Research Article

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CHARACTERIZATION OF BIOPOLYMER PRODUCING *STREPTOMYCES PARVULUS*, OPTIMIZATION OF PROCESS PARAMETERS AND MASS PRODUCTION USING LESS EXPENSIVE SUBSTRATES

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Abstract: PHB is attracting much attention as substitute for non-degradable petro-chemically derived plastics because of their similar material properties to conventional plastics and complete biodegradability under natural environment upon disposal. Out of ten actinomycetes isolates obtained from different soil samples, the PHB accumulating isolate was identified by Rapid screening method and based on the morphological, physiological, biochemical and molecular studies, the isolate was identified as *Streptomyces parvulus* RL01 (GenBank Accession no: JX457345. The influence of incubation time, carbon source and nitrogen source on the PHB accumulation was studied. PHB accumulation was maximum in 48 h of incubated in a medium with 1.5% lactose as carbon source and 1% casein as nitrogen source yielded maximum PHB accumulation of 1.7g/L and 2.9g/L respectively. For the production of PHB various inexpensive substrates such as Casava podwer, Coconut oil cake Ground nut oil, Jack fruit seed powder, Potato starch, Rice bran and Wheat bran were used and it was found that PHB production was maximum in whey (2.8 g/L) and minimum in other substrates (1 g/L, 0.1 g/L, 0.2 g/L, 1 g/L, 1 g/L, 0.3 g/L and 0.3 g/L).

Keywords: Polyhydroxybutyrate, Actinomycetes, Rapid screening, *Streptomyces parvulus*.

INTRODUCTION

Modernization and progress had its share of disadvantages and one of the main aspects of concern is the pollution it is causing to the earth- be it land, air and water. The disposal of plastic waste is a growing problem across the country. Most of today's plastic and the synthetic plastics are produced from petrochemicals. As conventional plastics are persistent in the environment, improperly disposed plastic materials are a significant source of environmental pollution, potentially-harming life (Nir *et al*., 1993). The plastic has both environmental and health hazards. There is a need of technical guidelines for protecting human health and the environment from the improper management and disposal of plastic wastes.

Biodegradable polymers or bioplastics are important and interesting areas that are being looked out as alternatives for synthetic plastics. Microbial polymers which has evolved significantly over the past fifteen years and received increased attention for possible application as biodegradable, melt processable and biocompatible (Byrom, 1987). PHAs are the most investigated among biodegradable polymers in recent years. They are superior to other biodegradable polymers because of the large number of different monomer constituents that can be incorporated.

Biodegradable polymer plays a predominant role in the functioning of as biodegradable plastic due to their potentially hydrolysable ester bonds Poly- hydroxy alkanoates (PHAs) are one such polymer produced by a variety of microorganisms and accumulated in the cytosol as Poly-β-hydroxy- butyrate (PHB) during unbalanced growth condition. The microorganisms such as *Bacillus sp, Azotobacter sp, Pseudomonas sp, Rhizobium sp, Escherichia coli, Klebsiella sp, Alcaligenes sp, and Nocardia sp* are known to produce PHB effectively. *Alcaligenes eutrophus* is the prime PHB producer that accumulates PHB up to 80% of its dry weight.

The first PHA detected and studied was PHB. It was first observed by Lemoigne in 1926 as a constituent of bacterium *Bacillus megaterium* (Lemoigne, 1926). Since this first report, PHB accumulation has been found in many microorganisms: representatives of Gram-negative and Gram-positive species (i.e., autotrophs, heterotrophs, phototrophs, aerobes, anaerobes) and archae bacteria**.** Bacteria synthesize and accumulate PHAs as carbon and energy storage materials or as a sink for redundant reducing power under conditions of limiting nutrients in the presence of excess carbon source. When the supply of the limiting nutrient is restored, the PHA can be degraded by intracellular depolymerases and subsequently metabolized as a

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carbon and energy source.

The accumulation of PHA by microorganisms can be stimulated under unbalanced growth conditions, *i.e.*, when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C: N ratio of the feed substrate is higher. PHB is accumulated by numerous microorganisms and is the best characterized PHA (Madison and Huisman, 1999). It is a unique intracellular polymer accumulated under stress conditions but with excess carbon source. During starvation, PHA serves as carbon and energy source and is rapidly oxidized thereby retarding the degradation of cellular components, combating the adverse conditions as in rhizosphere (Okon and Itzigsohn, 1992).

