Determination of optimum condition for Poly-β-Hydroxybutyrate Production from *Bacillus* spp .

[1] Najeh Hashem Kadhim and [1] Noor Abd- ulamer Oda

[1]Karbala University College of science Department of biology

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Abstract

The optimum cultural and ecological condition for Poly-β-Hydroxybutyrate Production from *Bacillus* spp. Isolate which was isolated from soil samples at holy Kerbala governorate were studied .

These condition represented by the type and the concentration of carbon , nitrogen and mineral salts source ,in addition to the incubation period temperature , shaking speed , initial pH of production medium and occulation volume . Results revealed that , using production medium containing Date syrup at 2% as a source of carbon and ammonium chlorate at 0.2 % as a source of nitrogen , accompanied with Potassium phosphate and magnesium sulphate at 0.105 % as a total concentration for mineral salts source . The pH was adjusted to 7 with 2% v/v occulation volume . The medium was inoculated by using shaking inocular with 40 ˚C at 200 rpm /min for 72 hr.

تحديد الظروف المثلى لأنتاج متعدد بيتا هيدروكسي بيوتريت من بكتريا الباسلس

ناجح هاشم كاظم [1] و نورعبد الأمير عوده [1]

[1] قسم علوم الحياة / كلية العلوم/جامعة كربلاء

الكلمات المفتاحية : متعدد بيتاهيدروكسي بيوتريت , شراب التمر ,الباسلس , بولمر حيوي

الخلاصة

تمت دراسة الظروف الزرعيه والبيئيه المثلى لأنتاج Poly-β-Hydroxybutyrate من عزلة بكتيرية *Bacillus* spp. عزلت من نماذج تربه في محافظة كربلاء المقدسه .

تمثلت هذه الظروف بنوع وتركيز مصدر كل من الكاربون والنايتروجين والأملاح المعدنيه وكذلك مدة ودرجة حرارة الحضن وسرعة الرج والأس الهايدروجيني الأبتدائي للوسط الأنتاجي وحجم اللقاح واسفرت النتائج عن استخدام وسط انتاج يحتوي على عصير التمر بتركيز 2% بوصفه كمصدر للكاربون وكلوريد الأمونيوم بتركيز 0.2% وهو مصدر للنتروجين ودعم هذا الوسط بـ(فوسفات البوتاسيوم وكبريتات المغنيسيوم) بتركيز كلي مقداره 0.105% مصدراً للأملاح المعدنيه وعدل الأس الهيدروجيني (pH) الى 7 وحجم لقاح 2% حجم/ حجم من الوسط , وتم الحضن بالحاضنه الهزازه بدرجة حرارة 40˚ م وبسرعة رج 200 دورة /دقيقة ولمدة 72 ساعه.

1. Introduction

Dependence on conventional plastics and their boundless usage have resulted in waste accumulation and greenhouse gas emissions. Recent technologies are directed towards the development of bio-green materials that exert negligible sideeffects on the environment. A biologically-synthesized plastic, polyhydroxyalkanoate (PHA), has been attracting major interests due to its similar physical properties to synthetic plastics. Unlike synthetic plastics, PHA is produced from renewable resources and is degraded aerobically by microorganisms to CO2 and H2O upon disposal.(1)

PHAs are a family of optically active biological polyesters, containing approximately 150 different hydroxyalkanoic acids are at present known, synthesized by various bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells.(2) produced by various bacterial strains under conditions of nutrient shortage such as nitrogen, phosphate, sulphur or oxygen(3)

PHA can be degraded by abiotic degradation, i.e., simple hydrolysis of the ester bond without requiring the presence of enzymes to catalyze this hydrolysis. During the biodegradation process, the enzymes degrade the residual products untill final mineralization (biotic degradation) (4).

PHAs are generally classified into short-chain-length PHA (sCL-PHA) and medium-chain-length PHA (mCL-PHA) by the different number of carbons in their repeating units. For instance, sCL-PHAs contain 4 or 5 carbons in their re-peating units, while mCL-PHAs contain 6 or more carbons in the repeating units. The term mCL was coined because the number of carbons in the monomers roughly corresponds to those of medium-chain-length carboxylic acids. PHA nomenclature and classification may still evolve as new structures continue be discovered. The main biopolymer of the PHA family is the poly hydroxybutyrate homopolymer (PHB)(5)

PHB is water insoluble and relatively resistant to hydrolytic degradation but soluble in chloroform and other chlorinated hydrocarbons (6). PHB shows good oxygen permeability , good UV-resistant but has poor resistance to acids and bases (7). PHB has its melting point at 175 °C and glass transition temperature of 15 °C. PHB sinks in H2O, while polypropylene floats. But sinking of PHB facilitates its anaerobic biodegradation in sediments (8) . The Molecular weight (Mw) of PHB can influence many properties, such as degradation rate and mechanical strength(9)

Widespread production of PHB has far been limited due to high production costs. For PHB production to become more economically feasible, better bacterial strands as well as cheaper feedstock and purification methods are needed. Genetically modified bacteria will allow the use of cheap and abundant sources, such as household waste, agricultural and industrial waste, waste water, etc. for producing large amounts of PHB. Cheap, safe and efficient purification methods can then be used to recover PHB with high purity.(5)

PHB is suitable for medical applications like bone plates, nails, screws (9,10) and in the treatment of osteomyelitis (11) and used in the following productions; Shampoo bottles; Food containers; Suitable , dishwasher use due to the good impact and temperature resistance of the material; Disposable razors; Disposable utensils; Fishing nets; Composite-glial growth factors; Drug carriers; PHB coated papers; Golf tees, *etc*.(12)(6).

