ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 04, pp.29224-29229, April, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

SCREENING AND PRODUCTION OF BIODEGRADABLE PLASTICS FROM MARINE *Bacillus* Spp

***,[1]Subashini, G., [2]Bhuvaneswari, S., [3]Chitradevi, K. and [4]Kanmani, N.**

Department of Microbiology, Shrimati Indira College, Trichy-2

ARTICLE INFO

Article History:
Received 18th January, 2016
Received in revised form
08th February, 2016
Accepted 15th March, 2016
Published online 26th April, 2016

Key words:

Poly-β-hydroxybutyrate,
Bioplastic,
Sudanblack,
Biodegradation,
B. Subtilis,
B. cereus.

ABSTRACT

Plastic is considered an individual gift of modern science and technology to mankind. Nowadays, plastics and synthetic polymers are mainly produced from petro chemical elements, which do not decompose, thus resulting in the environmental pollution. Bacterial plastic is usually defined as an exciting new area of research, where naturally synthesized bacterial polymer, such as the lipid storage material PHB is being used as raw materials for plastic based packaging materials. Poly-β-hydroxybutyrates (PHBs) are one such biopolymer, which are commonly found in soil and synthesized by a broad range of bacteria, during the limitation of nitrogen, calcium, magnesium, iron or essential vitamins. The present investigation includes isolation and identification of marine *Bacillus* sp. from seawater and screening of media components for maximum production of Poly-β-hydroxybutyrates. Twenty *Bacillus* sp. was isolated from seawater. Among 20 isolates, nine showed the maximum Sudan black absorption, which indicated the highest production of Poly-β-hydroxybutyrates. Among nine isolates, 3 isolates were selected best isolates for the maximum production of poly-β-hydroxybutyrates, 2 isolates are *B. Subtilis* and one isolate identified as *B. Cereus.* The highest PHB production was observed at pH 8 by all three isolates, at 40°C for *B. subtilis* and 35°C for *B.cereus*. Glucose was found to be sole carbon source for the production of PHB by *B. subtilis*. Maltose was found best carbon source for the maximum production of PHB for B. *cereus*. The maximum amount of PHB production in ammonium sulphate for *B. subtilis*and and yeast extract for *B. cereus*. *B. subtilis* isolates were produced the maximum PHB at 3% salinity while B. cereus was produced at 2% salinity. Poly-β-hydroxybutyrateis biodegradable plastics. So definitely they can support quality lifespan of all living creatures including human being due to non-pollution environment.

Citation: Subashini, G., Bhuvaneswari, S., Chitradevi, K. and Kanmani, N. 2016. "Screening and production of biodegradable plastics from marine *Bacillus* spp.", *International Journal of Current Research*, 8, (04), 29224-29229.

INTRODUCTION

Plastics materials have become an integral part of contemporary life because of their many desirable properties including durability and resistance to degradation. These non-degradable plastics accumulate in the environment at a rate of millions of ton per year causing several problems. Recently, issues concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics (Anderson *et al*., 1990). Biodegradable plastic is new and very interesting because of its actual utilization of bacteria to form a biopolymer. Bioplastics are a special type of biomaterials, derived from plant sources or microbial sources, rather than traditional petrochemical. Though bioplastics have been made from both plants and microbes, we focus on microbial bioplastics. (Ningthoujam, 2009) Bacterial plastic is usually defined as an exciting new area of research, where naturally synthesized bacterial polymer as, lipid storage material. PHB is being used as raw materials for plastic based packaging materials (Madigan *et al*., 1997). Poly-β- hydroxybutyrate (PHB) (bio plastic), the best-known poly hydroxyl alkanoates (PHA), is an energy and/or carbon storage material synthesized and accumulated by numerous microorganisms (Lee *et al*., 1999). The main advantage of this polymer (Poly-β-hydroxybutyrate) is that, since they are of biological origin, they degrade naturally and completely to CO2 and H2O under natural environment by enzymatic activities of microbes. PHB is considered to be an ideal storage material because of its highly reduced and water insoluble character. Therefore, no osmotic pressure effects are induced inside the cell. PHB is also a family of biodegradable and biocompatible polymers having many interesting properties, such as piezoelectricity and nonlinear optical activity, which may be useful in some high-value applications (Miller *et al.*,

