Optimization and characterization of bioplastic produced by *Bacillus cereus* SE1

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CERTIFICATE

This is to certify that the project report entitled **"Optimization and characterization of bioplastic produced by *Bacillus cereus* SE1"** submitted by **Ms. Shreema Pradhan** to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in **LIFE SCIENCE** is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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Shreema Pradhan

DECLARATION

I, Shreema Pradhan, hereby declare that this research project report entitled **Optimization and characterization of bioplastic produced by *Bacillus cereus* SE1**
is the original work carried out by me under the supervision of Dr. Surajit Das, in the Laboratory of Environmental Microbiology and Ecology (LEnME), Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge and belief the work reported here or any part thereof has not been presented to any other Institute or University for the award of any degree or diploma.

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Abstract

Bioplastics can be defined as plastics made of biomass such as corn, sugarcane etc. These substances have been increasingly spotlighted as means to saving fossil fuels, reducing CO2 emission and plastic wastes. Biodegradability of Bioplastics has been widely publicized in society and the demand for packaging is rapidly increasing among retailers and the food industry at large scale. The plastic which is available in market is very dangerous as it is non-biodegradable. Therefore, it is the demand of the day that biodegradable plastics should be produced and used. The present review highlights all these points regarding the applications, production, types, challenges, sustainability, fermentation, process development and use of cheap substrates for bioplastics production.

The bacterial isolate *Bacillus cereus* SE-1 was found to possess a higher level of PHB production in presence of various carbon sources used. However, a higher level of production was obtained with maltose (5.63 g/l) and a lower production was obtained when dextrose (0.4 g/l) was used as sole carbon source. FTIR analysis reveals that, the extracted PHB possesses the same pattern of functional groups as that of the standard PHB i.e. the characteristics presence of –OH and –C=O stretching. In order to increase the potential use of the extracted PHB, it was blended with thermoplastic starch under experimental conditions. After blending, the crystalline nature of the product was highly increased as the diffraction patterns were observed at 2θ values of 26.80°, 31.74°, 45.59°, and 56.22°. Thus, the bacterial mediated PHB synthesis can be used as a better alternative to deal with the currently available practices of plastic use and its gradually increasing pollution level.

Keywords: polyhydroxybutyrate, acetoacetylcoA, acetyl coA, bio plastic.

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LIST OF ABBREVIATIONS

g	Gram
µl	Micro liter
ml	Milliliter
l	Liter
h	Hour
°	Degree
C	Centigrade
min	Minute
%	Percentage
No.	Number
sp.	Species

1. INTRODUCTION

1.1 Environment

The natural environment encompasses all living and non-living thing occurring naturally on Earth. The natural environment is contrasted with the built environment, which comprises the areas and components that are strongly influenced by humans. Environment is the biotic and abiotic surrounding of an organism or population, and includes the factors that have an influence in their survival, development and evolution.

All life that has survived must have adapted to conditions of its environment. Temperature, light, humidity, soil nutrients, etc., all influence any species, within any environment. Some long term modifications along the history of our planet have been significant, such as the incorporation of oxygen to the atmosphere. This process consisted in the breakdown of carbon dioxide by anaerobic microorganisms that used the carbon in their metabolism and released the oxygen to the atmosphere. This led to the existence of oxygen-based plant and animal life, the great oxygenation event. Other interactions are more immediate and simple, such as the smoothing effect that forests have on the temperature cycle, compared to neighbouring unfrosted areas.

1.2 Environmental Pollution

Pollution is the introduction of contaminants into the natural environment that causes adverse change. Pollution can take the form of chemical substances or energy, such as noise, heat or light. Pollutants, the components of pollution, can be either foreign substances/energies or naturally occurring contaminants. Pollution is often classed as point source or nonpoint source pollution.

A point source of pollution is a single identifiable source of air, water, thermal, and noise or light pollution. A point source has negligible extent, distinguishing it from other pollution source geometries. The sources are called point sources because in mathematical modelling, they can be approximated as a mathematical point to simplify analysis.

Nonpoint source (NPS) pollution refers to both water and air pollution from diffuse sources. Nonpoint source pollution affects a water body from sources such as polluted runoff from agricultural areas draining into a river, or wind-borne debris blowing out to sea. Nonpoint source air pollution affects air quality from sources such as smokestacks.

Pollutions may lead to critical problems in the global geochemical cycles as well as the sustainable habitation of humans as well as other organisms. Even though other organisms suffer from the adverse effects of natural changes, however, the main culprit is human. Various types of hazardous substances can enter the natural environment by a number of natural and/or anthropogenic activities, disturbing the living systems along with many adverse changes in the environment (Kampa and Castanas, 2008). In different urban areas huge mega lexes have been constructed which are not sustainable and they experience problems with waste management, heat islands, increasing pollution and crowding of increasing population etc. (William, 2011). CO2 is toxic for pregnant women and when exposed, the fetus may be harmed. Likewise, car exhaust gases damage health of both adults and children, leading to change in behavior and psycho-social development of children (Chelala, 2010; Markert et al., 2011).

1.3 Plastic

Plastics play an important role in almost every aspect of our lives. Plastics are used to manufacture everyday products such as beverage containers, toys, and furniture. The widespread use of plastics demands proper end of life management. The largest amount of plastics is found in containers and packaging (e.g. soft drink bottles, lids, shampoo bottles), but they also are found in durable (e.g. appliances, furniture) and nondurable goods (e.g. diapers, trash bags, cups and utensils, medical devices).

The use of plastics is widespread within our society; this is primarily due to the favourable thermal and mechanical properties of plastics making it a stable and durable material. The extensive global use of plastics has contributed heavily to environmental pollution; as plastics are not always properly discarded or recycled and consequently persist within the environment. The manufacturing processes required to produce plastic also creates large quantities of chemical pollutants. In recent years there has been a shift in public opinion, with people becoming more ecologically aware. The shift in public opinion and political influence combined with the increasing price of oil, has driven industries to investigate biodegradable alternatives to plastic, which are not manufactured using petrochemical methods. Materials produced from synthetic polymers are widely used for a diverse range of applications in modern society. The production of biodegradable alternatives with greater compatibility in the environment is necessary if the applications continue to grow.

