Production of Polyhydroxyalkanoate by Local Strain of *Bacillus megaterium* AUMC b 272 Utilizing Sugar Beet Wastewater and Molasses

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Abstract

Development of polyhydroxyalkanoates (PHAs) as a potential substitute material to some conventional plastics has drawn much attention due to their biodegradable and compatible properties. The potential applications of PHA in various industries and in the medical field are encouraging. Nevertheless, the production cost of PHA has been a major drawback. Consequently, scientific effort have been made to overcome the high cost of the substrates used in the bio-production. In this study, sugar beet industry wastewater without and with beet molasses was used as potential low cost substrate for production of the biopolymer PHA by a local bacterial strain. This strain was selected after screening of 30 bacterial isolates for PHA production and was identified according to 16S rRNA gene sequencing as *Bacillus megaterium* AUMC b 272. The maximum PHA formed by this strain reached to 4.053 g/L with a recovery yield equal to 41.79 % of the bacterial biomass using modified mineral salt medium (MSM) medium containing 6 % beet molasses as sole carbon source and 0.5 g/L ammonium oxalate as a nitrogen source. The initial pH of the medium was adjusted at 8.5. Cultures were incubated at 200 rpm and 37° C for 24 hrs. On the other hand, the bacterial strain produced negligible levels of PHA when grown on the same medium constituents under the same conditions with replaced distilled water and molasses by sugar beet wastewater. While the PHA concentration reached to 0.828 g/L with

recovery yield 20.58 % of biomass in the same medium under the same conditions after replacement of distilled water by sugar beet wastewater. It is worthy to mention that the COD in the sugar beet wastewater medium at the end of fermentation period was removed by 69 %. Characterization of the obtained PHA was achieved using Fourier transform-infra-red spectroscopy (FT-IR) and gas chromatograph mass spectrometric (GC-MS). Accumulation of considerable levels of PHA as well as high levels of COD removal from sugar beet wastewater strongly introduced this biotechnological process as valuable method for production of PHA as biodegradable biopolymer from sugar beet industry wastewater in presence of beet molasses as potential low cost substrates and, at the same time, for biological treatment of industry wastewater.

Keywords: Polyhydroxyalkanoates, Production, Sugar Beet Wastewater, Sugar Beet Molasses, Bacillus megaterium.

Introduction

Organic plastic – usually known as bio-polymer- is an environment friendly form of plastics that is produced by renewable biomass sources and that isn't fossil-fuel plastics derived from petroleum and natural gas. Such type of plastic can both conserve fossil resources and reduces dangerous effect of non-degradable plastics.

Polyhydroxyalkanoate (PHAs) is a polyester resamples to ordinary plastic that can produced by some bacterial strains in order to store carbon and energy reserves (1,2). Early discovering of PHA was in 1926 by Maurice Lemoigne as inclusions in both *Bacillus megaterium* and *Azotobacter chroococcum* which is accumulated as intracellular granules (3).

Khanna and Srivastava (4) mentioned that the PHAs are accumulated as granular inclusions in the cytoplasm of bacterial cell with diameter of 0.2: 0.5 µm, molecular weight ranges from 2×10^5 to 3×10^5 Da (Figure 1) and that varies according to microbial species or the growth conditions such as type and concentration of the carbon source, fermentation conditions and mode of fermentation (batch, fed-batch, continuous). PHAs are biodegradable, exhibit thermoplastic properties and can be produced from different renewable carbon sources (5) . They have a wide range of applications, such as in the manufacture of bottles, packaging

materials; films for agricultural purposes as in well as in biofuel and several medical applications (6,7).

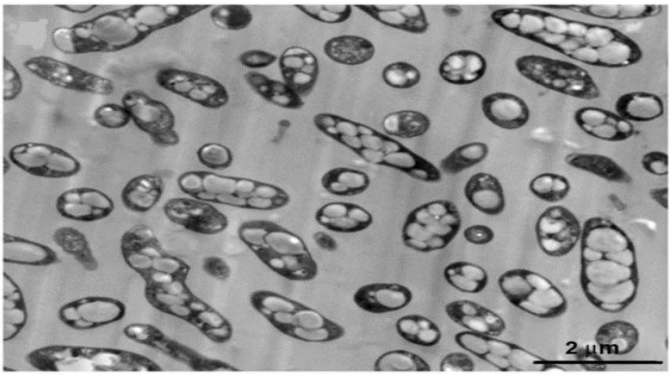

Figure (1): Transmision electron micrograph of accumilation of PHA in bacterial cell [4].

The major cost in the PHAs production is the cost of carbon feedstock for cultivation of PHA producing bacteria. So, selection of a suitable, renewable and low cost waste material for the production of PHAs has become an important objective for the commercialization of bioplastics and can significantly affect the PHAs production cost. Therefore, the simplest approach is to choose renewable, inexpensive and most readily available carbon substrates that could support both microbial growth and PHAs production efficiently.

Industrial wastewater from all dietary factories contains many of dissolved organic matter that need to be removed by wastewater treatments, that is undesirable valuable matters especially those containing simple carbohydrates. Wastewater treatment plants are normally designed to comply with the environment regulations that vary according to each country (8) **.** Because of its contents, this wastewater is ideal environment for growth of micro-organisms. Normally in beet sugar factories, the main contaminant of the effluent is sucrose which is not toxic, soluble carbohydrate and ideal substrate for such microbial growth. The microorganisms fed on the organic material and water soluble carbohydrates causing the consumption of dissolved oxygen in water as well as raising the level of Biological Oxygen Demand (BOD). These growing microbes may

be pathogenic or toxinogenic sources. Dangers of high levels of BOD resulting from the accompanied decrease in the dissolved oxygen in water are the consumption by those microbes. This shortage of dissolved Oxygen seriously harm aquatic organisms which may not survive (9, 10) **.** A large amount of wastewater are discharged from beet sugar factories and these wastewater represent a potential renewable feedstock for PHAs production. Utilizing these waste materials as carbon source for PHA production not only reduces the substrate cost, but also saves the cost of waste disposal in these factories.

So, the sugar beet wastewater was chosen as the feedstock for production of PHAs by selected PHA producer *Bacillus megaterium* strain in this study for two reasons. The readily biodegradable, high-organic-content composition of these wastewater meets criteria for PHA production and secondary treatment of sugar beet wastewater alleviates many potential environmental concerns associated with these waste material. The overall goal of this study was to evaluate the feasibility of producing PHA, while simultaneously treating sugar beet wastewater. The specific objectives were to: select the highly PHAs producer bacterium; optimization of PHAs production conditions; evaluate the efficacy of the selected bacterium to use sugar beet wastewater for producing PHA; and using sugar beet molasses as a supporting nutrient to increase the PHAs production. Finally, characterization of the obtained PHAs was also achieved.