PHA is generally biodegradable, with good biocompatibility, making them attractive as tissue engineering biomaterials. PHAs are high molecular mass polymers with properties similar to conventional plastics such as polypropylene. Therefore, they have a wide range of applications, such as in the manufacture of bottles, packaging materials; films for agriculture and also in medical applications (Oliveira et al., 2004). The main advantage is that, the biodegradable polymers are completely degraded to water, carbon dioxide and methane by anaerobic microorganisms in various environments such as soil, sea, lake water and sewage and hence, is easily disposable without harm to the environment (Brandl *et al*., 1991).

The present was carried out to investigate the ability of some actinomycetes isolates to produce PHB, optimize the growth conditions for maximium PHB accumulation and use of inexpensive substrates for mass production of PHB.

MATERIALS AND METHODS

Soil samples were collected from gardens of CMS College of Science and Commerce, Coimbatore.10.0 g sample was weighed out and mixed with equal amount of $CaCO_3$. The mixture was then transferred to a sterile petri plate moisturized with wet filter paper discs and incubated at 28° C for 7 days. Following the incubation, 1.0g of the sample mixture was weighed out, transferred to 99.0ml of sterile distilled water and boiled at 48° C for 5 minutes. The sample was serially diluted, plated onto Starch Casein agar medium using spread plate technique and incubated at 28° C for 3 to 5 days.

Rapid screening of native bacterial isolates for PHB production:

Growth on nutrient agar with 1% glucose: Nearly 10 Actinomycetes isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan black B dye (Juan *et al.*, 1998). Nutrient agar medium supplemented with 1% glucose was poured into sterile petriplates and allowed for solidification. The plate was divided into 6 equal parts and in each part an actinomycete isolate was spotted. The plates were incubated at 30°C for 24 h.

Following incubation ethanolic solution of (0.02%) Sudan black B was spread over the colonies and the plates kept undisturbed for 30 min. They are washed with ethanol (96%) to remove the excess stain from the colonies, and the results were observed. From the result the soil isolate which showed positive result for PHB production was taken for further studies.

Identification of PHB producing soil isolates by phenotypic methods:

Growth on Nutrient agar with 1% Nile Blue A (Ostle & Holte, 1982): Nutrient agar was prepared with 1% Nile blue A and dispensed in sterile Petri plate and bacterial isolate was streaked. The plates were incubated at 30°C for 1-2 days. The appearance of colonies was noted.

Characterization of selected soil isolates:

After 24 h of incubation the colony showing positive result for PHB production was inoculated onto nutrient agar slants and incubated for 24 h to study the morphological, biochemical, physiological and molecular characteristics. Bacterial smear was prepared on a clean slides and heat fixed. The smear was treated with crystal violet for 1 min and washed with tap water. Grams iodine was added for 1 minute, washed and alcohol was added and left for 30 s. Finally, safranin was added and left for 45 s washed, air dried and the smear was observed under microscope. Biochemical tests such as indole test, methyl red test, voges proskauer test, citrate utilization test, urease test, catalase test, oxidase test, carbohydrate fermentation test, nitrate reduction teat, hydrogen sulfide production test, starch hydrolysis test, casein hydrolysis test, casein hudrolysis test and tween 20 test were carried out.

Molecular characterization:

16S rDNA amplification: The genomic DNA was isolated by using Marmur's method given for gene sequencing in Sri Gowri Biotech Research Academy, Thanjavur. This purified DNA was used in PCR for amplification of 16S rDNA gene. The primers used for amplification of the bacterial 16S rDNA were

518f (5'- CCAGCAGCCGCGGTAATACG-3') and
800r (5'- TACCAGGGTATCTAATCC-3')

The initial denaturing step was carried out for 5 min at 95°C and 0.25 µl of Taq polymerase was added to the sample and kept in PCR Kit for denaturation at 95°C for 1 min. Primer annealing performed at 56°C for

1 min and primer extension at 72°C for 3 min. This was repeated for 35 cycles. DNA amplification was performed in a thermal cycler with an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 56°C), and extension (3min at 72°C).

