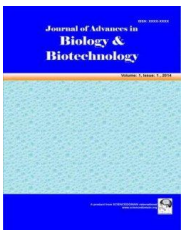

Journal of Advances in Biology & Biotechnology
1(1): 40-54, 2014; Article no. JABB.2014.004

SCIENCEDOMAIN *international*
www.sciencedomain.org

Screening, Optimization and Extraction of Polyhydroxyalkanoates (PHAs) from *Bacillus thuringienesis*

S. E. Desouky[1*], H. H. El-Shiekh[1,2], M. A. Elabd[3] and A. M. Shehab[1]

[1]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Egypt.
[2]The Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt.
[3]National Research Centre, Cairo, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author SED designed the study, wrote the protocol and performed editing of the manuscript. Authors HHES and MAE managed the analyses of the study and managed the literature searches. Author AMS executed the experimental protocol and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

Received 25th June 2014
Accepted 5th August 2014
Published 19th August 2014

ABSTRACT

Aims: This study illustrated obtaining bacterial isolates capable to produce polyhydroxyalkanoates (PHA) under stressed conditions and also using cheap wastes for production process.
Study Design: Two-step throughput screening of 50 bacterial isolates by lipophylic stain (Sudan black B) and flouroscence stain (Nile red) will be carried on, then the best condition for PHA production followed by identification of most potent isolate by molecular charterization will be investigated.
Place and Duration of Study: The study were performed in physiological lab in botany & microbiology department in faculty of science, Al-azhar university and NRC from October 2012 until April 2014.
Methodology: Bacterial isolates will be investigated using two different indicator stains, Sudan Black and Nile Red, for PHA productivity then the highly producing isolate will be identified. Factors controlling PHA production and stressed condition will be studied

**Corresponding author: Email: dr_saidesouky@yahoo.com;*

alternatively depends on increasing of PHA production which will be assayed quantitatively by converting PHA to crotonic acid. Biopolymer will be extracted using successive solvents.
Result: Five soil samples were collected from different localities contaminated with industrial wastes and 50 isolates of bacteria were purified then screened for PHA production by using Sudan black B as a primary screening and confirmed by fluorescent Nile red staining. Eleven promising bacterial isolates were selected based on their PHA yields. Out of eleven natural promising isolates, BS11 was found to be the most efficient PHA producer and identified as *Bacillus thuringiensis* (KJ206079). The effect of different conditions on PHA produced by *B. thuringiensis* including carbon sources, nitrogen sources, incubation temperatures, pH and incubation periods were studied. The highest amount of PHA was obtained from this isolate using medium containing 30 g/l molasses, 0.8 g/L ammonium sulphate at pH 7.5 and incubation temp at 35ºC and after 72 hours. In conclusion, after the optimization of PHA synthesizing conditions, *B. thuringiensis* accumulate up to 69 % of its dry weight and can be employed for industrial production.
Conclusion: The experiment indicate the efficiency of two-throughput screening systems targeting PHA producing isolates. Also optmiziation of PHA producing factors for the most potent producing strain, *Bacillus thuringiensis* (KJ206079), was increased from 2.5 g/L to 4.1 g/L and represent about 69% cell dry weight.

Keywords: Polyhydroxyalkanoates; biopolymer; Bacillus thuringiensis.

ABBREVIATIONS

UV: ultaviolte, DMSO: dimethyl sulfoxide, PHA: polyhydroxyalkonate, MSM: mineral salt media, NRC: National Reacerch Center.

1. INTRODUCTION

Petroleum based plastics are almost exclusively made from a nonrenewable resource and a large amounts of wastes that creating a risk to human health and to the environment [1]. In response to problems associated with plastic wastes, there has been considerable interest in the development and production of biodegradable plastics specially from utilization of Agro-industrial materials in that will not only ensure the low production cost but also solve the problem of management of waste material to a certain level [2]. Polyhydroxyalkanoates (PHAs) is a biodegradable polymer possesses a criteria of plastics and produced by microbes to serve as a food stock which could serve as an alternative that will not be affected by the depleting fossil fuels, rise in crude oil prices [3]. PHAs are generally divided into two groups, short-chain-length (SCL) PHAs and medium-chain-length (MCL) PHAs. SCL-PHAs consist of (R)-hydroxyalkanoates of C_3-C_5, while MCL-PHAs are comprised of aliphatic and/or aromatic (R)-hydroxyalkanoates of C_6-C_{14}. PHAs, microbial copolyesters consisting of SCL- and MCL- hydroxyalkanoates have been reported in some bacterial strains [4]. SCL-PHAs are thermoplastics with a high degree of crystallinity, while MCL-PHAs are elastic or tacky materials with a low degree of crystallinity and a low melting temperature MCL- PHAs with functional groups are of great interest, because the functional groups can improve the physical properties of the polymers. Moreover, some functional groups can be modified by chemical reactions to obtain more useful polymers and extend the potential application of MCL-PHAs as environmentally biodegradable polymers and functional biomaterials for biomedical uses [5,6]. In a addition, many grafting reactions have

been reported to improve the thermal and mechanical properties of MCL-PHAs [7] PHA is insoluble in water and thus the polymers are accumulated as intracellular granules inside the bacterial cells. It is beneficial for bacteria to store excess nutrients inside the cytoplasm [8]. A wide variety of microorganisms are able to naturally accumulate PHA as intracellular energy storage materials under an excess of carbon source and conditions of limiting nutrients such as oxygen, nitrogen and phosphate [9-11]. PHA synthesized by a large number of bacteria and have received great attention as sources for biodegradable, biocompatible, and thermoprocessible plastic materials [10,12]. Many researchers have explained that, soil bacteria generally produce PHA specially species that belonged to genus Bacillus which have been reported and extensively studied [13-16].The genus Bacillus was identified as one of the first Gram positive bacteria capable of producing PHA [17]. This genus has been widely used for a long time in industry and academia, due to the stability of its replication and maintenance of plasmids [18]. In a current study, we aimed to run an efficient two-step throughput screening for soil bacteria targeting PHA producer strains subsequently optimize PHA production conditions for the most potent identified strain.