Material and methods

Isolation and collection of bacterial isolates

A total of 35 samples were collected from different regions of Bilqass sugar factory of Daqahlya Company for Sugar & Refining in Daqahlya, Egypt as follow: 15 soil samples (500 g each), five collected from each of region of molasses tanks, soil of beet-roots and the waste of melted raw sugar; 20 wastewater samples (500 ml each), also five samples of each of sludge of anaerobic water treatment station, sludge of aerobic water treatment station, refinery wastewater and water basin in addition to 5 samples of dairy wastewater. These samples were serially diluted (10^{-1} – 10^{-4}) and aliquots of 1.0 mL of the 10^{-3} and 10^{-4} dilutions were plated on

mineral salt medium (MSM) plates supplemented with 2% sucrose as sole carbon source. Plates were incubated at 37°C for 72 hrs. The bacterial colonies were purified. Purity was checked-up microscopically and morphologically using Gram's stain. Only pure isolates were sub-cultured onto slants of nutrient agar (NA) medium and kept at 4° C for further investigations (11) .

Composition of growth media

Mineral salt medium (MSM) (12,13). contains (g/L): $Na_2HPO_4.12H_2O$: 9.0; KH_2PO_4: 1.5; $MgSO_4.7H_2O$: 0.2; NH_4Cl: 1.0; $CaCl_2.2H_2O$: 0.02; Fe^{+3} NH_4 citrate: 0.0012; in addition to 1ml of trace elements solution. (g/L): EDTA: 50; $FeCl_3$: 8.3; $ZnCl_2$: 0.84; $CuCl_2.2H_2O$: 0.13; $CoCL_2.6H_2O$: 0.1; $MnCl_2.6H_2O$: 0.016; H_3BO_4: 0.1. Sucrose, molasses, or wastewater of Bilqass sugar factory were, individually, incorporated as carbon sources at different concentrations as mentioned in each experiment. The pH value of the medium was initially adjusted to 7.0 and autoclaved at 121°C for 20 min.

Nutrient agar medium (NAM) (12,15). was used for bacterial purification, that is composed of the following concentrations (g/L): beef extract: 3; peptone: 5; sodium chloride: 5; agar: 15. Medium was autoclaved at 121°C for 20 min. The pH was adjusted at 7.0.

The composition of sugar beet molasses is, generally, as follow: 80 - 81 % dry substances, 20 - 19 % water, 47 - 48 % sucrose, 1 % glucose, 0.25 % fructose, 0.25 % raffinose, about 10 % ash, non-sugars content enfold minerals and trace elements mainly K, Na, Ca, Mg, Fe, and Cu, beside compounds like crude proteins, vitamin-B complex, non-nitrogen substances, biotin, and their pH range from 7.5 - 7.8 (8,14). Sugar beet wastewater used in this study was collected from Bilqass sugar factory of Daqahlya Company for Sugar & Refining in Daqahlya, Egypt. This wastewater contains, as recorded in the factory follow up sheet, BOD, COD, NH_3, PO_4 and NO_3 at 1800, 3600, 2, 9 and 13 ppm, respectively.

Screening of bacterial isolates for PHA production

Primary screening of bacterial isolates was done using Sudan black B plate assay (16,17). Secondary screening of the positive PHA

producers was done using Nile blue A plate assay(17). The positive bacterial isolates for PHA production were cultivated onto mineral salt broth medium supplemented with 2% sucrose as a sole carbon source. The cultures were incubated for 72 hours at 37^{o} C and 200 rpm. The bacterial biomass obtained was harvested and then PHA polymer was precipitated, washed, extracted and identified (12).

Characterization and identification of PHA producing bacterial isolate

The selected PHA producing bacterial isolate was subjected to some morphological and biochemical tests. The morphological characteristics of the bacterium colonies were described according to Williams and Davies (18). It was examined for KOH test as method described by ***Halebian et.al.(19).*** Also, this isolate was examined for Gram stain reaction as standard procedures given by ***Barthalomew and Mittewer(20).***

Molecular identification was performed at Solgent Company, South Korea. The genomic DNA was extracted from the isolated bacterium using the genomic DNA Prep kit according to the manufacturer's instructions after glass bead beating to disrupt the cell walls. The extracted DNA was then used as a template for PCR to amplify the 16S rRNA gene. For identification of the isolated bacterium, the partial 16S rRNA gene sequence was compared with full sequences available in the GenBank database using the BLAST search tool (NCBI).

Optimization of PHA production

Several environmental and nutritional factors were tested for selecting the optimal conditions for PHA production by the selected producer strain of *Bacillus megaterium* (AUMC b 272). These factors included concentration of sugar beet molasses (1.0 – 15.0%) as sole carbon source in MSM. At optimum concentration of molasses, the effect of incubation temperatures (30 - 45°C) was also tested. Taking the last results in consideration, the role of incubation periods (24, 48, 72 & 96 h) was studied and at optimum incubation period, ammonium chloride in the basal MSM-molasses medium was replaced at nitrogen equimolecular weight with organic (ammonium

oxalate and yeast extract), or inorganic (ammonium sulfate, sodium nitrate and di-ammonium hydrogen orthophosphate) nitrogen sources, separately, in order to select the best nitrogen source for PHA production. Also, effect of pH values (pH 7- 9) in MSM was tested for achievement of optimum pH value. Furthermore, the effect of different concentrations of ammonium oxalate (0.25, 0.50, 1.0, 1.3, 1.5 and 2.0 g/L) on PHA was also investigated. Finally, at the optimum conditions of all the previous factors, effect of various agitation speeds (0. 150. 200 & 250 rpm) on the growth and PHA production of the bacterial strain was investigated.

Production of PHA using sugar beet wastewater and molasses

Sugar beet wastewater and molasses from Daqahlya Company for sugar manufacturing and refining were used for PHA production by the selected bacterial strain. The selected bacterial strain was cultured on basal MSM in which wastewater was the sole carbon source replaces both distilled water and molasses content and still using other additives at the optimum conditions. Also, a comparison was done with the treatment in which the wastewater replaced only distilled water and the molasses still being used. The well-known Chemical Oxygen Demand (COD) analysis as a measurement of the organic load was estimated using Potassium Di-Chromate ($K_2Cr_2O_7$) method, COD test of closed reflux and Colorimetric method, for the raw wastewater and wastewater medium after fermentation.

