



Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network



RESEARCH ARTICLE

BIOSCIENCE RESEARCH, 2020 17(4): 2551-2557.

OPEN ACCESS

Optimum conditions for β -hydroxybutyrate production by *Azotobacter chroococcum* 9

Suaad Ali Ahmed* and Shatha Salman Hassan

Department of Biology, College of Science, University of Baghdad, Iraq

*Correspondence: suaad_ali82@yahoo.com Received 16-07-2020, Revised: 08-10-2020, Accepted: 19-10-2020 e-Published: 30-10-2020

Azotobacter chroococcum 9 was isolated from corn field in Agricultural College in Abu Ghrab, The effect of different conditions included medium composition, incubation period, pH, temperature and aeration on accumulation of β -hydroxybutyrate from *A. chroococcum* 9 were determined. The maximum productivity appeared when Stockdale nitrogen free medium containing 2% glucose as carbon source and 1% peptone as nitrogen source at pH 7.5 was inoculated with 3.8×10^4 cell/ml and incubated in static incubator at 30 °C for 48 h, PHB productivity at these conditions was 40.3%.

Keywords: *Azotobacter chroococcum*, β -hydroxybutyrate, Polyhydroxyalkanoates

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are bacterial polyesters that have received great interests in recent years. PHAs have been considered to be good substitutes for petroleum derived synthetic plastics showing similar properties as synthetic polymers and furthermore they degrade after disposal (Sharifzadeh Baei, M. et al. 2010).

Polyhydroxyalkanoates (PHA) production by wild type bacterial strains occurs under nutrient depletion conditions. In the PHA production phase, the cell growth is limited owing to the depletion of essential nutrients such as nitrogen, phosphorus, magnesium, among others. This depletion in the presence of excess carbon source triggers the metabolic shift from growth to PHA production phase (Mahmoudi, M et al. 2010).

Poly- β -hydroxybutyrate (PHB) belongs to the class of biodegradable plastics PHAs. PHB was the first among the family of PHAs to be detected by Lemoigne in 1926 as a constituent of bacterium *Bacillus megaterium* (Lemoigne, M. 1926).

The characteristics of this compound is similar to synthetic plastic or petrochemical-based

plastics (such as polypropylene, polyurethane, vinyl chloride and hexachloroethane etc.). Therefore, PHB and its copolymer can be used as Biodegradable plastic, which can reduce the current problems with decreasing fossil resources and environmental impact caused by plastic garbage (Sangkharak, K. and Prasertsan, P. 2008). In addition, it has a promising application in medicine, material science and agriculture, etc. (He, G.Q. et al. 2004). The aim of this study was to obtain an efficient isolate of *Azotobacter* producing PHB and optimization of favorable conditions for PHB production.

MATERIALS AND METHODS

Activation medium (Garrity, G.M. 2005):

This medium is composed of: 0.3 g K_2HPO_4 , 0.7 g KH_2PO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.05 g $FeSO_4 \cdot 9H_2O$, 0.005 g $Na_2MoO_4 \cdot 2H_2O$, 20 g Sucrose and 5 g Yeast extract, pH was adjusted to 7.3.

Sucrose mineral salts medium (Garrity, G.M. 2005):

This medium is composed of : 10 g Sucrose , 3 g CaCO_3 , 0.5 g K_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g CaSO_4 , 0.02 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.01 g MoO_3 , 0.01 g KI , pH was adjusted to 7.2-7.3, sterilized by autoclave at 121 °C for 15 min. For preparing of solid medium 2% agar was added .

PHB Production medium (Stockdale nitrogen free medium) (Stockdale, H. et.al., 1968) :This medium is composed of :

Solution A : 20 g Glucose , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.11 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.012 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$.

Solution B: 2 g K_2HPO_4 , 0.4 g NaCl .

Solution A and B were autoclaved at 121 °C/15 pounds for 10 min. and equal volumes were mixed after cooling ,the pH was 7.7 . Solid medium was prepared by addition 2% agar to the medium.

Collection of sample :

Twenty soil specimens were collected by sterile instrument, these specimens were obtained from soil near to plant roots at depth 10-15 cm and mixed together in clean sterile sacs and transported to laboratory .The bacteria used in the experiments was an isolate of *Azotobacter chroococcum* isolated from corn field in Agricultural College in Abu-Ghrab .