2. MATERIALS AND METHODS

2.1 Isolation of Bacterial Strains

Five Soil samples 3.0-4.0 cm deep from surface were used for isolation of the bacteria. Around 1.0 g of sample was serially diluted in sterile distilled water and plated onto nutrient agar plates and incubated at 30ºC for 24 hours. Various colonies of different morphologies were individually picked and subcultured 3-4 times on nutrient agar plates. The bacteria were streaked on nutrient agar slants, incubated at 30ºC overnight and then stored at 4ºC for further use.

2.2 Culture Medium

Minimal mineral medium (M medium) [19], containing (per liter) $Na_2HPO_4.2H_2O$, 4.5 g; KH_2PO_4, 1.5 g; MgSO4.$7H_2O$, 0.2 g; NaCl, 0.9 g; $(NH4)_2$ SO4, 2g; $CaCl_2.2H_2O$, 0.02 g; NH_4 Fe (III) Citrate, 0.05 g; agar, 15; trace element solution SL6, 1 ml; glucose, 20 g. M medium was used to as a preculture for all experiments.

2.3 Screening for PHA-producing Bacteria

2.3.1 Rapid screening of isolates for PHA production by plate assay method

The bacterial colonies were examined for PHA accumulation by staining with Sudan Black (0.3 g in 70% ethanol) by using rapid screening method. The bacterial isolates were grown on nutrient agar medium supplemented with 1% glucose. Every plate was divided into 6 equal parts and in each part a bacterial isolate was spotted. Then plates were incubated at 30ºC for 72 hours. Ethanolic solution of Sudan black B was spread over the colonies and the plates were kept undisturbed for 30 minutes. Finally the plates were washed with ethanol (96%) to remove the excess stain from the colonies. The PHA producing colonies giving dark blue colored were taken as positive for polyhydroxyalkanoates production [20].

2.3.2 Screening of PHA producing microorganisms by viable - colony staining method

For routine analysis, 0.002 mL of a solution composed of 0.25 mg Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) per mL dimethyl sulfoxide (DMSO) was added to 1 mL

sterilized medium to give a final concentration of 0.5 µg dye/mL medium. Then agar plates were exposed to ultraviolet light (312 nm) after appropriate cultivation period (2-5 days) of the screened microoganisms to detect the accumulation of PHAs. The lighted colonies were recorded as positive [21].

2.4 Extraction and Assay of PHA

Bacterial cells containing polymer were collected after centrifugation at 4000 rpm for 10 min. Then pellet was resuspended in equal volume of 4% sodium hypochlorite and incubated at 37ºC for 24 hour. Pellet was washed with acetone, ethanol and water to remove the unwanted materials. The whole mixture was centrifuged again and the supernatant was discarded. Finally polymer granules were dissolved in hot chloroform [22].

2.4.1 Quantifications of bacterial growth and dry weight

Cell growth was monitored by measuring the optical density (O.D) at 600 nm using spectrophotometer Ten milliliter culture medium was centrifuged at 10,000 rpm, 4ºC for 15 min and cell pellet was washed with 10 mL distilled water. Cell pellet was harvested by centrifugation and dried at 105ºC for 48 h, or till constant weight was obtained [23] Cell mass concentration was determined by the standard calibration curve between OD 600 and cell dry weight.

PHA accumulation (%) = Dry weight of extracted PHA (g/L) / DCW (g/L) × 100%

2.5 Quantifications for Standard PHA

Standard PHA sample (0.02-0.1g) was digested by heating in concentrated H2SO4 at 100ºC for 10 min estimated at 235 nm in U-V visible spectrophotometer to determine slope and easily calculate factor = 1/slope By referring to the standard curve, the quantity of PHA produced was determined [20].

2.6 Identification of Most Producer Isolate

Identification of most potent isolate was based on 16S rRNA sequence analysis and also by comparing its morphological, physiological and biochemical charteristics using the identification keys described by [24,25]. Partial 16S rRNA sequence of bacterial isolate was carried out in Sigma Research, Cario, Egypt and were analyzed by members of the Sigma .DNA was extracted using protocol of GeneJet genomic DNA purification kit (Fermentas) and amplified using Maxima Hot Start Master Mix (Fermentas). PCR product was purified using Gene Jet PCR Purification Kit (Fermentas). The forward and reverse used for PCR amplification were 27f(5-AGAGTTTGATCCTGGCTCAG-3)and 1492r (5-GTTACCTTGTTACGACTT-3) (16S rRNA universal primer). Sequencing of the PCR product was carried out in GATC (Guanin Adenin Thymin Cytosine) German Company using ABI 3730 X1 DNA sequencer.

2.6.1 Phylogenetic tree analysis

By using 16S rRNA gene sequence, the strain were identified by BLAST search (blast.ncbi.nlm.nih.gov/Blast.cgi) The sequence of closely related type strains were retrieved

for constructing the phylogentic trees to confirm similarities of most potent strais with other related groups.

2.7 Optimization of Culture Conditions for PHA Production by *Bacillus thuringiensis* (KJ206079)

Starting culture were prepared by inoculating the selected strain in 100 ml MSM medium and incubating on a shaker (150 rpm) at 37ºC for 48 hrs. The starting culture containing 1.6x 10^4 viable cells/ml was inoculated (10%) to MSM medium. For optimization of best incubation periods to production of PHA, different incubation periods (24h, 48h, 72h, 96h, 120h) were studied. Effect of media ingredients like carbon and nitrogen sources on PHA production was determined by simply replacing the carbon source with other carbon sources i.e (fructose, glucose, arabinose, mannose, galactose, maltose, soluble starch, glycerol, sucrose, mannitol, agricultural wastes as bagasses and renewable resources as molasses. Sugars were added at equivalent weights. Also nitrogen source was replaced by (peptone tryptone, ammonium phosphate, ammounium chloride, potassium nitrate and potassium nitrite) in equivalent to 0.5 g/l concentration. The inoculated culture in production medium was incubated at different temperature viz. 25ºC, 30ºC, 35ºC, 37ºC and 40ºC for temperature optimization and optimum temperature was selected. For pH optimization, culture was incubated in carbon rich nutrient medium with different pH i.e.(3.0,5.5,7.0,7.5,8.0 and 10.0.) Each culture were taken from different flask and PHA production was determined. For inoculum size optimization, culture was incubated in carbon rich nutrient medium with different inoculum size concentration i.e. (1%,3%,5%,7.5%,10%and12.5%) and PHA production was calculated according to 2.5 part.