2 - Materials and Methods

2.1 Microorganism used in the study : PHB accumulating *Bacillus* spp. isolate in which study was used which isolated from soil samples at holy Kerbala governorate wer studied .The strains identification were examined for their colony morphology, cell shape , gram reaction and Spore

staining as per the standard descriptions of the Bergey's Manuals of Determinative Bacteriology (13).

2.2 Production of PHB From *Bacillus* spp.

The *Bacillus spp* stran was growth in N2 deficient medium (pH 7.0) containing 1% glucose , 0.02% $MgSO_4$, 0.01% NaCl, 0.05% KH_2PO_4, 0.25% peptone, and 0.25% yeast extract. Production studies were carried out in 100ml flasks containing 19.96 ml culture medium and incubated at 37°C on a shaker incubator at 150 rpm for 48h and inoculated with inoculum sizes(400µ) (14)

2.3 Extraction and Determination of PHB :

After incubation time in N2 deficientmedium broth at 37°C, 10 mL of culture was collected and centrifuged at 6000 rpm for 15 min. The washed pellet twice with steraile deionized water was resuspended in 1mL of deionized water, transferred to pre weighed boat and dried to constant weight at 80°C for drying incubator . the dried pellet was treated with 10 mL sodium hypochlorite and incubated 60°C for 1hrs. After incubation, the mixture was centrifuged at 6000 rpm for 15 min and washed steraile deionized water , 10 ml ethanol was added the mixture was centrifuged at 6000 rpm for 15 min and 10 ml acetone the mixture was centrifuged at 6000 rpm for 15 min and was added 10 ml boiling chloroform and filtered through pre-wetted Whatmann No.1 filter paper. chloroform extract was evaporated to dryness (15).

2.4 Quantification of PHB production and selection of isolates:

The quantification of PHB production due to the method of John and Ralph (1961)(16).

2.4.1 Estimation of cell dry weight:-

After centrifugation of the culture medium, the supernatant was discarded and the pellet was washed with distilled water. The washed pellet was resuspended in 1 ml distilled water, transferred to preweighed plates and dried to constant weight at 60˚C (17) .

2.4.2 Estimation of PHB by crotonic acid assay:

The amount of PHB in a sample taken can be determined by spectrophotometric assay. This assay was facilitated by the conversion of PHB into crotonic acid by sulphuric acid treatment. Crotonic acid standard solution was prepared with different increasing concentrations (10 to 40 µg). Absorbance of crotonic acid was measured at 235 nm and a standard curve was plotted. Then, the sample containing 5 to 50 µg polymer in chloroform was taken in a clean test tube and the solvent was evaporated by heating in a boiling water bath. Then, 10 ml of concentrated H_2SO_4 was added to the tube and heated for 10 min at 100°C in a water bath. The solution was then cooled and thoroughly mixed. The sample was then transferred to a quartz cuvette and the absorbance was measured at 235 nm against a sulphuric acid blank . The amount of crotonic acid was then calculated by plotting graph (18,19)

2.5 Optimization of Culture Conditions for PHB Production by *Bacillus* spp. isolate :

2.5.1 Incubation Time: The inoculated cultures in production medium were incubated at different time ranging from 24 to 96 h in optimized condition. At each time point (24, 48, 72, and 96 h), cultures were tested for growth and PHB production and the growth and PHB productions were recorded.

2.5.2 Incubation Temperature: The inoculated cultures in production medium were incubated at different temperature 25, 30, 35, 40 and 45°C for temperature optimization. After 72 h of incubation, growth and PHB production were determined and optimum temperature was selected.

2.5.3 agitation speed (rpm) : These cultures were incubated with agitation ranging from 50 to 300 rpm in shaker incubator. After 72 h and incubation in 40°C the growth and PHB production were determined and optimum agitation speed was selected.

2.5.4 Type of Carbon Sources and the preferred concentration: Effect of different carbon sources on PHB production .The selected bacterial were grown in N2 deficient medium with different carbon sources Dates juice , glucose, starch , sucrose, maltose and lactose at 1 per cent level The flasks were incubated at 40°C on a rotary shaker (200 rpm) for 72h. The effect of preferred concentration studed (0 , 0.5 , 1 , 1.5 , 2 ,2.5 ,3) % from Dates juice and quality Reduced-sugar by miller method (20) .

2.5.5 Type of Nitrogen Sources and the preferred concentration : Effect of media ingredients like nitrogen sources on PHB production was determined by simply replacing nitrogen source with other nitrogen sources (peptone, yeast extract, peptone and yeast extract , Ammonium chloride, ammonium sulphate , urea) and used 2% Dates juice Carbon Source. The flasks were incubated at 40°C on a rotary shaker (200 rpm) for 72h. The effect of preferred concentration studed (0.0 , 0.1 , 0.15 , 0.2 ,0.25 . 0.3 ,0.35) % from Ammonium chloride and used same other condition **.**

2.5.6 The type of multivalent minerals and the preferred concentration: Initially formulated N2 deficient medium contain (Sodium Chloride , Magnesium sulphate and Potassium monohydrogen phosphate). To study the effect of these minerals both on biomass and PHB productivity, eight flasks each containing 19.96 ml N2 deficient medium used 2% Dates juice Carbon Source and 0.2% Ammonium chloride nitrogen sources were prepared as follows: One contained only Sodium Chloride ; second contained only Potassium monohydrogen phosphate ; third contained only Magnesium sulphate ; fourth contained Potassium monohydrogen phosphate and Sodium Chloride ; fifth contained Sodium Chloride and Magnesium sulphate ; sex contained Magnesium sulphate and Potassium monohydrogen phosphate seven contained N2 deficient medium devoid of all minerals ; eight contained media with out multivalent minerals. The flasks were incubated at 40°C on a rotary shaker (200 rpm) for 72h. The effect of preferred concentration studed (0.000 , 0.035 , 0.070 , 0.105 , 0.140 ,0.175 , 0.210) % from bath Magnesium sulphate and Potassium monohydrogen phosphate and used same other condition.