Isolation of *Azotobacter* :

Soil dilutions were prepared by adding 10 g of soil to 90 ml of sterile D.W in 250 ml flask and shaken for 20 min , serial dilutions (10^{-1} - 10^{-8}) were made . One ml from each dilution was used to inoculate Sucrose mineral salts medium (SMS) (Garrity, G.M. 2005), and incubated at 28-30 °C for 3 days. 0.1 ml of liquid culture was spreaded out on plate medium (triplicate plates) , the plates were incubated at 28-30 °C for 3 days. The growing bacterial colonies were purified by streaking on SMS medium for several times . One isolated colony was picked and recultured on maintenance medium.

Estimation of PHB % (Henderson, R. A. and Jones ,C. W. 1997) :

Total dry weight (total biomass) was determined by harvesting of bacterial cells, washing, drying to constant volume and weighting of cells , then PHB was extracted and the content

was estimated as % of biomass after calculated of PHB and bacterial cells dried weight.

$$\text{PHB \%} = \frac{\text{weight of PHB (gram)}}{\text{weight of cell dry mas (gram)}} 100 \times$$

Determination of factors affecting PHB production

In all the experiments mentioned below bacteria was cultured in activation medium and incubated at 28- 30°C for 24h , fifty ml (in 250 ml flasks) of the production medium were inoculated with 2% of activated culture and incubated at different environmental conditions to determine the optimum nutritional and environmental factors for PHB production which included the following :

Inoculum size (cell number)

From 24h activated culture serial dilutions (10^{-1} - 10^{-7}) were prepared , optical density of culture and dilutions was measured at 600 nm .Viable count was performed by spreading 0.01ml of dilutions on activation agar medium , and incubated at 28-30 °C for 24 h , the colonies were calculated and the number of cells was estimated . The production medium was inoculated with 2% of each dilution .

Incubation period

Production medium was inoculated with 2% of bacterial culture and incubated at 28-30 °C for different periods (24 ,48,72 ,96 h).

pH

The production medium prepared with different pH values (6 ,6.5 ,7 , 7.5 , 8 ,8.5) was inoculated with 2% of bacterial culture and incubated at 28-30 °C for 48h.

Temperature

Bacteria was inoculated 2% into the production medium and incubated at different temperatures (25 ,30,37,40 °C) for 48h , pH was 7.5 .

Carbon source

Production media containing 2% of different carbon sources (glucose ,fructose ,sucrose ,lactose , mannitol) ,were prepared then inoculated with bacterial culture and incubated at 30 °C for 48h , pH was 7.5 .

Nitrogen source

The production media containing 1% of different nitrogen sources (yeast extract, meat

extract, peptone ,trypton or casein) , were inoculated with 2% of bacterial culture and incubated at 30 °C for 48h , pH was 7.5.

Shaking

The production medium was inoculated with 2% of bacterial culture pH was 7.5 and incubated at 30 °C for 48h , in shaking incubator (100 rpm) ,other flask was incubated at the same conditions but without shaking .

RESULTS AND DISCUSSION

Isolation of *Azotobacter* :

Twenty samples were collected from different places in corn field in Agricultural College in Abu- Ghrab near the plant roots . Eighteen bacterial isolates were grown on Sucrose mineral salt medium (selective media for *Azotobacter* genus which contain mineral salts without nitrogen source that facilitate the growth of this bacteria). Twelve isolates were belonged to *Azotobacter* depending on the morphological and microscopic examination (Garrity, G.M. 2005).

Effect of inoculum size

It was observed that PHB increases with increasing of cells (initial inoculum) up to 3.8×10^4 cell/ml , the content of PHB was 10.8% of dry cell weight (DCW) (Figure 1)

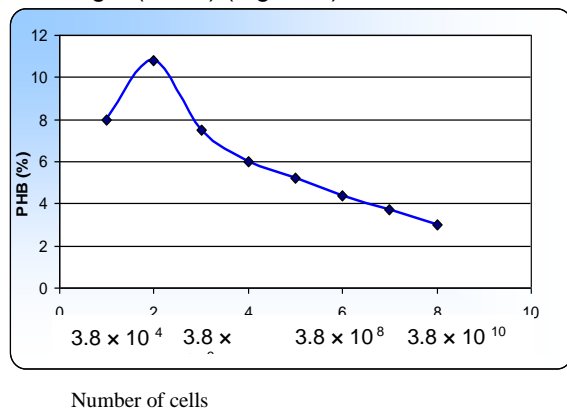


Figure 1 : PHB production by *A. chroococcum* 9 inoculated in Stockdale nitrogen free medium

with different inoculum sizes ,incubated at 28-30 °C for 48 h , at pH 7.7.