3. RESULTS AND DISCUSSION

3.1 Isolation of Representive Bacteria, Screnning by Sudan Black B & Nile Red Staining and Quantitative Estimation for Polyhydroxyalkanoates by Spectroscopy

Different bacterial isolates (50) were obtained from soils collected from different regions. All the bacterial isolates were screened with two-steps screening system on solid agar medium using Sudan black (Figs. 1,2) and flouroscence nile red staining, out of 50 isolates, 11 were suggested to be PHA producers as detected by Sudan black in first step. In the second step, the promising isolates were confirmed by flouroscence Nile red staning. Promising isolates were grown in liquid medium for 48 hr. and PHA was extracted and quantified (Table. 1). The percentage of PHA from cell dry weight was determined. It was ranged from (16-44%) of the cell dry weight. The most active isolate BS11 was produced 0.89 g/l PHA about 44% of cell dry weight. The highest prodicing isolate was selected for more detailed studies.

Out of the 50 bacterial isolates screened for PHA production 11 showed excellent staining by sudan black in Plate assay method confirmed by nile red staning.

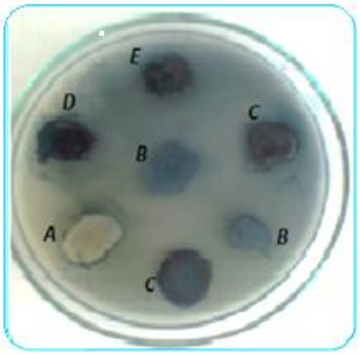

Figure : 1

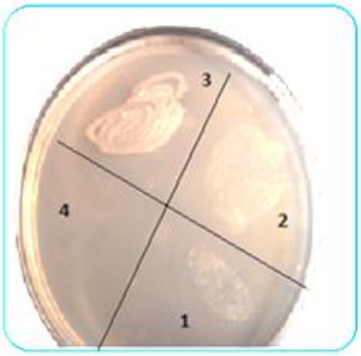

Figure : 2a

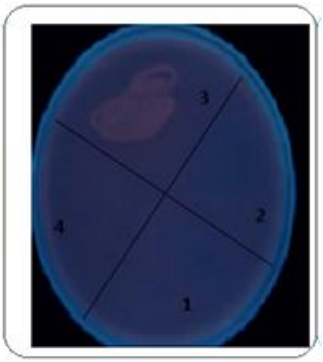

Figure :2b

Figs. 1, 2. Two-step screening method for PHA producing bacterial isolates

(Fig. 1) indicate Sudan black-stained isolates were ranked as, Negative result showing in (A) taking symbol (-) in [Table.1] , Poorly stained colonies as showing in (B) taking Symbol (+), medium stained colonies as showing in (C) taking Symbol (++), strongly stained colonies as showing in (D) taking Symbol (+++), Excellently stained colonies as showing in (E) taking Symbol (++++).(Fig. 2a) showing in colonies No.(1,2,4) negative result under visible light while (Fig. 2b) showing in No (3) positive bacterial isolate showing fluorescence beam on agar plate under UV light

Table 1. Screening steps for PHA producing bacterial isolates related to source of isolation and percentage of PHA percentage/ cell dry weight

No.	Isolate code	Gram Stain	Shape	Qualitative estimation for biopolymer		Quantitative estimation for biopolymer	% PHA/cell dry weight
				Sudan black	Nile red staining	Cortonic acid methods g/l	
1	BS1	G +ve	bacilli	++	Positive	0.15	24.1
2	BS2	G +ve	bacilli	-	-	-	-
3	BS3	G +ve	bacilli	+	Positive	0.11	29.7
4	BS4	G +ve	bacilli	+++	Positive	0.46	28.7
5	BS5	G +ve	Cocci	+	Negative	-	-
6	BS6	G -ve	bacilli	-	Negative	-	-
7	BS7	G -ve	bacilli	-	Negative	-	-
8	BS8	G -ve	bacilli	-	Negative	-	-
9	BS9	G +ve	Cocci	-	Negative	-	-
10	BS10	G +ve	bacilli	-	Negative	-	-
11	BS11*	G +ve	bacilli	++++	Positive	0.89	44
12	BS12	G +ve	bacilli	-	Negative	-	-
13	CF1	G +ve	bacilli	-	Negative	-	-
14	CF2	G -ve	Cocci	-	Negative	-	-
15	CF3	G +ve	bacilli	-	Negative	-	-
16	CF4	G +ve	bacilli	-	Negative	-	-
17	CF5	G +ve	bacilli	-	Negative	-	-
18	CF6	G +ve	bacilli	+	Positive	0.10	20.4
19	CH1	G +ve	bacilli	-	Negative	-	-
20	CH2	G +ve	bacilli	-	Negative	-	-
21	CH3	G -ve	bacilli	-	Negative	-	-
22	CH4	G +ve	Cocci	-	Negative	-	-
23	CH5	G +ve	bacilli	-	Negative	-	-
24	CH6	G +ve	bacilli	-	Negative	-	-

Table 1 continued……..

