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Vol. 7(43), pp. 5025-5035, 25 October, 2013
DOI: 10.5897/AJMR2013.6060
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Optimization of bioplastic (poly-β-hydroxybutyrate) production by a promising *Azomonas macrocytogenes* bacterial isolate P173

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Accepted 24 September, 2013

An extensive screening program was previously done to isolate a promising bacterial isolate *Azomonas macrocytogenes* isolate P173 capable of polyhydroxybutyrate (PHB) production. It produced 24% PHB per dry weight after 48 h. In this study, several experiments were designed to optimize the composition of the culture medium and environmental factors for maximizing PHB production by the respective isolate. Results show that 60% aeration, incubation temperature 37°C and an initial pH 7.5 were optimum for PHB production. A modified culture medium for PHB production was designed containing 0.7% glucose and 100 mg/L potassium nitrate as a carbon and nitrogen sources, respectively. Using this modified medium together with optimum environmental conditions, PHB production was increased from 24 to 42% per dry weight after 24 h of incubation rather than 48 h. Acriflavin-induced mutation resulted in one variant (173A2) which produced 47% PHB per dry weight after 24 h of incubation using the same modified culture medium except for glycerol 1.5% as carbon source.

Key words: *Azomonas macrocytogenes*, poly-β-hydroxybutyrate, optimization of microbial bioplastics bioplastic.

INTRODUCTION

Polyhydroxyalkanoates (biopolymers; PHA) are precious gifts of biotechnology to mankind. Since polyhydroxybutyrate (PHB) was discovered by Lemogine in *Bacillus megaterium* in 1926, there is extensive research towards industrial PHB production to substitute synthetic polymers (Anderson and Dawes, 1990). PHA resembles synthetic polymers in many chemical and physical properties, however being biodegradable and produced from renewable source makes it superior to its rival. They have a lot of applications, for example they are preferred candidates for developing controlled/sustained release drug delivery vehicles (Nair and Laurencin, 2007) and also can be used in biomedical implants and biofuels (Bonartsev et al., 2007; Zhang et al., 2009). However, PHB production is more expensive than synthetic polymer production so there is a need to explore its production from locally available and renewable carbon sources such as horticultural, agricultural waste, corn, cassava, etc. This would be of economic value considering the gains that would result from PHB application (Steinbüchel et al., 1998). Moreover optimization of other culture conditions such as the appropriate time to harvest polymer, aeration levels and incubation temperature is important to manage.

PHB has widespread occurrence in both Gram-positive and negative bacteria (Naranjo et al., 2013; Khanna and Srivastava, 2005; Anderson and Dawes, 1990). A lot of studies regarding PHB existence and optimization

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specifically in nitrogen fixing organisms were carried out. For example in free-living, N_2-fixing *Rhizobium* cells PHB synthesis has been described ranging from 35 to 50% per cell dry weight (Tombolini and Nuti, 1989). Moreover, Senior and his coworkers (1972) studied PHB production in *Azotobacter beijerinckii* which produced 50% PHB per dry weight with oxygen limitation conditions. Stockdale and his coworkers (1968) confirmed 22% PHB production per dry weight using 2% glucose as carbon source in *Azotobacter macrocytogenes*. Even mutant strain of *Azotobacter vinelandii* produced 65% PHB using glucose and ammonium acetate (Page and Knosp, 1989). An overall conclusion of PHB production is widespread in nitrogen fixing organisms.

In our previous study, a bacterial isolate from the soil was selected and its PHB production reached 24% per dry weight after 48 h of incubation. This isolate was fully identified using microscopical examination, culture characteristics, biochemical reactions and 16S ribosomal RNA gene sequencing (accession code KC685000) as *A. macrocytogenes* isolate P173 (Elsayed et al., 2013). *Azomonas* is nitrogen fixing Gram negative rod bacteria. To our knowledge, this is the first time to optimize PHB production in *Azomonas* species. Therefore, the resent work aims to study various environmental conditions and culture characteristics required to optimize PHB production by this isolate as well as strain improvement by mutation.

MATERIALS AND METHODS

Microorganisms

A. macrocytogenes isolate P173 (accession code KC685000) is a promising PHB producer obtained through an extensive screening from soil. The organism was maintained on nutrient agar slants at 4°C and renewed monthly.

Chemicals

Different chemicals used in the present study were of highest quality available and obtained mainly from Sigma-Aldrich (Munich, Germany), El-Nasr chemical Co. (Adwic, Cairo, Egypt) and other local suppliers. Ready-made culture media and media ingredients were obtained from Lab M (Topley house, England), Oxoid (USA) and Difco (Detroit, USA).

Culture Media

In the laboratory, formulated basal mineral salts medium (MSM; Berlanga et al., 2006), the modified MSM (M1, M2, M3 and M4) were used in this study and their compositions are listed in Table 1.

Fermentative production of PHB

The pre-culture was prepared by transferring a loopful from a culture grown onto nutrient agar into 5 ml LB broth (Miller, 1972), incubated at 37°C, at 160 rpm for 20 h. The production process was carried out in Erlenmeyer flasks (100 ml) containing 20 ml aliquots of MSM medium. The flasks were inoculated with the seed culture at 5% v/v (OD_{640nm} of 0.3) and incubated in a shaking incubator (200 rpm) at 37°C for 2 days. At different time intervals (time course experiments) or at the end the incubation period (other experiments), the fermentation broth was sampled to determine biomass and PHB concentration.

Analytical methods

Biomass determination

Cellular growth was expressed in terms of dry cell weight which was calculated from the equation of a calibration curve constructed between optical density (OD 640 nm) and dry cell weight of the tested isolate *A. macrocytogenes* isolate P173:

$$Y = 19.202X + 0.0782$$

Where, Y is OD 640 nm, X is dry cell weight (g %).

PHB concentration determination

For PHB extraction, an aliquot of 1 ml was withdrawn from MSM in Eppendorf tubes (1.5 ml) previously washed with alcohol followed by hot chloroform (to remove plasticizers) (Law and Slepeckey, 1961). The cells were harvested by centrifugation at 12,000 rpm for 5 min. The cell pellets were digested with a sodium hypochlorite solution (equivalent to active chlorine 4-6%; density at 20°C of 1.12) at 37°C for 1 h with stirring at 160 rpm. The insoluble materials containing PHB were collected by centrifugation at 12,000 rpm for 10 min. The pellets were washed with 1 ml aliquot each of water, ethanol and acetone, respectively. About 1 ml hot chloroform was added to extract PHB. After that 10 ml concentrated sulphuric acid (98%) were added to residue and the tubes were kept in a water bath at 100°C for 15 min. The solution was left to cooland the final solution was measured with a spectrophotometer at 235 nm.