Fig. 1.Plastic pollution

1.4 Dangers of Using Plastic Bags

1.4.1 Environmental Damage

Plastic bags have been known to cause a lot of environmental damage. A single plastic bag can take up to 1000 years, to decay completely. This makes the bags stay in environments longer, in turn leading to great build-up on the natural landscape (much more than degradable materials like paper). In other words, the more plastic bags used, the greater the chances of environmental damage.

1.4.2 Threat to Animal Life

As per Marrickville Council of Australia, as many as 100,000 whales, turtles and birds die have been reported to die every year, mainly because of plastic in their environment. Plastic bags not only have adverse effects on our natural habitats, but have also been found to be responsible for the death of many animals, mainly on account of the suffocation encountered on eating them.

Suffocation

Not only animals, infants and young children have also been reported to have lost their life, on account of plastic bags. Since plastic bags are thin and airtight as well, children often end up blocking their mouths and nostrils with them. In case they are not being monitored by an adult, this leads to suffocation and, in some cases, even death.

1.5 Pollution

Plastic bags are extremely durable. In most probability, majority of the pollution present there will comprise of plastic bags only. In other words, plastic bags have led to a great increase in the pollution levels.

One of the main disadvantages of plastic bags is that they are not renewable. The reason behind this is that they are made of petrochemicals, a non-renewable source of energy. They can be recycled, but not as easily as paper bags. Plastic bags can last for as much as hundreds of years.

1.6 Bioplastic

Bio plastics are plastics derived from renewable biomass sources, such as vegetable fats and oils, corn starch. Biodegradable bio plastics can break down in either anaerobic or aerobic environments. Plastic is one of the major pollutants of earth. To replace synthesis plastic bio plastic is develop and also to decrease pollution. Microorganisms and algae are the mostly degraded organic waste to form bio plastic.

Fig. 2. Biodegradable utensils, bottles, packaging materials made from bioplastics

Several bacteria species like *Actinobacillus*, *Azatobacte*, *Agrobacterium*, *Rhodobacter* possess the ability to convert organic waste to bacterial PHB (Poly hydroxy butyrate). PHA (poly hydroxyalkonates) is naturally occurring polyester synthesized by bacteria. For industrial production of PHB some bacterial species like *Bacillus* sp., *Pseudomonas* sp., *Aeromonas* sp. have been used. Biodegradable bio plastics are used for disposable items, such as packaging and catering items (crockery, pots, bowls, straws).They are also often used for bags, trays, containers for fruit, vegetables, eggs and meat, bottles for soft drinks and dairy products. Non disposable applications include mobile phone casings, carpet fibres, and car interiors, fuel line and plastic pipe.

Fig. 3. An illustration of the time taken for biodegradable plastic to degrade

1.7 The present status of Bio-plastics and its future

Since the large scale production of Bio-plastic in industry is very much costly so it has not been used extensively. During 20th century the bioplastics production was mainly dominated by the developed countries like North America, Japan, and Western Europe etc. On the basis of this study, it has been assumed that, by 2013, Brazil will become one of the world's leading bioplastics producers. In Japan, the demand of bioplastics will reach a value six times more than 178000 metric tons in 2013. China has planned to produce 100,000 metric tons of bioplastics by 2013. The market of bioplastics is in the nascent stage in Southeast Asia. A research work carried out by BCC has revealed a fact that the bioplastics market value has reached 541 million pounds in 2007. By 2012, this value is expected to reach a level of 1.2 billion pounds. In 2008, a number of biodegradable plastics like polylactic acid, resins, polyesters etc. accounted for about 90% of total bioplastics demand. Biodegradable plastics are environment friendly and can replace all plastics products available at this time. Production of bioplastics will definitely result in reduction in emission of CO_2 compared to traditional plastics. A fear of damaging already existing recycling projects by the bioplastics is one of the major concerns. The cost of production of bioplastics is also too high. This is one of the major problems related to bioplastics development. The cost is around 1.3 to 4 Euro per Kg now.

1.8 Polyhydroxy Butyrate (PHB)

Renewable and bacterially synthesized, poly (3- hydroxybutyrate) (PHB) is a completely biodegradable, highly hydrophobic thermoplastic polyester material containing a crystalline fraction of almost 80%. The chemical structure and physical properties of PHB are fairly similar

to those of certain petroleum- based synthetic polymers. Therefore, PHB has been the subject of extensive studies as an environmentally friendly polymeric material. Because of its high crystalline, PHB is stiff and brittle, and this results in very poor mechanical properties with a low extension at break, which limits its range of applications. Until now, there has been no large commercial production of PHB products because of its higher cost with respect to commercial polymers, high brittleness, and difficult processing.

Polyhydroxybutyrate (PHB) is a biopolymer that can be used as a biodegradable thermoplastic material for waste management strategies and biocompatibility in the medical devices (Steinbüchel and Valentin, 1995). The viability of microbial large scale production of PHB is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to petrochemical plastics (Doi, 1994)

PHB was produced by a variety of microorganism under the environmental stresses such as nutrient limitation i.e. nitrogen, phosphorus or oxygen limitation (Spiekermann et al., 1999; Thakor et al., 2005). The microorganism and the strategy of production were affected on duration of fermentation, growth rate, carbon source concentration, etc.

PHB is a highly crystalline thermoplastic polymer with a relatively high melting temperature (in the range of 170-180°C) and a glass transition temperature in the range of 0-5°C. Itundergoesthermal degradation at temperature around the melting temperature

The current cost of the PHB production is considerably more than that of the synthetic plastics. Studies on process analysis and economic evaluation showed that PHB productivity.