****Corresponding author: Subashini, G.***
Department of Microbiology, Shrimati Indira College, Trichy-2

1987). Advantages of bioplastics, over petroleum based polymers are thermoplastic or elastic properties with melting points ranging from 40ºC to 180°C and degradation in microbe active environments in 5-6 weeks. The released carbon dioxide and water are absorbed during photosynthesis in nature. PHB is fully biodegradable polyester with the very good barrier properties among various available biopolymers, PHB is highly recommendable, since it shows unique chemical and biological properties and its solubility in acidic solutions makes it easily available for industrial purposes. The maximum production of PHB from soil microorganism helps to reduce the petroleum based plastic products mainly for packaging. Especially, when low density and price are taken into account, the design and the use of products that is reusable, recyclable and compostable. Apart from advantages mentioned above, several reasons can be proposed to explain for selecting PHB producing *Bacillus sp.,* because of its ubiquitous presence in seawater, resistance to heat and ability to germinate and grow in favourable conditions.

MATERIALS AND METHODS

Collection of water sample

Water sample was collected from the various parts of Tuticorin coastal area ie 1km and 5km away from the seashore in polyethylene plastic bottles

Isolation of PHB Producing Organisms from sea water Samples

Tenml of sea water was mixed in 90 ml sterile distilled water and shaken vigorously for 2 min. The diluted samples were heated at 60ºC for 30 min in water bath. Then the liquid was serially diluted and plated on nutrient agar medium. The plates were incubated at 37°C for 24–48 hrs. The isolated colonies were selected and sub cultured on minimal agar medium for further studies.

Qualitative Screening for the Production of PHB using Sudan Black Staining Technique (Williamson and Wilkinson, 1958)

The isolated bacterial strains were screened for PHB production. As a preliminary step, screening of PHB producers was carried out using viable colony staining technique. The cultures were grown on minimal media supplemented with glucose (2%) as a sole carbon source, incubated at 40ºC for 48hrs. After incubation, the plates were flooded with Sudan black B solution for the detection of microbial intracellular lipid granules and kept undisturbed for 20 minutes.. Viable colony staining technique was selected in order to reveal the different pattern of Sudan black absorption seen on the agar plates such as Maximum, Moderate and Minimum absorption.

Preparation of Sudan Black B Solution (parshad *et al.*, 2001)

The Sudan Black B solution was prepared by dissolving 0.3 gm of Sudan black B powder in 75 ml of 95% ethanol. The solution was made up to 100 ml with distilled water and mixed thoroughly and the filtrate was stored for further use. The effective PHB producers were selected the optimization studies.

Quantitative screening for PHB producing isolates

The selected strains were grown on minimal broth (pH 7) under standard conditions and incubated at 37°C. During incubation, samples were retrieved after every 24 hrs for 4 days (24-72 hrs) to quantify the production of PHB (µg/ml) by chloroform extraction method.

Extraction of Poly-β-hydroxybutyrate (Ishizaki and Tanaka, 1991)

PHB produced from the selected and standard isolates were extracted by the following procedure. About 10ml of the bacterial cultures (24-96 hours) grown in minimal broth was retrieved at an interval of 24 hrs and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet was suspended with 2.5ml of 4% sodium hypochlorite solution and 2.5ml of chloroform. The pellet suspension was incubated at 30ºC for 1 hour. After incubation, the suspension was centrifuged at 1,500 rpm for 10 minutes. After centrifugation, three phases were obtained. The upper phase consisted of hypochlorite solution which was removed and the middle phase (chloroform containing undisturbed cells) was separated by filtration from the bottom phase (chloroform with PHB). The extracted chloroform phase was used to quantify the production of PHB by measuring the absorbance at 230nm.