Studying the different environmental and physiological conditions

For each condition, the biomass, PHB concentration and itsproduction percent per dry weight for the test isolate were measured as previously mentioned.

The effect of aeration

This was done using Erlenmeyer flasks (250 ml) with the following varying volumes of MSM: 25, 50 and 100 ml corresponding to 90, 80 and 60% aeration (Park et al., 1997).

The effect of inoculum size

Erlenmeyer flasks (100 ml) each containing 20 ml MSM were prepared and inoculated with pre-culture with different inoculum sizes (0.5, 2, 5 and 10 % v/v).

The effect of initial pH

Erlenmeyer flasks (100 ml) each containing 20 ml MSM medium were prepared at different pH values of 4, 5, 7, 8 and 9.

Table 1. Chemical composition of MSM and modified media.

Name of ingredient	Basal medium (MSM)	Modified media			
		M1	M2	M3	M4
Carbon source (amount/liter)	Glucose (7 g)	Glucose (7 g)	Glycerol (15 ml)	Glucose (7 g)	Glycerol (15 ml)
Nitrogen source (amount/liter)	Ammonium chloride (100 mg)	Potassium nitrate (100 mg)	Potassium nitrate (100 mg)	Potassium nitrate (100 mg)	Potassium nitrate (100 mg)
Minerals (amount/liter)	$MgSO_4 \cdot 7H_2O$(0.2 g); $CaCl_2$ (0.01 g); Ferrous ammonium sulphate (0.06g); Trace elements solution* (1 ml)	No tested minerals		Same minerals as in MSM	
Common ingredients (amount/liter)	$Na_2HPO_4 \cdot 12H_2O$ (10.2 g); KH_2PO_4 (1.5 g); NaCl (10 g)	The same as in MSM			

*Trace elements solution contains (amount/liter) ($CoCl_2.6H_2O$ (0.2 g), H_3B0_3 0.3 g, $ZnSO_4.\ 7H_2O$ 0.1 g, $MnCl_2\ .4H_2O$ 30 mg, $NiCl_2$ 10 mg, $CuSO_4.\ 5H_2O$ 10 mg) (Berlanga et al., 2006); MSM and modified media were sterilized by autoclaving. Glucose and trace elements solution were filter sterilized and were aseptically added to the autoclaved media with the indicated concentration.

The effect of incubation temperature

Erlenmeyer flasks (100 ml) each containing 20 ml MSM were prepared, inoculated and incubated at different temperatures (28, 37 and 40°C).

The effect of different media components

Effect of replacement of glucose in MSM with other carbon sources: A set of flasks with MSM media were prepared with same ingredients except for replacement of glucose with other carbon sources (0.7%). The used carbon sources were classified as follows: monosaccharaides sugars (fructose, galactose and, arabinose), disaccharides sugars (maltose, lactose, sucrose), sugar alcohol (mannitol, glycerol), polysaccharides as starch, oils (paraffin, corn oil), unrefined carbon sources as Malt extract and others as potassium acetate. All carbon sources were prepared as stock solution (10%) and sterilized by autoclaving except for fructose and arabinose which were sterilized by membrane filtration.

At the end of incubation, samples were removed to measure biomass and PHB concentration. Except in the case of using oils as carbon source, biomass was measured as optical density and expressed in terms of dry weight as previously described. In case of oils, formation of emulsion between oil and medium confers turbidity to medium which affects optical density. In this case, biomass was measured directly by drying 5 ml culture in pre-weighed centrifuge tubes at 37°C for 24 h.

Effect of variable concentration of some selected carbon sources in MSM: The carbon sources showing promising PHB percentage per dry weight without abrupt decrease in biomass were tested in different concentration to determine the most suitable concentration to be used in further experiments. The concentrations used were 0.4, 0.7, 1.5, 2 and 4% w/v.

Effect of replacement of ammonium chloride in MSM with other nitrogen sources: Erlenmeyer flasks (100 ml) each containing 20 ml MSM were prepared with the same ingredients except for replacement of ammonium chloride with other nitrogen sources (100 mg/L). The nitrogen sources used were organic ones as yeast extract, peptone, tryptone, beef extract, urea and amino acids and inorganic ones as ammonium nitrate and potassium nitrate.

All nitrogen sources were sterilized by autoclaving except for urea which was prepared as stock solution and sterilized by membrane filtration.

Effect of variable concentration of some selected nitrogen sources

The nitrogen sources showing promising results were tested at different concentrations 50, 100, 200, 500 and 1000 mg/l to determine the best suitable concentration to be used in further experiments.

Effect of different multivalent minerals

Initially formulated MSM contain ferrous ammonium sulphate, calcium chloride, magnesium chloride and trace elements. To study the effect of these minerals both on biomass and PHB productivity, five flasks each containing 20 ml MSM were prepared as follows: One is MSM devoid of all minerals; second contained only ferrous ammonium sulphate; third contained only calcium chloride; fourth contained only magnesium chloride and fifth contained only trace elements solutions.

The used mineral concentrations were the same as in MSM medium.