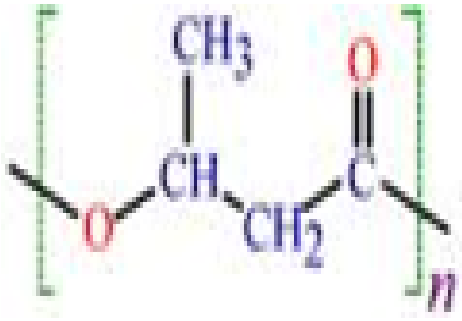

Fig. 4. Structure of Polyhydroxybutyrate(PHB)

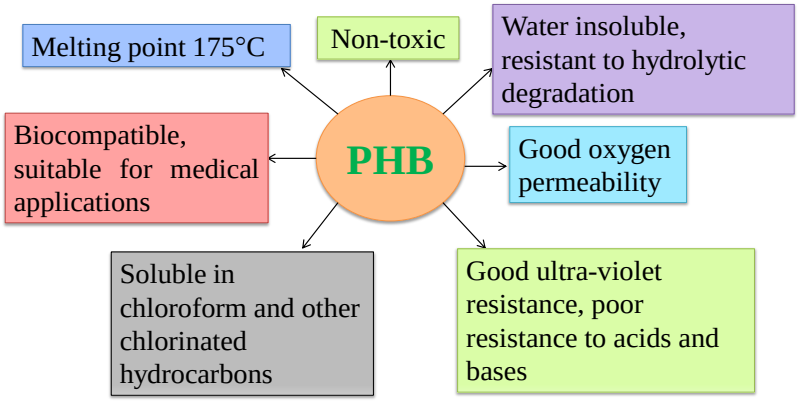

Fig 5. Useful properties of PHB

1.9 *Bacillus cereus*

Bacillus cereus is an endemic, soil-dwelling, Gram-positive, rod-shaped, beta hemolytic bacterium. Some strains are harmful to humans and cause food borne illness, while other strains can be beneficial as probiotics for animals.

Bacillus cereus is a spore-forming bacterium that occurs naturally in many kinds of foods and can cause illness in humans. It can form spores that are resistant to heating and dehydration and can therefore survive cooking and dry storage. When foods containing *B. cereus* spores are in the 'temperature danger zone' the spores may germinate, and the bacteria may grow, produce toxins, and make people sick. Such illness is frequently linked with starchy foods of plant origin such as rice, pasta, potatoes, pastry and noodles. *B. cereus* can cause vomiting or diarrhoea and, in some cases, both. This depends on the kinds of toxin it produces. When food containing live *B. cereus* is eaten, the bacteria may grow and produce another toxin, 'diarrheal toxin', in the gut. This can result in diarrheal symptoms. Illness from *B. cereus* can be prevented by making certain that hot foods are kept hot and cold foods are stored cold. It is important to remember that re-heating food that has been 'temperature abused' will not make it safe.

Fig. 6. *Bacillus cereus* under microscope

OBJECTIVES

The current project aimed to investigate the PHB production by Bacillus cereus bacterial isolates and to optimize PHB production with various carbohydrate concentrations. The main focus of the work includes:

- Optimization of PHB production by bacterial isolate for different carbon sources
- Characterization of produced PHB
- Blending of the crude PHB extract to increase its usability

2. REVIEW OF LITRATURE

2.1 Converting renewable resource into bio plastic

Polyhydroxy alkanoate (PHA) is similar physical properties to synthetic plastics. PHA is produced from renewable resources and is degraded aerobically by microorganisms to CO_2 and H2O.polyhydroxyalkanoate (PHA), which belongs to the group of polyoxoesters, and it possesses biodegradable thermoplastic properties. PHA is synthesized by bacteria under extreme growth conditions.

PHA serves as storage compounds of carbon and energy sources, due its insolubility inside bacterial cytoplasm. Poly (3-hydroxybutyrate) [P(3HB)] is the most common PHA and was first described by Lemoigne, a French scientist in year 1925. Natural PHA-producing bacteria include *A. latus, B. megaterium, C. necator and P. oleovorans,* which are capable of utilizing various carbon sources including plant oils or wastes to produce PHA. Among these *C. necator* has been the most extensively studied and commonly used bacterium for PHA production. Depending on the culture conditions bacteria that are used for the production of PHA can be classified into two groups.

The first group of bacteria requires limitation of essential nutrients such as nitrogen, oxygen and presence of excess carbon source for the efficient synthesis of PHA. Bacteria include *C. necator, Protomonas extorquens and Protomonas oleovorans.*

The second group of bacteria does not require nutrient limitation for PHA synthesis. Bacteria included in this group are *A. latus,* a mutant strain of *Azotobacter vinelandii* and recombinant *E. coli* harbouring the PHA biosynthetic.

Plant oils such as soybean oil, palm oil and corn oil are desirable carbon sources for PHA production. They are relatively cheaper than most sugars. Glycerol is a by-product of the palm oil refining process. Applications of glycerol include oral-care products, cosmetics, food and beverages.

Naturally occurring CO_2 and sunlight serve as carbon and energy sources, obtaining PHA from genetically modified crop plants. Cyanobacteria are also PHA producers that utilize CO_2 and sunlight as carbon and energy sources. Development of PHA as potential substitute material to some conventional plastics has drawn much attention due to the biodegradable and biocompatible properties of PHA. The potential applications of PHA in various industries and in the medical field are encouraging.

2.2Microalgae as bioreactors for bioplastic

Poly-(R)-3-hydroxybutyrate (PHB) is aliphatic polyester with thermoplastic properties, which is naturally produced by bacteria such as *R. eutropha* H16 and *Bacillus megaterium* as storage compound and is 100% biodegradable. PHB is synthesized from acetyl-CoA by the action of three enzymes: a ketothiolase, an acetoacetyl-CoA reductase and a PHB synthase.

The three bacterial enzymes were expressed in the cytosol. *Arabidopsis thaliana* (up to 40% of dry weight) due to stunted growth and infertility; these plants were not suitable for large-scale cultivation. The highest levels of PHB synthesis in plants with fertile offspring are obtained in the plastid of *Nicotiana tabacum*.

Microalgae share all the advantages of photosynthetically driven eukaryotic systems, they possess high growth rates, are easy to handle and do not need much more than light and water for cultivation. Thus, microalgae are thought to have great potential as novel low-cost expression systems.

The enzymes PhaA (ketothiolase), PhaB (acetoacetyl- CoA reductase) and PhaC (PHB synthase) of the Gram negative bacterium *R. eutropha H16* were expressed in the cytosol of the diatom. Microalgae like the diatom *P. tricornutum* have a great potential not only as biosynthetic factory for recombinant proteins but also as photosynthetically fuelled bioreactors for synthesizing polymers like PHB.

