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EFFECT OF CARBON, NITROGEN, PROTEIN SOURCES AND INCUBATION TIMES AND TEMPERATURE ON POLYHYDROXY BUTYRATE SYNTHESIS BY *BACILLUS SUBTILIS* MTCC 1790.

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ABSTRACT

Polyhydroxybutrate(PHB), polyhydroxyalkanoate a polymer belonging to the polyesters class that was first isolated and characterized in 1925 by Indian microbiologist Abhilash singh. To study of this paper identified the PHB granules in may be either renewable(based on agricultural, plants or animal products) or synthetic. There are four types of biopolymer based respectively on starch, sugar, cellulose and synthetic materials. The maximum growth of *Bacillus subtilis KO* strain was from 0.608 to 0.780g/10ml broth medium recorded at molasses concentration of 10%w/v at 45˚calcias and 24hrs.Inoculation period when molasses broth medium supplement with 0.1%w/v of ammonium sulphate as a nitrogen source. Our findings confirm the fact that *Bacillus subtilisKO* strain succed to growth strongly in the absence of any of the inorganic nitrogen source supplied to the molasses broth medium. *Bacillus subtils MTCC1790* culture was used for the production of PHB a different media composition. A number of *Bacillus subtilis* have been reported to accumulate 9 to 67% cell dry weight(CDW) of PHB. Anaerobic system also have the added benefit of potentially co-producing other valuable by products like ethanol, lactate, succinate or hydrogen.

KEYWORDS: Polyhydroxybutarate(PHB), Polyhydroxyalkanoate(PHA), *Bacillus subtilis.*

INTRODUCTION

The extensive usage of petrochemical plastics due to their versatile properties especially durability is causing serve problem in waste management affecting the aesthetic quality of

cities, water bodies and natural areas by Full et.al. 2006, As a result, lot of research is now focused on the production of biodegradable plastics, polyhydroxyalkonoates(PHAs) are the only naturally occurring polymers that are 100%biodegradable it defined to Khanna and Srivastava, 2005.

Gram positive bacteria such as *Bacillus.sp* are ideal candidates for industrial scale PHA production due to the lack of LPS layer. Member of this genus are known to grow rapidly, posses various hydrolytic enzyme and produce co-ploymers from structurally unrelated carbon sources (Valappil et.al. 2007b Halami, 2007).

The environmental benefits that could occur in replacing conventional polymers with biopolymers may however come at an economic loss (Zinn et.al, 2001; Godbole. et. al 2003).

PHBs are considered strong candidates as they have very similar properties to synthetic polymers, as seen in but degrade completely to water and carbon-dioxide under aerobic conditons(lee,1996).

Plastics can be easily molded into almost any desired shaped including fibers and thin films by C.S.K Reddy, R. Ghai, Rashni V. CKalia.

Excessive molecular size seems to be mainly responsible for the resistance of these chemicals to biodegradation and their persistence in soil for a long time(Atlas,1993). In the recent years there has been increasing public concern over the harmful effects of petrochemical-derived plastic materials in the environment, Nature'sbuilt-in mechanisms and self-regulation ability cannot tackle novel pollutants since these are unfamiliar to it. This has prompted many countries to it. This has prompted many countries to start developing biodegradable plastic.

Kalia. et. al. 2000a, according to an estimate more than 100 million tonnes of plastics are produced every year. The per captia consumption of plastics in united states of America is 80,60kg in the European countries and 2kg in India 40%of the 75 billion pounds of plastics produced every year is discard into landfills.

Incinerating plastics has been one option in dealing with non degradable plastics but other than being expensive it is also dangerous. Harmful chemicals like hydrogen chloride and hydrogen cyanide are released during incineration it disease by Johnstone 1990, Atlas, 1993.

Recycling also presents some major disadvantages as it is difficult sorting the wide variety of plastics material such that is further application range is limited by Johnstone, 1990, Flecter, 1993.

PHB is widely distributed interacellular reserve substance typical of prokaryotes,PHB exists in the cytoplasmic fluid in the form of cytoplasmic fluid in the form of crystalline granules about 0.5mu.m in diameter and can be isolated as native granules or by solvent extraction(1-3). various research have explained that soil bacteria generally produce PHB by determination Belmaaslim.

Poly-hydroxybutyrate(PHB) is an interacellular microbial thermoplastics that is widely produced by many bacteria (Branuegg et, al., 1998) in terms of molecular weight, bittleness, stiffness, melting point and glass transition temperature, the PHB homopolymer is comparable to some of the more common petrochemical more derived thermoplastics such as polypropylene. Therfore in certain application, PHB can directly replace some more traditional non biodegradable polymers (Poirier.et.al., 1995).