Analytical methods

Detection of dry weight biomass:

The broth cultures were centrifuged at 10,000 rpm, 4°C for 5 min and the cell pellets were, individually, washed with distilled water. Cell pellets were harvested by centrifugation and dried at 105°C until constant weight to estimate the dry cell weight.

PHA extraction and detection:

After incubation time, bacterial cells were harvested by centrifugation at 10,000 rpm for 10 minutes. The pellet was then treated with 5% NaOCl solution and incubated at 37 °C for one hour, and then centrifuged at 5000 rpm for 15 minutes. Residual bacterial cells were washed with distilled water and acetone, respectively. The

pellet was dissolved in 5 ml of boiling $CHCl_3$ and allowed to evaporate after filtration [21]. The residual biomass was dissolved in 10 mL of concentrated H_2SO_4 and heated for 10 minutes at 100 °C in a water bath to convert the polyhydroxyalkanoate into crotonic acid, which was brown colored. The solution was cooled and measured at 235 nm against a sulfuric acid blank using UV Spectrophotometer (SHMADZO UV-1800 Spectrophotometer). Standard curve was established with PHB (Sigma, Aldrich) concentrations ranging from 1.0: 8.0 mg/ml PHB [22]. The quantity of PHA produced was determined by comparison with the standard.

COD estimation: The COD test of closed reflux, colorimetric method [23] was done using the Chekit Direct COD tintometer and photometer COD vario, according to the standard methods of water analysis, the absorbance at 430 nm, result was monitored as mg Oxygen /l.

Characterization of PHA:

The extracted PHA bio-polymer was purified, air dried and kept for further characterization according to their physico–chemical properties using the following techniques: Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography-Mass Spectroscopy (GC-MS).

FTIR analysis:

The extracted PHA was dissolved in chloroform and added to KBr and then evaporated. This prepared sample was subjected to FTIR spectroscopy at Faculty of Science, Mansoura University, Egypt, to analyze the PHA structure and purity. The peaks were observed from 4000-400 cm^{-1} (24,25).

Monomer identification using GC-MS (26) :

The purified PHA polymer was subjected to Thermo-Scientific GC-MS DIP (ISQ7000) at Faculty of Science, Assiut University, Assiut, Egypt, to identify the composition and chemical structure of the basic monomer, using direct inlet prop (DIP), electron ionization mode, full scan (30:800 m/z), 50 °C initial temp, 400 °C final temp and heating rate 30 °C/min.

Results

A total of pure 30 bacterial isolates collected from the different sources were screened for PHA production using Sudan black-B stain technique. Bacterial isolate SNS5 isolated from sludge of an-aerobic water treatment station in Bilqass Beet-Sugar factory, Egypt showed a remarkable positive stain on agar plates upon exposing to visible light. This isolate was considered as a positive for PHA production and was selected for characterization and identification. Capability of this bacterial strain for PHA production was confirmed according to method described by (17). using Nile blue-A dye, and examination under UV light (254 nm). The result confirmed that this strain produced PHA**.**

Bacterial isolate SNS5 was identified based on some morphological, biochemical and genetical criteria. It is Gram-positive and rod shape. The sequence similarity of the 16S rRNA gene was compared with other data obtained from the National Center for Biotechnology Information (NCBI) website. The results revealed that the isolate showed similarity of 100% with *Bacillus megaterium* ATCC 14581 (NR_117473.1) and *Bacillus megaterium* NBRC 15308 (NR_112636.1). So, it was identified as *Bacillus megaterium* SNS5. This strain was deposited in Assiut University Mycological Center, Assiut, Egypt under number AUMC b 272 and their nucleotide sequences have been deposited in the GenBank database under accession number MK494946. The phylogenetic tree of this strain was shown in Figure (2).

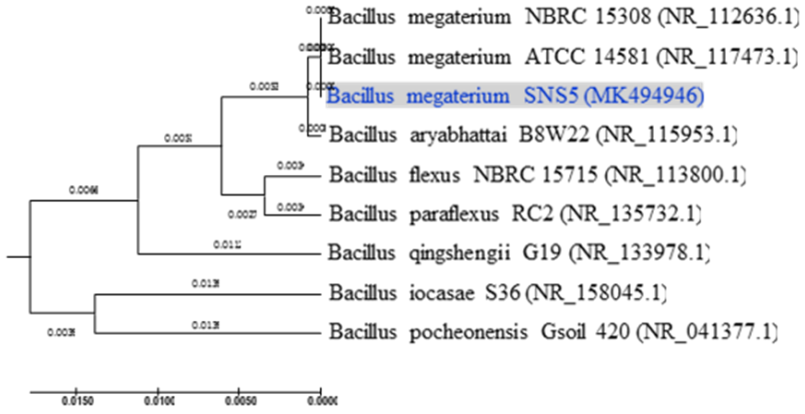

Figure (2): Phylogenetic analysis of 16S rRNA sequences of the bacterial isolates (SNS5) with the sequences from NCBI.

An MSM molasses medium was used basically in a series of experiments to optimize the PHAs production by the selected bacterial strain. To investigate the influence of molasses concentration on PHA production by this strain, it was cultivated in MSM medium with the initial concentrations of beet sugar molasses at 1.0, 3.0, 6.0, 9.0, 12.0 and 15.0 % as sole carbon source at 37^{o} C and pH 7.0 in incubator checker at 200 rpm for 48 hrs. Results in **Table 1)** showed that the maximum dry biomass and PHA produced by *B. megaterium* AUMC b 272 were 3.683 and 1.213 g/L, respectively, obtained in MSM with 6 % molasses. The recovery yield of PHA under these conditions was 32.92% (w/w). So, MSM with 6 % molasses was chosen for further experiments.

Table 1. Effect of molasses concentrations on biomass (DW) and Polyhydroxyalkanoate (PHA) produced by the tested bacterial strain cultured in MSM.

Molasses % in MSM	DW±SD* (g/L)	PHA ±SD (g/L)	Recovery yield %
1.0	0.423± 0.013	0.115± 0.003	27.20
3.0	0.473± 0.005	0.126± 0.009	26.73
6.0	3.683± 0.036	1.213± 0.038	32.92
9.0	2.306± 0.035	0.989± 0.076	42.87
12.0	2.174± 0.032	0.742± 0.04	34.12
15.0	2.124± 0.015	0.746± 0.04	35.14

*** SD = Standard Division**

The effect of different incubation temperatures (30, 37, 40 & 45°C) on PHA production by this bacterial strain was tested taking into our consideration the later proper value of molasses concentration. The best PHA value by *B. megaterium* AUMC b 272 was 1.869 g/L obtained from molasses MSM at 37°C with recovery yield equal to 40.94 % of dry biomass (Table, 2).