Biochemical studies have revealed two different pathways for synthesis of PHB.

(i) In organisms like *Azotobacter beijerinckii* and *Zoogloea ramigera*, a three-step metabolic pathway is seen. The first step is catalyzed by enzyme 1-ketothiolase, which condenses acetyl coenzyme A (acetyl-CoA) to acetoacetyl-CoA. This intermediate is then reduced to D-(-)-P3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA reductase (Nishimura et al., 1978; Schubert et al., 1988). The last step is catalysed by the enzyme PHB synthase and cause head-to-tail polymerization of the monomer to PHB.

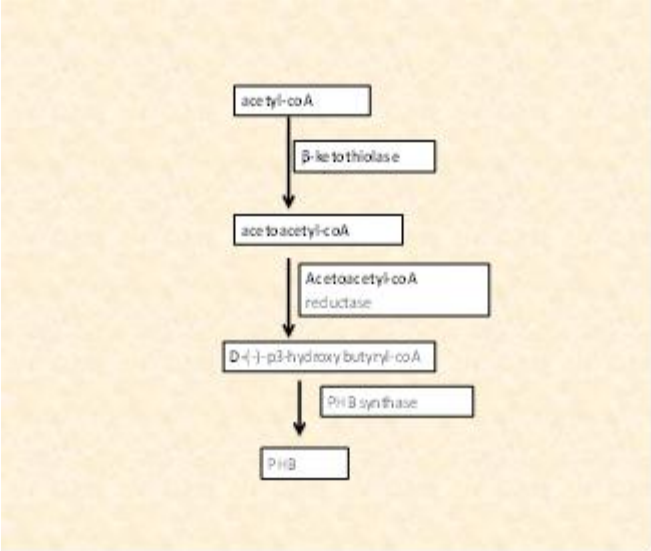

Fig. 7. *Azatobacter beijerinckii* and *Zoogloea ramigera:* a three step metabolic pathway

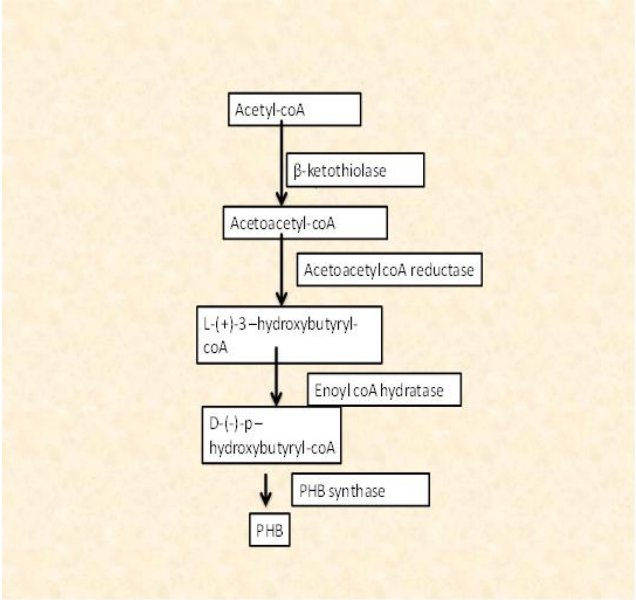

Fig. 8. *Rhodospirillium rubrum* PHB synthesis carried out through five step poly hydroxybutyrate (PHB) synthesis pathway.

In *Rhodospirillum rubrum*, PHB synthesis is carried out through five-step synthetic pathway. An NADH-dependent acetoacetyl-CoA reductase enzyme catalyses the formation of L-(+)-3-hydroxybutyryl-CoA, which is then converted to D-(-)-P-hydroxybutyryl-CoA by two stereospecific enoyl-CoA hydratases before polymerization (Moskowitz and Merrick, 1969; Schubert et al., 1988).

In contrast to β-ketothiolase and acetoacetyl CoA reductase, PHB synthase is the most important enzyme of the synthetic pathway.

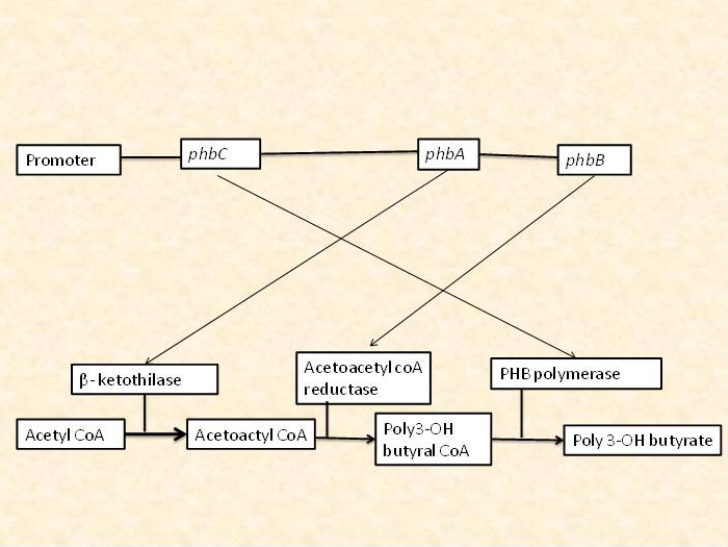

Fig. 9. Operon model of genes responsible for polyhydroxybutyrate (PHB) production.

It has been observed from experiments that acetyl CoA acyltransferase is the main enzyme which regulates the synthesis of PHB.

The PHB synthesis begins with the condensation of two acetyl-CoA molecules to acetoacetyl-CoA by enzyme ketothiolase, encoded by the *phaA*gene (Fig. 9). This intermediate is then reduced to D-(-)-3-hydroxybutyryl-CoA by the enzyme named as acetoacetyl- CoA reductase, which is a product of the *phaB*gene. Finally, the enzyme PHA synthase encoded by *phaC*gene catalyses the polymerization of 3-hydroxybutyryl CoA to Polyhydroybutyrate by joining PHB monomers through the use of two thiolate groups (Steinbuchel and Hein, 2001).