25	CH7	G -ve	bacilli	-	Negative	-	-
26	CH8	G +ve	bacilli	-	Negative	-	-
27	CH9	G +ve	Cocci	-	Negative	-	-
28	CH10	G -ve	bacilli	-	Negative	-	-
29	CH11	G +ve	bacilli	-	Negative	-	-
30	CH12	G +ve	bacilli	-	Negative	-	-
31	CH13	G +ve	bacilli	-	Negative	-	-
32	AS1	G +ve	bacilli	++	Positive	0.64	35.3
33	AS2	G +ve	Cocci	+++	Positive	0.77	43
34	AS3	G +ve	bacilli	-	Negative	-	-
35	AS4	G +ve	bacilli	++	Positive	0.31	32.9
36	AS5	G +ve	bacilli	-	Negative	-	-
37	AS6	G +ve	Cocci	-	Negative	-	-
38	AS7	G -ve	Cocci	-	Negative	-	-
39	AS8	G +ve	bacilli	-	Negative	-	-
40	AS9	G +ve	Cocci	-	Negative	-	-
41	PC1	G +ve	bacilli	-	Negative	-	-
42	PC2	G +ve	bacilli	++	Positive	0.14	16
43	PC3	G +ve	bacilli	-	Negative	-	-
44	PC4	G +ve	bacilli	-	Negative	-	-
45	PC5	G +ve	bacilli	-	Negative	-	-
46	PC6	G +ve	bacilli	++	Positive	0.32	39.5
47	PC7	G -ve	bacilli	-	Negative	-	-
48	PC8	G +ve	Cocci	-	Negative	-	-
49	PC9	G +ve	bacilli	-	Negative	-	-
50	PC10	G +ve	Cocci	++++	Positive	0.64	31.8

** : significant result , BS; Benzene stations wastes CF; Clothes factory wastes CH; Coke Helwan wastes AS; Agricultural soil PC; Paints company wastes + ; The poorly stained colonies ++ ; medium stained colonies +++ ; strongly stained colonies ++++ ; excellently stained colonies*

3.2 Identification of Bacterial Isolates

3.2.1 Identification of the most potent isolate by using traditional method

BS11 is a Gram positive with rod shape and appears on agar plate with white color, spore former and grow under aerobic condition. Summary of morphological, physiological, and biochemical properties for isolate BS11 showed in (Table. 2).

3.2.2 Identification of the most producer isolate (BS11) by 16srRNA

The selected isolates were identified by partial sequencing of the PCR amplified 16S rDNA gene (Fig.3). The obtained sequences were submitted to the BLAST in order to find a homology with other 16S rDNA sequences. Comparing the sequence of the 16S rDNA gene of the isolate BS11 that we coded as AM365815 with the sequences in GenBank revealed that the isolate are similar to *Bacillus thuringiensis* (KJ206079) with 99% similarity according to the phylogenetic tree (Fig. 4) based on 16S rDNA sequences.

The phylogentic, morphological, physiological and biochemical characteristics of isolate BS11 suggested that; this isolate have high similarity with reference strain *Bacillus thuringiensis (KJ206079).* A comparative studies of the identification properties for isolate BS11 in relation to the reference strain was shown in (Table 2) according to identification manual [24,25].

Table 2. Summary of morphological, physiological, and biochemical properties for isolate *BS11* that matching with *Bacillus thuringiensis*

Organism Characters	*BS11*	*Bacillus thuringiensis*
Cell shape	Rod shape	Rod shape
Gram reaction	+	+
KOH (3%)	-	-
Spore formation	+	+
Growth at 50ºC	-	25-45
Urease	D	-
Catalase	+	+
Indole production	D	-
Methyl red	-	-
Voges-Proskuaer	+	+
Citrate utilization	+	+
Nitrate reduction	+	+
Carbohydrate fermentation		
Glucose	+	+
Xylose	-	-
Arabinose	-	ND
Manitol	-	ND
Lactose	+A	+A
Maltose	+A	+A
Sucrose	-	ND
Extracellular enzymes		
Amylase	+	+
Casinase	+	+
Lipase	+	+

+: positive, −: Negative, D: Doubtful, +A: Acid production, ND: Not Detected

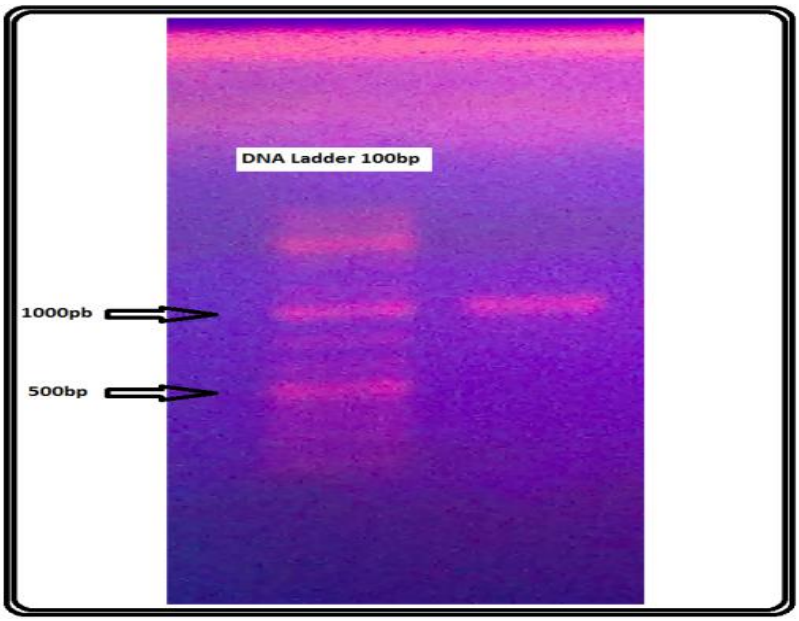

Fig. 3. PCR product of 16S rRNA gene for BS11 isolate (right lane) against DNA ladder (100 base pair)