Estimation of Poly-β-hydroxybutyrate by spectro photometric method

According to Khanafari *et al.* (2006) the standard curve was derived by preparing the PHB standard solution at different concentration (100-1000µg/ml). About 2ml of concentrated sulphuric acid was added to all the tubes, and kept in boiling water bath for 10 min. for the conversion of PHB into crotonic acid. After cooling, the absorbance was measured at 230 nm using UV spectrophotometer (Systronics 180) and standard graph was plotted. About 2ml of concentrated sulphuric acid was used as blank. Similar procedure was carried out for all the samples. The readings were plotted in standard graph of PHB in the sample were determined.

Optimization of production of PHB

The selected bacterial isolate was grown in 250 ml conical flasks containing 100 ml MSM broth at different pH (5, 6, 7, 8 and 9), different temperature 25°C, 30°C, 35°C, 40°C and 45°C, different time ranging from 24 to 120 h, different carbon sources like glucose, fructose, sucrose, maltose, starch, cellulose, xylose, mannitol, lactose and galactose at 20g/L concentration, different 'N' sources were used like ammonium sulphate, ammonium chloride, ammonium nitrate, peptone, tryptone and yeast extract, all at 1.0 g/l concentration, the different percentage of NaCl such as 1 to 5%. The flasks were incubated at best pH and temperature for optimum time. After incubation, culture was tested for PHB production.

Selection of significant variable for PHB production

The significant variable were selected for PHB production by *Bacillus subtilis*T2 using the Plackett–Burman statistical experimental design. This is a very economical design with the run number a multiple of four and comprises of two level screening designs. This design is extremely useful in finding importance of the factors affecting the production of PHB production

Statistical analysis of Plackett-Burman Design

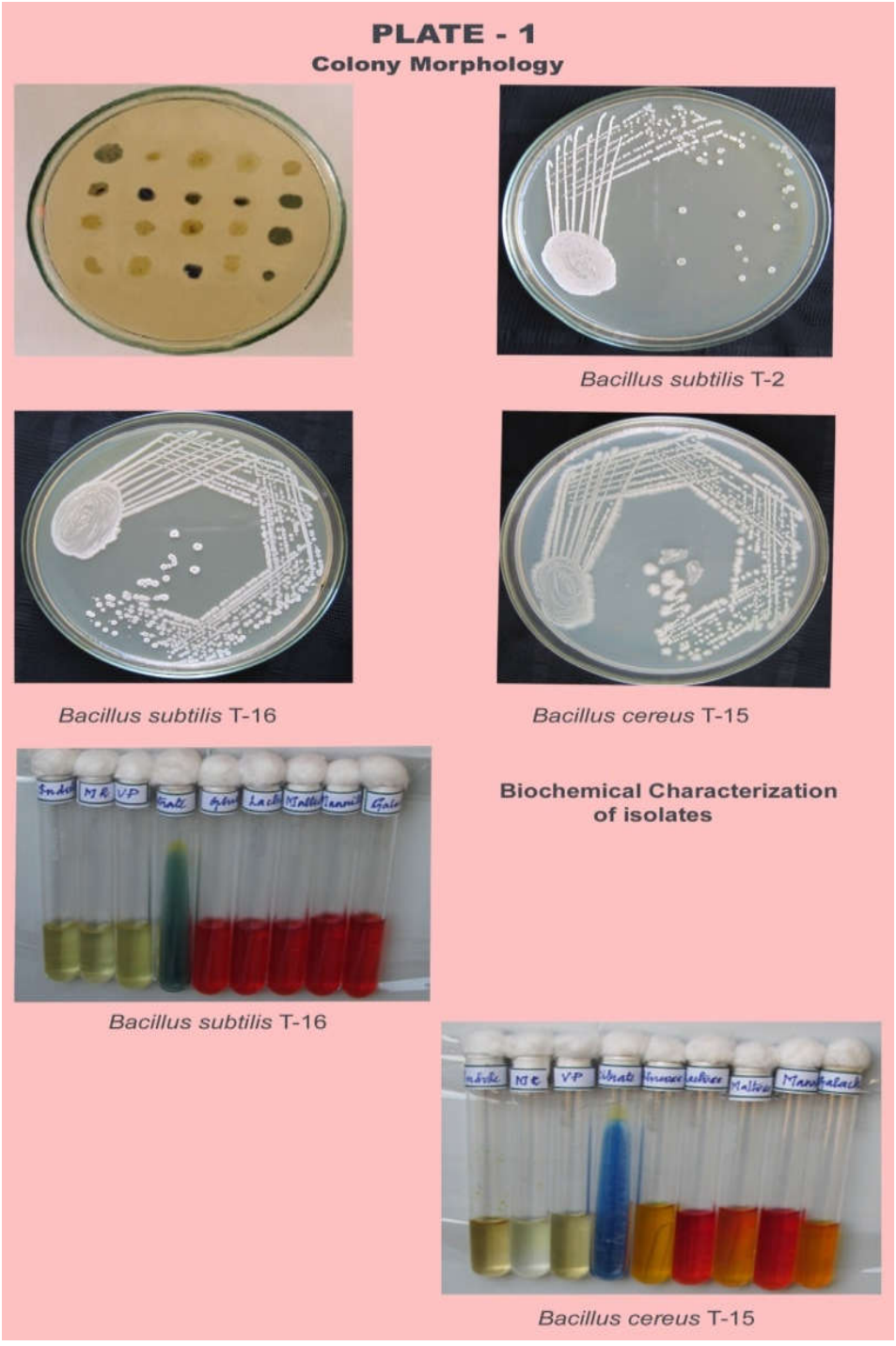
The statistical software package Minitab version 17.0 (Minitab Ltd., Coventry CV3 2TE, UK) was used for analyzing the experimental data. The PB experimental design is based on the first order model using stepwise regression analysis as given in equation 1.