The Solution for Plastics

The three types of biodegradable plastics introduced are photodegradable, semibiodegradable and completely biodegradable. photodegradable plastics have light sensitive groups incorporated directly into the background of the polymer as additives. extensive ultra violet radiation (several weeks to month) can disintengrate their polymeric structure rendering them open to further bacterial degradation(Kalia et al, 2000a). However, landfills lack sunlight and thus they remain non-degraded. semi bio degradable plastics are the starch linked plastics where starch is incorporated to hold together short fragments pf polyethylene. The idea behind starch linked plastics is that once discarded into landfills, bacteria in the soli will attack the starch and release polymer fragments that can be degraded by other bacteria. Bacteria indeed attack the starch but are tured off by the polyethylene fragments, which thereby remain non degradable(Johnstine,1990). The third type of biodegradable plastics is rather new and promising because of its actual utilization by bacteria to form a biopolymer. Includedarepolyhydroxyalkanotes (PHA)polylactides (PLA), aliphatic polymers polysaccharides, copolymers and/or blenders of the above.

MATERIAL AND METHODS

Culture Used: *Bacillus subtilis* MTCC1790 culture was used for the production of PHB at different media composition.

Media and Growth Conditions

The strain was grown in nutrient broth culture medium (Atlas, 1997) contained (g/l) peptone, 2.5; Nacl, 2.5; yeast extract, 1.0; beef extract, 0.5. Culture(100ml in 250ml Erlenmeyer flasks) were inoculated with a 2%(v/v) and incubated at 37°C,47°C,57°C with vigorous orbital shaking at 225-250 rpm.

Culture Procedure

Effect of production of PHB in different carbon, nitrogen and protein sources and at different temperature and incubation times.

The ratio of 2% neem oil, castor oil, mustard oil, Ammonium nitrate, Potassium nitrate, Urea, Rice bran, Wheat bran and gingerly fodder cake were added individually at each nutrient medium instead of carbon, nitrogen and protein sources in nutrient broth. To all these different media formulate *B.subtilis* was inoculated and incubated in shaker at 250 rpm for 24h at 37°C,47°C,57°C and also at different incubation time(6,21,25,30,45,48h).

Analytical Procedure

Effect of temperature on growth

This was performed at different temperatures(37°C,47°C,57°C) to study how temperature influence high PHB production.

Effect of Incubation Period on Growth

This was also determined on different broth medium that have used through determination of DCM under different incubation periods (6, 21, 25, 30, 45,48h).

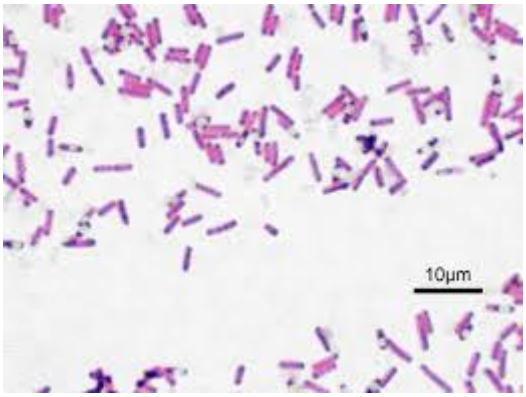
Screening of PHB

- Smear prepared by heat fixing, few drops of 0.25g of Sudan block solution in 60% ehanol was added.
- It was leaved for 5-10 minutes then the slide was decolourised by adding xylene. Finally saffranin was added.
- After 10sec, the stain removed by running water.

- The slide was observed under microscope (oil immersion lens).
- The PHB content was observed after Sudan black staining technique.

Determination of Dry Cell Mass (DCM)

- This was performed by growing Bacillus strain in nutrient broth under tested factors.
- Cells were harvested by centrifugation at 9000rpm for 20minutes.
- The cell pellet was washed with phosphate buffer and recentrifuged.
- After that the cell pellet was dried until constant weight was obtained and DCM was calculated.
- DCM was the mean for determination of optimum growth conditions.