2.5.7 Initial pH: The medium used contain Dates juice 2% as Carbon Source , Ammonium chloride 0.02% as Nitrogen Source , Magnesium sulphate and Potassium monohydrogen phosphate 0.105% as minerals salt source . Different initial pH of the medium (5.5, 6, 6.5, 7, 7.5, 8 and 8.5) was used to check whether pH has any noticeable effect on PHB production. The initial pH of the medium was adjusted by 1N hydrochloric acid or sodium hydroxide . The flasks were incubated at 40°C on a shaker incubater (200 rpm) for 72hr.

2.5.8 Inoculum size: The inoculated cultures in production medium by difrent inoculum size (1, 1.5, 2, 2.5 ,3 ,3.5, and 4) % v/v. The medium used contain Dates juice 2% as Carbon Source , Ammonium chloride 0.2% as Nitrogen Source , Magnesium sulphate and Potassium monohydrogen phosphate 0.105% as minerals salt source at initial pH 7 and incubated the flasks at 40°C on a rotary shaker (200 rpm) for 72 hr.

3- Result and Discussion

1- incubation time In Figure (1) showed effect of incubation time on PHB production by the *Bacillus* spp. The PHB yields increase with time dependent manner and highest yield 0.232 mg/ml was obtained after 72h of incubation of isolate in nutrient medium under stationary conditions of growth and decreased thereafter. This reducing in PHB production after 72 h may be due to lack of micronutrients as well as increase in metabolites that might have negative effect on the PHB production. Produced PHB from *Bacillus thuringiensis* and *Bacillus shackletonii K* 5 at 24 h incubation time (21) ww Produced from *Bacillus.species* at 72 h(14).

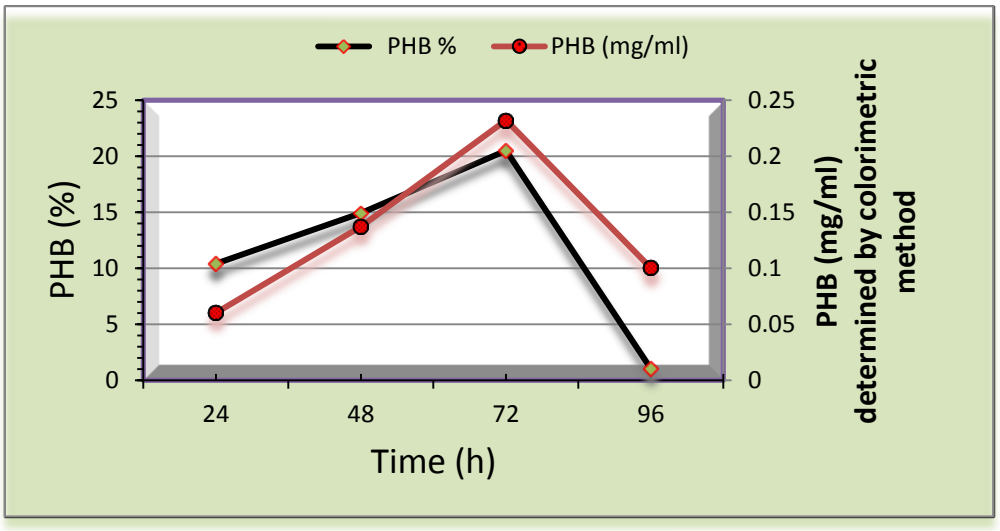

Fig. 1: Effect of Incubation time in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.1).

2- Incubation temperature

The preferred habitat of a given bacterium can provide a hint of which types of enzymes of potential industrial interest it might produce. These might include enzymes that are stable and active at very high or very low temperatures. Being able to accurately predict this based on a genomic sequence (22) In Figure (2) showed the effect of temperature on PHB production from *Bacillus* spp, it found that 40 °C incubation temperature is optimum for PHB synthesis under fermentation condition . The data from our study also concluded that 40°C was the optimum temperature for PHB production from *Bacillus subtilis NG220* (23) and Produced from *Alcaligenes eutrophus* that 37°C (24).

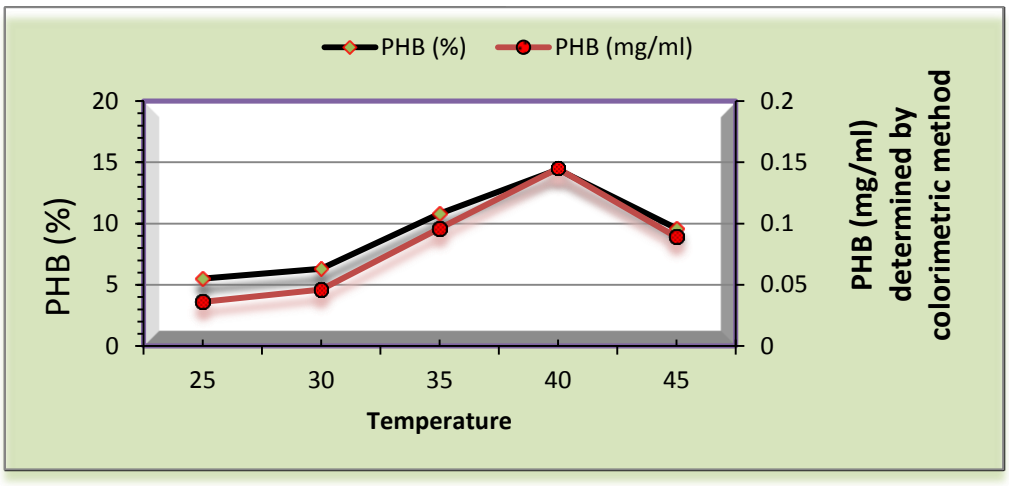

Fig.2: Effect of Incubation Temperature in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.2).