$$Y = \beta 0 + \Sigma \beta i \, xi \ldots\ldots (1)$$

Where, Y is the response (PHB production), β_0 is the model intercept, β_i is variable estimates and X_i are independent variables. The variables whose confidence levels were higher than 95 % were considered that significantly influences the PHB production.

RESULTS

From analysis, *Bacillus* sp. was isolated from seawater collected at Tuticorin. Twenty *Bacillus* sp. were isolated using the heating method to induce spore germination where the samples were heated at 60°C for 30 min in water bath that were likely to destroy most vegetative bacteria without destroying the spores.



Qualitative Screening for PHB Producing Isolates

Table 1.

S.No	Isolates	Sudan black absorption
1	T-1	++
2	T-2	+++
3	T-3	+
4	T-4	+++
5	T-5	++
6	T-6	+++
7	T-7	+++
8	T-8	++
9	T-9	+
10	T-10	+
11	T-11	++
12	T-12	+++
13	T-13	+
14	T-14	+
15	T-15	+++
16	T-16	+++
17	T-17	++
18	T-18	+
19	T-19	+++
20	T-20	+++

Growth pattern (+++) seen on minimal agar plate showed the maximum absorption
Growth pattern (++) showed moderate Sudan black absorption
Growth pattern (+) showed less Sudan black absorption

Table 2.

S.No	Isolates	PHB production ± SEM (µg/ml)
1	T-2	143.00 ± 2.64575
2	T-4	124.67± 1.85592
3	T-6	117.00 ± 1.73205
4	T-7	108.00± 1.73205
5	T-12	98.67 ± 1.76383
6	T-15	158.33 ± 3.75648
7	T-16	137.00 ± 2.64575
8	T-19	122.33 ± 2.02759
9	T-20	117.00 ± 2.08167