Table 2. Effect of incubation temperature on DW and PHA produced by the tested bacterial strain cultured in MSM.

Temp of MSM	DW±SD* (g/L)	PHA ±SD (g/L)	Recovery yield %
30.0	5.11± 0.055	1.81± 0.04	35.42
37.0	4.565± 0.04	1.869± 0.044	40.94
40.0	3.6± 0.071	1.686± 0.065	46.82
45.0	5.334± 0.062	0.696± 0.038	13.05

*** SD = Standard Division**

At optimum molasses concentration and temperature, the effect of different incubation periods (12, 24, 48, 72 and 96 hrs) on PHA production by this strain was tested and the result showed that the best PHA value was 1.898 g/L after 24 h of incubation with recovery yield reaching to 43.60 % of dry biomass (Table, 3**)**.

Table 3. Effect of incubation period on DW and PHA produced by the tested bacterial strain culutred on MSM.

Incubation (hrs.)	DW±SD* (g/L)	PHA ±SD (g/L)	Recovery yield %
12.0	0.614± 0.025	0.242± 0.026	39.40
24.0	4.354± 0.129	1.898± 0.021	43.60
48.0	3.629± 0.01	1.217± 0.036	33.54
72.0	1.609± 0.012	0.498± 0.025	30.97
96.0	2.074± 0.029	0.279± 0.012	13.43

*** SD = Standard Division**

To evaluate the influence of pH on growth and PHA production by bacterial strain under study, the initial pH of the sterilized MSM medium with 6 % beet molasses was adjusted at different pH values as 6.0, 7.0, 7.5, 8.0, 8.5 and 9.0 using NaOH 5N and HCl 5N and inoculated with the bacterial inoculum. Cultures were incubated at 37^{o}C and 200 rpm for 24 h. Results in Table (4) showed that *B. megaterium* AUMC b 272 formed the best PHA value (1.982 g/L) from molasses MSM at pH 8.5 with recovery yield attaining to 41.09 % of dry biomass (w/w).

Table 4. Effect of initial pH value of MSM on DW and PHA produced by the tested bacterial strain

pH of MSM	DW±SD* (g/L)	PHA ±SD (g/L)	Recovery yield %
6.0	3.235± 0.017	0.83± 0.012	25.66
7.0	4.354± 0.129	1.898± 0.021	43.60
7.5	4.733± 0.017	1.903± 0.04	40.21
8.0	4.766± 0.032	1.933± 0.021	40.56
8.5	4.824± 0.01	1.982± 0.05	41.09
9.0	5.165± 0.038	1.404± 0.09	27.18

*** SD = Standard Division**

The effect of some organic and inorganic nitrogen sources was investigated for the PHA production by this bacterial strain in MSM medium with the optima molasses percentage, incubation temperature, incubation time and initial pH. Ammonium oxalate was recorded as the best nitrogen source. The highest PHA value was 2.697 g/L with recovery yield reaching 35.84 % of the dry biomass (Table, 5).

Table 5. Effect of nitrogen source used in MSM on DW and PHA produced by the tested bacterial strain

Amm. source in MSM	DW±SD* (g/L)	PHA ±SD (g/L)	Recovery yield %
$Amm.SO_4$	2.851± 0.054	1.522± 0.007	53.40
$diAm.PO_4$	3.368± 0.068	1.352± 0.018	40.13
$NaNO_3$	4.181± 0.046	1.583± 0.02	37.86
Amm. Oxalate	7.525± 0.054	2.697± 0.038	35.84
Yeast extract	3.318± 0.031	1.825± 0.01	55.00
Amm. Cl	4.854± 0.129	1.988± 0.021	40.96

*** SD = Standard Division**

Furthermore, the effect of different concentrations of ammonium oxalate (0.25, 0.50, 1.0, 1.3, 1.5 and 2.0 g/L) on PHA formation by the tested strain was investigated and the results showed that concentration of 0.5 g/L was the best concentration and formed very high level of PHA reaching to 4.053 g/L with recovery rate achieving 41.79 % of the biomass (Table 6)

Table 6. Effect of ammonium oxalate concentration in MSM on DW and PHA produced by the tested bacterial strain

Amm. oxalate concn. in MSM	DW±SD* (g/L)	PHA ±SD (g/L)	Recovery yield %
0.25 g/l	7.405± 0.021	2.257± 0.006	30.49
0.50 g/l	9.699± 0.056	4.053± 0.015	41.79
1.0 g/l	7.520± 0.14	2.704± 0.049	35.96
1.3 g/l	7.157± 0.054	2.697± 0.038	37.68
1.5 g/l	6.802± 0.012	2.569± 0.042	37.77
2.0 g/l	7.065± 0.023	2.349± 0.025	33.25

*** SD = Standard Division**

Agitation rates at static, 150, 200 and 250 rpm were investigated to determine which rate is preferred for the tested strain under the optima nutritional and environmental conditions which recorded in the previous experiments in this study. The results showed that the agitation rate of 200 rpm was the best one. The highest PHA value by *B. megaterium* AUMC b 272 was 4.053 g/L with recovery yield equal to 41.79 % of dry biomass at this agitation rate (Table 7).

Table 7 . Effect of agitation rate on DW and PHA produced by the tested bacterial strain

Rpm.	DW±SD* (g/L)	PHA ±SD (g/L)	Recovery yield %
Static	1.344± 0.01	0.293± 0.015	21.81
150 rpm	6.4± 0.1	2.426± 0.04	37.91
200 rpm	9.699± 0.056	4.053± 0.015	41.79
250 rpm	6.869± 0.021	3.152± 0.006	45.88

*** SD = Standard Division**

Production of PHA by *B. megaterium* (AUMC b 272) using wastewater with and without molasses of sugar beet from Bilqass, beet sugar factory, Egypt at optimized conditions was studied. MSM with sugar beet wastewater replaced both distilled water and molasses with still using other additives under the optima conditions recorded in this study. The results showed that the bacterial strain grow well under these conditions but produced a negligible level of PHA (Table, 8). It is worthy to mention that the chemical oxygen demand (COD) as g/L was determined for sugar beet wastewater

before and after fermentation by the bacterial strain and the results revealed that the COD level declined from 10 g/L in sugar beet wastewater before fermentation to 3.1 g/L after fermentation by *B. megaterium* AUMC b 272**.**

On the other hand, when the sugar beet wastewater replaces only distilled water in the MSM - 6% beet molasses, a remarkable PHA was produced. PHA value reached 0.828 g/L with a recovery yield of 20.58 % of dry biomass. Although this yield of PHA was less than those recorded when using basal MSM-6% molasses with distilled water (Table, 8), but it is a considerable result for production of PHA from sugar beet wastewater as potential low cost substrate in addition to their effect as biological treatment method for industrial wastewater. The relative decrease in PHA yield by the tested bacterial strain may be due to the interference of the wastewater components that alternates the other applied concentrations.