Then the reaction mixture was kept at 72°C for 5 min and then cooled at 4°C. The amplified DNA was detected by using gel electrophoresis. Five 5 µl of amplified DNA was added with 1 µl of loading dye and loaded in lane 1. Marker DNA (2 µl) (HIND III digested DNA) was kept in lane 2. The tray was filled with 0.5% Tris-borate-EDTA (TBE) buffer. The gel loaded with sample DNA and marker DNA were placed submerged in buffer. And a potential difference was applied via electric field. After 45 min, the DNA bands present in the gel were visualized by staining with ethidium bromide (10 mg/ml in H_2B) and examined under the UV light. The purified DNA fragments were sequenced using sequencer model ABI 3100 sequencer according to manufacturers' instructions (ABI PRISM 3100 Genetic Analyzer User's Manual).

Optimization

Optimization of time course for PHB accumulation:

Disruption of cells by chemical methods and PHB estimation (Law & Slepecky, 1961): Nutrient broth was prepared in test tubes, sterilized and inoculated with selected soil isolate of Actinomycetes. The medium were incubated at 30°C for 24-96 h and the PHB was estimated at every 24 h interval.

About 5 ml of the culture centrifuged at 10,000 rpm for 10 min at room temperature and the supernatant was discarded. The pellet was suspended in 2.5 ml of sodium hypochlorite and 2.5 ml of chloroform and it was incubated at 30°c for 1 h. The above contents were centrifuged at 1500 rpm for 10 min at room temperature. The upper hypochlorite phase, the middle chloroform containing undisturbed cells and the bottom chloroform phase with PHB were separated using micropipette.

The contents were again centrifuged at 1500 rpm for 10 min at room temperature and the phase other than chloroform with PHB was removed carefully using micropipette. Concentrated sulphuric acid was added to the chloroform phase containing PHB, was then boiled at 100°C in a water bath for 10 min. the absorbance of the samples was read at 230 nm using UV Spectrophotometer. The readings obtained were extrapolated on a standard graph of crotonic acid.

Optimization of Carbon source:

Nitrogen free broth tubes were prepared with different carbon sources (Dextrose, Glycerol, Lactose and Mannitol) of different concentrations (0.5%, 1%, 1.5% and 2%), Sodium chloride (0.3%) and distilled water at pH 7.2. The tubes were inoculated with Actinomycetes isolate. and incubated at 30°C for 24 h to 96 h. The accumulated PHB in the form of crotonic acid was estimated by UV spectrophotometer and the absorbance was read at 230nm. The readings obtained were extrapolated on standard graph of crotonic acid.

Effect of Nitrogen source in PHB production and its optimization:

Broth tubes were prepared of different concentration (0.5%, 1%, 1.5%, 2%) of Nitrogen sources Casein and Peptone, 1% w/v of Carbon sources (Dextrose, Glycerol, Lactose and Starch) and Sodium chloride (0.3%) at pH 7.2 were inoculated with 24 h old cultures of selected actinomycete isolate and incubated at 30°C for 48 h. The accumulated PHB in the form of crotonic acid was estimated by UV spectrophotometer and the absorbance was read at 230nm. The readings obtained were extrapolated on standard graph.

Extraction and estimation of PHB by using less expensive substrates

For the production of PHB various inexpensive agro-industrial residues such as Casava podwer, Coconut oil cake Ground nut oil, Jack fruit seed powder, Potato starch, Rice bran and Wheat bran were used

RESULTS

Rapid screening test for PHB accumulation:

Among 10 isolates of actinomycetes screened for PHB production, only one isolate A3 showed positive result for PHB accumulation. The isolates appeared bluish black in color due to the PHB granules and were taken for further studies.

Identification of PHB producing soil isolates by phenotypic methods:

Growth on Nutrient agar with 1% Nile Blue A (Ostle & Holte, 1982): Actinomycetes isolate formed grey colored colonies on nutrient agar with 1% Nile Blue A indicating that they accumulate polyhydroxybutyrate.

Characterization of the isolate:

Gram staining showed that they are Gram positive long filamentous rods. Biochemical and physiological test results are shown in the table.1.