Excess carbon source and exhaustion of any nutrient in the culture media like N2, O2, PO4 increases production of PHB. Under normal growth conditions, acetyl CoA is used up in the TCA cycle, and resulting CoA inhibits the enzyme acetyl Co A acyl transferase as well as PHB synthesis. But during carbon excess and nutrient limitation NADH concentration increases by decreasing the activity of NADH oxidase. Increase NADH concentration decreases the activity of citrate synthase and isocitrate dehydrogenase and acetyl CoA level increases. Condensation of acetyl CoA to acetoacetyl CoA initiates PHB synthesis (Oeding and Schlegel, 1973; Jackson and Dawes, 1976; Page and Knosp, 1989). Increased NADH/NAD ratio is adjusted by PHB synthesis and PHB performs the role of electron acceptor (Oeding and Schlegel, 1973; Page and Knosp, 1989).

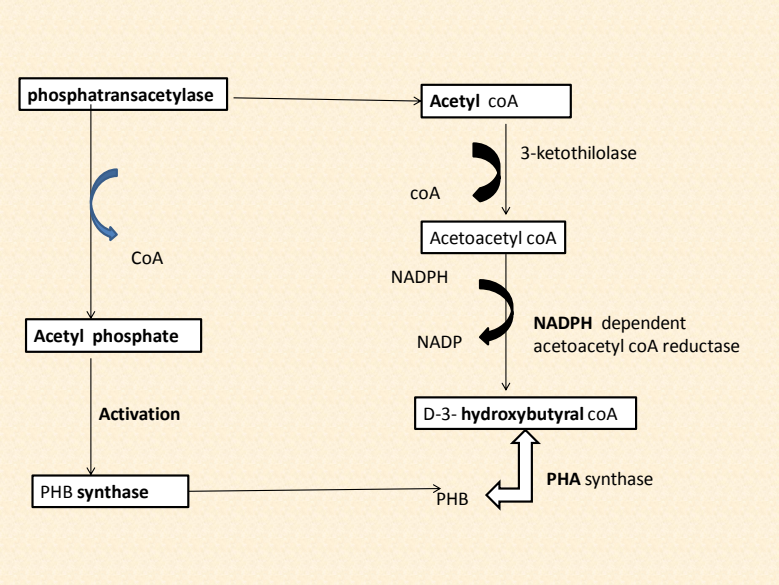

Fig. 10. Regulation in poly hydroxyl butyrate(PHB) metabolism

During PHB accumulation, in the first step acetyl-CoA flux increases because of reduced amino acid synthesis derived from nitrogen starvation and phosphor acetyltransferase activity also increases. Increase concentration of acetyl phosphate, activates PHB synthase to synthesize PHB (Asada et al., 1999).

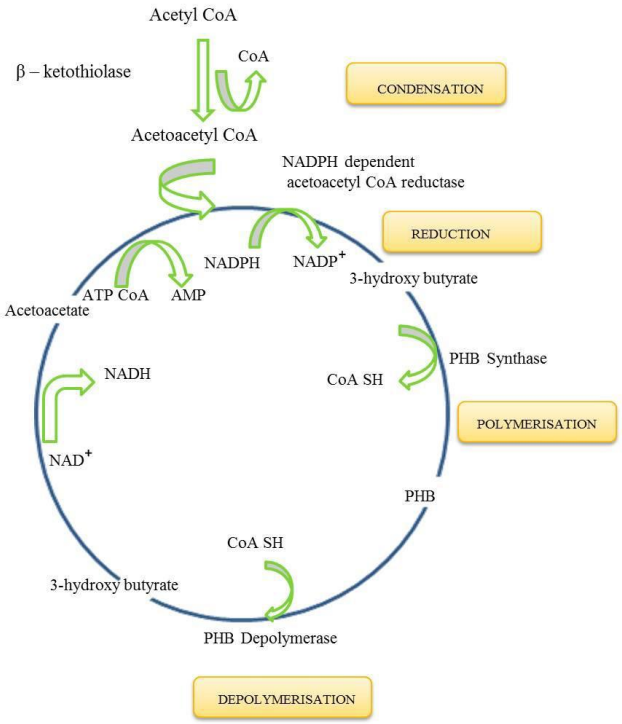

Fig. 11. The cycle representing the synthesis and degradation of poly-hydroxy-butyrate (PHB)

2.3 The history of bioplastic production

Poly-3-hydroxybutyrate was first detected by Lemoigne in 1926 from the Pasteur Institute, France (Lemoigne, 1926; Schubert et al., 1988). Poly3-hydroxybutyrate (PHB) is produced by joining of β- Hydroxybutyrate monomers by ester bonds. Since 1926, over 100 PHAs have been identified from different microbial species present in the environment (DiGregorio, 2009). Until 1980s, scientists were not able to find out any alternative for petroleum based plastics to reduce the pollution. In the late 80s, Anthony Sinskey from Massachusetts Institute of Technology (MIT) and his colleagues successfully isolated the first enzyme 'thiolase' which plays a major role in the biological process to produce bioplastics followed by the discovery of the genes required for the synthesis. The first patent applications of bioplastics were made in 1987 and finally accepted in 1993 (DiGregorio, 2009).

2.4 Microbes as the bioplastic producers

Microbes have been reported to be the potent producers of PHB due to their high adaptability in various extreme environmental conditions. Out of these, *Bacillus* spp., *Pseudomonas* spp. and *Vibrio* spp. are found to be more efficient for PHB production due to their higher stability and reproducibility under environmental stress.

2.5 The recovery of poly (3- hydroxybutyrate) using dispersions of sodium hypochlorite solution and chloroform

A number of solvent extraction processes have been developed to recover PHB from biomass. For example, PHB can be extracted from bacterial cells with methylene chloride, propylene carbonate, dichloroethane, or chloroform.