Table 8. Biomass (DW) and PHA produced by the tested bacterial strain using wastewater with and without molasses of sugar beet from Bilqass Beet-Sugar factory, Egypt at optimized conditions

Medium	DW±SD* (g/L)	PHA ±SD (g/L)	Recovery yield %
MSM in wastewater without molasses	2.149± 0.033	0.036± 0.003	1.68
MSM in wastewater with 6%molasses	4.024± 0.01	0.828± 0.049	20.58
MSM in distilled water with 6% molasses	7.936± 0.056	4.053± 0.015	51.07

*** SD = Standard Division**

Characterization of produced PHA

Characterization of the obtained PHA was achieved using Fourier transform-infra-red spectroscopy (FT-IR) and gas chromatograph mass spectrometric (GC-MS).

Fourier Transform-Infrared Spectroscopy

FTIR was performed to identify the functional groups, for the produced polymer and % transmittance was measured for the wavenumbers between 4000- 400 cm^{-1} in order to analyze the functional groups in the monomeric unit for this polymer. The polymer showed absorption bands as in Figure (3). The absorption

bands at 3436 cm^{-1} is related to OH stretching, at 2975, 2933 cm^{-1} related to C-H stretching, at 1725 cm^{-1} referring to C=O stretching, at 1276 cm^{-1} related to C-O stretching at 1379 cm^{-1} related to CH_3 stretching, and at 1453 cm^{-1} due to CH_2 stretching as recorded in several studies (27,29).

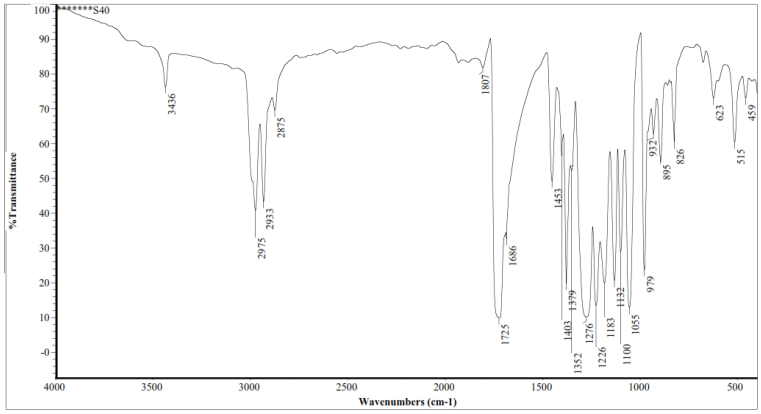

Figure (3): FTIR analysis of PHA produced by *Bacillus megaterium* AUMC b 272

Mass spectrometric analysis for monomer units of PHA: Upon applying mass spectro-metric analysis Using G-C. DIP, for a purified micro-sample from the polymer produced by *Bacillus megaterium* AUMC b 272, we get simple peak corresponding to the molecular weight of PHB monomer (Figure, 5). As illustrated in Figure (5) in which the peak appeared at time of 11.87 min. The composition and chemical structure for the monomer was identified using the standard libraries and mass spectrum. Mass spectrum of PHB showed the following peaks: at m/e= 103.1 that corresponding to hydroxybutyric ion, at m/e= 86.1 that correspond to crotonic acid without hydroxyl group which give the base peak at m/e= 69.1 (Figure, 5). The above facts prompted us to assign the structure of the produced polymer as poly (-(D)-β-hydroxybutrate (PHB) (Figure, 6). This structure was confirmed by formation of (D)-β-hydroxybutrate (PHB) as a result of thermal degradation (30,31).

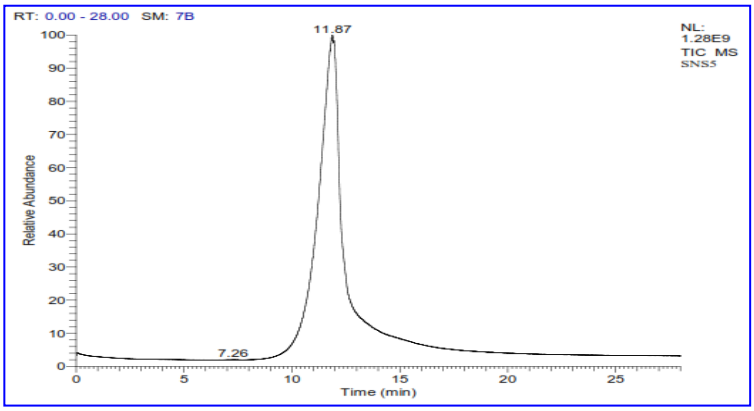

Figure (4): GC-Mass Spectrum for polymer produced by produced by *Bacillus megaterium* AUMC b 272

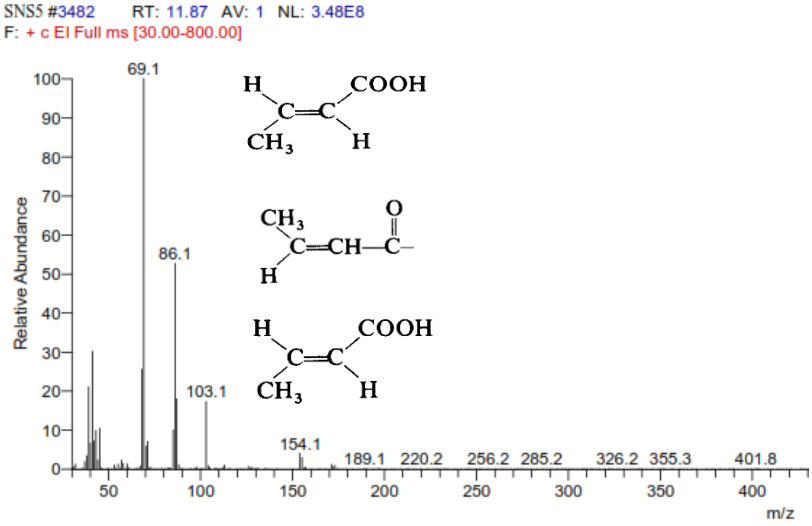

Figure (5): Mass spectrum for analysis of polymer. Produced by *Bacillus megaterium* AUMC b 272