Table.1: Characterization of selected soil isolate

Sl.No	Biochemical and Physiological Test	Result
1	Indole test	Negative
2	Methyl red test	Positive
3	Voges proskauer test	Negative
4	Citrate utilization test	Positive
5	Urease test	Positive
6	Catalase test	Negative
7	Oxidase test	Positive

8	Carbohydrate fermentation test	Positive
9	Hydrogen sulfide test	Negative
10	Nitrate reduction test	Negative
11	Starch hydrolysis test	Positive
12	Casein hydrolysis test	Negative
13	Tween 20 hydrolysis test	Negative
14	Gelatin hydrolysis test	Negative

Molecular characterization:

The soil isolate was identified as *Streptomyces parvulus* RL01, its evolutionary relationship was studied (fig. 1) and deposited in the GenBank under the accession number JX457345.

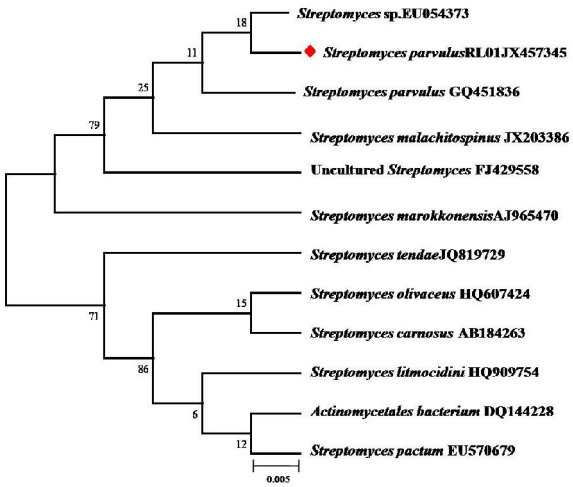

Figure.1: Evolutionary relationship of 12 taxa

Optimization of time course for PHB production:

The intracellular storage granule, PHB was estimated by a conventional method (Kim *et al*.,1994) revealed that the polymer could be converted quantitatively to crotonic acid by heating in concentrated sulphuric acid and ultraviolet absorption was shifted to 230nm. So crotonic acid was used as standard in this study to quantify PHB. In this study it was observed that PHB accumulation was peak at 48 h and declined at 96 h as shown in fig. 2, which might be due to the utilization of PHB storage granules by the microorganism as a carbon and energy source.

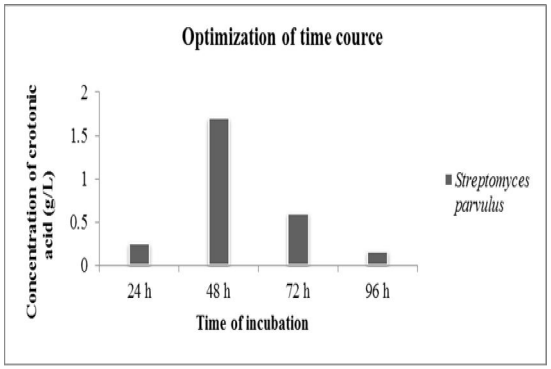

Figure.2: Optimization of time source

Similar studies were reported in *Rhizobium japonicum, Rhizobium cicer* (Nazime *et al*., 2002) and in *Bacillus megaterium* NCIM 2475 (Otari and Ghosh, 2009).

Optimization of Carbon source:

Optimization of carbon source was done for *Streptomyces parvulus* to get a better yield of PHB. The various carbon sources (1% w/v) used were Dextrose, Glycerol Lactose and Starch. In this study *Streptomyces parvvulus* efficiently produced the maximum concentration of 1.7 g/L with 1.5% Lactose when compared to other carbon sources. But at the same time Starch has also shown good results. The effect of different carbon sources in the production of PHB by S. *parvulus* is shown in fig. 3.

