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Biopolymer production by some marine bacterial strains isolated from Egypt

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Abstract

PHAs (Polyhydroxyalkanoates) are considered as an energy and carbon reserve that accumulated in bacteria. PHAs are substances converted into CO2 and water within a year by a variety of microbes. Polyhydroxybutyrate (PHB) is the most widely studied PHA. This work was conducted to isolate bacteria that produce PHB from seawater (the new Suez Canal, the city of Qantara, Ismailia, Egypt), screening using Sudan Black B (SBB) and Nile blue A (NbA). A number of twenty isolates stained with SBB gave positive results (black- blue coloring), while sixteen of them were considered as PHB producers when using NbA dye. The strains were identified based on their 16S rRNA gene sequencing and deposited in the GenBank. The temperature and aeration were studied to obtain the high productivity of PHB. The results revealed that the use of 30°C temperature with the use of aeration is more suitable for the production of PHB, where the highest productivity was obtained 2.30 g /l when growing Bacillus megaterium strain YSBM6 at 30°C and shaker speed of 110 rpm for 48 h. FT-IR and GC-MS were used to identify the PHB produced. Results of FTIR and GC-MS analysis confirmed the polymer as PHB and the ability of isolated bacterial strains to produce PHB.
Key words: *Bacillus megaterium,* Polyhydroxybutyrate (PHB), FTIR, Bioplastic, marine, and GC-MS

1. Introduction

Plastic products, which are ubiquitous in our daily lives, are increasingly producing severe environmental issues. Every year, millions of tons of these non-biodegradable polymers end up in the environment. Recycling is one approach for the efficient handling of used plastic materials. Biodegradable plastics are another way to reduce plastic waste, among which polyhydroxyalkanoate (PHAs) are getting a lot of attention. PHAs are prevalent intracellular moleculesd [1].

Polyhydroxyalkanoates (PHAs) are common intracellular compounds formed by a wide range of species of bacteria, such as *Alcaligene eutrophus, Azotobacter Beijierinckia, Rhizobium sp, Pseudomonas oleovorans, Bacillus sp*, and some fungi and archaea, in nutrient-limiting conditions such phosphorus, nitrogen, and sulfur with excess carbon [2]. A characteristic of storage polymers is that they are insoluble and biodegradable in water and even by renewable carbon thermal plastics that can be manufactured from sources. Therefore, It has attracted considerable attention to its exploitation for commercial aspirations [3]. It enters widely different industries such as medicines, long term dosage of drugs, cosmetic world, cosmetic containers, shampoo bottles, insecticides, fertilizers, packing materials [4].

The most popular polymer family of PHA is polyhydroxy butyrate (PHB), which conceded the ideal alternative to non-degradable synthetic plastic, due to its capacity to be degraded rapidly in natural environmental conditions. These polymers can make up to 90 % of the cell's dry weight in internal membrane-enclosed inclusions [5]. Corporations accumulate like reserves in various stress environments as the bacteria grow [6]. Some microorganisms are fully capable of degrading PHAs into CO_2 and water within a year unlike plastics based on petrochemicals that require many decades to fully downgrade [7].

The marine environment is one of the world's largest ecosystems; it has not yet to be fully studied. So the aim of this work is: 1) Isolation and screening of microorganisms have the ability to produce PHB. 2) Identification and characterization of the most promising PHB producer isolates using molecular

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techniques. 3) Studying the effect of incubation temperature and aeration on PHB production. 4) Characterizations of PHB structure using FTIR and GC-MS analysis.

2. MATERIAL AND METHODS

2.1. Samples collection

Marine water samples were collected in sterilized clean bottles from the new Suez Canal, the city of Qantara, Ismailia, Egypt, during July 2018.

2.2. Isolation procedures

Serial dilutions from each marine sample were prepared to get dilutions up to 10^{-4}. For isolation of organisms, 1ml of each dilution was plated into Sucrose/Yeast extract agar medium (SYA) [8] supplemented with 3% NaCl (SYANa). The plates were incubated for 24 h at 30°C.