3- Agitation speed (rpm)

The effect of agitation rate on cell growth and PHB production was also studed. The experimental results showed that the PHB production increased from 0 to 200 rpm. Therefore, an elevated agitation rate apparently enhanced both cell growth and PHB production from 0.005 mg/mL to 0 .250 m g/mL (Figure3). Limiting agitation rate to 250 rpm also slightly decreased PHB production to 0.135 mg/mL, probably because of the excessive shear force produced at agitation speeds exceeding 300 rpm.

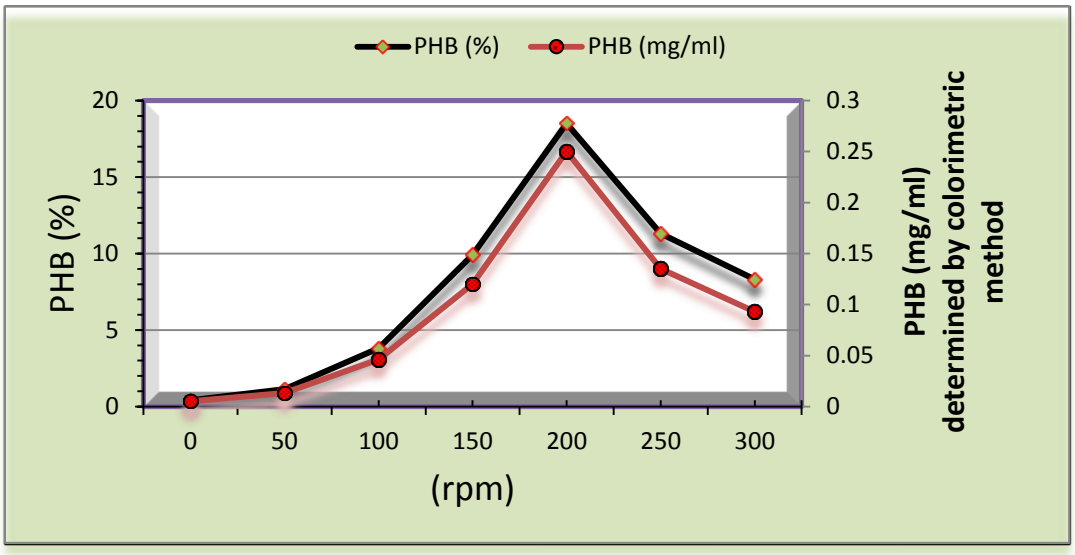

Fig.3: Effect of rotational speed in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.3).

4- The type of carbon source and the preferred concentration One of the most crucial variables affecting PHB biopolymer production is the carbon source. In this concern, the effect of different carbon sources on PHB production by *Bacillus* spp.were investigated. The strain exhibited nutritional versality in terms of varied growth and PHB production when tested on various carbon sources. Results in Figure (4) showed the growth and PHB accumulation measured after 72h

incubation. Obviously, the maximum PHB production was attained 0.120 mg/ml when Dates juice was used as a sole carbon source. Amount of PHB was clearly decreased when used other source such glucose (0.086) mg/ml , starch (0.058) mg/ml , sucrose (0.083) mg/ml, maltose (0.037) mg/ml and lactose (0.083) mg/ml . the preferred carbon source to PHB production was different Dependence on type of bacteria such *Bacillus sp*. JMa5 preferred carbon source (Molasses) *B. sphaericus and B. brevis* preferred carbon source (Sucrose*) and B. cereus* ATCC14579 preferred carbon source (Hexanoate) (25) .The optimum carbon source concentration require for the production of PHB was 2% from Dates juice Figure (5)

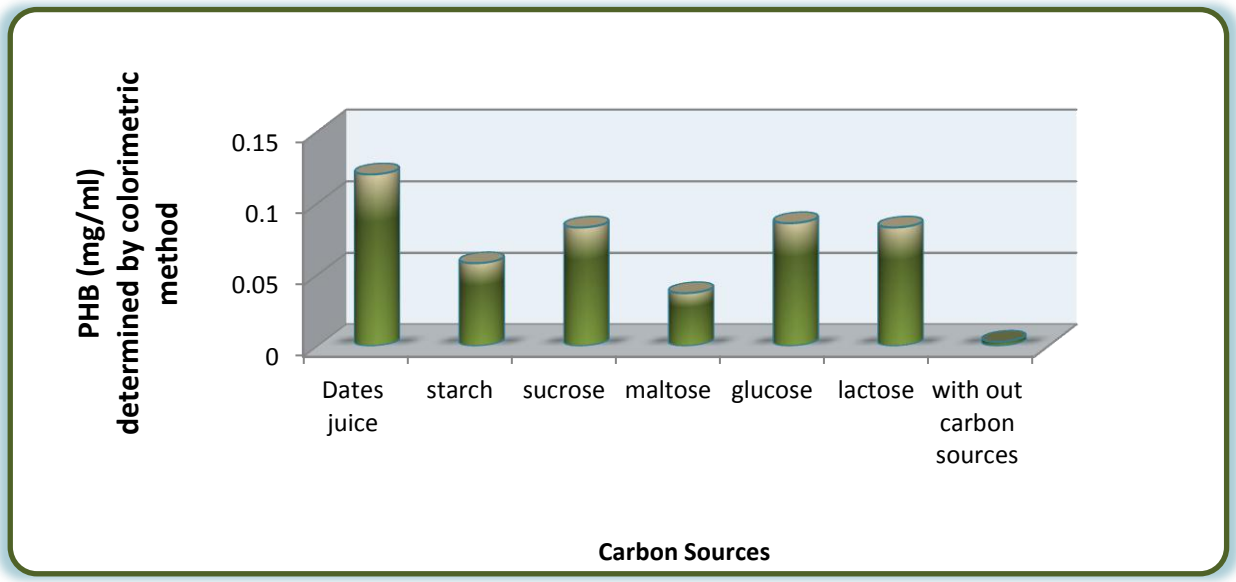

Fig.4: Effect of Carbon Sources in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.4).

