



## Bioplastic (PHB) production by *Vibrio mimicus* isolated from Vellar estuary

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### Abstract

In the present investigation different strains which showed the accumulation of PHB in the nitrogen deficient medium were isolated from sediment samples collected from Vellar estuary (Lat 11°29'24"N and Long. 79°45' 36" E). Among these *Vibrio mimicus* which showed the maximum PHB accumulation was selected for further study. The optimum growth conditions of *V. mimicus* were 36 hrs of incubation period, 35°C, pH-8, 1% NaCl, 4% glucose and 0.03% yeast extract. Mass scale was done by keeping the above ideal parameters; which resulted in 18.6g/L of PHB production on dry weight basis.

**Keywords:** Bioplastic, PHB, PHA, *Vibrio mimicus*, Vellar estuary.

### Introduction

Bioplastics are defined as plastics that degrade without any processing/ facilities and go back to the carbon cycle in the form of basic elements. They are environment friendly new generation polymers. They have a number of advantages over other biodegradable products and synthetic polymers which are petroleum based. The most widely produced microbial bioplastics are PHB, PHA and their derivatives (Witholt and Kessler, 1999). Poly 3-hydroxybutyric acid (PHB) is the most common natural microbial PHA. In some microbial species, accumulation of PHA occurs during the presence of excess carbon and a limitation of nitrogen sources. PHAs produced in response to stressful conditions serve as energy storage molecules to be utilized when common energy sources are absent (Solaiman and Ashby, 2005).

Polyhydroxybutyrate (PHB) is a polyhydroxy alkanate (PHA), a polymer belonging to the polyesters class that are of interest as bio-derived and

biodegradable plastics. The poly-3-hydroxybutyrate is probably the most common type of polyhydroxyalkanoate. Typically, PHAs are intracellular and accumulated as energy storage granules when the microbial cells encountered under nutrient imbalance condition such as a limitations of nitrogen and phosphorus meanwhile also in the presence of excess carbon source (Anderson and Dawes, 1990). PHB is a commonly found substance and readily biodegradable aerobically and anaerobically. Microbes can use PHB. It exists in the cytoplasmic fluid in the form of crystalline granule about 0.5 µm in diameter. -Hydroxybutyrate is connected by ester linkage and form PHB (Verlinden *et al.*, 2007).

Biodegradable plastics are completely degradable and their properties can be easily manipulated. They can be produced from a number of microorganisms preferably bacteria apart from yeast and fungi. Hence

the objective of the present study was to isolate a potential PHB (bioplastic) producing strain from natural marine environment.

## Materials and Methods

### Collection of samples

Soil sample was collected from Vellar estuary (Lat 11°29'24"N and Long. 79°45' 36" E) in Parangipettai. Clean, sterile screw capped bottles were used for collecting and transporting the sample to the laboratory.

### Isolation of PHB producing strain

Soil samples were diluted using standard volume (9ml) of sterile distilled water in series 0.1ml of diluted samples were inoculated by spread plate technique on the surface of Mineral agar medium (g/l): (NH<sub>4</sub>)<sub>2</sub> HPO<sub>4</sub> .4H<sub>2</sub>O- 3.5g, K<sub>2</sub>HPO<sub>4</sub>.3H<sub>2</sub>O- 7.5g, KH<sub>2</sub>PO<sub>4</sub> - 3.7g, MgSO<sub>4</sub> - 0.17g, yeast extract - 0.04g, glucose- 2g, Agar -1.8g, pH - 7 ± 0.2 and 10ml of Microelement stock solution (g/l): contains FeSO<sub>4</sub> .7H<sub>2</sub>O- 2.7 mg, MnCl<sub>4</sub> .4H<sub>2</sub>O- 1.98 mg, CoSO<sub>4</sub> .7H<sub>2</sub>O-2.8 mg, CaCl<sub>2</sub> . 2H<sub>2</sub>O- 0.17 mg and ZnSO<sub>4</sub> .7H<sub>2</sub>O- 29 mg.