A less complex procedure is the use of a differential digestion method employing sodium hypochlorite. Although simple and effective, this method has been avoided because it had been reported to cause severe degradation of PHB (Ramsay et al., 1990) reported that by optimizing the conditions of sodium hypochlorite digestion and by balancing the ratio of hypochlorite to non-PHB biomass, PHB of 95% purity with a weight average molecular weight of 600,000 was recovered from *Alcaligenes eutrophus.*

PHB is hydrophobic, while lyophilized cells are hydrophilic. When PHB is isolated from the cell by the action of hypochlorite, it will immediately migrate to the chloroform phase avoiding severe degradation. Chloroform can, at least partially, protect the PHB molecules from further destructive action of the hypochlorite.

2.6 Improving thermal properties bio plastic by blending

PHB yield and the cost of carbon substrate considerably affect the final price of PHB. Development of an efficient recovery method is also important to lower the price of PHB. The cost of carbon source accounts for 70 to 80% of total raw material cost, the price of PHB can be significantly lowered if cheap carbon substrate can be used.

The cost of industrial production of bioplastics is very high in comparison to synthetic plastics now-a-days and basically the cost of production depends on the cost of biomass for fermentation (Sindhu et al., 2011), but at the same time its production in large scale is also essential. So to reduce the cost to some extent blending of PHB can be performed with other polymers. According to other studies, if the ratio of starch blending to PHB is maintained at 30:70 % it would be advantageous to reduce the cost of PHB.

3. MATERIAL AND METHODS

3.1 Bacterial strain used

The bacterial strain used during this was previously isolated and characterized from the solid waste dumping site of NIT, Rourkela (Fig. 12). It was preserved at low temperature under standard conditions and was revived prior to this study. The pure colony of the culture was used during throughout the study

Fig 12.Solid waste dumping sites of NIT, Rourkela

3.2 Optimization of polyhydroxybutyrate (PHB)

3.2.1 Extraction of PHB from carbon sources

Bacillus cereus SE-1 was cultured in Minimal Davis Media supplemented with dextrose as carbon source for 3 days at 37°C at 150 rpm in a rotary shaker. After 3 days of incubation, extraction of PHB was performed following sodium hypochlorite-chloroform method (Fig. 12). 20 ml of culture was centrifuged at 6,900 g for 20 minutes and supernatant was discarded. The pellet was suspended in 2.5 ml of 4 % sodium hypochlorite for digestion and 2.5 ml of hot chloroform and was incubated at 37°C for 1 hour. The suspension was centrifuged at 1500 g for 10 minutes (The upper phase contains hypochlorite solution and the middle phase contains chloroform with cell debris). The bottom phase containing PHA with chloroform was collected and further was followed by extraction with hot chloroform and precipitated with ethanol and acetone (1:1). The precipitate was allowed to evaporate for dryness at 30°C to obtain PHA crystals (Singh and Parmar, 2011).

This method was repeated for other carbon sources like dextrose, lactose, sucrose, maltose, fructose, galactose.

3.3 Characterization techniques

3.3.1 Fourier transform infrared spectroscopy (FT-IR)

Extracted PHB granules were dissolved in isotonic saline solution (30kg/m3) and then 20ml of the solution was deposited on KBr disc. The depositors were then dried and IR spectra was recorded with a Bruker model IFS-55 FTIR spectrometer, at 27°C from 400 to 4000cm-1range, coupled to a Bruker IR microscope fitted with an IBM compatible PC running OPUS, Version 2.2 software

3.3.2 X ray diffraction (XRD)

The crystalline structure of PHA was studied using X-ray diffractograms, recorded on a Siemens D500 instrument with Ni-filtered Cu Kα (40 KV, 40 mA) radiation source employed by powder method. The freeze dried sample was used in a 2mm diameter capillary tube. Every scan was recorded from 10to 60º (for 2θ) in step-by-step mode with intervals of 10º between each scan and the intensities were recorded

3.4. PHB- Thermoplastic starch blends

Thermoplastic starch (TS) was obtained by mixing starch powder, water and glycerol in the composition 50:15:35 (w/v/v), respectively (Ramsay et al., 1993). The contents were mixed for 15-30 min. to obtain a paste. The paste was transformed into TS by heating at 100°C in water bath with continuous stirring for 15 min. This product so obtained was mixed with PHB in the ratios 58:52 (w/w) and 100:20 (w/w) and solvent cast films were obtained from chloroform.

4. RESULTS

4.1 Bacterial isolate

The bacterial isolate used during this study was identified as *Bacillus cereus* by 16S rRNA gene sequencing. It showed positive reaction towards Gram's reaction and has distinguished characteristic feature on petriplate (Fig. 13).

Fig. 13. *Bacillus cereus* SE-1 grown on Luria bertani agar plate

4.2. Extraction of PHB

PHB producing strain showed peculiar characteristics upon extraction by sodium hypochlorite. After cell lysis, sodium hypochlorite remains in the upper layer, cell debris are present in the middle phase and the PHB forms precipitate upon addition of chloroform (Fig. 14).

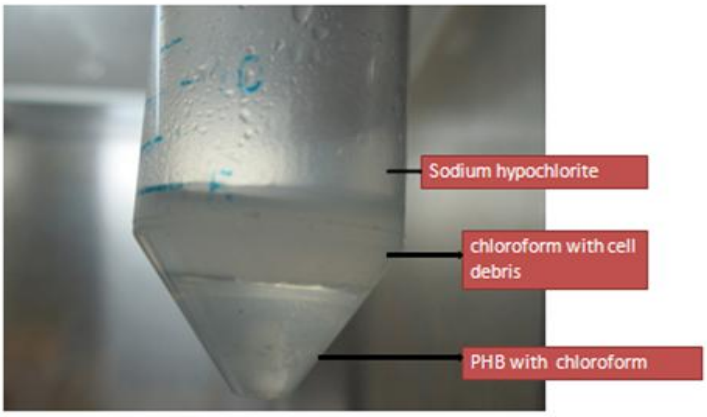

Fig. 14. Three layers obtained during extraction of PHB by sodium hypochlorite method

4.3 Optimization of different carbon sources

Carbon sources used during this study were dextrose, sucrose, maltose, fructose and galactose. After extraction, the PHB were lyophilized and dry weight was taken (Fig. 15). Out of the carbon sources used, in presence of maltose a higher level of PHB production was observed (0.563 g), whereas, a lower level of production was obtained with dextrose (0.04 g). The details of the amount of PHB produced have been given in Table 1.