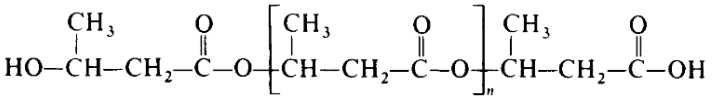

Figure (6): Molecular structure of poly (-(D)-β-hydroxybutyrate (PHB) produced by *Bacillus megaterium* AUMC b 272

Discussion

Recently, biodegradable and biocompatible green thermoplastics has continuously received much attention, since they are an alternative to petroleum nonrenewable plastics. Polyhydroxyalkanoates (PHAs) are polyesters that can be obtained from some bacterial strains under conditions of limited nitrogen and excess of carbon and oxygen. In this study, the optimum production conditions and the active bacterial strain were investigated for maximization of PHA accumulation in bacterial cells grown on Egyptian sugar beet wastewater without and with beet molasses as cheap and available carbon sources.

From 30 different bacterial isolates collected from different samples of Bilqass sugar factory, Egypt and screened for PHA production using Sudan black-B stain technique, the bacterial isolate SNS5 (from sludge of an-aerobic water treatment station in Bilqass beet-sugar factory, Egypt) showed a remarkable positive stain on agar plates upon exposing to visible light. This isolate was considered as positive for PHA production. Also their ability to PHA production was confirmed using Nile blue-A dye. Sudano-philic nature of the PHA granules was previously observed[32]. Ostle and Holt [2] reported that Nile blue A appears to be a more specific stain for poly-, B-hydroxybutyrate than Sudan black B.

Characterization and identification of the selected bacterial strain (SNS5) was performed. Morphologically, the colonies of this bacterial isolate was white, raised, circular, entire margin and opaque. Microscopically, this isolate was negative for KOH test and positive for Gram staining with rod shaped cells. The 16S rRNA gene sequencing revealed that this isolate was 100 % identical to *Bacillus megaterium* ATCC 14581 (NR_117473.1) available in Gen-bank database. The nucleotide sequence of *Bacillus megaterium* SNS5 was deposited in the Gen-Bank database with accession number MK494946.

Nutritional and environmental factors affecting PHA production by the selected bacterial strain were tested for maximization of PHA production. The results showed that the maximum PHA formed by this strain reached to 4.053 g/L with

recovery yield equal to 41.79 % of the bacterial biomass using modified MSM medium supplemented with 6 % sugar beet molasses as sole carbon source and 0.5 g/L ammonium oxalate as nitrogen source at 37°C and initial pH 8.5 incubated at 200 rpm for 24 hrs. Production of PHA from medium with 3:5 % molasses was recommended by several studies (33,35). Wang *et al* [36] reported that the maxima dry weight and PHB produced by *Alcaligenes latus* ATCC 29714 grown on sugar beet juice medium reached 10.30 ± 1.01 and 4.01 ± 0.95 g/L, respectively, with recovery yield of PHB equal to 38.66 ± 7.28 % of dry cell weight.

Recently, ***Hassan et al***.(37) reported that *Clostridium beijerinckii* ASU10 (KF372577) produced PHA at 37.93% of their dry biomass using 6 % molasses medium. Also, many studies reported a pH range from neutral to slightly acidic or slightly alkaline (pH 6.5–8.0) as the optimum for PHAs accumulation by various bacterial strains (34,38-40).

According to this study, agitation rate was a critical factor for PHA accumulation by the tested strain. The lowest growth was at static condition, while a maximum growth rate with higher accumulation of PHA was gradually increased with increasing agitation rate (up to 200 rpm) perhaps due to the increase in oxygen availability and transferring mass as a requirement of aerobic condition for bacterial growth (41).

Results of the present study showed also that the bacterial strain produced negligible levels of PHA when grown on the same medium constituents under the same optima conditions replacing distilled water and molasses by sugar beet wastewater.

While the PHA concentration reached to 0.828 g/L with recovery yield 20.58% of biomass in the same medium (MSM-6% molasses) under the same optimum conditions with only replacement of distilled water by sugar beet wastewater. It was found that, accumulation of PHA was significantly dependent on how richness of carbon source required for PHA storage in bacterial cells (40,42).

It is worthy to mention that the COD in the sugar beet wastewater medium at the end of fermentation period was reduced by 69 %. This result is better than that recorded by ***Khardenavis et al. (41).*** Who used molasses based distillery spent wash for production

of PHB by waste activated sludge and reported that the added advantage of PHB production was the 60% COD removal.

The produced polymer was characterized by Fourier transform infrared (FTIR) spectroscopy (12,17) and mass spectroscopy (30,31,44) .by studying molecular structure and monomer molecular weight and confirmed as polyester. In this polymer the monomeric unit is hydroxybutyric acid and the produced polymer is polyhydroxybutrate (27,29-40).

Conclusion

PHA accumulation was greatly affected by environmental and nutritional conditions during bacterial growth. The highest level of PHAs accumulation by the selected bacterial strain was achieved by using modified MSM medium with 6 % beet molasses as sole carbon source and 0.5 g/L ammonium oxalate as nitrogen source, initial pH 8.5, agitation at 200 rpm and incubation at 37°C for 24 hrs. The carbon level in sugar beet wastewater is insufficient for accumulation of considerable level of PHAs in bacterial cell during fermentation. So, the addition of other carbon source such as molasses with 6 % concentration into the fermentation medium in order to increase the level of PHA accumulation is very important. It is worthy to mention that the COD in the sugar beet wastewater medium was removed by 69 % at the end of fermentation period. Accumulation of considerable level of PHA as well as high levels of COD reduction from sugar beet wastewater strongly introduced this biotechnological process as valuable and economic method for production of PHA as biodegradable biopolymer from sugar beet industry wastewater in presence of beet molasses as potential low cost substrates and, at the same time, for biological treatment of industrial wastewater.