### Screening for potential PHB producing species

A total of 10 strains of were randomly selected for screening of PHB accumulation. Strains were grown on Mineral medium. The presence of PHB granules in bacterial cells was primarily identified by staining with Sudan Black B. Specimen was prepared from the well grown culture and was immersed in a filtered solution of 0.3% (w/v) Sudan Black in ethylene glycol. Smear was stained for 10min and the slide was drained and dried. Then the slide was immersed and withdrawn several times in xylene and blot dried with an absorbent paper. Finally, the slide was counter stained for 5min. with 0.5% (w/v) aqueous safranin. After drying, the stained slide was examined in a microscope for the presence of PHB granules. Potential strains were differentiated based on the microscopical observation of PHB granules.

### Identification and confirmation of potential strain

The most potent strain based on highest PHB granule accumulation was identified based on morphological, cultural, biochemical and physiological characteristics based on Bergey's Manual of Determinative Bacteriology (Buchanan *et al.*, 1974).

### Qualitative analysis of PHB producers

The potential isolate was grown in E<sub>2</sub> broth in 50 ml flask, and incubated at room temperature on an orbital shaker at 150rpm. At regular interval growth was observed, the bacterial cells were harvested and the polymers extracted using alkaline hypochlorite reagent and followed by solvent extraction method from the whole cells (Williamson and Wilkinson, 1958).

### Optimization for growth

The shake-flask culture of potential strain was optimized for the effect of different environmental parameters like incubation period, pH, temperature, salinity and different carbon and nitrogen sources on the growth of the potential strain.

Potential strain was tested against various incubation period 0-48 hrs at a interval of 6 hrs, pH (6.0 - 10.0), temperature (25°C - 45°C), NaCl concentrations (0.5%- 2.5%, w/v), different carbon sources like glucose, xylose, sucrose, mannose and fructose. The ideal carbon source was tested at concentrations ranged from 1-5%. Similarly various nitrogen sources like beef extract, ammonium nitrate, peptone, yeast extract and potassium nitrate were tested on bacterial growth. The ideal nitrogen source was tested at 0.01%- 0.05% concentration. The cells were cultivated on a shaker (150 rpm) under aerobic condition until 48 hrs. Growth was assessed for every 6 hrs using spectrophotometer.

### Mass scale production

*Vibrio mimicus* was cultivated in high sucrose (4%) and nitrogen (peptone 0.03%) medium with all the above optimized parameters in 500ml volume in 1L conical flasks in a shaker at 150 rpm for 36hrs which was based on the optimized incubation period. Growth and PHB content were determined at the end of cultivation.

### Quantification of PHB from whole cells

PHB extraction was done as previously. Quantification of PHB was made by the percentage of total cell dry weight. Both the CDW (cell dry weight) and PHB yield were expressed in (w/v) i.e. g/L.

### Determination of cell concentration

Total cell concentration was determined by weighing the cell dry weight (CDW) obtained as follows.

Ten ml culture samples were centrifuged at 12,000 rpm for 15min. at 4°C. The pellet was re suspended in distilled water (10 ml) and centrifuged again for washing. The washed cells were dried at 105°C for 24 hrs in a hot air oven and cooled down. The drying was repeated until constant weight was obtained.

## Results and Discussion

PHB was first discovered in bacteria. It is a unique intracellular polymeric material accumulating under unbalanced growth conditions in a wide variety of bacteria, but with excess carbon sources. It is regarded as source of potentially useful biodegradable natural

plastic since its physical characteristics are similar to those of petrochemical polyesters polypropylene (Sujatha *et al.*, 2005).

In the present investigation different strains showed the accumulation of PHB in the nitrogen deficient medium isolated from water samples collected from Vellar estuary. Screening for the potent strain was done by the maximum PHB accumulation when observed under microscope after staining with Sudan black was selected for further study (Fig. 1) and it was identified as *Vibrio mimicus* (Table 1).