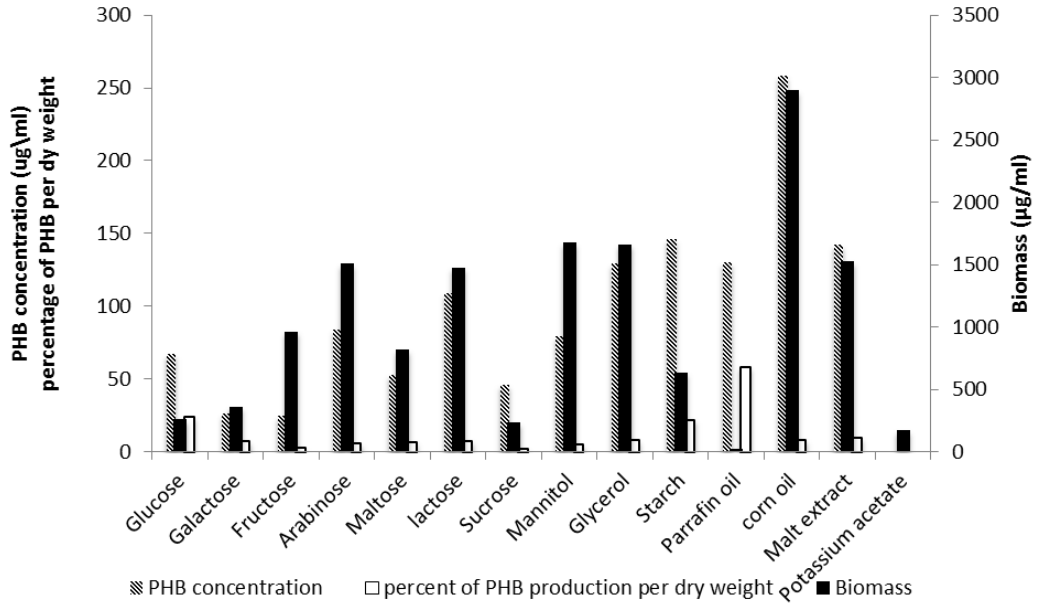

Figure 1. Effect of different carbon sources on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

PHB production under combined selected pre-tested conditions

From the previously studied factors/conditions that proved to be optimium for PHB production by our isolate, four modified media were created. In most cases, the selection criteria for the chosen factors/conditions depended on high PHB percentage per dry weight and reasonable amount of biomass formation not less than 50% of the maximum biomass attained by different factors within the same tested category. The modified media were M1, M2, M3 and M4 (Table 1). Time course of PHB production was done after 24, 48 and 72 h.

Induction of mutation using UV rays

This was done by Direct-Plate Irradiation (Lin and Wang, 2001). A loopful of isolate from a nutrient agar slant was inoculated in LB broth for overnight at 37°C and 200 rpm. A series of dilutions of the obtained culture were used to prepare two sets of nutrient agar plates. One set for UV irradiation and the other for estimating viable count in the bacterial culture. All UV irradiations were done in a custom-built UV chamber with a glass front (germicidal lamp 15 W UV lamp of 254 nm). The majority of the experiments were done in the dark to avoid photo reactivation. All the plates were grown in the 37°C incubator for 24 h before scoring the number of colonies. This treatment resulted in 99.9% kill as determined by viable count of the survivors. The resultant colonies were collected for PHB production assessment.

Induction of mutation using chemical mutagens

This was done according to Lopes et al. (2010) with minor modifications. Stock solutions of acriflavin and proflavin (each of 50 mg/ml) were prepared in phosphate buffer (50 mM, pH7.5) and sterilized by membrane filtration. The isolates were grown by inoculating a loopful of nutrient agar slant in LB broth (10 ml final volume) for overnight at 37°C and 200 rpm. Aliquots of 0.5 ml from stock solutions of acriflavin and proflavin were separately added to equal aliquots of cell suspension of isolates. The contact time of reaction of chemical mutagen and cell suspension was 30 min. Then, the reaction mixtures were centrifuged at 12000 rpm for 10 min and the cells were washed with normal saline. The mutagen treated cells were re-suspended in 200 µl sterile saline and surface inoculated on nutrient agar plates.The plates were incubated at 37°C for 24 h. The grown colonies were collected to measure PHB production.

Testing the PHB productivity of the collected variants

Screening for PHB was done for the collected variants of our isolate as previously described. Comparison of the PHB production of the collected variants to its wild type organism was carried out.

RESULTS

Effect of environmental factors on PHB production

As shown in Table 2, maximum PHB percentage per dry weight was achieved at 80% aeration, 5% inoculum size, pH of 7.5 and 37°C incubation temperature.

Effect of different media components on PHB production

Effect of carbon sources

As shown in Figure 1, the highest PHB percentage per dry weight was attained using paraffin oil (42%). Paraffin

Table 2. Effect of aeration, inoculum size, initial pH and incubation temperature on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

Factor	PHB (µg/ml)	Biomass (µg/ml)	PHB percentage per dry weight(%)
60% Aeration	24	383	6
80% aeration	184	1400	13
90% aeration	155	366	42
0.5% inoculum size	55	580	9.5
2% inoculum size	123.5	615	15.7
5% inoculum size	169	770	24
10% inoculum size	148	840	17.6
28°C	74	820	9
37°C	169	667	25
40°C	92	870	10.5
pH 4	0	257	0
pH 5	71	766	9.2
pH 7.5	169	667	25.3
pH 8	121	603	21.7
pH 9	127	988	20

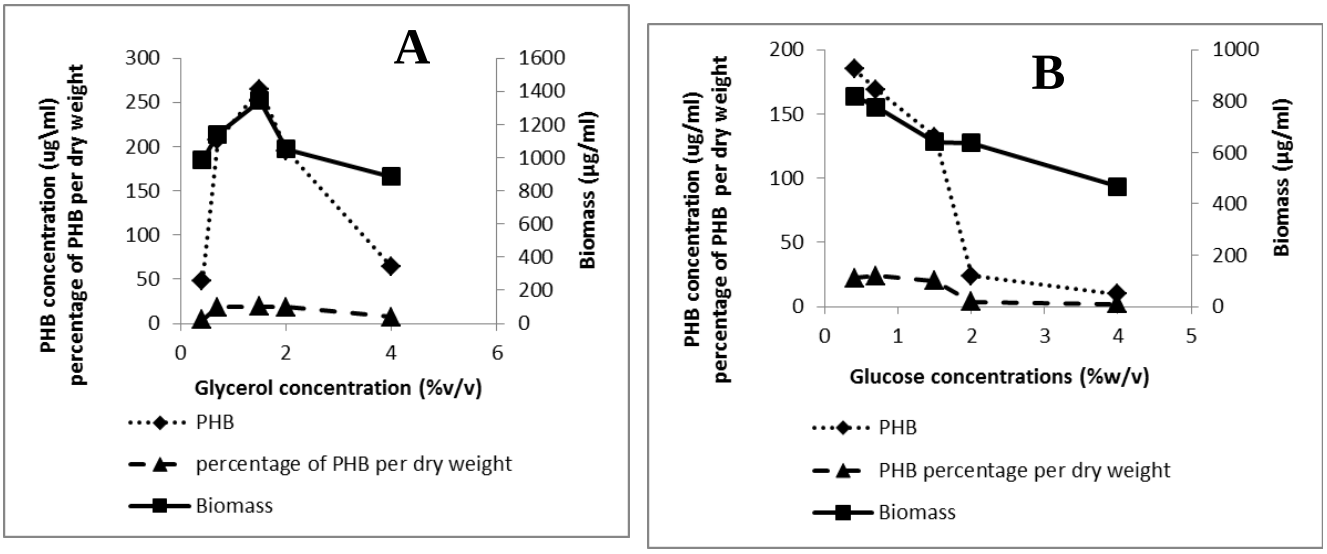

Figure 2. Effect of different concentrations of glycerol (A), glucose (B) on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