SEM- Standard Error Mean

Table 3. Optimization of pH for the production of PHB by *Bacillus* isolates

S.No	pH	PHB production ± SEM (µg/ml)		
		*Bacillus subtilis*T-2	*Bacillus cereus* T-15	*Bacillus subtilis*T-16
1	5	167.00±2.08167	124.00±2.08167	156.00±4.16333
2	6	182.33±1.45297	147.67±1.76383	173.67±2.33333
3	7	212.00±1.1547	181.67±1.76383	190.67±3.4801
4	8	224.33±2.60342	204.00±4.16333	212.67±1.76383
5	9	202.00±2.3094	178.67±1.45297	198.33±2.02759

SEM- Standard Error Mean

Table 4. Optimization of temperature for the production of PHB by *Bacillus* isolates

S.No	Temperature (°C)	PHB production± SEM (µg/ml)		
		*Bacillus subtilis*T-2	*Bacillus cereus* T-15	*Bacillus subtilis*T-16
1	25	127.67±1.45297	113.00±1.52753	128.33±1.66667
2	30	148.67±1.33333	127.67±1.45297	137.33±1.45297
3	35	192.67±1.76383	174.33±2.33333	165.00±2.88675
4	40	226.33±1.85592	162.00±1.52753	202.00±1.52753
5	45	215.67±2.33333	145.67±2.33333	182.67±1.20185

SEM- Standard Error Mean

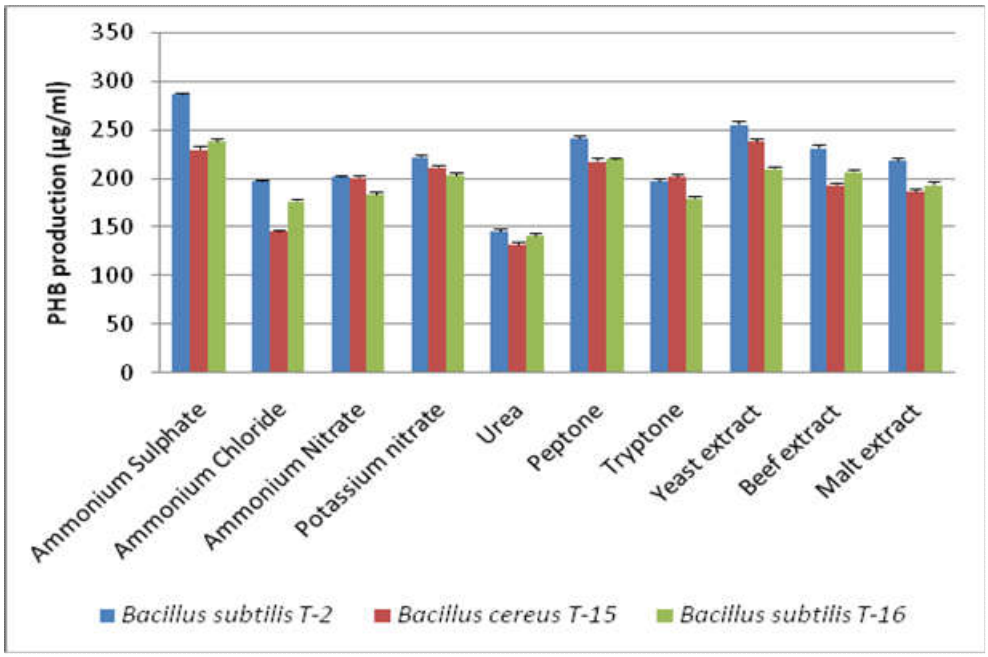


Figure 1. Optimization of nitrogen sources for the production of PHB by *Bacillus* isolates

Table 5. Optimization of carbon sources for the production of PHB by *Bacillus* isolates