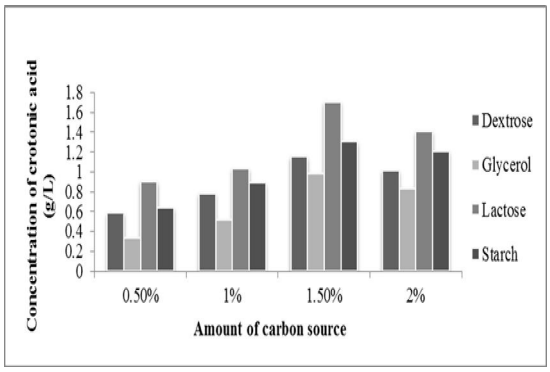

Figure.3: Optimization of carbon source

Poul *et al.* (1990) studied the effect of different carbon sources at a fixed C: N ratio of 15:1. The PHA accumulation was the most significant in the late logarithmic to stationary phase of growth, with the highest quantities of PHB synthesized on malate, as compared with acetate, pyruvate, lactate and fructose.

Effect of Nitrogen source in PHB production:

To increase the yield of PHB from *Streptomyces parvulus,* it is essential to understand the effect of Nitrogen source in the production. In this study different concentration of both Peptone and casein were used to estimate the changes in the amount of PHB produced and it was estimated that medium with casein, carbon sources Lactose and Starch has accumulated PHB of 2.9 g/L and 2.6 g/L respectively. At the same time medium with peptone and the carbon sources Lactose and Starch also accumulated PHB of 1.9 g/L and 2.6 g/L respectively. The effect of nitrogen sources in the production of PHB by *Streptomyces parvulus* is shown in fig. 4 and 5.

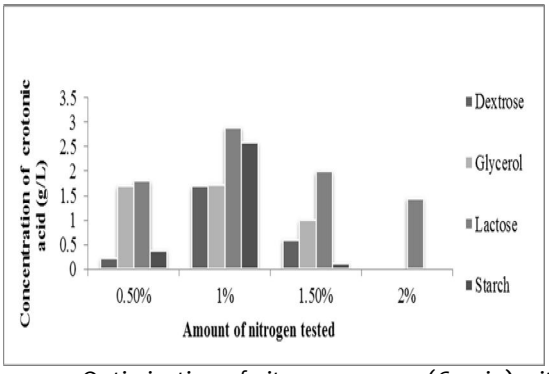

Figure.4: Optimization of nitrogen source (Casein) with carbon source

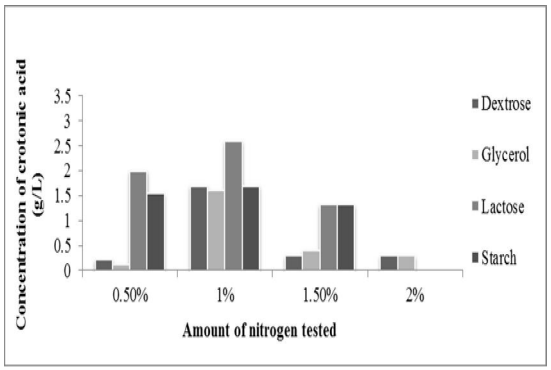

Figure.5: Optimization of nitrogen source (Peptone) with carbon source

From these findings it is clear that medium containing lactose with nitrogen source (casein) can favour *Streptomyces* to accumulate more PHB. Starch with nitrogen also gives desirable results. Similar study was done by Aysel *et al.* (2002). In their study, PHB was produced by a selectant of *Streptomyces* isolates in media containing different nitrogen sources (KNO_3, glycine, peptone, proteose peptone, L-asparagine, tryptone and malt extract), each combined with 2 g/L glucose as the carbon source. The most effective PHB production was observed on glycine/glucose medium with a 7.6% dry mycelial weight. Nitrogen limiting conditions were inhibitory to *Streptomyces* growth, but stimulated PHB accumulation.

Extraction and estimation of PHB by using less expensive substrates:

Streptomyces parvulus showed maximum accumulation of PHB in concentrated whey (2.8 g/L) and remaining less expensive substrates such as Casava podwer, Coconut oil cake Ground nut oil, Jack fruit seed powder, Potato starch, Rice bran and Wheat bran (1 g/L, 0.1 g/L, 0.2 g/L, 1 g/L, 1 g/L, 0.3 g/L and 0.3 g/L) showed minimum accumulation. The results are shown in fig. 6.