oil decreased biomass to a great extent while producing a moderate amount of PHB thus increasing PHB percentage per dry weight. Glycerol, glucose, malt extract and corn oil increased amount of PHB produced much more than paraffin oil but they did not decrease biomass so they gave lower PHB percentage per dry weight. Therefore, different concentration of glucose and glycerol were tested as they produced the largest PHB percentage per dry weight without decreasing biomass to less than 50% attained with other carbon sources. Figure 2A reveals that 0.7% w/v glucose led to highest PHB percentage per dry weight (24%) without affecting biomass, also 1.5% v/v glycerol gave highest amount of PHB but the biomass was increased to a large extent leading to obvious decrease in PHB percentage per dry weight (19%) (Figure 2B).

Effect of nitrogen sources

Figure 3 shows that maximum PHB production percentage per dry weight was achieved using ammonium

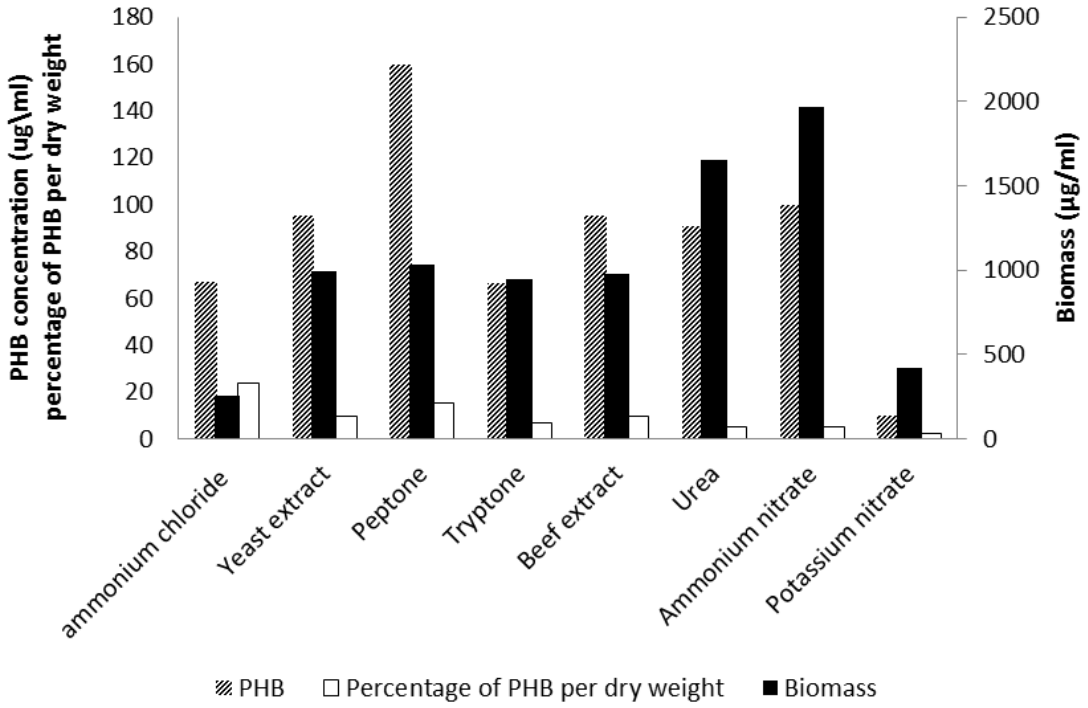

Figure 3. Effect of different nitrogen sources on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

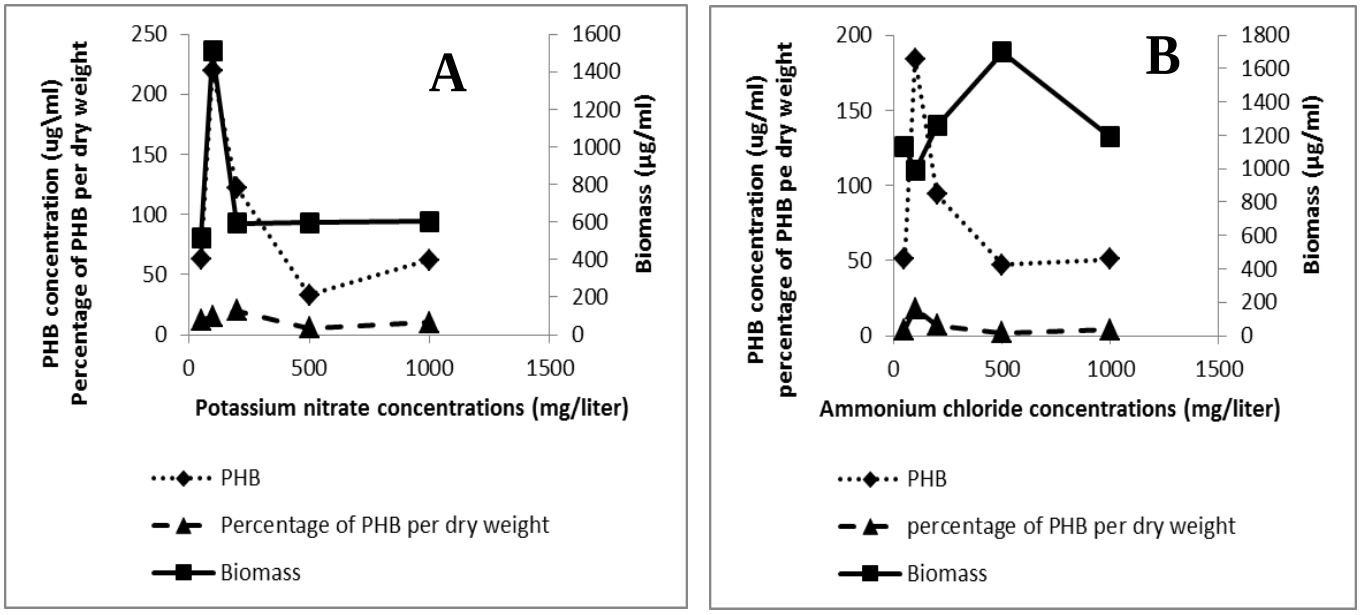

Figure 4. Effect of different concentrations of potassium nitrate (A), ammonium chloride (B) on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

chloride followed by potassium nitrate. Therefore, different concentrations of both potassium nitrate and ammonium chloride were tested (Figure 4). Results revealed that 100 mg/L potassium nitrate gave the highest PHB percentage per dry weight without decreasing biomass.