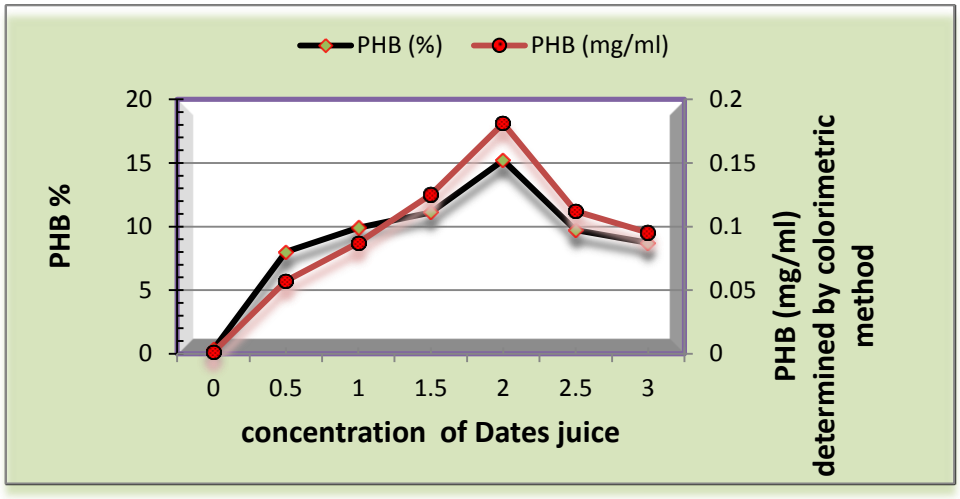

Fig.5: Effect of Carbon Sources (Dates juice) concentration in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.4).

5- Effect of Nitrogen Sources for PHB Production.

PHB production has been previously shown to be. affected by the Nitrogen Sources used and concentration (7). In this study, six Nitrogen sources were tested. These include (peptone, yeast extract, peptone and yeast extract , Ammonium chloride, ammonium sulphate , urea. In (Figure 6) showed the growth and PHB accumulation measured after 72h incubation, Ammonium chloride is the preferred source in the PHB Production (0.183) mg/ml. The synthesis of intracellular PHA is significantly increased by the addition of various complex nitrogen sources. The importance of the presence of acetyl CoA and NADPH, a cofactor of the reductase in the PHA synthetic pathway (25) (7). The optimum Nitrogen Sources concentration require for the production of PHB was 0.2% from Ammonium chloride (Figure 7).

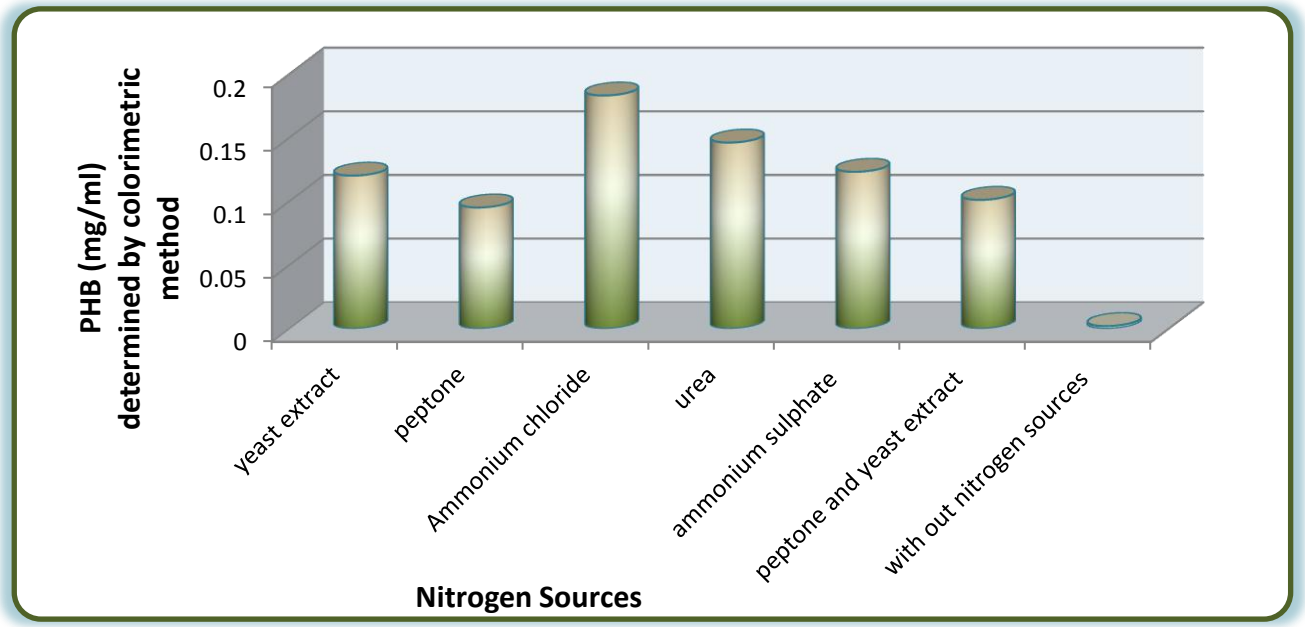

Fig.6: Effect of Nitrogen Sources in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.5).