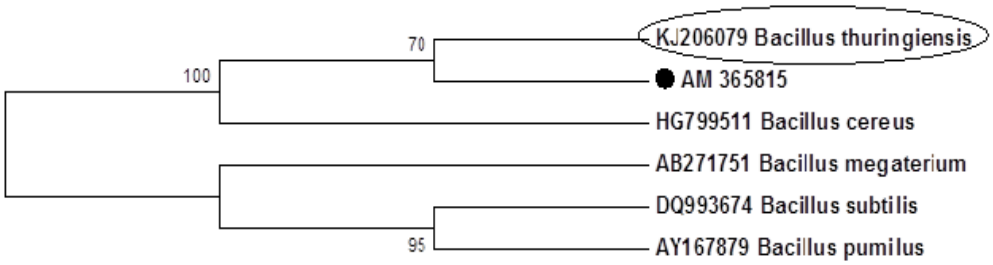

Fig. 4. Neighbor-joining tree based on 16s rRNA gene sequences, showed the phylogentic relationships of the isolate BS11 and related taxa

3.3 Standard Graph of PHA Concentration

Relationship between optical density and concentration of standard PHA illustrated in [Fig. 5] and through this can calculate slope and factor to determine amount of extracted PHA.

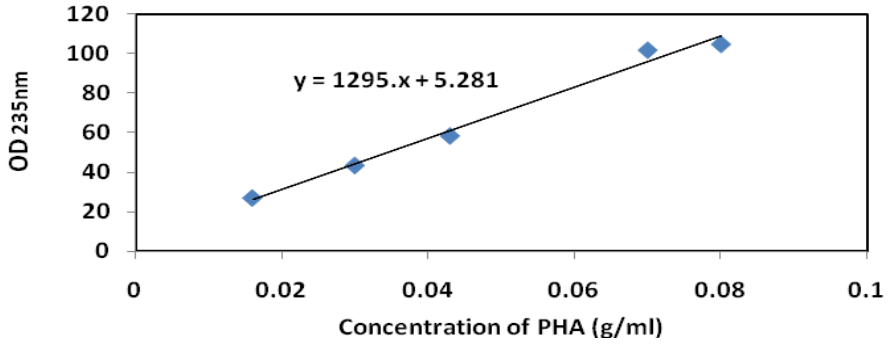

Fig. 5. Standard graph of PHA concentration

3.4 Optimization of Culture Conditions for PHA Production by *Bacillus thuringiensis*

[Fig. 6A] illustrating the effect of incubation periods on PHA production by the *B. thuringiensis.* It was clear from figure that, The PHA yields was increased with time dependent manner and the highest yield 2.5 g/l was obtained after 72 h incubation time. Suddenly after this point the growth pattern sharply decreased. [Fig. 6B] showing the effect of different carbon sources on PHA production. Yield of PHA was 2.9 g/l in case of using sugar cane molasses as a carbon sources at conc. 20 g/L. In [Fig. 6C], production of PHA yield 3.6 g/L was obtained after 72 h of growth when ammonium sulphate was used as nitrogen source that already one component of MSM medium. After selection of Molasses to be the best carbon source, we studied different concentration of it on PHA production *by B thuringiensis* and the highest PHA yield determined as 3.5 g/L was obtained at concentration 30 g/L [Fig. 6D]. [Fig. 6E] showing the effect of different concentration of best nitrogen sources (ammonium sulphate) on PHA production, 3.9 g/L, that was obtained at concentration 0.8 g/L. [Fig. 6F] showing the effect of different incubation temperature on PHA production and the highest PHA yield,4g/L, was obtained at 35ºC. [Fig. 6G] investigating the effect of different pH as PHA yield 4.1 g/L was obtained at pH 7.5. [Fig. 6H] clearly determined the the best inoculum size suitable for PHA production that was obtained at 10% inoculum size.