Determination of cells

- 10ml of culture broth was centrifuged at 15,000rpm for 10 minutes.
- It was washed with phosphate buffered saline then air dried for 20 minutes at room temperature.
- 12.5ml of chloroform and 6% sodium hypochlorite was added and incubated overnight at 30°C.
- After incubation it was centrifuged at 8000rpm for 10 minutes.
- After centrifugation, there were 3 phases, on which upper two layers were removed.
- To the third layer, methanol with water (7:3w/w) was added. Then it was centrifuged at 15000rpm for 10 minutes.
- To the pellet 0.1 ml of concentrated sulphuric acid as added.
- They were heated at 100°C in a water bath for 20 min.
- After cooling, the amount of PHB was determined on a spectrophotometer at wavelength of 235nm (Kuniko *et al*., 1989; Bowker, 1981; Ishizaki and Tanaka, 1991).

Electron Microscopic Observation

- To observe electron microscopic view, concentrated sulphuric acid was added to the pellet
- Then heated at 100°C for 1h and incubated for overnight.
- After incubation concentrated sulphuric acid was removed by washing and kept at 4°C then air dried.
- Finally the powered form of PHB was obtained and observed under Electron Microscope.

Table 1: Effect of temperature on the production of PHB of the *B.subtilis* MTCC1790 on media with different carbon, nitrogen and protein sources.

Carbon, Nitrogen and Protein Sources	DifferentTemperature Cell Dry Weight(g/L)		
	37°C	47°C	57°C
Neem Oil	0.266	0.072	0.014
Castor Oil	0.260	0.110	0.091
Mustard Oil	0.194	0.120	0.087
Ammonium Nitrate	0.477	0.311	0.015
Potassium Nitrate	0.173	0.132	0.057
Urea	0.352	0.166	0.105
Rice bran	0.165	0.083	0.072
Wheat bran	0.254	0.204	0.105
Gingerly fodder cake	0.101	0.039	0.023
Nutrient Broth(control)	0.266	0.072	0.014

Table 2: PHB accumulation using different carbon, nitrogen and Protein substrates by *Bacillus subtilis* MTCC1790.

Substrate	Fluorescence intensity
Neem Oil	+++
Castor Oil	ND
Mustard Oil	++
Ammonium Nitrate	+++
Potassium Nitrate	++
Urea	++
Rice bran	+++
Wheat Oil	++
Gingerly fodder cake	ND

+,++,+++ degree of PHB accumulation, ND-Not determined.

RESULT AND DISCUSSION

In this study, production of the *Bacillus subtilis* MTCC1790 strain was detected between 6h and 48h in nutrient broth medium (Fig2). It was determinate that the PHB yield of the strain

increased (18.15%, 14.85%) until 45 th hours. It can be thought that until the sporulation time it produced PHB and used PHB. Spore were produced during the stationary phase of Bacillus cultures and at a time when PHB being produced and consumed (Benoit, 1990; Nam and Ryu, 1985). *Streptomyces griseorubinosus* DBCC 719 isolated was accumulated PHB amounting to 9.5% of mycelial dry mass in the early stationary phase when grown in chemically defined medium with 2%(wt/vol) glucose as the sole source of carbon (Manna *et al*.,1999). Expect 45 th h, others time was a decrease in PHB yield. For this strain, although dry weight increased at 48th h, the decrease of PHB might indicate that the bacteria used PHB as a source of carbon and nitrogen causing an unsuitable condition due to inadequate nitrogen and carbon sources in the medium. The highest level of PHB accumulation was observed in the medium with neem oil as carbon sources, as a nitrogen source, in ammonium nitrate containing medium and in wheat bran medium the maximum PHB was obtained. Thus, we have shown that depending upon the utilized sources of carbon and nitrogen, PHB synthesis may be selectively induced in Bacillus strains.

PHB synthesis in *Bacillus subtilis* MTCC 1790 strain was constantly reaching its peak levelat 24th h. (Nazan and Ugur sidal, 2011). In the present study, the maximum growth rate obtained at 48th h. The decrease in PHB level after a definite time despite the increase in cell number may be due to insufficient carbon and nitrogen levels in the environment owing to the uptake of PHB by the bacteria (Yuksekdag *et al*., 2004).

From the results of factors investigated in the present study an ideal medium and ideal condition for cultivation and growth of *Bacillus subtilis* MTCC 1790 strain was determined and used to further studies. From the data obtained in the present study, it could be recognized that this study may be the unique to use neem oil and mustard oil not only to isolate the bacterial strain *Bacillus subtilis* MTCC 1790, but also to grow and maintain it. Moreover, it used a medium contain only neem oil to produce considerable amount of valuable products with economic importance by *Bacillus subtilis* MTCC 1790(Younis *et al*., 2009).

On the basis of data obtained from this study, *Bacillus subtilis* MTCC 1790 strain may be used for industrial production after the optimization of the PHB synthesis conditions.

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