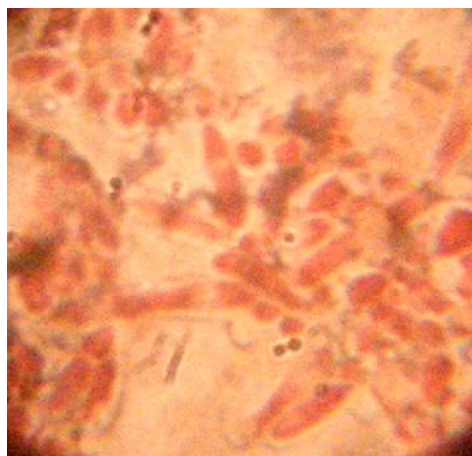


Fig.1: PHB granules microscopic observation (1000X)

Table: 1 Biochemical identification of *Vibrio mimicus*

Tests	Result
Hydrogen sulphide(TSI agar)	-
Urea hydrolyzed	+
Indole	+
Methyl Red	+
Voges- proskauer	-
Citrate	+
Catalase	+
Grams reaction	-
Motility	-
Maltose	+
Lactose	+
Oxidase	-
Nitrogen reduction	+
Simmons citrate	+
Urea broth	+
Sucrose	-
Polymyxin	S

Throughout the experiment mineral medium was used for quantitative analysis of PHB production under of varied incubation period 0-48hrs (at an interval of 6hrs), pH (6, 7, 8, 9 and 10), temperature (25°C, 30°C,

35°C, 40°C and 45°C), salinity (0.5%, 1.0%, 1.5%, 2.0% and 2.5%), and carbon sources (glucose, xylose, sucrose, mannose and fructose), nitrogen sources (beef extract, ammonium sulphate, peptone, yeast extract

and potassium nitrate) and different concentrations of carbon and nitrogen sources (Figs. 2-9).

PHB granules are synthesized and intracellularly accumulated in most bacteria under unfavorable growth condition such as limitation of nitrogen, phosphorus, oxygen or magnesium in the presence of excess supply of carbon source (Lee, 1996 and Du *et al.*, 2001). Strategies are still being developed to simulate conditions for efficient production of PHAs (Du *et al.*, 2001 and Yu, 2001).

In the present study 36hrs of incubation was found to be optimum for maximum growth of *V. mimicus*

(Fig. 2). Sharma and Mallick (2005) found maximum PHB accumulation at 21<sup>st</sup> day of incubation. In this work pH-8 favored maximum growth of *V. mimicus* (Fig. 3) whereas Sharma and Mallick (2005) found maximum PHB accumulation at pH 8.5 (8.9%, w/w of dry cells). Khatipov *et al.*, 1998 observed that PHB accumulation in *Rhodobacter sphaeroides* was significantly enhanced when pH of the culture medium was increased to 7.5 from 6.8. Quillaguam *et al.*, 2006 observed that the pH of the medium was increased during cultivation from 7.5 to about 9.7±0.2. PHB accumulation was found to be the maximum at pH 8.5 followed by pH 7.5. Acidic pH was not found suitable for PHB accumulation (Panda *et al.*, 2006).