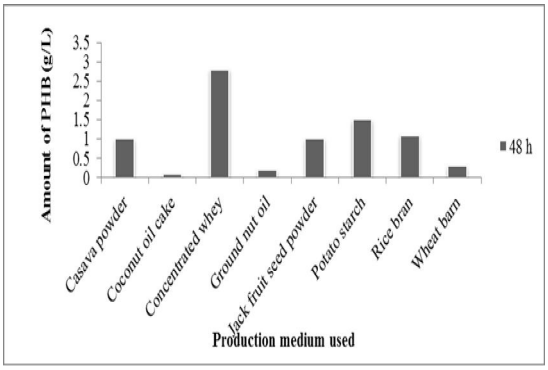

Figure.6: Estimation of Production medium for PHB production

DISCUSSION

In the present study maximum accumulation was attained in a time period of 48 h. In a study done *Agrobacterium radiobacter* by Klüttermann *et al.* (2002) to optimize the time period for maximum accumulation of PHB, the highest PHB level (60% PHB of cell dry weight) was achieved in the stationary growth phase (after 96 h). This shows that the time course is different for different organism and it cannot be generalized for all. It may depend on their individual physiological requirements of the organisms. Steinbuchel and Pieper (1992) studied the production of PHB-PHV copolymer by *R. eutropha* strain R3 under nitrogen limitation. PHA contents were 47%, 35.7%, 29.5%, 21.5% and 43.2% when fructose, gluconate, acetate, succinate and lactate were used as carbon sources, respectively. In a nitrogen limiting condition, in the present study, PHB production was maximum in medium with 1.5% carbon source.

From the present investigation, it is clear that medium containing lactose with nitrogen source (casein) favoured *Streptomyces parvulus* to accumulate more PHB. Starch with nitrogen also gives desirable results. Similar study was done by Aysel *et al.*, (2002). In their study, PHB was produced by a selectant of *Streptomyces* isolates in media containing different nitrogen sources (KNO_3, glycine, peptone, proteose peptone, L-asparagine, tryptone and malt extract), each combined with 2 g/L glucose as the carbon source. The most effective PHB production was observed on glycine/glucose medium with a 7.6% dry mycelial weight. Nitrogen limiting conditions were inhibitory to *Streptomyces* growth, but stimulated PHB accumulation. Studies on effect of nitrogen and carbon sources on production of PHB are also done by Godbole *et al.* (2000) in *Alcaligenes eutrophus.*

Studies have been done in *Rhizobium* and it was estimated that *Rhizobium* can only produce 0.1 g/L of PHB in YEM medium (Nazime *et al.*, 2002). From the present study it is determined that *Streptomyces parvulus* can accumulate a good amount of PHB in

whey. Yellore and Desai (1995) used cheese whey to minimize the cost of production of PHB using the newly isolated *Methylobacterium*, approximately 45% PHB (w/w) was produced.

It is clear that *Streptomuces* parvulus accumulates maximum PHB in medium lactose with nitrogen source casein (1%). Lactose, milk sugar (Carbon source) is the most abundant constituent of whey and it forms at least 78% (w/w) of the whey's total solids. Whey also contains casein as a source of nitrogen. These factors make whey a good substrate for *Streptomyces parvulus* for the accumulation of PHB. Whey also contains both NPN (Non protein nitrogen) and casein as nitrogen sources. Even though the casein coagulation happens during milk coagulation in cheese making, some percentage of the casein will remain in the whey. These reasons explain why whey is a suitable production medium for PHB. Production of poly-β hydroxyl-butyrate by using whey as a medium looks promising, since the use of inexpensive feed-stocks for poly-β hydroxyl-butyrate is essential if bioplastics are to become competitive products.

It is concluded that the parameters such as incubation time, presence of carbon and nitrogen source and their concentration in the growth medium influences the accumulation of PHB by microorganisms. In case of *Streptomyces parvulus* maximum PHB accumulation was attained at an incubation period of 48 h and also when provided with 1.5% lactose as carbon source and 1% casein as nitrogen source. It was previously reported that the presence of nitrogen limits the PHB accumulation but in our study the presence of nitrogen enhanced the accumulation. Maximum PHB production was attained in a medium with nitrogen source and carbon source. This shows that the findings regarding the influence of limitation of nitrogen source on PHB accumulation cannot be generalized for all PHB producing organism. The present study also shows that among the industrial waste concentrated whey act as a very good carbon source for PHB accumulation.