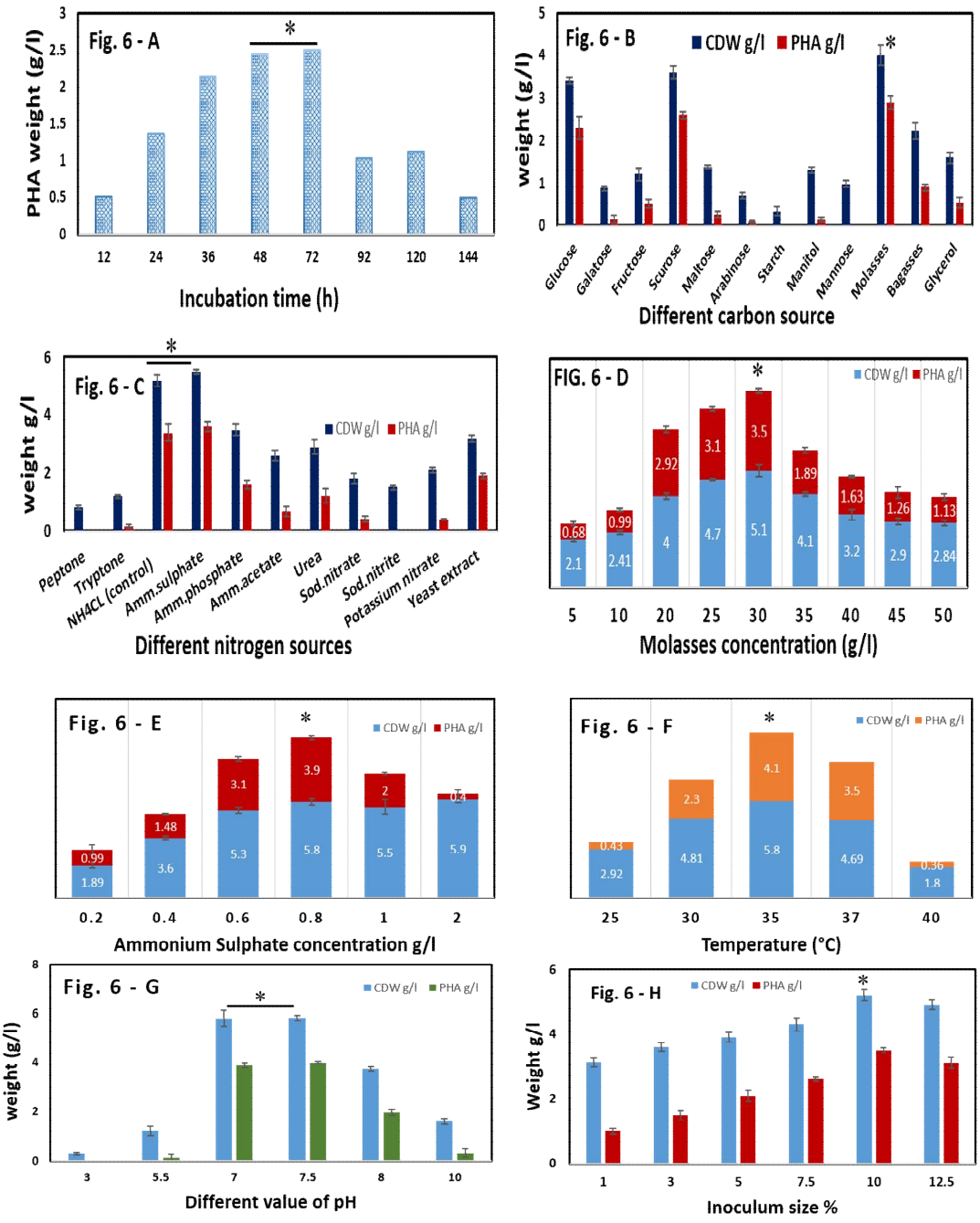

Fig. 6. Effects of different enviromental parameter that affected on PHA productivity (A) incubation periods , (B) different cabon sources , (C) Different nitrogen sources , (D) different conc. From best carbon sources, (E) different conc. From ammonium sulphate, (F) Diifferent incubation temprature (G) Different pH,(H) Different inoculum size . All experiments were done in triplicate and data represented as an average ± standard deviation. (*); optimum or best conditions, horizontal bars are denoted to represent a range

After optimization of growth conditions, *B. thuringiensis* was grown in two liter conical flask containing 1000 ml of the modified production medium as follow; 3% molasses, 0.8 g/L ammonium sulphate at pH 7.5. After 72 hours of incubation at 35ºC and 120 rpm, PHA was extracted, purified, dried and then weighted. It was clear that, after optimization of growth factors, productivity of PHA was enhanced from 2.5 g/L to 4.1 g/L. We can speculate from mentioned result that, increasing the productivity mainly due to increasing of total cell dry weight rather than biopolymer percentage to cells (Fig. 7).

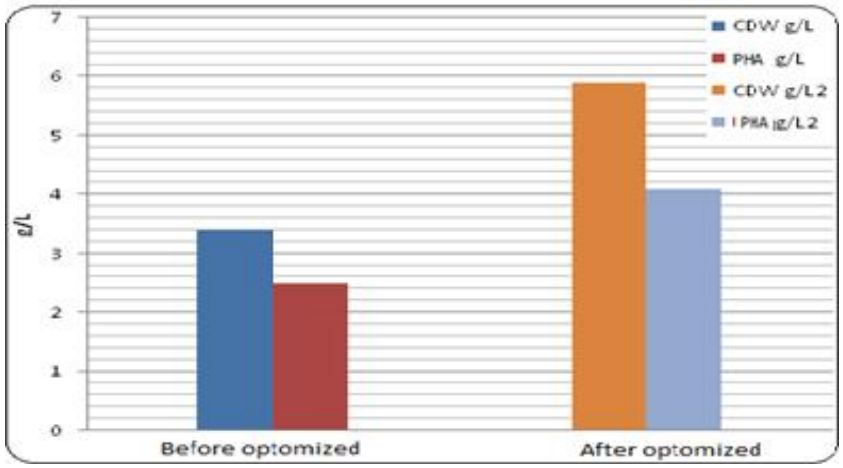

Fig. 7. Production of PHA before and after optimization from *B. thuringiensis.* The experiment was done in duplicate and data represented as an average

It was interesting to note that bacteria belonging to *Bacillus* accumulated a high concentration of PHAs [26,27]. In addition, reports suggesting the use of *Bacillus* sp for the production of a range of different PHAs by utilizing different carbon sources [28]. In this study, new polyhydroxyalkanoic acid producing isolate was selected and it was identified as *a Bacillus thuringiensis (KJ206079)* using sequencing of 16S rDNA as molecular test (and microbial tests like Gram stain and other morphological and biochemical properties (As it is well known, PHA accumulation in the bacterial cell can be determined quite easily with Sudan Black using rapid screening plate method [29,20] or by microscopic screening [9,30,31] and all the two methods were previously used to select PHA producing bacteria. In general, PHA polymer is synthesized by the bacterial cells under limiting growth conditions, when the carbon source is in excess and nitrogen, phosphorus, magnesium, sulfur or oxygen is present in a limiting concentration [15]. Medium we used in this study is the nitrogen-deficient medium; it was the best medium for *Bacillus sp.* in terms of both the cellular growth and PHA accumulation [32,33]. The carbon source is one of the most factor affecting PHA biopolymer production. In this study, the effect of different carbon sources like (Glucose, sucrose, Lactose, Maltose, molasses, and others.) on PHA production by *Bacillus sp* were investigated. Results showed that maximum PHA production was attained when molasses was used as a sole carbon source which considered inexpensive substrste to lowering cost of production of PHA. By comparison our results in relation to other Bacillus sp or Mycobacterium as well as Azotobacter sp which have the ability to produce PHA, the best amount of growth and PHA accumulation was measured after 72 h incubation same like condition of *Bacillus cereus* [34,11]. The formation of PHA biopolymer depends on starvation and stress conditions that almost differ from species to other like Bacillus subtilis, megaterium and Mycobacterium [35,36,37,38]. In some cases under controlled conditions,

Bacterial cells can form biopolymer in highest rate within 24 h [39,40,41,42,4] Also, our results showed that the optimum temperature is 35ºC and pH 7.5 resulted in a maximum PHA production. Many points can be taken to control PHA production e.g. Spo A that seems to be a master transcription factor for PHA formation [43]. Economically, it s favorable to use agro-industrial wastes as a carbon source for biopolymer formation [44]. Production of PHA might be occur under harsh condition like high salt concentration which might be better for large scale production [45].