S.No	Carbon source	PHB production± SEM (µg/ml)		
		*Bacillus subtilis*T-2	*Bacillus cereus* T-15	*Bacillus subtilis*T-16
1	Glucose	278.67± 2.33333	227.00±1.1547	216.00±1.73205
2	Fructose	153.00± 4.04145	167.67±1.45297	143.00±2.64575
3	Sucrose	281.67± 2.02759	214.67±2.33333	157.00±2.64575
4	Maltose	194.00± 5.1316	252.00±2.3094	182.33±2.02759
5	Starch	200.67± 1.76383	201.33±1.76383	186.67±2.33333
6	Cellulose	232.33± 4.33333	182.33±2.18581	203.00±2.64575
7	Xylose	208.00± 2.3094	154.00±2.88675	194.00±2.88675
8	Mannitol	197.33± 2.90593	101.33±1.76383	182.67±2.02759
9	Lactose	83.00± 2.3094	208.00±2.3094	73.33±2.96273
10	Galactose	176.33± 2.60342	83.33±2.4037	164.33±2.02759

SEM- Standard Error Mean

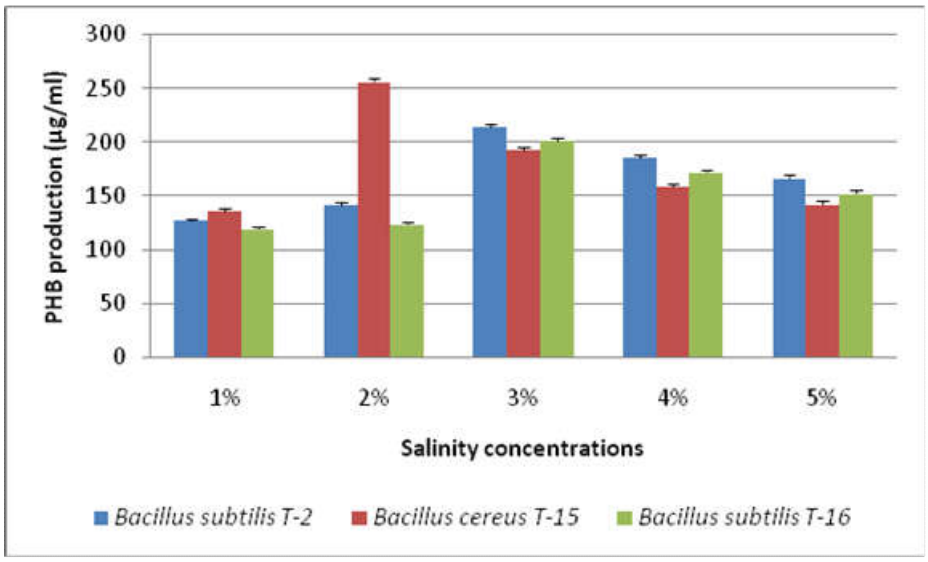


Figure 2. PHB production on different salinity by *Bacillus* isolates

On Nutrient Agar, the colonies usually were circular with smooth edges, brightness, and yellowish white and were convex. Their size varied from small to 4 mm in diameter. The isolates were designated as, T-1 to T-20. (Plate 1) Among 20 isolates, T-2, T-4, T-6, T-7, T-12, T-15, T-16, T-19 and T-20 showed the maximum Sudan black absorption, which indicated the highest production of Poly-β-hydroxybutyrates. The remaining isolates showed moderate and minimum observation of Sudan block in the medium. Among the nine isolates, T-2, T-15 and T-16 were selected best isolates for the maximum production of poly-β-hydroxybutyrates at the rate of 143.00± 2.64575, 158.33 ± 3.75648 and 137.00 ± 2.64575 respectively by chloroform extraction method. The remaining isolates showed less production of Poly-β-hydroxybutyrates. (Table 1,2). Microscopic and biochemical tests performed on the isolates according to an established protocol for the identification of *Bacillus* species given in the Bergey's Manual of Systematic Bacteriology. All isolates were aerobic with straight rods, motile, endospore forming, gram positive, strongly catalase and oxidase positive. All three isolates showed positive results on urease, indole test while VP and citrate utilization showed negative reaction. MR reaction showed negative with T-2 and T-16 isolates while positive reaction with T-15 isolates. *Bacillus* sp. T-2 and T-16 showed positive results of glucose, galactose, mannitol and xylose utilization and tentatively identified as *B. subtilis* T-2 and T-16 while *Bacillus* sp. T-15 showed negative results of above said sugar fermentation test except glucose and tentatively identified as *B. cereus* T-15.