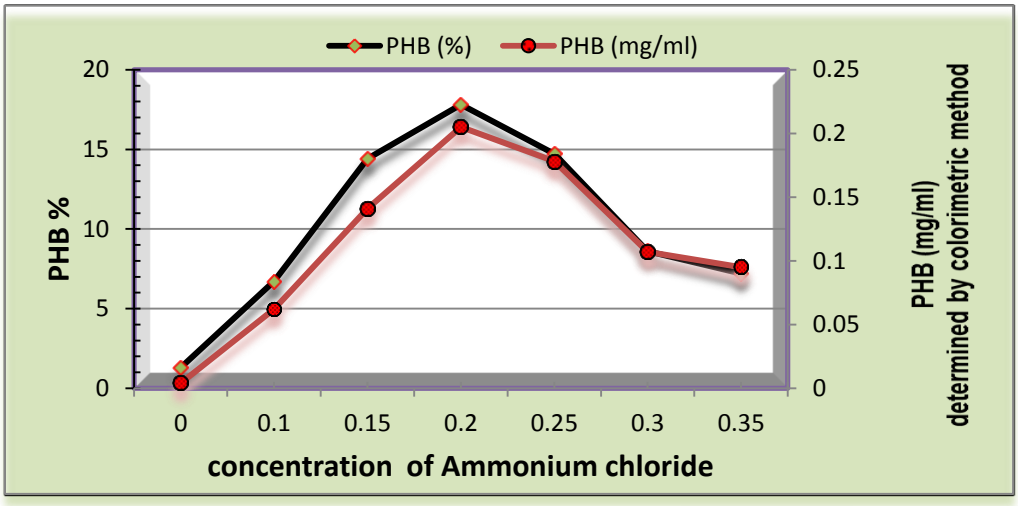

Fig.7: Effect of Nitrogen Sources (Ammonium chloride) concentration in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.5).

6- Effect of Mineral Salts on PHB Production

Adding mineral salts individually to the production medium had interesting effects on PHB production . The highest production was obtained in flask 6 contain from of (0.207) mg/ml (KH_2PO4 and $MgSO_4$) (Figure 8). Phosphate limiting condition (in presence of KH_2PO_4) was important factor for PHB production . However, addition of phosphate was also required for (ADP phosphorylation (7) , nuclic acid generated (26) and energy transport reaction) (27). The optimum Mineral Salts concentration require for the production of PHB was 0.105 % from (KH_2PO_4 and $MgSO_4$) (Figure 9)

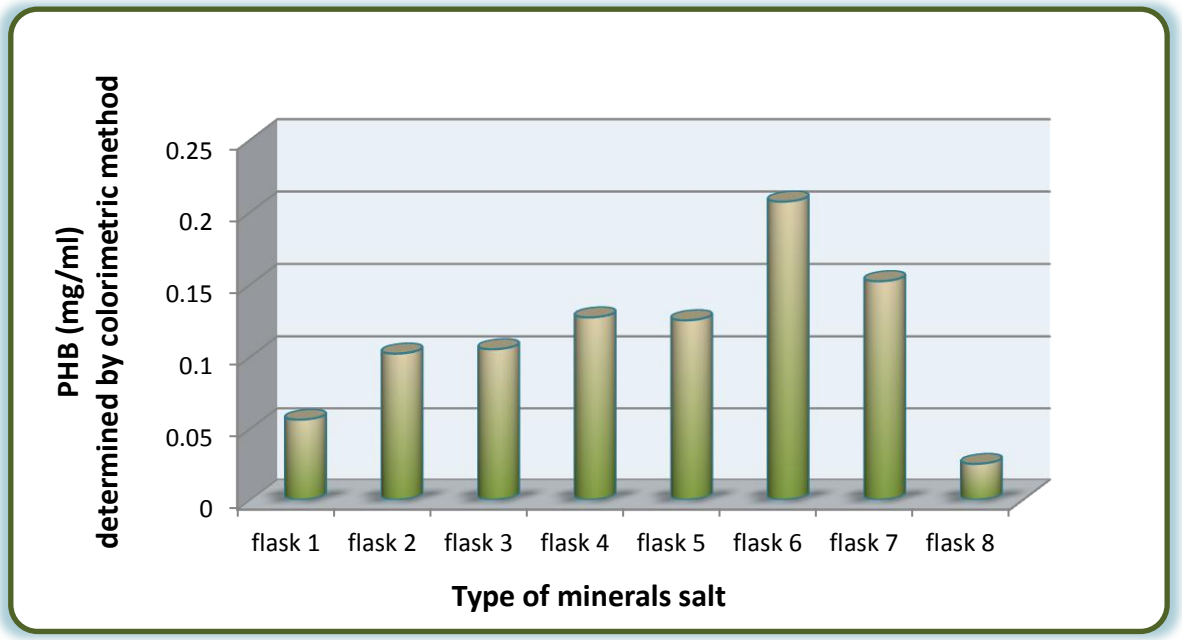

Fig.8: Effect of of different multivalent minerals in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.6).