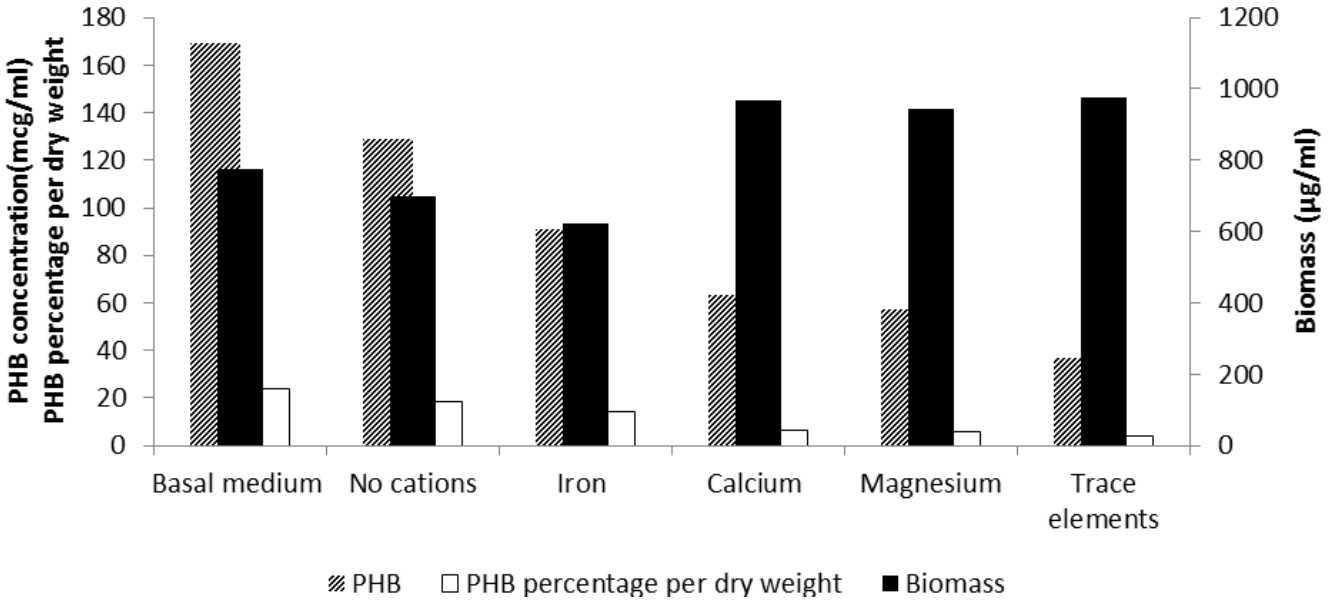

Figure 5. Effect of minerals on PHB concentration, biomass and PHB percentage per dry weight in *A.* macrocytogenes isolate P173.

Table 3. Comparison of maximum productivity and PHB production percentage per dry weight of *A. macrocytogenes* isolate P173 in basal and newly formulated media.

Name of medium	Maximum PHB production (µg/ml) (time in hours)	Maximum PHB percentage per dry weight (%) (time in hours)
Basal medium (MSM)	172 (48)	23 (48)
173M1	230 (24)	42 (24)
173M2	143 (24)	10 (24)
173M3	177 (48)	13.8 (48)
173M4	137 (48)	15.8 (48)

Effect of minerals

Highest PHB production percentage per dry weight was attained using MSM containing all minerals rather than their absence or their separate usage in medium (Figure 5).

PHB production under combined selected pre-tested conditions

As depicted in Table 3, PHB production in medium 1 (M1) reached 42% per dry weight after only 24 h of incubation in comparison with MSM medium. This means two fold increase in PHB percentage per dry weight. The other modified media M2, M3 and M4 show no significant effect on PHB production. The time course of PHB production using the best modified medium 173M1 is shown in Figure 6.

Effect of mutation

Acriflavin-induced mutation resulted in variant 173A2 which produced more PHB than wild type (P173). However, UV induced mutation as well as proflavin-induced mutation resulted in decreasing PHB production in the collected variants (Figure 7). Therefore, variant P173A2 was chosen for further experiments.

Testing PHB productivity by *Azomonas macrocytogenes* variant P173A2 in the four modified media

The variant P173A2 accumulated PHB up to 47% per dry weight after 24 h of incubation in medium 2 (M2). This differs from wild type which produced only 10% PHB per dry weight using this medium. The other modified media showed no significant effect on PHB production by this