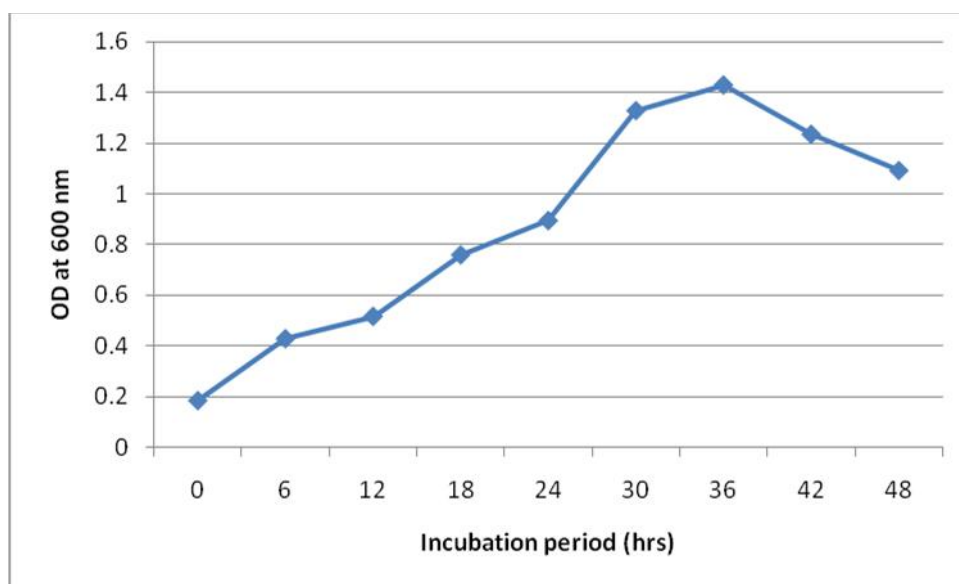


Fig. 2: Effect of incubation period on growth

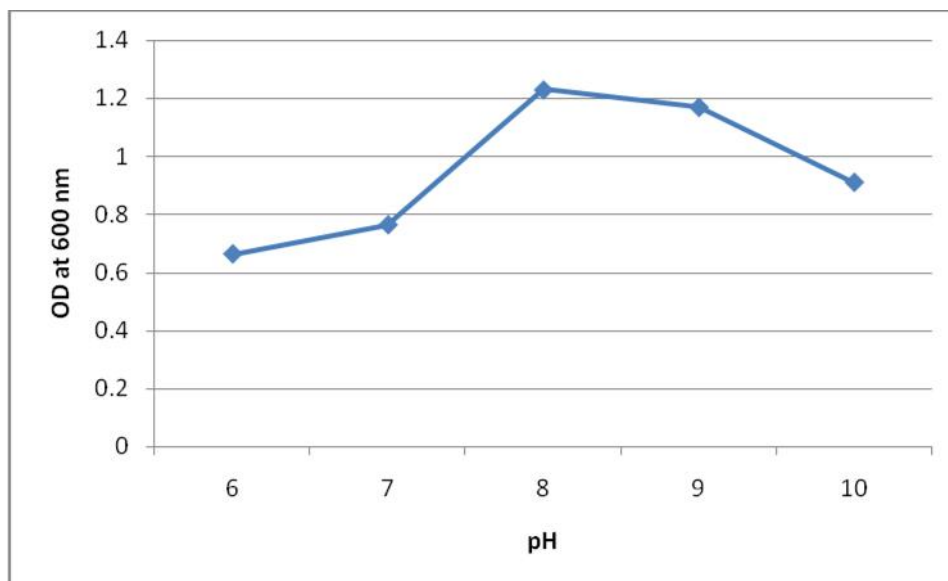


Fig. 3: Effect of pH on growth

In this study temperature 35°C was found to be ideal for the growth of *V. mimicus* (Fig. 4). Flora *et al.*, 2010 observed 33°C as the optimum for growth and PHB synthesis, however over the 25–37°C range, the temperature effect was negligible in view of the reproducibility. It was clear that the temperature range

of 30–35°C was more suitable for PHB production. In the present work 1% salinity was found to be ideal for growth (Fig. 5). Quillaguam *et al.*, 2006 found that the sodium chloride concentration of 0.5–4.5% (w/v) provided the highest cell densities and also the PHB accumulation of about 54%.