The inoculum size is one of the important factors affecting the yield of any product, it represents the number of initial cells to be added to the medium. The optimum inoculum size is depend on the medium composition and concentration of nutrients , furthermore the

cultural conditions. Starter culture (10%) of *R. sphaeroides* N20 containing 1.5×10^4 cells/ml was inoculated into glutamate - malate medium and gave maximum PHB content 53.9% of DCW (Sangkharak , K . and Prasertsan, P . 2008) Ramadas *et al.* (2009) reported that maximum production of PHB by *Bacillus sphaericus* NCIM 5149 was obtained with 2 ml (8×10^8 cell/ml) of preinoculum of 16 h age, which gave about 25% PHB.

Effect of incubation period:

A.chroococcum 9 was cultured in Stockdale nitrogen free medium and incubated for 24-96 h to determine the optimum time for PHB production . Maximum PHB production 19.5% was obtained after 48h of growth (Figure 2).

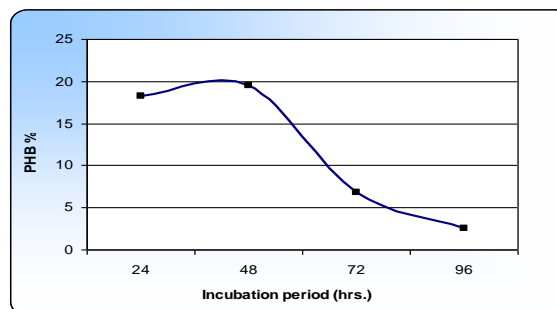


Figure 2 : PHB production by *A. chroococcum* 9 after growing in Stockdale nitrogen free medium at pH 7.7 and incubated at 28-30 °C for different periods of incubation,

The lipid inclusions accumulate by many bacteria when they enter the stationary phase of growth (Page , W. J. 1995) , after 48 h the unfavorable conditions of the medium caused decreasing in PHB yield, due to increasing in medium viscosity accompanying with exopolysaccharide production (Mercan , N. ; Aslim , B etal. 2002) , and depletion of essential nutrients and carbon sources in the medium lead to consuming of PHB by bacteria as a sources of carbon and energy (Yüksekdağ, Z. N. et al.2004).

The maximum content of PHB (74.03%) produced by *Rhizobium* spp. 2426 was obtained after 48h of growth in yeast extract mannitol with L-cysteine medium (Mercan , N. et.al., 2002). Maximum production of PHB by *B. subtilis* 25 (18.03%) and *B. megaterium* 12 (14.79%) was obtained after 45 h of incubation and decreased to (7.98% , 6.55%) respectively after 48 h (Yüksekdağ, Z. N. et al. 2004) .

Effect of pH

A. chroococcum 9 was grown in Stockdale nitrogen free medium with different pH values (6 - 8.5) , the optimum pH for PHB production was between (7-7.5) and maximum PHB production (29.4%) revealed in the medium with pH 7.5 (Figure3).

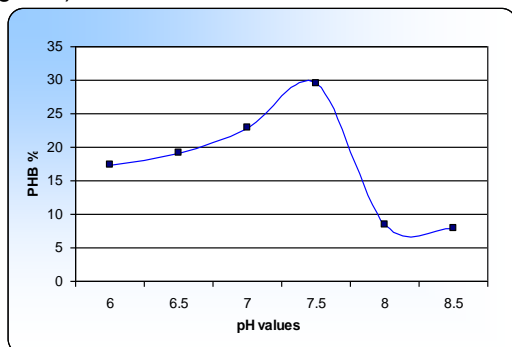


Figure 3 : PHB production by *A. chroococcum* 9 inoculated with 2% in Stockdale nitrogen free medium with different pH values and incubated at 28-30 °C for 48 h

The drastic change in production of PHB in bacteria seems to be due to the effect of initial pH on the bioavailability of trace elements (Ramadas , N. V. et al.2009) , pH change of the environment will change the electric charges of various molecules involved in multiple biological activities such as its effect on the solubility of proteins , enzyme activities and other molecules , pH also affect the permeability of cytoplasmic membrane of cells and metabolic activity of carbohydrates (Yüksekdağ, Z. N. et al. 2004). Maximum production of PHB (46%) by *Bacillus sphaericus* NCIM 5149 was obtained in medium prepared from 2% jackfruit seed powder with initial pH 7.5 (Ramadas , N. V.et.al., 2009) . The PHB productivity of *Rhizobium* spp. 2426 was 40 % at pH 7 (Mercan , N. et al. 2002).