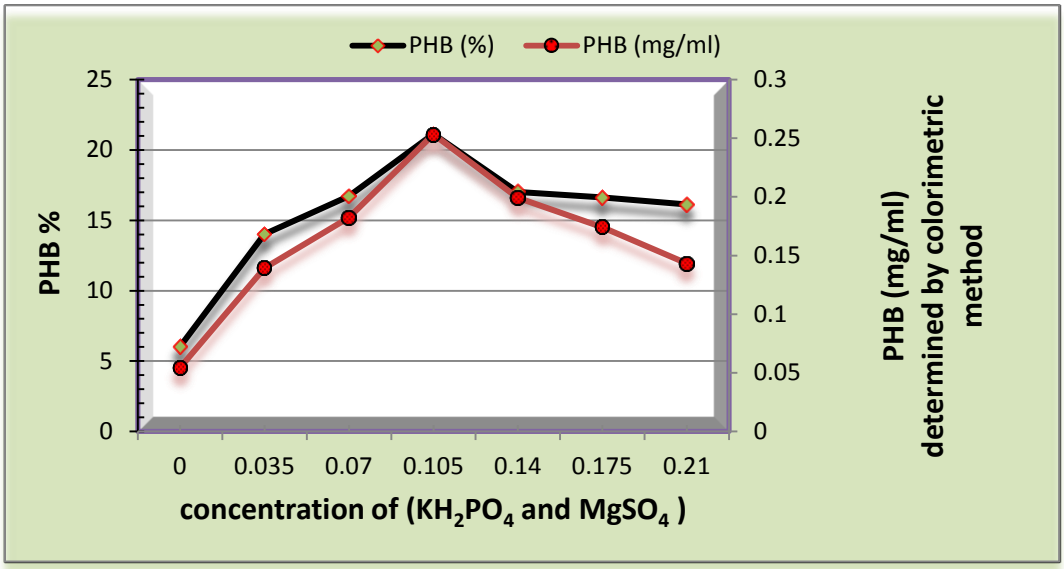

Fig.9: Effect of different (KH_2PO_4 and $MgSO_4$) concentration in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.6).

7- Effect of medium pH on PHB production

pH of the medium from 5.5 to 8.5 supported PHB production by this strain ranging from (0.054 to 0.145) mg/ml (Fig.10). *Bacillus* spp.is reported to produce more PHB at pH (7) (0.252) mg/ml .The influence of pH of culture media on PHB production was also optimized and highest production was obtained at pH range of (6.0 -7.0).

pH 7.0 being neutral, is the most favorable pH for bacterial growth and hence, would have contributed to higher PHB production. that PHB production lack of polymer accumulation at higher pH value can best be explained by an effect on the degenerative enzymes of polymer breakdown, so that the PHB is utilized at the rate almost equal to the rate of its synthesis (23).

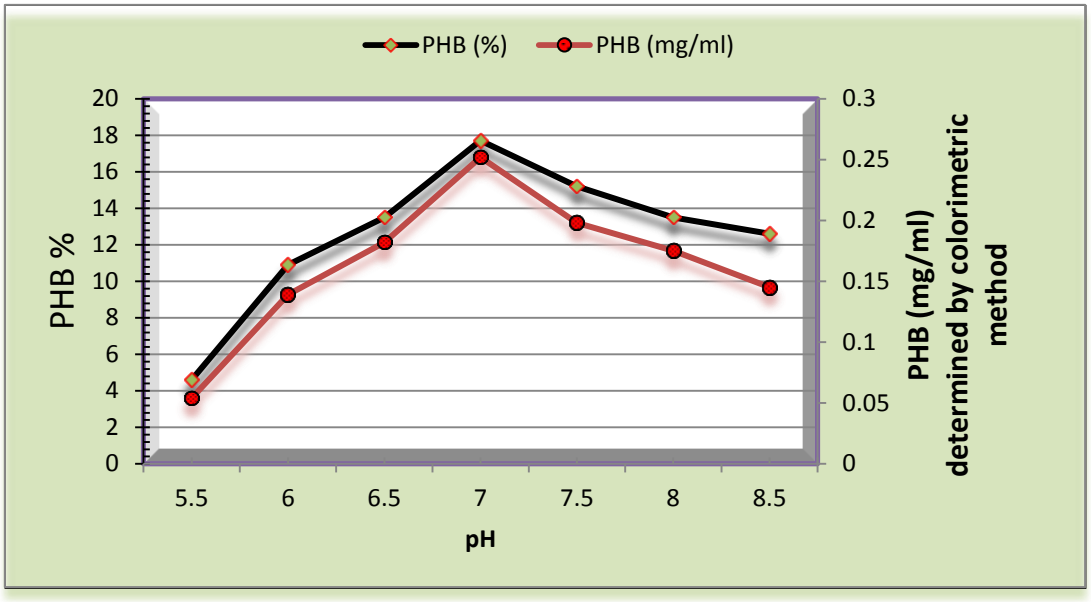

Fig.10: Effect of pH in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.7).

8- The effect of inoculum size

PHB production increased with increasing cell density from 1% to 2,5 % of inoculum size in the biphasic growth condition (0.181 to 0.225) mg/ml and decrease in PHB production from 0.289 to 0.147 at 4% inoculum (Fig.11). Low inoculum size required longer time for cells to multiply and produce the desired product and A small amount of inoculum can lead to insufficient number of microbial cells and a reduced amount of the secreted enzymes while a much higher inoculum could lead to or cause a lack of oxygen and depletion of nutrients in the culture media (28)

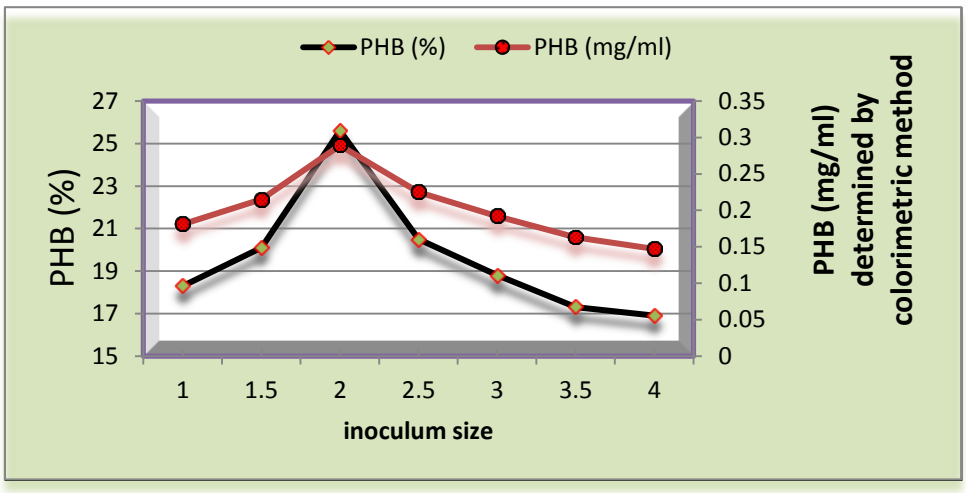

Fig.11: Effect of inoculum size in bacteria growth and PHB accumulation by *Bacillus* spp. in production medium and condition according of find in (2.5.8).