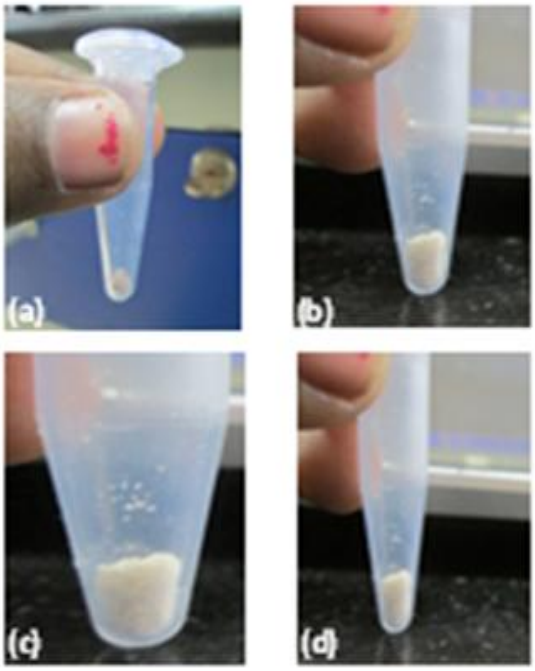

Fig. 15. Production of PHB from different carbon sources like (a) dextrose (b) sucrose (c) maltose (d) fructose

Table 1. Optimization of PHB from different carbon sources

Carbohydrate source	Cell biomass/ 2 ml (in g)	PHB production/ 100 ml (in g)
Dextrose	0.024	0.04
Sucrose	0.026	0.281
Maltose	0.097	0.563
Fructose	0.079	0.361
Galactose	0.074	0.362

4.4 FTIR analysis

The spectrum of FTIR revealed the presence of marked peaks at wave numbers 3410 cm^{-1} corresponding to the hydroxyl (-OH) stretching, C=O stretching at 1728 cm^{-1}aliphatic stretching of carbonyl group of RCOA of that of the PHB. Other absorption bands were found at 1288 cm^{-1} were that of the aliphatic -CH stretching groups. The bands at 1320 cm^{-1} to 1000 cm^{-1} were characteristic to that of carbon oxygen stretch of the esters(C-O-C bond).The strong band at 1100 cm^{-1} was C-O-C stretching and 1650 cm^{-1} for CH_2 stretching. PHB with different backbones and different chemical groups are previously studied. The result was tabulated (Table 2) and graph shown in Fig.16.

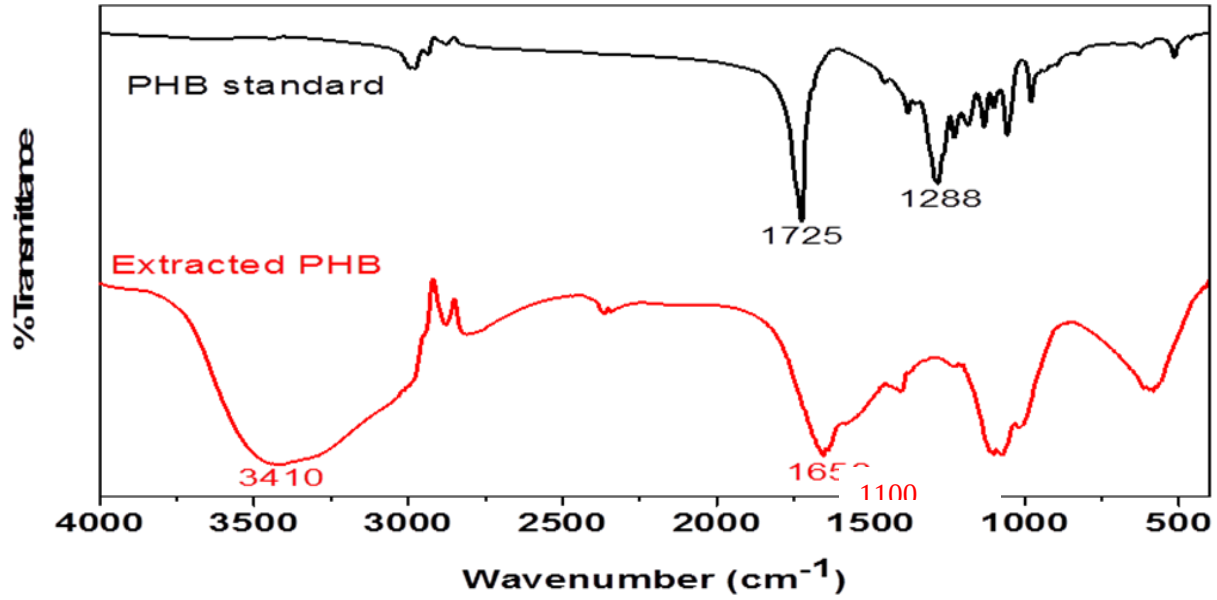

Fig. 16. Showing flourier transforms infrared spectroscopy (FTIR) of standard and extracted PHB. FTIR analysis performed for characterization of extracted PHB from the *Bacillus cereus* SE1 bacteria resulted some peaks showing the presence of functional groups like CH_2, CH and C=O, O-H, which are also present in PHB structure.

Table 2. Functional groups identified by Fourier transform- Infrared spectroscopy analysis of PHB

Wave number (cm-1)	Functional groups
1320–1000	C-O-C stretching of esters
1300–1150	-CH stretching
1470–1450	-CH_3 vibration
1730–1715	C=O stretch of ester
1680–1640	-CH_2 stretching
3500–3200	-OH stretching

4.5 XRD analysis

The XRD studies was carried out to check if the polymer had a crystalline structure or amorphous. On looking at the diffraction patterns of PHB, it showed 2degree values of 26.80°, 31.74°, 45.59°, and 56.22° at the intensities 1800a.u., 3100a.u., 4700a.u., and 700a.u.(Figure: 17). The increased intensity of peaks showed that the polymer have more organized packed crystalline structure.