Effect of temperature

The effect of different temperatures (25 , 30 , 37 , 40 °C) on PHB production by *A. chroococcum* 9 were determined , the results showed that maximum PHB production by this bacteria was 32.2% at 30 °C (Figure 4) .

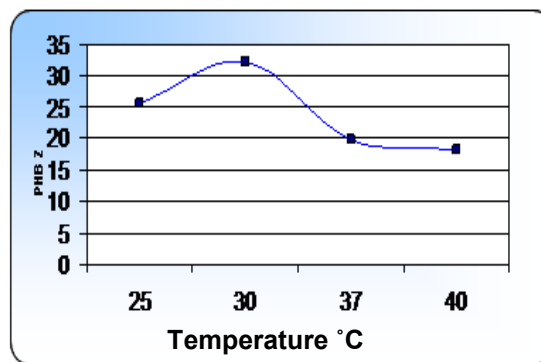


Figure 4 : PHB production by *A. chroococcum* 9 inoculated with 2% in Stockdale nitrogen free medium and incubated at different temperatures for 48h, at pH 7.5

Temperature is one of the environmental factors influencing the chemical reaction , enzymatic activity , growth rate of bacterial cells and fatty acids composition of cytoplasmic membrane (Yüksekdağ, Z. N. et.al., 2004). El-sayed *et al.*(2009) reported that *R.eutropha* ATCC 17697 and *A.latus* ATCC 29712 produce maximal content of PHB 48.43 % and 14.29 % , respectively at 30 °C in two stage batch fermentation . Other bacteria also produce maximum PHB at 30 °C such as *Halomonas boliviensis* LC1 (Quillaguaman, J. et. al. 2005) and *Azohydromonas lata* DSMZ 1123 (Sharifzadeh Baei , M. et al. 2010) .

Effect of carbon source

The effect of different carbon sources on PHB production in *A. chroococcum* 9 was studied. The results showed that glucose gave the highest content of PHB (29.8%) followed by fructose (17.3%), while the disaccharides lactose and sucrose gave lower PHB content (15.9%) and 914.7%) respectively (Figure 5).

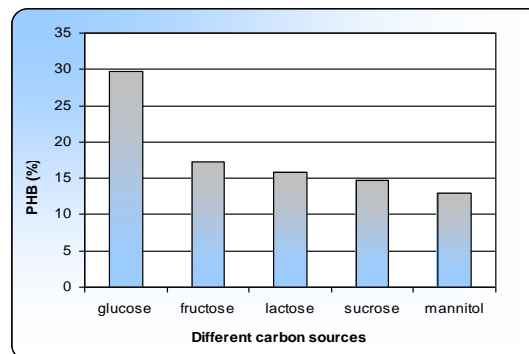


Figure 5 : PHB production by *A. chroococcum* 9 after growing on Stockdale nitrogen free medium containing different carbon sources

and incubated at 30 °C for 48 h at pH 7.7

Azotobacter cells grow well in media containing glucose as carbon source (Lin, L. P. and Sadoff, H. L. 1968). Maximum PHB production by *A. chroococcum* strains NCIB 8002, NCIB8003 and NCIB9125 in nitrogen free medium with 2% glucose was 42.4%, 30.7% and 42.4% respectively (Stockdale, H.; et al., 1968). Beaulieu et al. (1995) reported that PHB yield of *A. eutrophus* in the presence of glucose as a carbon source is 40%, while PHB over 90% of dry cell weight have been reported in case of *Methylobacterium* using glucose and lactose as carbon sources (Ghatnekar, M. S. et al. 2002).

Effect of nitrogen source

Maximum PHB (40.3%) was obtained in medium containing peptone while the inorganic nitrogen source ammonium chloride reduced the accumulation of PHB (Figure 6).