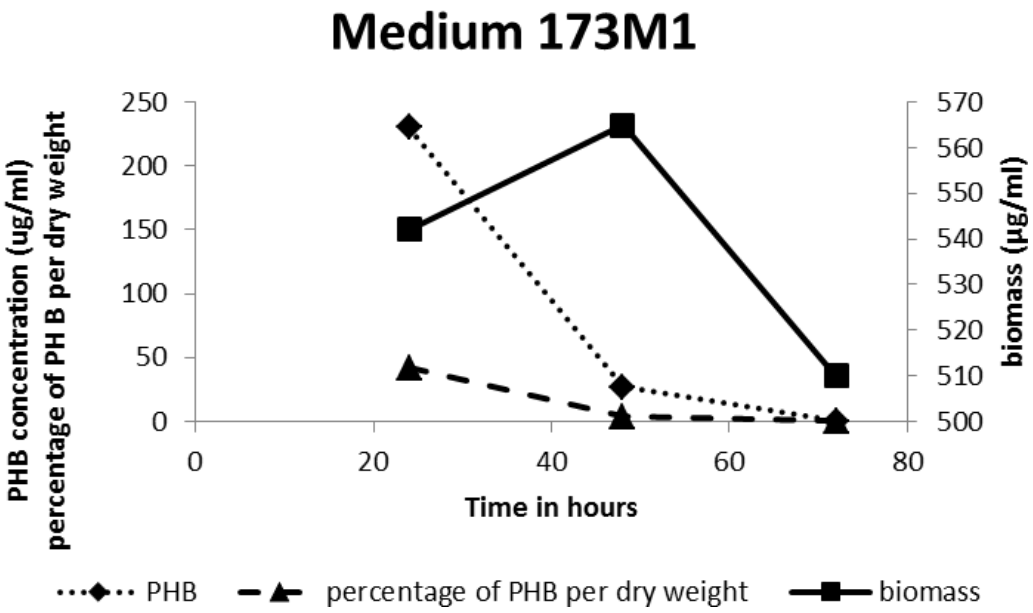

Figure 6. Time course of PHB production, biomass and PHB percentage per dry weight using the best modified medium (173M1) for *A. macrocytogenes* isolate P173.

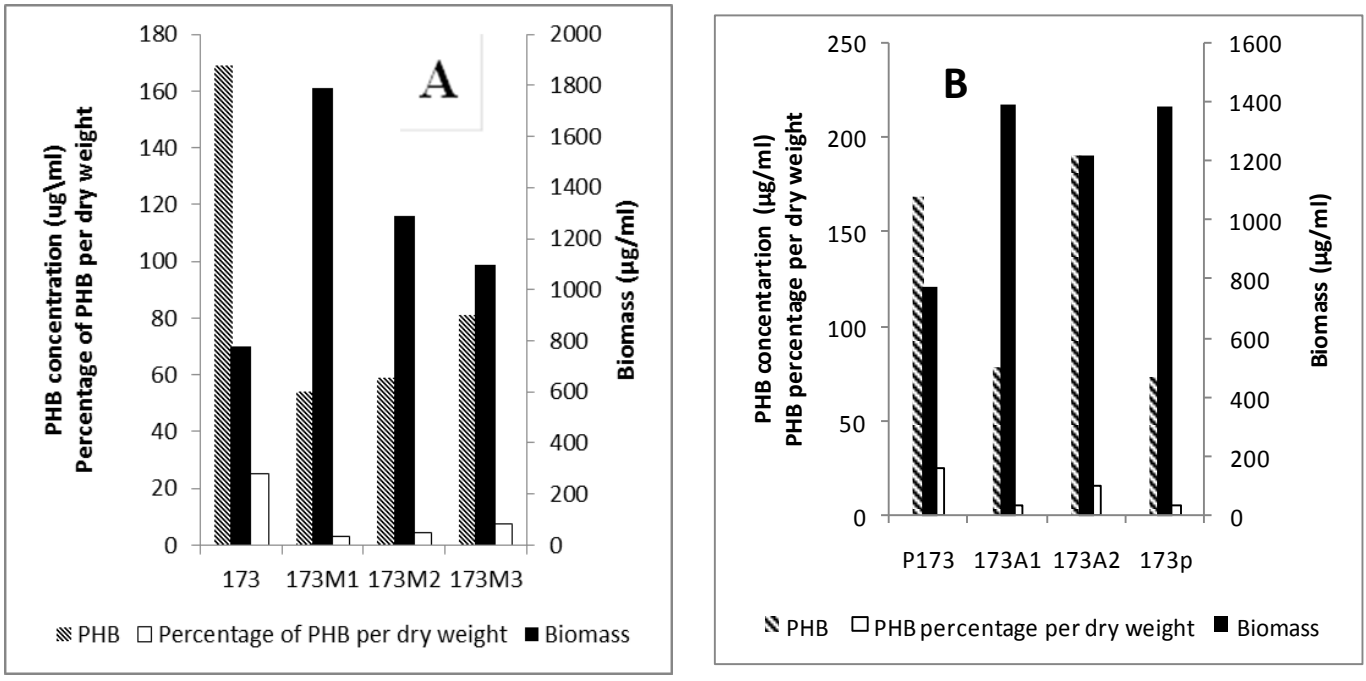

Figure 7. Results of screening of PHB production of *A. macrocytogenes* isolate P173 variants after exposure to UV (A), chemical mutagens (B).

variant. The comparison between four modified media in terms of maximum PHB productivity is shown in Table 4. Time course of PHB production using best modified medium for the variant 173M2 is shown in Figure 8.

DISCUSSION

A. macrocytogenes is ellipsoidal to rod-shaped Gram negative cell that is more than 2 mm in diameter, usually

Table 4. Comparison of maximum productivity and PHB production percentage per dry weight of *A. macrocytogenes* variant 173A2 in newly formulated media.

Name of medium	Maximum PHB production (µg/ml) (Time in hours)	Maximum PHB percentage per dry weight (%) (time in hours)
173M1	124 (24)	23.9 (24)
173M2	212 (24)	47 (24)
173M3	156 (72)	13.6 (72)
173M4	187.5 (24)	15 (72)

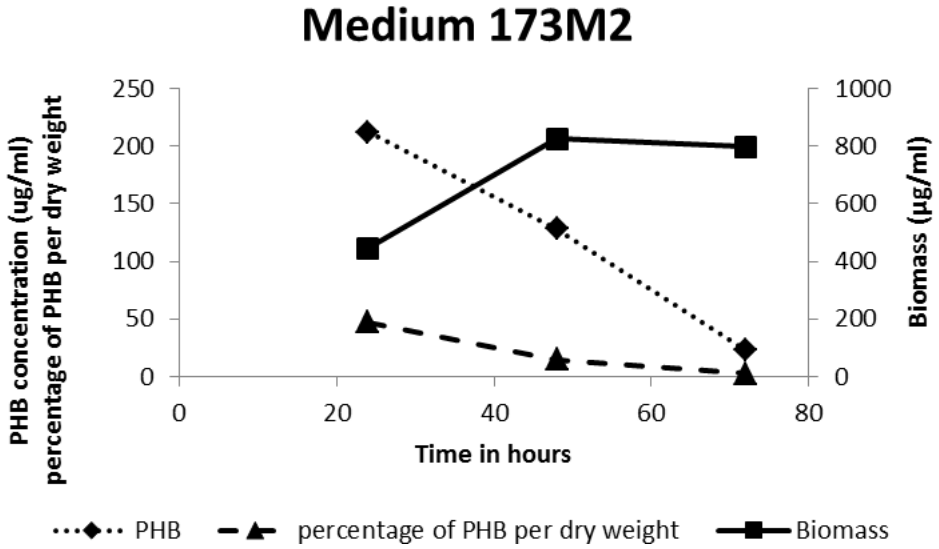

Figure 8. Time course of PHB production, biomass and PHB percentage per dry weight using the best modified medium for *A. macrocytogenes* variant P173A2.