The highest PHB production was observed at pH 8 by *B. subtilis*T-2(224.33±2.60342 µg/ml), followed by *B. cereus* T-15 (212.67±1.76383µg/ml) and *B. subtilis* T-16 (204.00±4.16333 µg/ml).(table3,4) The highest PHB production was observed at 40°C for *B. subtilis* T-2 (226.33±1.85592 µg /ml) and *B. subtilis* T-16 (202.00±1.52753µg /ml) and at 35°C for *B. cereus* T-15 (174.33±2.33333µg /ml). The higher PHB production occurred at 2nddays for *B. subtilis*T-2 (372.33±1.20185 µg/ml) and *B.cereus* T-15(287.67±1.85592µg/ml) and at 3rdfor *B. subtilis*T-16 (320.33±1.45297µg/ml). After those periods, PHB production was decreased.

Glucose was found to be sole carbon source for the production of PHB by both *B. subtilis* T-2 and T-16 at the rate of 278.67± 2.33333 and 216.00±1.73205 µg/ml respectively. Maltose was found best carbon source for the maximum production of PHB for B. *cereus* T-15 (252.00±2.3094 µg/ml).(table5) The maximum amount of PHB production in ammonium sulphate for *B. subtilis*T-2 (287.00±1.52753 µg/ml) and *B. subtilis* T-16

(238.67±2.33333µg/ml) and yeast extract for *B. cereus* T-15 (238.67±2.33333 µg/ml). (Fig. 1) There is gradual increase in production with increase the salinity and after optimal level PHB production was decreased. *B. subtilis* isolates were produced the maximum PHB at 3% salinity while B. cereus was produced at 2% salinity.*B.subtilis* T-2(Fig.2) isolate produced the maximum amount of PHB. T-2 isolate was selected for screening of significant variables such as pH, temperature (ºC), incubation periods (h), glucose (g/L), ammonium sulphate (g/L) and NaCl (%) (fig1) using Plackett–Burman statistical experimental design with stepwise regression analysis. The results indicated that there was a variation of PHB production in the twelve trials in the range from 162.8 to 221.67µg /ml. These variations reflected the importance of medium optimization to obtain higher PHB yield. . Among the factors, glucose, temperature and pH found the most significantly help to maximum production of PHB (Table-5). The regression equation was Y= PHB production (µg/ml) = 190.77 + 7.18 pH + 8.16 Temperature (°C) + 12.20 glucose (g/L) in relation to the PHB production data in this design. Pareto chart of effects was plotted (Figure-9) for identifying the factors that are important for PHB production and showed the factors main effect estimates on the horizontal axis. The chart also showed a vertical line indicating statistical significance ($p = 0.05$).

