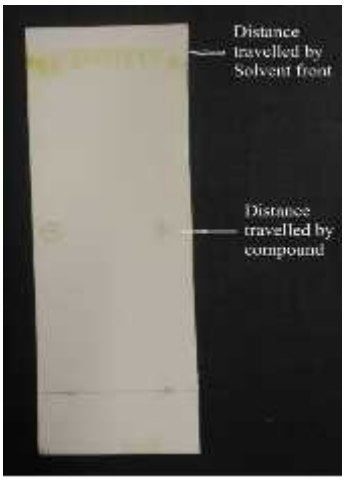

Figure 7 Thin Layer Chromatography performed for obtained product

Fourier transform-infrared spectrometer (FTIR) analysis

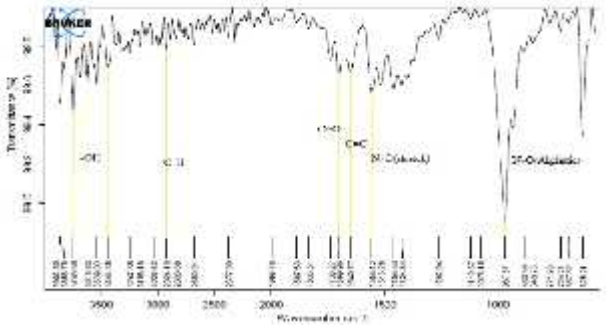

Figure 8 FTIR spectrometer analysis result of obtained product

3745.36/cm corresponds to stretch and free -OH group while 3444.38/cm corresponds to stretch, H-bounded -OH group. 2924.19 corresponds to presence of C-H group. 1699.96/cm and 1649.77/cm corresponds to C=O and C=C respectively. 1556.42/cm and 967.67/cm corresponds to –NO group, but 1556.42/cm corresponds to stretch –NO group while 967.67/cm corresponds to aliphatic –NO group. (Figure 8)

In current project only one type of media was used for optimization while in another project worked by Elsayed B. Belal and Mona A. Farid used different types of media for optimization medium 1 (Sucrose / yeast extract broth medium (Bormann *et al.*, 1998), medium 2 (nutrient broth medium (Atlas, 1997), medium 3 (Bänziger and Tobler 2001), medium 4 (synthetic medium, Burdman *et al.*, 1998) were used. (Elsayed B *et.al.*, 2016) For optimization in current project only 72 hrs incubated production media was used while Priyanka Sharma and Bijendar Kumar Bajaj used 24, 48, 72, 96 hrs production for optimization purpose. (Sharma P. *et.al*., 2014) After production, media was centrifuged and PHB was extracted and absorbance was recorded. Produced PHB was further used for Characterization purpose. Two methods were used for characterization and they were Thin layer Chromatography and Fourier transform-infrared spectrometer (FTIR). In Thin Layer Chromatography, a drop of sample was placed near the bottom silicon plate and was kept in the solution to run. By the calculation R_f value of current study was found to be 0.46. While in another experiment performed by Priscilla Anitha and Priya Iyer, (Anitha P. *et.al.*, 2015) they worked on *Bacillus sp*. and *Salmonella sp*. In their work, they obtained 0.57 as R_f value for PHB produced by *Bacillus* sp.

Another characterization test was done was FTIR. Current project's result is shown in Figure 10 and is explained below the figure, while in another project work done by Priyanka Sharma and Bijendar Kumar Bajaj, FTIR results showed that band was obtained at 1721.95/cm corresponding to aliphatic ester carbonyl group and 2926.43/cm corresponding to C-H stretch, nearly similar to present results (Fig. 8). They also obtained an absorption band at 3433.35/cm showing the presence of –OH bonding similar to this results (Fig. 8). (Sharma P. *et.al*., 2014)

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