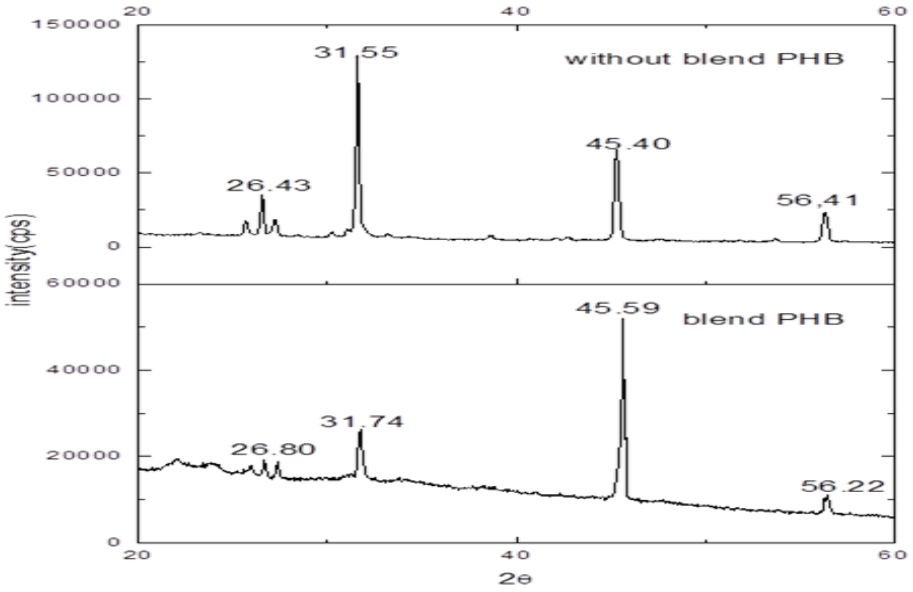

Fig. 17. Showing XRD of pure and blend poly hydroxybutyrate(PHB)

4.6 PHB blend with thermoplastic starch

The PHB blend produced was found to be brittle and breaks easily. Blending PHB with other polymers is an economic way to improve its mechanical properties. To this effect PHB was blended with thermo stable starch. PHB and starch was not completely miscible and the blend showed insoluble particle aggregation on the surface (Fig. 18).

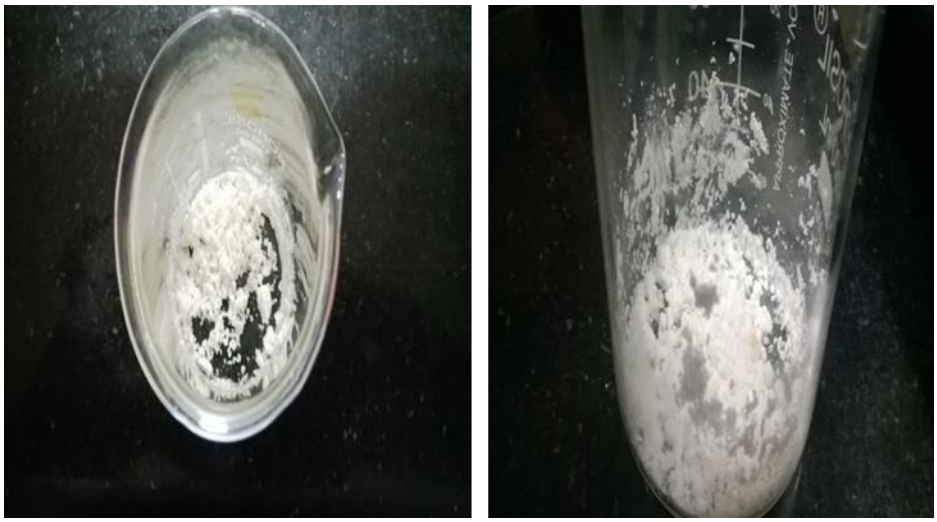

Fig. 18. Thermoplastic starch blend with PHB

5. Discussion

The amount of plastic waste increases every year and the exact time needed for its biodegradation is unknown. Ecological awareness has impelled the development of new biodegradable materials.

Bio-plastics can be defined as plastics made of biomass such as corn and sugarcane. These substances have been increasingly highlighted as means for saving fossil fuels, reducing CO_2 emission and plastic wastes. Biodegradability of bio plastics has been widely publicized in society and the demand for packaging is rapidly increasing among retailers and the food industry at large scale.

Population growth has led to the accumulation of massive volume of non-degradable waste materials across our planet. The accumulation of plastic waste has become a major concern in terms of the environment (Saharan and Badoni, 2007). Conventional plastics not only take many decades during decomposition, but also produce toxins while degradation. Hence, there is need to produce plastics from materials that can be readily eliminated from our biosphere in an "eco-friendly" fashion (Gross and Kalra, 2002). Bioplastics are natural biopolymers synthesized and catabolized by various organisms. These get accumulated as storage materials in microbial cells under stress conditions. However, the high production cost and the availability of low-cost petrochemical-derived plastics led to bioplastics being ignored for a long time.

Poly (3-hydroxybutyrate) (PHB) is a homopolymer of 3-hydroxybutyrate, partially crystalline with a high melting temperature and a high degree of crystallinity. PHB is a thermoplastic and one of the most widely investigated members of the family of poly hydroxyalkanoates (PHAs).

During this work, dispersions of sodium hypochlorite solution and chloroform were used in the recovery of microbial PHB. The treatment with hypochlorite alone caused such severe degradation and the molecular weight decreased drastically with increasing hypochlorite. Different carbon source were affected on amount of bacterial growth and PHB extraction.

6. Conclusion

Using conventional plastics comes with a multitude of drawbacks: the large amount of energy that is required to produce the plastic, the waste that is a result of plastic production, and the use of materials that do not biodegrade readily. In order to shift the production of plastics towards a more sustainable path, research is being conducted to determine the types of renewable bioplastic resources that could be converted into plastic form.

PHB was extracted from different carbon sources (e.g. dextrose, lactose, fructose, maltose, galactose), where maltose produce more amount of PHB (0.562 g /100 ml culture). Characterization of extracted PHB was carried out by FT-IR, and XRD. Results showed that the bacterial culture accumulated about 7.75g of PHB was extracted from different carbon sources. The extracted PHB was blended with thermoplastic starch to improve its physical characteristics.

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