2.5-3.5 µm in length, motile by peritrichous, lophotrichous or polar flagella and is capable of fixing nitrogen under aerobic conditions (Young and Park, 2007). In this study, an optimum medium for PHB production for this species was created using a series of experiments. We found that 80% aeration, pH of 7.5, incubation temperature 37°C were the best environmental conditions for PHB production in this bacteria. Also, the best modified culture medium should consist of 0.7% glucose, 100 mg/L potassium nitrate and absence of tested minerals. These conditions encouraged bacteria to produce 42% PHB per dry cell weight after only 24 h of incubation. These minimum requirements in culture medium and short time of incubation make PHB production in *A. macrocytogenes* a cost-effective process. Moreover, after exposing this bacterium to acriflavin as a chemical mutagen, the collected variant (P173A2) showed improvement in PHB production. This variant (P173A2) produced 47% PHB per dry cell weight after 24 h of incubation using the same culture medium as for the wild type except for glycerol as carbon source.

Among the investigated parameters was aeration percentage, it was found that maximum biomass as well as PHB production occurred at 80% aeration, however, both decreased at higher aeration percentage. Barron (1955) proposed that oxygen may have harmful effect on biochemical materials due to nonspecific oxidation of enzymes (Barron, 1955).

After studying the effect of pH on PHB production, we found that maximum PHB percentage per dry weight occurred at pH of 7.5. This agreed with many previous studies (Grothea et al., 1999; Tamdogan and Sidal, 2011). At extremes of pH, PHB production decreased in spite of increasing biomass (Tavernier et al., 1997). We speculated that *Azomonas* directed its energy for growth rather than PHB production.

Maximum PHB production was achieved at temperature 37°C. A lot of studies confirmed maximum PHB production at range of 33-37°C (Grothea et al., 1999; Tabandeh and Vasheghani, 2003). It was pointed out that PHB production decreased at temperature extremes due to low enzyme activity at such temperatures (Tamdogan and Sidal, 2011).

The inoculum size of seed culture was investigated to

stabilize initial microbial load. 5% v/v was the best initial inoculum size. Furthermore, different classes of carbon source were studied to point out the best carbon to be used in optimized medium. *Azomonas* produced maximum PHB from monosaccharides followed by sugar alcohols followed by oils. Moreover, *Azomonas* showed a low efficacy in utilizing disaccharides sugar and polysaccharides. Maximum PHB was achieved using 0.7% glucose without affecting biomass. A lot of studies are comparable to our results; glucose is an easily assimilated carbon source and encouraged bacteria to produce PHB (Ramadas et al., 2009; Hori et al., 2001; Borah et al., 2002).

It is well known that any bacteria capable of producing PHB needs excess carbon source in addition to a limited other source such as nitrogen or phosphate (Naranjo et al., 2013; Santhanam and Sasidharan, 2010). Here, we used nitrogen source as the limiting one. Our bacteria synthesized PHB in large amounts using inorganic nitrogen sources rather than organic ones. The best nitrogen source used was potassium nitrate. This agreed with some studies especially using glucose as carbon source (Pal et al., 2008; Rohini et al., 2006).

It is fascinating that our newly modified medium helped the bacteria to produce double its initial PHB production from MSM medium from 24 to 42% per dry cell weight. Also, maximum PHB as well as biomass occurred after 24 h of incubation. Afterwards, PHB decreased dramatically due to its consumption by the bacteria. Naheed et al. (2011) stated that 66% PHB per dry weight was achieved by *Enterobacter* after 24 h of incubation. Also, Rohini et al. (2006) reported 19.7% PHB per dry weight using *Bacillus thuringiensis*. Kim (2000) mentioned that *Azotobacter* produced 46% PHB per dry weight using starch.

In order to complete our study, strain improvement by mutation was tried both physically and chemically. Physical mutation was done by UV where UV induces a complex spectrum of mutations (Miller, 1985). We exposed our isolate to UV rays but unfortunately no improvement in PHB production has occurred, although a lot of studies evaluated UV as a mutagen for PHB production and successfully managed to reach its target (Pal et al., 2008; Sreeju et al., 2011). On the other hand, after exposure of our isolate to chemical mutagen especially acriflavin, one of the collected variant improved PHB production. Chemical mutations not only by acriflavin, but also nitrosoguanidine or ethylmethylsulphonate are known for improving PHB production (Lakhawat et al., 2012; Pal et al., 2008; Sreeju et al., 2011). We tested the four optimized media with this variant, medium 2 (M2) proved to be effective with it which composed of glycerol instead of glucose. It is known that glycerol is cheaper than glucose (Naranjo et al., 2013). This strengthens our target to decrease cost of PHB production. Moreover, it produced 47% PHB per dry weight after 24 h of incubation. Therefore, the prospective work will be directed to large scale production of PHB using *A. macrocytogenes* isolate P173 and molecular characterization of the genes involved in the biosynthesis of PHB.

REFERENCES

Anderson AJ, Dawes EA (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54(4):450-472.

Barron ESG (1955). Oxidation of some oxidation-reduction systems by oxygen at high pressures. Arch. Bioche. Biophy. 59:502-507.

Berlanga M, Montero MT**,** Hernández-Borrell J, Guerrero R (2006). Rapid spectrofluorometric screening of poly-hydroxyalkanoate-producing bacteria from microbial mats. Int. Microbiol. 9:95-102.

Bonartsev AP, Myshkina VL **,** Nikolaeva DA, Furina EK , Makhina TA, Livshits APB, Ivanov EA, Iordanskii AL, Bonartseva GA (2007). Biosynthesis, biodegradation, and application of poly(3-hydroxybutyrate) and its copolymers - natural polyesters produced by diazotrophic bacteria. Communicating Current Research and Educational Topics and Trends in Applied Microbiology. 1:295-307.