4. CONCLUSION

Fifty bacterial isolates were isolated from five samples of soil from different regions contaminated with different wastes, two-step throughput screening was done for investigated isolates by using by Sudan black B staining and flouroscence Nile red staining and alternatively quantified by converting to crotonic acid. Out of effective screening, Isolate BS11 identified as *Bacillus thuringiensis (KJ206079)* showed highest efficiency for PHA formation. Successfully, we were improved the productivity of PHA from 2.5 g/L to 4.1 g/L.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Yu J, Heiko S. Microbial utilization and biopolyester synthesis of bagasse hydrolysates. Bioresour. Technol. 2008;99:8042-8048.
2. Bhairavi G, Prashant P, Chandrashekhar K, Deepti DM, Twisha SP. PHA production using novel agro-industrial sources from different *Bacillus* species. Int J Pharma and Biosci. 2008;2(3):242-249.
3. Ceyhan N, Ozdemir G. Poly-βhydroxybutyrate (PHB) production from domestic wastewater using Enterobacter aerogenes12Bi strain. Afr J Microbiol Res. 2011;5(6):690-702.
4. Steinbüchel A, Hein S. Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. Adv. Biochem. Eng. Biotechnol. 2001;71:81-123.
5. Tan, Giin-Yu Amy, Chia-Lung Chen, Ling Li, Liya Ge, Lin Wang, Indah Mutiara Ningtyas Razaad, Yanhong Li, Lei Zhao, Yu Mo, Jing-Yuan Wang. Start a research on biopolymer polyhydroxyalkanoate (PHA): A review. Polymers. 2014;6(3):706-754.
6. Hazer B, Steinbüchel A. Increased diversification of polyhydroxyalkanoates by modification reactions for industrial and medical applications. Appl. Microbiol. Biotechnol. 2007;74:1-12.
7. Curley JM, Hazer B, Lenz RW, Fuller RC. Production of poly(3-hydroxyalkanoates) containing aromatic substituents by Pseudomonas oleovorans. Macromolecules. 1996;29:1762-1766.
8. Verlinden R, Hill DJ, Kenward MA, William CD. Bacterial synthesis of biodegradable polyhydroxyalkanoates. J Appl Microbial Rev. 2007;102:1437-1449.
9. Wang HH, Li XT, Chen GQ. Production and characterization of homopolymer polyhydroxyheptanoate by a fadBA knockout mutant *Pseudomonas putida* KTOY06 derived from *P. putida* KT2442. Process Biochem. 2009;44(1):106-111.

10. Hyakutake M, Saito Y, Tomizawa S, Mizuno K, Tsuge T. Polyhydroxyalkanoate (PHA) synthesis by class IV PHA synthases employing *Ralstonia eutropha* PHA as host strain. Bioscience, Biotech and Biochem. 2011;75(8):1615-1617.
11. Rodriguez-Contreras A, Koller M, De Sousa Dias MM, Calafell M, Braunegg G, Marqués-Calvo MS. Novel Poly [(R)-3-hydroxybutyrate]-producing bacterium isolated from a Bolivian hypersaline lake. Food Technology and Biotechnology. 2013;51(1):123-130.
12. Cao Y, Zhang X. Production of long-chain hydroxy fatty acids by microbial conversion. Appl Microbiol Biotechnol. 2013;97(8):3323-31.
13. Adwitiya P, Prabhu A, Kumar A, Rajagopal B, Dadhe K, Ponnamma V. Optimization of process parameters for maximum poly-Beta-hydroxybutyrate (PHA) production by *Bacillus thuringiensis* IAM 12077. Pol. J. Microbiol. 2009;58:149-154.
14. Gurunathan S, Pandian SR, Deepak V, Kalishwaralal K, Rameshkumar N, Jeyara M. Optimization and fed-batch production of PHA utilizing dairy waste and sea water as nutrient sources by *Bacillus megaterium* SRKP-3. Bioresour Technol. 2010;101:705711.
15. Reddy VST, Mahmood KS. Production of PHB and P(3HB-co-3HV) biopolymers of *Bacillus megaterium* strain isolated from municipal sewage sludge. W J Microbiol Biotechnol. 2009;25:391-397.
16. Valappil S, Misra S , Boccaccini A , Keshavarz T , Bucke C. and Roy I. Large-scale production and efficient recovery of PHA with desirable material properties, from the newly characterized *Bacillus cereus SPV.* J. Biotechnol. 2007;132:251-258.
17. Lemoigne M. Produit de déshydration et de polymérisation de l'acide β-oxybutyrique. Bull. Soc. Chim. Biol. 1926;8:770-782.
18. Biedendieck R, Gamer M, Jaensch L, Meyer S, Rohde M, Deckwer WD, and Jahn D. Asucroseinducible promoter system for the intra- and extracellular protein production in *B. megaterium*. J Biotechnol. 2007;132:426–430.
19. Kung W. Wachstum und Poly-D-(-)-hydroxybuttersa¨ure Akkumulation beiAlcaligenes latus .Diploma Thesis. Graz University of Technology. Austria; 1982.
20. Mohamed T, Shaaban M, Azza Turky and Mowafy El. Production of some biopolymers by some selective Egyptian soil bacterial isolates. Journal of Appl Sci Research. 2012;8(1):94-105.
21. Spiekermann P, Rehm B, Kalscheuer R, Baumeister D, Steinbiichel A. A sensitive viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol.1999;71:73-80.
22. Aarthi N, Ramana KV. Identification and characterization of Polyhydroxybutyrate producing *Bacillus cereus* and *Bacillus mycoides* strains. Interl J of Environmental Sci. 2011;1(5):744-756.
23. Naheed N, Jamil N, Hasnain S. Screening of contaminated soils for biodegradable plastic producing bacteria and profiling of their resistance markers. Afr J of Microbiology Research. 2011;5(24):4097-4104.
24. Bergey's manual of systematic bacteriology. The Proteobacteria and Archaea, 2nd Edition, Volume 2, Editor-in-Chief: George M. Garrity Editors: Don J. Brenner, Noel R. Krieg and James T. Staley; 2005.
25. Cowan ST. Cowan and steel's manual for the identification of medical bacteria. 3th Ed. Cambridge Univ. Press, London; 1993.
26. Sangkharak K, Prasertsan P. The production of polyhydroxyalkanoate by *Bacillus licheniformis* using sequential mutagenesis and optimization. Biotechnology and Bioprocess Engineering. 2013;18(2);272-279.