REFERENCES

1. Aysel U, Nurettin, P, Yavuz B, Accumulation of Poly-3-Hydroxybutyrate in *Streptomyces* sp. During Growth with Different Nitrogen Sources. Turkish Journal of Biology, 2002, 26, 171-174.
2. Brandl H, Hanselmann KW, Evaluation and application of dialysis pore water samples for microbiological studies at sediment water inter faces. Aquatic Sciences, 1991, 5, 55-73.
3. Byrom D, Polymer synthesis by microorganisms: technology and economics. Trends Biotechnology, 1987, 5, 246-250.
4. Godbole S, Daginawala HF, Chakrabarti T, Effect of carbon and nitrogen sources on production of poly-?-hydoxybutyrate from whey by *Alcaligenes eutrophus.* In: 59th Annual Conference of Association of Microbologists of India, 2000, p. 59.
5. Juan ML, Gonzalez LW, Walker GC, A Novel Screening Method for Isolating Exopolysaccharide deficient Mutants. Applied Environmental Microbiology, *1998*, 64, 4600-4602.
6. Kim GJ, Lee IY, Yoon SC, Shin YC, Park YH, Enhanced yield and a high production of medium-chain-length poly (3-hydroxyalkanoates) in a two-step fed-batch cultivation of *Pseudomonas putida* by combined use of glucose and octanoate. Enzyme Microbial Technology, *1997*, 20, 500-505.
7. Kluttermann K, Tauchert H, Kleber HP, Synthesis of Poly-β-hydroxybutyrate by *Agrobacterium radiobacter* after growth an DCarnitine. Acta Biotechnology, *2002*, 22, 261-269.
8. Law J, Slepecky, R A, Assay of poly-3-hydroxybutyric acid. *Journal of Bacteriology*, 1961, 20,345-348.
9. Lemoigne M, Products of dehydration and of polymerization of P-hydroxybutyric acid, Bull. Soc. Chem. Biol, 1926, 8, 770-782.
10. Madison, LL, Huisman GW, Metabolic engineering of poly-3-hydroxyalkanoates from DNA to plastic, *Microbial Molecular Biology Reviews*, 1999, 63, 21-53.
11. Nazime, Production of PHB by some *Rhizobium* bacteria, Turkish Journal of Biolology, 2002, 26,215-219.
12. Nir MM, Miltz J, Ram A, "Update on Plastics and the Environment Progress and Trends, Plastic Engineering, *1993, pp*: 75-93.
13. Okon Y, Itzigsohn R, Poly-3-hydroxybutyrate metabolism in *Azospirillum brasilense* nad the ecological role of PHB in the rhizosphere. FEMS Microbiology Reviews, 1992, 103: 131-140.
14. Oliveira, FC, Freire DMG, Castilho LR, Production of Poly (3-hydroxybutyrate) by solid state fermentation with *Ralstonia eutropha*. Biotechnology Letters, 2004, 26, 1851-1855.
15. Ostle AG, Holt JG, Nile blue A as a Fluorescent Stain for Poly-3-Hydroxybutyrate. Applied Environmental Microbiology, *1982*, 44 (1), 238-241.
16. Otari SV, Ghosh JS, Production and characterization of the polymer Polyhydroxy Butyrate-copolyhydroxy valerate by *Bacillus Megaterium* NCIM 2475. Current Research Journal of Biological Sciences, *2009*, 1(2), 23-26.
17. Poul E, Mulard D, Blanc P, Fages J, Homa G, Parelleux A, Effects of partial 'O' pressure and agitation on growth kinetics of *Azospirillum lipoferum* under fermentor conditions. Applied Environmental Microbiology, 1990, 56, 3235-3239.
18. Steinbuchel A, Pieper U, Production of a copolyester of 3-hydroxybutyric acid and 3 hydroxyvaleric acids from single unrelated carbon sources by a mutant of *Alcaligenes eutrophus*. Applied Microbiology and Biotechnology, 1992, 37: 1-6.
19. Yellore V, Desai A, Production of poly-?-hydroxybutyrate from lactose and whey by *Methylobacterium* sp. 2P24. Applied Microbiology, 1998, 26, 391-394.

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