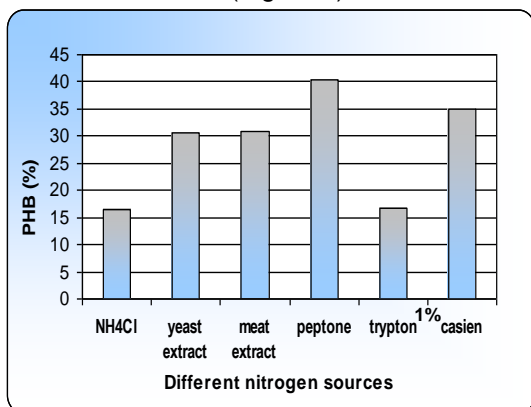


Figure6: PHB produced by *A. chroococcum* 9 after growing on Stockdale nitrogen free medium containing different nitrogen sources and incubated at 30 °C for 48 h, pH 7.5

PHB accumulation by *A. vinelandii*, *A. chroococcum*, *A. beijerinckii* and *E. coli* increased when the organisms were grown in media containing organic nitrogen sources (Page W.J. and Cornish, A. 1993). The effect of different nitrogen compounds (NH₄NO₃, bactopectone, casein, yeast extract, meat extract, protease peptone and trypton) on PHB production showed that incorporation of nitrogenous compounds into the medium increased PHB production by *A. chroococcum* 1723 maximum productivity about 75.7% was observed in the presence of 1% meat extract (Khanafari, A. et al. 2006).

Effect of agitation

It was found that the maximum PHB production occurred without agitation condition,

PHB content was 36.7% in static condition, while in agitation (100 rpm) the yield of PHB was 32%. On the other side the results showed that cellular dry weight of *A. chroococcum* 9 was higher in agitation condition than in static condition. Aeration has different influences on PHB production among different bacteria, *A. beijerinckii* produces PHB under oxygen limitation (Senior, P. J. et al. 1972). Extreme aeration (shaken at 225 rpm) decreased the PHB yield in *A. vinelandii* UWD to 0.4 mg/ml, while non-PHB residual mass was relatively unaffected (Chen, G. and Page, W. J. 1997). Polymer production by *chroococcum* strain H23 decreased at aeration rates from 50 to 300 rpm (Pozo, C. et al. 2002).

CONCLUSION

B-hydroxybutyrate was extracted from *Azotobacter chroococcum* 9. Many environmental and nutritional conditions that effect on production process were examined, included medium composition, incubation period, pH, temperature and aeration. Maximum production yield for B-hydroxybutyrate under the optimum conditions was 40.3%.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

SSH designed the experiments. SAA performed the experiments and also wrote the manuscript. All authors read and approved the final version.

Copyrights: © 2020@ author (s).

This is an open access article distributed under the terms of the [Creative Commons Attribution License \(CC BY 4.0\)](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author(s) and source are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

REFERENCES

- Beaulieu, M.; Beaulieu, Y.; Melinard, J.; Pandian, S. and Goulet, J. 1995. Influence of ammonium salts and cane molasses on growth of *Alcaligenes eutrophus* and production of polyhydroxybutyrate. *Appl.*