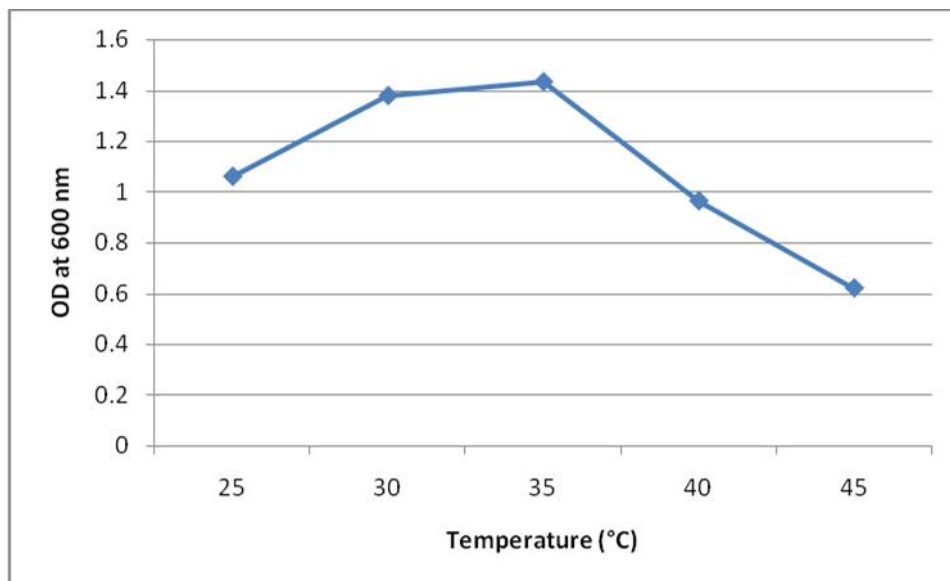


Fig. 4: Effect of Temperature on growth

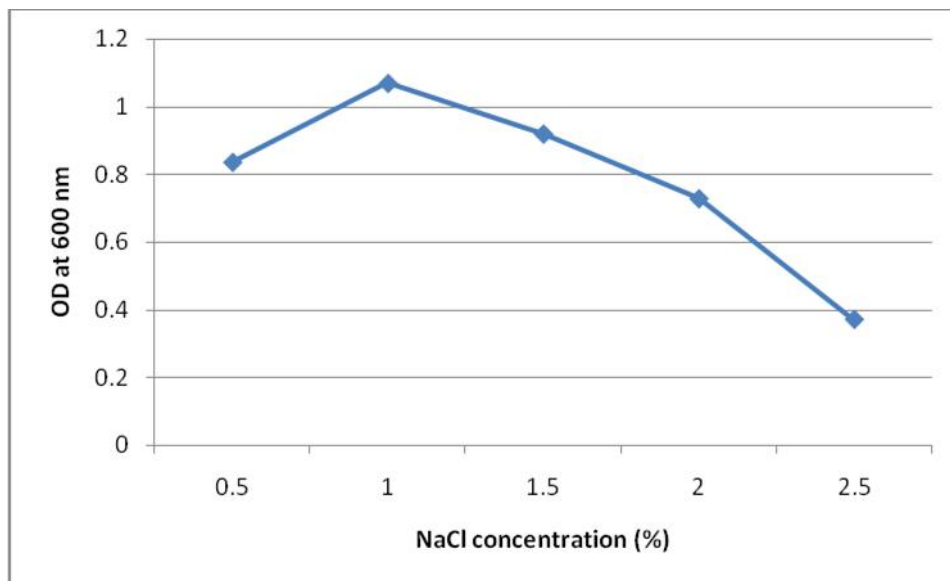


Fig.5: Effect of Salinity on growth

In the present work 4% glucose as carbon source favored 18.6g/L of PHB production (Figs. 6-7) whereas Rohini *et al.*, 2006 observed 0.13g/L of PHB when glucose was used as the substrate. Lee *et al.*, 1995 found that the glucose utilization in cyanobacteria occurs via pentose phosphate pathway

and the stimulation of PHB accumulation in glucose-supplemented cultures could be due to the production of reduced cofactor NADPH. Similar explanation could be valid for fructose- and maltose-supplemented cultures (Sharma and mallick, 2005).