27. Sangkharak K, Prasertsan P. Screening and identification of polyhydroxyalkanoates producing bacteria and biochemical characterization of their possible application. J of General and Appl Microbiology. 2012;58(3):173-182.
28. Halami PM. Production of polyhydroxyalkanoate from starch by the native isolates *Bacillus cereus* CFR06. World J of Microbiology and Biotechnology. 2008;24(6):805-812.
29. Chandrashekharaiah PS. Isolation, screening and selection of efficient poly-hydroxybutyrate (Phb) synthesizing bacteria. Thesis Submitted To The University of Agricultural Sciences, Dharwad, In Partial fulfillment of the requirements for The Master Degree of Science in Agricultural Microbiology; 2005.
30. Singh P, Parmar N. Isolation and characterization of two novel polyhydroxybutyrate (PHB)-producing bacteria. Afr J of Biotechnology. 2011;10 (24):4907-4919.
31. Dhingra HK, Priya K. Physiological and molecular identification of polyhydroxybutyrates (PHB) producing micro-organisms isolated from root nodules of leguminous plants. Afr J of Microbiology Research. 2013;7(30):3961-3967.
32. De Vries YP, Hornstra LM, de Vos WM, Abee T. Growth and sporulation of *Bacillus cereus* ATCC 14579 under defined conditions: Temporal expression of genes for key sigma factors. Appl and Environmental Microbiology. 2004;70(4):2514-2519.
33. Valappil SP, Boccaccini AR, Bucke C, Roy I. Polyhydroxyalkanoates in Gram-positive bacteria: Insights from the genera *Bacillus* and Streptomyces. Antonie van leeuwenhoek. 2007a;91(1):1-17.
34. Mizuno K, Ohta A, Hyakutake M, Ichinomiya Y, Tsuge T. Isolation of polyhydroxyalkanoate-producing bacteria from a polluted soil and characterization of the isolated strain *Bacillus cereus YB-4.* Polymer Degradation and Stability. 2010;95(8):1335-1339.
35. Singh M, Patel S, Kalia V. *Bacillus subtilis* as potential producer for polyhydroxyalkanoates. Microbial cell factories. 2009;8(1):38-40.
36. Kumar T, Singh M, Purohit HJ, Kalia VC. Potential of *Bacillus sp*. to produce polyhydroxybutyrate from biowaste. J Appl Microbiology. 2009;106(6):2017-2023
37. Shivakumar S. Accumulation of poly (3-hydroxybutyrate) by Microbacterium barkeri DSM 20145. Turk J Biol. 2012;36:225-232.
38. Berekaa M, Thawadi AM. Biosynthesis of polyhydroxybutyrate (PHB) biopolymer by *Bacillus megaterium SW1-2:* Application of Box-Behnken design for optimization of process parameters. Afr J of Microbiology Research. 2012;6(4):838-845.
39. Shamala TR, Chandrashekar A, Vijayendra SV, Kshama L. Identification of polyhydroxyalkanoate (PHA) producing *Bacillus spp*. using the polymerase chain reaction (PCR). J. App. Microbiology. 2003;94(3):369-374.
40. Khanafari A, Sepahei AA, Mogharab M. Production and recovery of poly-β-hydroxybutyrate from whey degradation by azotobacter. Iran J of Environmental Health Science Engineering. 2006;3(3):11-33.
41. Pal A, Prabhu A, Kumar AA, Rajagopal B, Dadhe K, Ponnamma V, Shivakumar S. Optimization of process parameters for maximum poly (-beta-) hydroxybutyrate(PHB) production by *Bacillus thuringiensis* IAM 12077. Polish J of Microbiology. 2009;58(2);149.
42. Omar R, Idris A, Yunus R, Khalid K, Aida Isma MI. Characterization of empty fruit bunch for microwave assisted pyrolysis. Fuel. 2011;90:1536-1544.
43. Chen HJ, Tsai TK, Pan SC, Lin JS, Tseng CL, Shaw GC. The master transcription factor Spo A is required for poly (3 hydroxybutyrate) (PHB) accumulation and expression of genes involved in PHB biosynthesis in *Bacillus thuringiensis*. FEMS Microbiology Letters. 2010;304(1):74-81.

44. Hamieh A, Olama Z, Holail H. Microbial production of polyhydroxybutyrate, a biodegradable plastic using agro-industrial waste products. Global Adv Res. J. of Microbiology. 2013;2(3):054-064
45. Van Thuoc D, Huu Phong T, Thi Binh N, Thi Tho N, Minh Lam D, Quillaguaman J. Polyester production by halophilic and halotolerant bacterial strains obtained from mangrove soil samples located in Northern Vietnam., Wiley Online Library, Microbiology Open. 2012;1(4):395-406.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=601&id=39&aid=5793