Borah B, Thakur PS, Nigam JN (2002). The influence of nutritional and environmental conditions on the accumulation of poly-β-hydroxybutyrate in *Bacillus mycoides* RLJ B-017. J. Appl. Microbiol. 92:776-783.

Elsayed NS, Aboulwafa M, Aboshanab K, Hassouna N (2013). PHB production in *Azomonas*, *Acinteobacter* and *Bacillus* species: isolation, screening and identification. Arch. Clin.microbiol. In press.

Grothea E, Murray M, Chistib Y (1999). Fermentation optimization for the production of poly(β-hydroxybutyric acid) microbial thermoplastic. Enzy. Microbial Technol. 25:132-141

Hori K, Kaneko M, Tanji Y, Xing X, Unnu H (2001). Construction of self-disruptive *Bacillus megaterium* in response to substrate exhaustion for polyhydroxybutyrate production. Appl. Microbiol. Biotechnol. 59(2-3):211-216.

Khanna S, Srivastava AK (2005). Recent advances in microbial polyhydroxyalkanoates. Process Biochem. 40:607-619.

Kim B (2000). Production of poly(3-hydroxybutyrate) from inexpensive substrates."Enzyme and Microbiol Technol. 27:774-777

Lakhawat S, Pathak AN, Kulkarni MV, Srikanth GV (2012). Production of Poly-beta-hydroxybuytrate from Distillery spent wash using mutant *Azotobacter Vinelandii*. Intern. J. Adv. Biotech. Res. 3(2):596-604.

Law J, Slepeckey R (1961). Assay of poly-beta-hydroxy butyric acid. J. Bacteriol. 82:33-36.

Lin K, Wang A (2001). UV mutagenesis in *Escherichia coli* K-12:Cell survival and mutation frequency of the chromosomal genes lacZ, rpoB, ompF, and ampA. J. Experm. Microbiol. immunol. 1:32-46.

Lopes BS, Hamouda A, Findlay J, Amyes SGB (2010). Effect of frameshift mutagen acriflavine on control of resistance genes in *Acinetobacter baumannii*. J. Med. Microbiol. 60(2):211-215.

Miller JH (1972). Experiments in molecular genetics. Cold spring harbor laboratory press, Cold Spring Harbor, New York. p. 443

Miller JH (1985). Mutagenic specificity of ultraviolet light. J. Mol. Biol. 182:45-68.

Naheed N, Jami N, Hasnain S, Abbas G (2011). Biosynthesis of polyhydroxybutyrate in *Enterobacter* sp. SEL2 and *Enterobacteriaceae bacterium* sp. PFW1 using sugar cane molasses as media. Afr. J. Biotechnol. 11(16):3321-3332.

Nair LS, Laurencin CT (2007). Biodegradable polymers as biomaterials. Prog. Polym. Sci. 32:762-798.

Naranjo JM, Posada JA, Higuita JC, Cardona CA (2013). Valorization of glycerol through the production of biopolymers: The PHB case using *Bacillus megaterium*. Biol. Technol. 133:38-44.

Page' WJ, Knosp O (1989). Hyperproduction of Poly-β-Hydroxybutyrate during Exponential Growth of *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. 55(6):1334-1339.

Pal A, Ashiwini P, Avinash AK, Badri R, Kajal D, Vomisi P, Srividya S (2008). Mutagenesis of *Bacillus thuringiensis* IAM 12077 for increasing poly(-β-) hydroxybutyrate (PHB) production. Turk. J. Biol.

33:225-230.
Park SK, Lee KT, Kim YB, Rhee YH (1997). Biosynthesis of polyhydroxybutyrate and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *Bacillus thuringiensis* R-510. J. Microbiol. 35:127-133.
Ramadas NV, Singh SK, Soccol CR, Pandey A (2009). Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149. Braz. Arch. Biol. Technol. 52(1):17-23.
Rohini D, Phadnis S, Rawal KS (2006). Synthesis and characterization of poly-β-hydroxybutyrate from *Bacillus thuringiensis* R1. Indian J. Biotech. 5:284-291.
Santhanam A, Sasidharan S (2010). Microbial production of polyhydroxyalkanotes (PHA) from *Alcaligens* spp. and *Pseudomonas oleovorans* using different carbon sources. Afr. J. Biotechol. 9(21):3144-3150.
Senior PJ, Beech GA, Ritchiet GAF, Dawes EA (1972). The role of oxygen limitation in the formation of poly-β-hydroxybutyrate during batch and continuous Culture of *Azotobacter beijerinckii*. Biochem. J.128:1193-1201.
Sreeju SN, Michael Babu, Mariappan C, Selvamohan T (2011). Effect of physical and chemical mutagens on biopolmer producing strains and RAPD analysis of mutated strains. Arch. App. Sci. Res.3(6):233-246.
Steinbüchel A, Füchtenbusch B (1998). Bacterial and other biological systems for polyester production. Trends Biotechnol. 16:419-427.
Stockdale H, Ribbons DH, Dawes EA (1968). Occurrence of poly-β-hydroxybutyrate in the *Azotobacteriaceae*. J. Bacteriol. 95(5):1798-1803.
Tabandeh F, Vasheghani-Farahani E (2003). Biosynthesis of poly-β-hydroxybutyrate as a biodegradable polymer. Iranian. Polymer. J. 12(1):37-42.
Tamodogan N, Sidal U (2011). Investigation of poly-β-hydroxybutyrate (PHB) production by *Bacillus subtilis* ATCC 6633 under different conditions. Kafkas.Univ. Vet. Fak. Derg. 17:176-179
Tavernier JP, Nava S, Courtois J, Courtois B, Barbotin J (1997). Exopolysaccharide and Poly-(beta)-Hydroxybutyrate Coproduction in Two *Rhizobium meliloti* Strains. Appl. Environ. Microbiol. 63(1):21.
Tombolini R, Nuti MP (1989). Poly(β-hydroxyalkanoate) biosynthesis and accumulation by different *Rhizobium* species. FEMS Microbiol. Lett. 60:299-304.
Young JM, Park DC (2007). Probable synonymy of the nitrogen-fixing genus *Azotobacter* and the genus *Pseudomonas*. International Syst. Evol. Microbiol. 57(12):2894-2901.
Zhang X, Luo R, Wang Z, Deng Y, Chen G (2009). Application of (R)-3-Hydroxyalkanoate methyl esters derived from microbial polyhydroxyalkanoates as novel biofuels. Biomacrolecules 10:707-711.