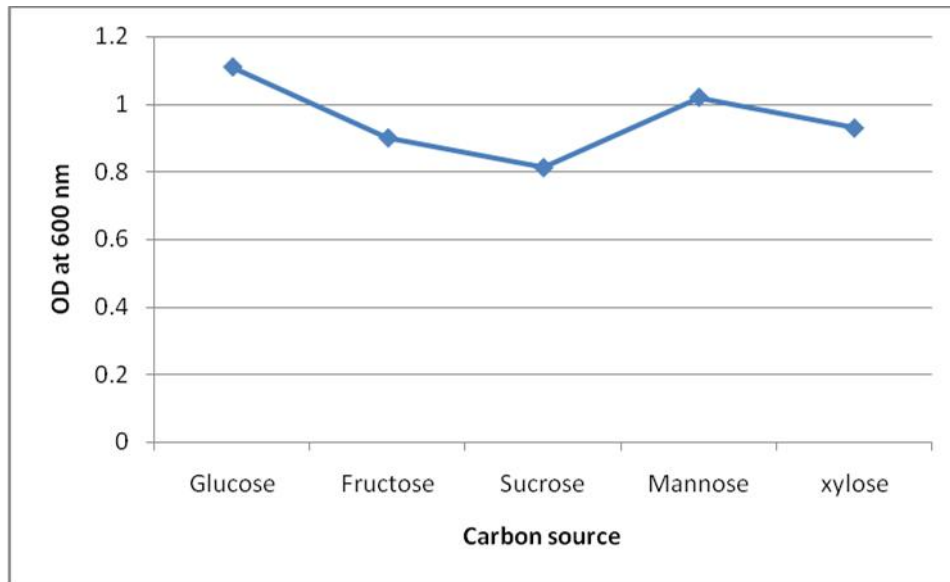


Fig. 6: Effect of Carbon sources on growth

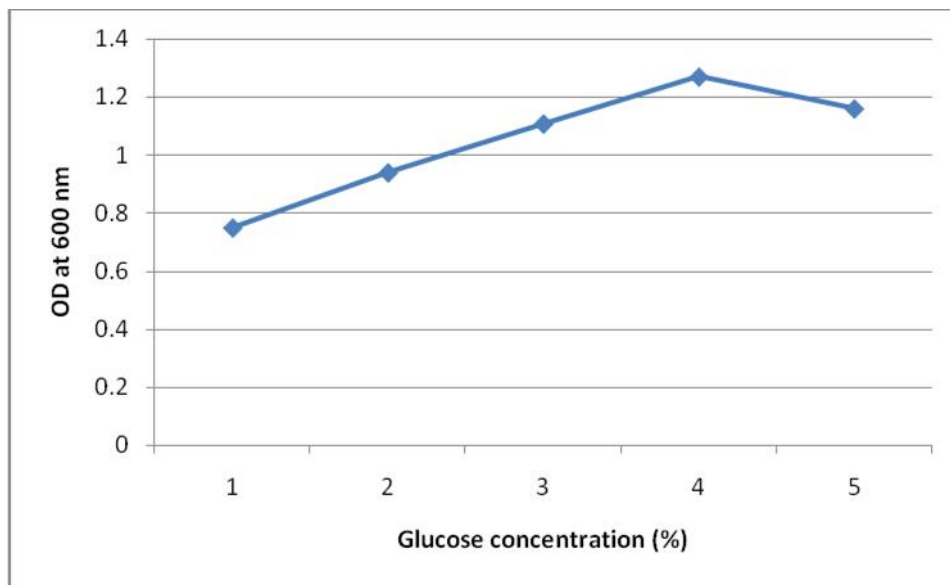
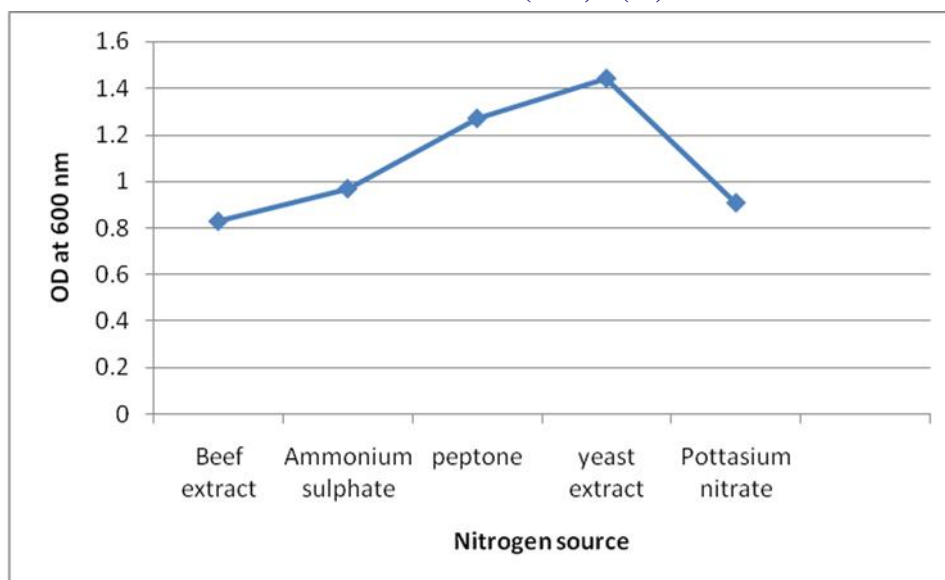