- Environ. Microbiol.*, 61(1): 165-169.
- Becking, J.H. 1981. The family - Azotobacteriaceae. In: Starr, M.P. The prokaryotes, vol. 1, Springer - verlag, Berlin, Heidelberg, New York. P. 795-817
- Çetin, D.; Gunduz, U.; Eroglu, I.; Yucel M. and Turker, L. 2006. Poly- β -hydroxybutyrate accumulation and releasing by hydrogen producing bacteria, *Rhodobacter sphaeroides* O.U.001. A transmission electron microscopic study. *African Journal of Biotechnology*. 5 (22): 2069-2072.
- Chen, G. and Page, W. J. 1997. Production of poly- β -hydroxybutyrate by *Azotobacter vinelandii* in a two-stage fermentation process. *Biotechnology Techniques*. 11(5) : 347-350.
- Das, Q.; Chowdhury, J.U. and Anwar, A.N. 2005. Isolation, purification and characterization of biodegradable polymer producing bacteria *Pseudomonas pseudomallei* *International Journal of Agriculture & Biology*. 7(1): 114-117.
- El-sayed, A. A.; Abdel Hafez, A.M.; Hemmat, M.A. and Khodair, T. A. 2009. Production of polyhydroxybutyrate (PHB) using batch and two-stage batch culture strategies. *Australian Journal of Basic and Applied Sciences*, 3(2) : 617-627.
- Garrry, G.M. 2005. *Bergeys Manual of systematic bacteriology*. 2th ed. Vol 2. part B. Springer.USA.
- Ghatnekar, M. S.; Pai, J. S. and Ganesh, M. 2002. Production and recovery of poly-3-hydroxybutyrate from *Methylobacterium* sp. V 49. *J. Chemical Technol. & Biotechnol.* 77: 444-448.
- He, G.Q.; Kong, Q. and Ding, L.X. 2004. Response surface methodology for optimization the fermentation medium of *Clostridium butyricum*. *Applied Microbiology*. 39(4) : 363-368.
- Henderson, R. A. and Jones, C. W. 1997. Physiology of poly-3-hydroxybutyrate (PHB) production by *Alcaligenes eutrophus* growing in continuous culture. *Microbiology*. 143 : 2361-2371.
- Khafaji, Z.M. 1987. *Biological activities of bacteria*. Office of Book home for printing and establishment, University of Mosul, Iraq. (In Arabic).
- Khanafari, A.; Akhavan Sepahei, A. and Mogharab, M. 2006. Production and recovery of poly-B hydroxyl butyrate from whey degradation by *Azotobacter*. *Iran. J. Environ. Health. Sci. Eng.* 3 (3) :193-198.
- Lemoigne, M. 1926. Products of dehydration and polymerization of β -hydroxybutyric acid. *Bull. Soc. Chem. Biol.* 8: 770-782.
- Lin, L. P. and Sadoff, H. L. 1968. Encystment and polymer production by *Azotobacter vinelandii* in the presence of 3-hydroxybutyrate. *Journal of Bacteriology*. 95 (6) : 2336-2343.
- Mahmoudi, M.; Sharifzadeh, M.; Najafpour, G. D.; Tabandeh, F. and Eisazadeh, H. 2010. Kinetic model for poly hydroxybutyrate (PHB) production by *Hydrogenophaga pseudoflava* and verification of growth conditions. *African Journal of Biotechnology*. 9(21): 3151-3157.
- Mercan, N.; Aslim, B.; Yüksekdağ, Z. N. and Beyatli Y. 2002. Production of Poly- β -Hydroxybutyrate (PHB) by Some *Rhizobium* Bacteria. *Turk. J. Biol.* 26 : 215-219.
- Page, W. J. 1995. Bacterial poly hydroxyalkanoates: natural biodegradable plastics with a great future. *Can. J. Microbiol.* 41: 1-13.
- Page W.J. and Cornish, A. 1993. Growth of *Azotobacter vinelandii* UWD in fish peptone medium and simplified extraction of poly- β -hydroxybutyrate. *Applied and Environmental Microbiology*. 59(12) : 4236-4244.
- Pozo, C.; Martı́nez-Toledo, M.V.; Rodelas, B. and González-López, J. 2002. Effects of culture conditions on the production of poly hydroxyalkanoates by *Azotobacter chroococcum* H23 in media containing a high concentration of alpehı́n (wastewater from olive oil mills) as primary carbon source. *Journal of Biotechnology* 97 : 125-131.
- Quillaguaman, J.; Hashim, S.; Bento, F.; Mattiasson, B. and Hatti-Kaul, R. 2005. Poly (β -hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1 using starch hydrolysate as substrate. *Journal of Applied Microbiology*. 99: 151-157.
- Ramadas, N. V.; Singh, S. K.; Soccol, C. R. and Pandey A. 2009. Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149. *International Journal*. 52(1) : 17-23.
- Sangkharak, K. and Prasertsan, P. 2008. Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under

- aerobic-dark condition . *Electronic Journal of Biotechnology*. 11(3) .
- Senior, P. J. ; Beech, G. A.; Ritchiet , G. A. F. and Dawes, E. A. 1972 . The role of oxygen limitation in the formation of poly -B-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii* . *Biochem. J.* 128: 1193-1201 .
- Sharifzadeh Baei , M. ; Najafpour, G. D.; Lasemi , Z.; Tabandeh, F.; Younesi , H. ; Issazadeh, H.and Khodabandeh ,M. 2010 . Optimization PHAs production from dairy industry wastewater (cheese whey) by *Azohydromonas lata* DSMZ 1123 . *Iranica Journal of Energy & Environmental* . 2 : 132- 136 .
- Stockdale, H. ; Ribbons , D. W. and Dawes , E . A. 1968. Occurrence of Poly-i-Hydroxybutyrate in the Azotobacteriaceae. *Journal of Bacteriology*, 95: 1798-1803 .
- Thompson , J . P. and Skerman, V. B . D. 1979. *Azotobacteraceae* . *The taxonomy and ecology of the aerobic nitrogen – fixing bacteria*. Academic Press. London. 419 .
- Yüksekdağ, Z. N. ; Aslım , B.; Beyatl, Y. and Mercan, N. 2004 .Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12 . *African Journal of Biotechnology* . 3 (1) : 63-66.