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Refrences

1. **Keshavarz T, Roy I**. **(2010).** Polyhydroxyalkanoates: bioplastics with a green agenda. *Current Opinion in Microbiology* **13** (3): 321 - 326.
2. **Ostle AG, Holt JG. (1982).** Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. *Applied and Environmental Microbiology*. **44** (1): 238 - 241.
3. **Sudesh K, Abe H, Doi Y. Synthesis, (2000).** structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in polymer science* **25**(10): 1503 - 1555.
4. **Khanna S, Srivastava AK. (2005);** Recent advances in microbial polyhydroxyalkanoates. *Process biochemistry* **40** (2): 607 - 619.
5. **Brandl H, Gross RA, Lenz RW, Fuller RC.** (**1990).** Plastics from bacteria and for bacteria: poly (β-hydroxyalkanoates) as natural, biocompatible, and biodegradable polyesters. Microbial Bioproducts: Springer; p. **77**-93.
6. **Khanna S, Srivastava AK. (2005);** Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. Process Biochemistry. **40** (6): 2173 - 2182.
7. **Oliveira F, Freire D, Castilho L. (2004)**; Production of poly (3-hydroxybutyrate) by solid-state fermentation with Ralstonia eutropha. Biotechnology letters. **26** (24): 1851 - 1855.
8. **Asadi M. Beet-(2006).**sugar handbook: John Wiley & Sons.
9. **El-Dean AMK.** (2012). Industrial wastewater treatment to Polyhydroxyalkanoates as biodegradable polymers. International Conference on: "New Role for the World Sugar Economy in a Changed Political and Economic Environment"; Novmper 2012; Aswan, Egypt Aswan, Egypt Sugar Technology Research Institute (STRI) Assiut University. p. **62**- 73.
10. **El_Shanshoury A, Hashim E, Zohri A, El_Tabake M, Hamad A**. **(2013).**Industerial waste water treatment and reuse in delta sugar company. *Egyptian Sugar Journal*.; **6** (3.1): 57 - 80.
11. **Atlas RM. (1993).** Handbook of microbiological media: CRC Press.

12. **Desouky SE-S, Abdel-Rahman MA, Azab MS, Esmael ME. (2017).** Batch and fed-batch production of polyhydroxyalkanoates from sugarcane molasses by *Bacillus flexus* Azu-A2. *J Innov Pharm Biol Sci*.; **4**: 55 - 66.
13. **Schlegel H, Kaltwasser H, Gottschalk G. (1961);** Ein submersverfahren zur kultur wasserstoffoxydierender bakterien: wachstumsphysiologische untersuchungen. *Archiv für Mikrobiologie* **38**(3): 209 - 222.
14. **Šarić LĆ, Filipčev BV, Šimurina OD, Plavšić DV, Šarić BM, Lazarević JM, (2016);** Sugar beet molasses: properties and applications in osmotic dehydration of fruits and vegetables. *Food and Feed Research* **43**(2): 135 - 144.
15. **Shirling ET, Gottlieb D. (1966);** Methods for characterization of *Streptomyces* species. *International journal of systematic bacteriology* **16**(3): 313 - 340.
16. **Phanse N, Chincholikar A, Patel B, Rathore P, Vyas P, Patel M. (2011);** Screening of PHA (poly hydroxyalkanoate) producing bacteria from diverse sources. *International journal of Bioscience* 1(6): 27 - 32.
17. **Godbole S. (2016);** Methods for identification, quantification and characterization of polyhydroxyalkanoates. *International Journal of Bioassays* **5**: 4977.
18. **Williams S, Davies F. (1965);** Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. *Microbiology* **38**(2): 251 - 261.
19. S, **Halebian S, Harris B, Finegold Rolfe R. (1981);** Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. *Journal of clinical microbiology* **13**(3): 444 - 448.
20. **Bartholomew JW, MITTwER T. A (1950);** Simplified bacterial spore stain. Stain technology. **25**(3): 153 - 156.
21. **Sathianachiyar S, Devaraj A. (2013);** Biopolymer production by bacterial species using glycerol, a byproduct of biodiesel. International *Journal of Scientific and Research Publications* **3**(8): 1 - 5.
22. **Dekwer D, Hempel D. (1999):** Microaerophilic production of alginate by *Azotobacter vinelandii*, Von der Gemeinsamen

Naturwissenscha ftlichen, Fakultat der Technischen UN. Wael Sabra, aus Alexandria, Agypten. **37** - 54.

23. **Cheremisinoff NP. (2001) ;** Handbook of water and wastewater treatment technologies: Butterworth-Heinemann.
24. **Oliveira FC, Dias ML, Castilho LR, Freire DM. (2007);** Characterization of poly (3-hydroxybutyrate) produced by Cupriavidus necator in solid-state fermentation. *Bioresource Technology* **98**(3): 633 - 638.
25. **Pandian SR, Deepak V, Kalishwaralal K, Rameshkumar N, Jeyaraj M, Gurunathan S. (2010);** Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by *Bacillus megaterium* SRKP-3. *Bioresource technology* **101**(2): 705 -711.
26. **Yeol Lee E, Yong Choi C. (1997);** Structural identification of polyhydroxyalkanoic acid (PHA) containing 4-hydroxyalkanoic acids by gas chromatography-mass spectrometry (GC-MS) and its application to bacteria screening. *Biotechnology Techniques* **11**(3):167-71.
27. **Hong K, Sun S, Tian W, Chen G, Huang W. A . (1999);** rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. *Applied Microbiology and Biotechnology* **51**(4): 523 - 526.
28. **Vishnuvardhan RS, (2009);**Thirumala M, Mahmood SK. Production of PHB and P (3HB-co-3HV) biopolymers by *Bacillus megaterium* strain OU303A isolated from municipal sewage sludge. *World Journal of Microbiology and Biotechnology* **25(**3): 391 - 397.
29. **Silverstein RM, Bassler GC. (1962);** Spectrometric identification of organic compounds. *Journal of Chemical Education*. **39**(11): 546.
30. **Grassie N, Murray E, Holmes P. (1984);** The thermal degradation of poly (-(d)-β-hydroxybutyric acid): Part 1—Identification and quantitative analysis of products. *Polymer degradation and stability* **6** (1): 47 - 61.
31. **Grassie N, Murray E, Holmes P. (1984);** The thermal degradation of poly (-(D)-β-hydroxybutyric acid): Part 3—The

reaction mechanism. *Polymer degradation and stability* **6** (3): 127 - 134.

32. **Razzaq A, Jamil N, Naheed N, Hasnain S. (2010);** Bacteria from contaminated urban and hilly areas as a source of polyhydroxyalkanoates production. *African Journal of Biotechnology*. **9**(13): 1919 - 1925.

33. **Desouky S, Shiekh H, Elabd M, Shehab A.** Screening, optimization and extraction of polyhydroxyalkanoates (PHAs) from *Bacillus thuringienesis*. Journal of Advances in Biology and Biotechnology. 20^{14}; 1: 40 - 54.