Fig. 7: Effect of concentration of carbon sources on growth

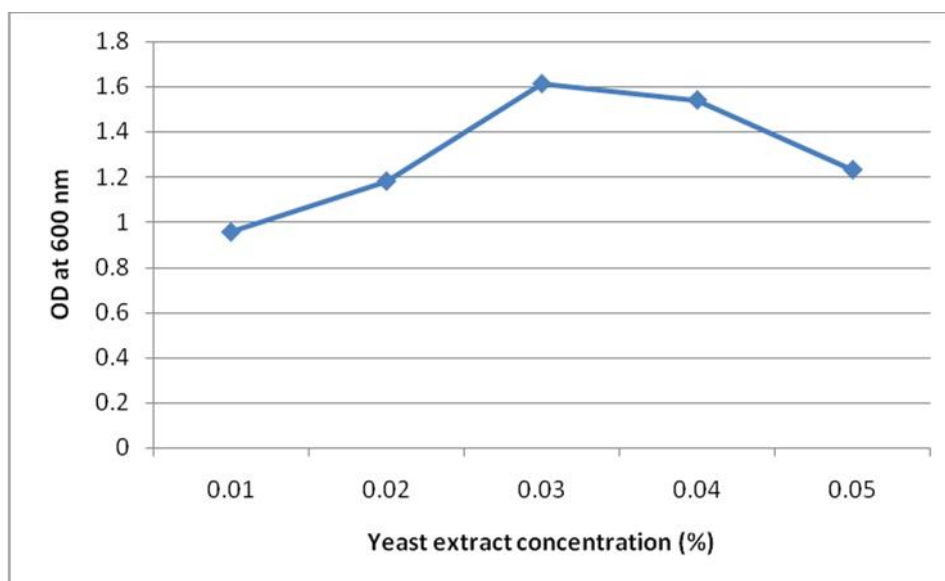
In this work 0.03% yeast extract as nitrogen source were found to be the ideal optimum growth parameters of the potent PHB producing *Vibrio mimicus* strain (Figs. 8-9). Lillo and Valera (1990) found that ammonium chloride as the nitrogen source was found to be the least supporter of PHB production whereas

Khanna and Srivastava (2005) observed the highest PHB production (2.2%) by *R. eutropha* on MSM medium supplemented with ammonium sulphate. Mulchandani *et al.*, 1989 worked on the accumulation of PHB by *A. eutrophus* with different salts of ammonium.





**Fig. 8: Effect of Nitrogen sources on growth**



**Fig. 9: Effect of concentration of nitrogen sources on growth**

In the present investigation, it was observed that PHB concentration was depended on the cell biomass and availability of low nutrient content in the media. It was observed that by using the optimized growth parameters 18.6g/L (dry weight) accumulation of PHB was obtained in mass scale production. PHB production of 16.23 g/L have been reported in a laboratory experiment on the co-culture *Lactobacillus delbrueckii* and *Ralstonia eutrophas* but theoretically under fed-batch fermentation with control of carbon and nitrogen feed rate could be increased up to 40 g/L (Ganduri *et al.*, 2005).

## References

- Anderson, A. and A.E. Dawes, 1990. Occurance, metabolism role and industrial uses of bacterial polyhydroxyalkates. *Microbiol.Rev.*, 54: 450-472.
- Buchanan, R. E., N. E. Gibbons, S. T. Cowan, J. G. Holt, J. Liston, R. G. E. Murray, C.F. Niven, A.W. Ravin and R. Y. Stanier, 1974. *Bergey's manual of determinative bacteriology*. 8<sup>th</sup> ed. William and Wilkins, Baltimore, p1268.
- Byrom, D., 1994. Polyhydroxyalkanoates. In: Mobley, D.P. (Ed.), *Plastics from Microbes: microbial synthesis of polymers and polymer precursors*. Munich, Hanser, pp 5–33.