Conclusion

The results of the present investigation provides basis for assessing a potential for using *Bacillus* spp. for PHB (a biodegradable plastic) production, which is an economically and environmentally important product, on large industrial scale, solving by this one of the problems of solid waste management that results from the accumulation of plastics and saving the environment from additional air pollution caused by its recycling and used Dates juice as renewable raw materials, since they were rich in carbon and nitrogen respectively, leading to develop a low cost process of PHB production .

Reference

1- Chee , J. ; Yoga , S. S. ; Lau , N. S. ; Ling , S.C. ; Abed , R. M. M. And Sudesh , K . A.Mendez – Vilas p.1395-1404. (2010) .

2- Chandrashekharaiah , P.S.. Thesis of Master science / University of Agricultural Sciences, Dharwad . (2005).

3- Reddy, C.S., Ghai, R., Rashmi, Kalia, V.C. Polyhydroxyalkanoates: an overview. Bioresource Technology. 2003;87: 137–146 .(2003).

4- Anderson, A.J., and Dawes, E.A.. Reviews. 54:450-472. (1990).

5- Chen , G.Q. Springer-Verlag Berlin Heidelberg 14 p. 17-34. (2010).

6- Kumaravel , S. ; Hema , R. and Lakshmi , R. E-Journal of Chemistry ISSN: 0973-4945. (2010).

7- Lakshman , K . University of Mysore , India. (2004).

8- Dawes , E. A. Bioscience Reports 8 (6) :537-547. (1988).

9- Rodrigues , K. M. S. ; Parra , D. F. ; Oliveira , M. J. A. ; Issa , M. G. ; Ferraz , H. G. and Lugão , A. B. XI International Macromolecular Colloquium , 6th International Symposium on Natural Polymers and Composites. (2007).

10- Bonartsev , A.P. ; Myshkina , V.L. ; Nikolaeva , D.A. ; Furina ,E.K. ; Makhina T.A. ; Livshits ,V.A. ; Boskhomdzhiev , A.P. ; Ivanov , E.A. ; Iordanskii , A.L. and Bonartseva , G.A. A.Mendez – Vilas vol(1) p.295-307. (2007).

11- Chan , R. T. H. ; Marçal , H. ; Russell , R. A. ; Holden , P. J. And Foster , L. J . R. International Journal of Polymer Science P. 9 . (2011).

12- Lee , J. E . ; Jung ,S.G. ; Park, C.S. ; Kim, H.Y. and Batt, C.A. . Bioorganic & Medicinal Chemistry Letters. 21(10): 2941-2944 . (2011).

13- Logan,N.A. and Vose,P.D. Genus I Bacillus In: Vos,D.P. ;Garrity,G.M.; Jones,D. ;Krieg, N.R. ; Ludwig ,W. ; Rainey,F.A. Schleifer K.H., and Whitman,W.B.editors 2009 Bergey's Manual Systematic Bacteriology Second Edition .Volume Three.Newyork :Springer P.(21-128).

14- Singh , P. and Parmar , N. African Journal of Biotechnology 10(24): 4907-4919. (2011).

15- Aslim, B.; Yuksekdag, Z. N. & Beyatli, Y. Turkish Electronic Journal of biotechnology.p. 24-30. (2002).

16- John, H.L. and Ralph, A.S. Journal of bacteriology, 82: 33-36. (1961).

17- Lakshmi , R .S. ; Hema ,T. A. ; Raj , D .T. and Shylaja ,S .T. J . IOSR Journal of Pharmacy and Biological Sciences.3 (2) : 21-25. (2012).

18- Selvakumar, K . ; Srinivasan , G. ; Baskar, V. and Madhan, R . European Journal of Experimental Biology 1 (3):180-187. (2011).

19- Berekaa , M. M. and Al Thawadi, A. M. African Journal of Microbiology Research 6(4): 838-845. (2012).

20- Miller,G.I.Anal.Chem.,3r.426-428. (1959).

21- Liu, Y. ; Huang , S.; Zhang , Y.and Xu, F.(2013). Isolation and characterization of a thermophilic Bacillus shackletonii K5 from a biotrickling filter for the production of polyhydroxybutyrate.Journal of Environmental Sciences 25(1):1-24.

22- Jensen , D. B. ; Vesth , T. C. ; Hallin , P. F. ; Pedersen , A. G. and Ussery , D. W . BMC Genomics 13(7) :S3. found online at:http://www.biomedcentral.com. (2012) .

23- Singh , G . ; Kumari , A. ; Mittal , A. ; Yadav , A . and Aggarwal , N. K. .BioMed Research International P. 10 .(2013).

24- Arun , A .; Murrugappan1, R ; Ravindran, A. D. D. ; Veeramanikandan , V. and Balaji , S . African Journal of Biotechnology 5 (17) : 1524-1527. (2006) .

25- Kumar , P.K.A. Thesis of Master science / Department of Food Microbiology. University of Mysore. (2007).

26- Divyashree , M. S. Thesis of Doctor of philosophy . Department of Food Microbiology/ University of Mysore , India.(2008) .

27- Sahalan , A.Z. ; Aziz , A.H.A. ; Lian , H. H. And Ghani , M.K.A. J. Sains Malaysiana 42(3): 301–306. (2013).

28- Hamieh , A. ; Olama , Z. and Holail , H. Global Advanced Research Journal of Microbiology . 2(3): 054-064. (2013).