34. **El-Sheekh MM, El-Abd MA, El-Diwany AI, Abdel-mohsen SI, Omar TH. (2015);** Poly-3-hydroxybutyrate (PHB) production by *Bacillus flexus* ME-77 using some industrial wastes. *Rendiconti Lincei.* **26**(2): 109 - 119.

35. **Kalaivani R, Sukumaran V. (2015);** Enhancement of technique for optimized production of PHA from marine bacteria, utilizing cheaply available carbon sources at Thanjavur district, India. *Int J Curr Microbiol Appl Sci.* **4**: 408 - 417.

36. **Wang B, Sharma-Shivappa RR, Olson JW, Khan SA. (2013);** Production of polyhydroxybutyrate (PHB) by *Alcaligenes latus* using sugarbeet juice. *Industrial crops and products*. **43**: 802-811.

37. **Hassan EA, Abd-Alla MH, Zohri ANA, Ragaey MM, Ali SM. (2019),** Production of butanol and polyhydroxyalkanoate from industrial waste by Clostridium beijerinckii ASU10. *International Journal of Energy Research*. In press.

38. **Ramadas NV, Singh SK, Soccol CR, Pandey A. (2009);** Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149. *Brazilian Archives of Biology and Technology*. **52**: 17 - 23.

39. **Sharma P, Bajaj B.** (**2015);** Cost-effective substrates for production of poly-β-hydroxybutyrate by a newly isolated *Bacillus cereus* PS-**10**. 1297 - 1304.

40. **Tripathi AD, Yadav A, Jha A, Srivastava SK. (2012);** Utilizing of Sugar Refinery Waste (Cane Molasses) for Production of Bio-Plastic Under Submerged Fermentation

Process. *Journal of Polymers and the Environment.* **20** (2) :446 - 453.

41. **Sasidharan R, Bhat S, Muthusamy C. (2015);** Biocompatible polyhydroxybutyrate (PHB) production by marine *Vibrio azureus* BTKB33 under submerged fermentation. 2014. *Ann. Microbiol*. **65**: 455 – 465.
42. **Serafim L, Lemos P, Albuquerque M, A M Reis M. (2008);** Strategies for PHA production by mixed cultures and renewable waste materials. **615**-628.
43. **Khardenavis AA, Vaidya AN, Kumar MS, Chakrabarti T. (2009);** Utilization of molasses spentwash for production of bioplastics by waste activated sludge. *Waste Management*. **29** (9): 2558 - 2565.
44. **Hiraishi A, Khan S. (2003);** Application of polyhydroxyalkanoates for denitrification in water and wastewater treatment. *Applied microbiology and biotechnology*. **61** (2): 103 - 109.
45. **Helleur R, MARCHESSAULT R**, **(1988);** editors. Pyrolysis-Gas Chromatography For The Rapid Characterization Of BacterialPoly(Beta-Hydroxybutyrate-co-Beta-Hydroxyvalerate). Abstracts Of Papers Of The American Chemical Society: Amer Chemical Soc 1155 16th St, Nw, Washington, Dc 20036.

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الملخص العربي

انتاج ألكانوات عديد الهيدروكسيل بسلالة بكتريا محلية هي *Bacillus megaterium AUMC b272* باستخدام مياه الصرف الصناعي و مولاس بنجر السكر

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صناعة البوليمرات الحيوية وتطويرها تستحوذ علي اهتمام كثير من الباحثين خاصة المشتغلين منهم فى مجال انتاج البولي هيدروكسي الكانواتPHA كمادة بديلة للبوليمرات التقليدية حيث انها تتفوق عليها فى المواصفات الطبيعية لأنها قابلة للتحلل حيويا وأنها متجددة المصادر. وكذلك لما لها من تطبيقات مشجعة في مختلف الصناعات و خاصة في المجالات الطبية حيث أنها تتحلل داخل الجسم الحي مع الوقت، إلا أن تكلفة الإنتاج لا تزال عائقاً كبيراً لانتاجها بطرق اقتصادية. وهذا شجع العلماء للسعي للتغلب على التكلفة العالية للمواد المستخدمة في إنتاجها حيويا. وفي هذه الدراسة تم استخدام مياه ال صرف الصناعي الناتجة من تصنيع سكر ال بنجر لإنتاج تلك البوليمرات الحيوي سواء بدون أو باضافة المولاس كونهما مواد متوفرة منخفضة التكلفة. وقد تم اختيار سلالة بكتيرية معزولةمحليّاً لهذه الدراسة. حيث تم اختيارها كسلالة منتجة للـــPHA بعد فحص 30 عزلة بكتيرية مختلفة. وتم تعريفها وفقاً لـ16S rRNA تحت الأسم والرقم التالي .Bacillus megaterium AUMC b272. وبلغ أعلي انتاج من PHA بهذه السلالة 4.053 جرام/لتر بنسبة 41.79% من الكتلة الحيوية للبكتريا عند

استخدام وسط تغذية MSM به 6% من مولاس البنجر كمصدر وحيد للكربون و0.5جرام/لترمن أوكسالات الأمونيوم كمصدر للنيتروجين ، وفترة تحضين 24 ساعة عند 37°م وأس هيدروجيني 8.5 و 200 لفة في الدقيقة . ومن ناحية أخرى فإن انتاج هذه السلالة من PHA يكاد لا يذكر عندما تنمو على نفس مكونات الوسط مع استبدال المياه المقطرة ومولاس السكر بمياه الصرف الصناعي لصناعة سكرالبنجر . في حين أن تركيز PHA قد وصل إلى 0.828 جرام/لتر بنسبة بلغة 20.58% من الكتلة الحيوية للبكتريا في نفس ظروف الوسط المغذي مع استبدال المياه المقطرة بمياه بنجرالسكرفقط. ومن الجدير بالذكرأن CODالموجود في وسط مياه الصرف الصناعي من صناعة سكر البنجر في نهاية فترة التخمر قد أزيلت بنسبة 69%. هذا وتم التعرف على مواصفات ال PHA المنتج باستخدام التحليل الطيفي (FT-IR) و تحليل الطيفي الكتلي(GC-MS). وتثبت تلك النتائج أنه من الممكن استخدام هذه التقنية في انتاج البوليمرات الحيوية (PHA) من مياه الصرف الصناعي المنتجة من صناعة بنجر السكر في وجود مولاس بنجرالبنجر كمواد مغذية منخفضة التكلفة وفي نفس الوقت معالجة مياه الصرف الصناعي تلك معالجة حيوية.