- Ganduri, V.S.R.K., S.Ghosh and P.R. Patnaik, 2005. Mixing control as a device to increase phb production in batch fermentations with co-cultures of *Lactobacillus Delbrueckii* and *Ralstonia eutropha*. *Pro. Biochem.*, 40, p. 257.
- Khanna, S., and A. K. Srivastava, 2005. Recent advances in microbial polyhydroxyalkanoates. *Process. Biochem.*, 40:607-619.
- Khatipov, E., M. Miyake, J. Miyake and Y. Asada. 1998. Polyhydroxybutyrate accumulation and hydrogen evolution by *Rhodobacter sphaeroides* as a function of nitrogen availability. In: Zaborsky, O.R., (edt.), *Bio Hydrogen*. New York and London: Plenum Press; p 157–62.
- Kim, S., W., P. Kim, H. S. Lee and J. H. Kim, 1996. High production of poly-beta-hydroxybutyrate (PHB) from *Methylobacterium organophilum* under potassium limitation. *Biotechnol. Lett.*, 18: 25-30.
- Lee, Y. W., Y.J. Yoo and J.W. Yang, 1995 Optimization of two stage continuous culture system for production of poly- -hydroxybutyrate. *Kor. J. Chem. Eng.*, 12:481-484.
- Lee, S.Y., 1996. Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.*, 49: 1-14.
- Lillo, J.G and F.R. Valera, 1990. Effects of Culture Conditions on Poly (3-Hydroxybutyric Acid) Production by *Haloferax mediterranei*. *Appl. Environ. Microbiol.*, 56(8): 2517-2521.
- Mulchandani, A., H. T. J. Luong, and C. Groom, 1989. Substrate inhibition kinetics for microbial growth and synthesis of poly- -hydroxybutyric acid by *Alcaligenes eutrophus* ATCC 17697. *Appl. Microbiol. Biotechnol.*, 30: p11.
- Panda, B., P. Jain, L. Sharma and N. Mallick, 2006. Optimization of cultural and nutritional conditions for accumulation of poly-3-hydroxybutyrate in *Synechocystis sp.* PCC 6803. *Bioprocess. Technol.*, 97: 1296-1301.
- Qiullaguaman, J., O. Delgado, B. Mattiasson and R. H. Kaul, 2006. Poly( -hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1. *Enz. Microbiol. Technol.*, 38: 148-154.
- Rohini, D., S. Phadanis, S.K. Rawal, 2006. Synthesis and characterization of poly-bhydroxybutyrate from *Bacillus thuringiensis* R 1. *Ind. J. Biotechnol.* 5: 276–283.
- Sharma, L and N. Mallick, 2005. Accumulation of poly- -hydroxybutyrate in *Nostoc muscorum*: regulation by pH, light-dark cycles, N and P status and carbon sources. *Biores. Technol.* 96; 1304-1310.
- Solaiman, D.K.Y. and R.D. Ashby, 2005. Rapid and specific identification of medium-chain-length polyhydroxyalkanoate synthase gene by polymerase chain reaction. *Appl. Microbiol. Biotechnol.*, 53:690-694.
- Sujatha, S., A. Mahalakshmi and R. Shenbagarathai, 2005. A study on accumulation of PHB in native *Pseudomonas* isolates LDC-5 AND LDC-25. *Ind. J. Biotechnol.*, 4: 216-221.
- Verlinden, R. A. J., D. J. Hill, M. A. Kenward, C. D. Williams and I. Radecka, 2007. Bacterial synthesis of biodegradable polyhydroxyalkanoates. *J. Appl. Microbiol.*, 102:1437-1449.
- Williamson. D.H and J. F. Wilkinson 1958. *F. gen. Microbiol.*, 19: 198.
- Witholt, B. and B. Kessler, 1999. *Curr. Opin. Biotechnol.*, 10:279-285.
- Yu, J., 2001. Production of PHA from starch waste water via organic acids. *J. Biotechnol.*, 86: 105-112.

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