DISCUSSION

Species of Bacillus pumilus, B. cereus, B. sphaericus, B. subtilis, B. flexus, B. amyloliquefaciens, B. megaterium, B. lentimorbus, B. coagulans, B. licheniformis, B. circulans, isolated from the coastal environment of Cochin, India (Parvathi *et al*., 2009). *Bacillus* sp. was isolated from seawater collected at Tuticorin. Twenty *Bacillus* sp. were isolated using the heating method to induce spore germination where the samples were heated at 60°C for 30 min in water bath that were likely to destroy most vegetative bacteria without destroying the spores. Among 20 isolates, T-2, T-4, T-6, T-7, T-12, T-15, T-16, T-19 and T-20 showed the maximum sudan black absorption, which indicated the highest production of Poly-β-hydroxybutyrates. The growth and the sudan black absorption pattern were viewed from viable colony staining technique. These isolates showed positive for the presence of lipophilic PHB granules observed as dark gray-black colour (Mekala *et al*., 2013). Effect of pH in the medium showed a strong influence on the production of PHB. The highest PHB production was observed at pH 8 by *B. subtilis*T-2(224.33±2.60342 µg/ml), followed by *B. cereus* T-15 (212.67±1.76383µg/ml) and *B. subtilis* T-16 (204.00±4.16333 µg/ml). The finding and the agreement with the work of Goepfert *et al*. (1972) who reported that pH 4.9 - 9.3 permitted the growth of *Bacillus cereus*. The maximum PHB production was obtained at pH range from 6.0 to 7.5 Goepfert *et al*. (1972). Next to the carbon, nitrogen source has to be controlled as nitrogen served as precursor for vitamins, amino acids and growth factors (Saranya and Shenbagarathai 2010). The maximum amount of PHB production in ammonium sulphate for *B. subtilis*T-2 and *B. subtilis* T-16 and yeast extract for *B. cereus* T-15.

The goodness of the fit of a model was checked by the coefficient of determination (R^2) which should be at least 0.80 for the good fit of a model (Guan and Yao, 2008). The present study, *B.subtilis* T-2 isolate was selected for further analysis based on maximum production of PHB. Plackett–Burman statistical experimental design with stepwise regression analysis was used for selecting significant variables

Conclusion

Poly hydroxybutyrate are biodegradable plastics. So definitely they can support quality lifespan of all living creatures including humanbeing due to non-pollution environment.

REFERENCES

Anderson, A.J. and Dewes, E.A. 1990. Occurence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanotes. *Microbiology Rev*., 54:pp450-472.

Goepfert I.M., Spira W.M., Kim H.U. 1972. *Bacillus cereus* food poisoning organisms. *A review* of *Milk Food Technology,* pp. 213.

Guan, X. and Yao, H. 2008. Optimization of viscozyme L-assisted extraction of oat bran protein using response surface methodology. *Food Chemistry*, 106: 345–351.

Ishizaki, J. and Tanaka, 1991. Production of poly beta hydroxybutyrir acid from CO2 by *Algaligenusbutrothcus* ATCC-197697. *Journal of fermentation and Bioengineering*, 71:354-257

Khanafari A., Sepahei A.A., and Mogharab M. 2006. Production of Polyhydroxybutyrate from whey degradation by *Azotobacter. Iran Journal of Environmental Health Science and Engineering*, 3(3): 193-198.

Lee, S.Y., and Choi, J.1999. Production and degradation of polyhydroxyalkanoates in waste environment. *Journal of Waste management,* 19: 133-139.

Madigan M.T., Martinko, J.M. and Parker, J. 1997. Broch Biology of Microorganisms (Bacterial plastics). Prentice Hall International, Ink. London. pp 588-592

Mekala M., Rajendran R. and Suganya K. 2013. Comparative studies on cell growth and production of PHB (Poly Hydroxy Butyrate) *International Journal of Environmental Sciences*, 3 (6): 2261-2276.

Miller, N.D and Williams, D.F.1987. On the biodegradation of Poly-β-hydroxybutyrate (PHB) homopolymer and Poly-β-hydroxybutyrate-hydroxyvalerate copolymers. *Biomaterials*, 8: 129-137.

Ningthoujam, D.S.2009. Bioplastics, Environment friendly biopolymers from microbes, *Science and Environment*, 7: 34-56.

Parshad I., Garson T., Patel J.J. and Wong M.N. 2001. The regulation of PHB metabolism in *Azotobacterbeijernickii. Applied Environmental Microbiology,*125: 75-78.

Saranya V. and Shenbagarathai R. 2010. Effect of nitrogen and calcium sources on growth and production of PHA of *Pseudomonas* sp. LDC-5 and it's mutant. *Curr Res J Biol Sci*., 23:164-167.

Williamson, D.H. 1958. Isolation and Estimation PHB inclusions of *Bacillus sp. Journal of General Microbiology*, 19:198-209.

****