Alcoholic solution of SBB (0.02%) was applied to stain bacterial colonies using the rapid detection and isolation of PHB producing bacteria. PHB producers colonies appeared bluish black while, colonies unable to incorporate the SBB appeared white [9].

Colonies with different characteristic features were maintained as pure cultures on SYANa medium by sub-culturing the isolates at an interval of 1-2 weeks.

2.3. Screening methods of PHB producing bacteria using two types of staining

1- Sudan Black B: Smears were prepared from pure culture of isolates and stained with SBB solution followed by counter stained with safranine [10]. The slides were examined using light microscope (× 100). The PHB granules appeared as blue-black granules inside pink cells for the cells that stained positive; and only pink cells for those that were negative.

2- Nile blue A: Smears were prepared from isolated strains and stained using Nile blue A stain according to the method described by [11]. The slides were observed under a fluorescence microscope at wavelength 490 nm. PHB granule producing bacterial isolates appear flourish bright yellowish-orange color.

2.4. Molecular identification

The promising isolates in PHB production were subjected to molecular identification as follows:

2.4.1. Bacterial DNA extraction

Sucrose/ yeast extract broth (SYBNa) was used for growing bacterial isolates. The conditions were set at 37°C for 24 h. Bacterial pellets were collected by centrifugation (12000 g for 5 min) and washed using Nacl saline. The gene JET Genomic DNA purification Kit (Thermo scientific, Lithuania) was the method used for DNA extraction [12]. Using Nanodrop spectrophotometer and agarose gel electrophoresis, the purity and DNA yields were ascertained.

2.4.2. Bacterial fingerprints and genotypic diversity

BOX-PCR fingerprints were produced using BOXA1R primer (CTACGGCAAGGCGACGCTGA CG) [13]. Dissociation of 8 µl of PCR products by 1.5% agarose gel electrophoresis in 0.5 X TBE-buffer for 4 h (50 V). Visual comparison of BOX-PCR fingerprints was performed.

2.4.3. Identification of bacterial isolates by 16S rRNA gene sequencing

The amplification of 19 isolates was performed. Universal primers were used *i.e.* F-27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1494 (5′-CTACGGYTACCTTGTTACGAC-3′) through PCR machine (Bio-Rad T100 thermal cycler). Inspection the purification of the PCR products was performed through agarose gel electrophoresis followed by gel extraction kit. Sequencing was done by Macrogen Koria.

2.4.4. Phylogenic analysis of bacterial isolates

The developmental history was deduced by the Neighbor-Joining method. The maximum Composite Likelihood method was the method of choice for computing the tree. Fifty two nucleotide sequences were analyzed. The current study provided 19 sequences of 16S rRNA gene amplified from bacterial isolates. While the NCBI gene bank data base provided the closet hits for 33 sequences. Mega software was used for conducting developmental analysis.

2.5. PHB Production and extraction

A set of two experiments were conducted to examine the chosen isolates (16) to produce PHB in 500 ml conical flasks containing 100 ml of Sucrose/Yeast extract broth (SYb) supplemented with NaCl (3%) were sterilized at 121°C for 20 min. Each flask was inoculated with 1ml of inoculum and incubated at the following condition:

1. Incubation at 25°C and 30°C for 48h to study the effect of temperature on PHB production. Incubation at 30°C under shaking condition (110 rpm) and static condition for 48 h to study the effect of aeration on PHB production.
2. At the end of fermentation period PHB dry weight and cell dry weight were determined. The PHB yield was calculated as well. The efficient isolates were selected according to the highest PHB productivity. Three replicates from each treatment were applied.

2.5.1 PHB dry weight, cell dry weight and yield of PHB measurement

Dry weight of extracted PHB was estimated as (g/L) by the hypochlorite and chloroform method [14, 15]. The total bacterial dry cell weight (DCW) was determined as (g/L) [16], 6]. The percentage of PHB yield was calculated from the following equation

Yield of PHB accumulation (%) = Dry weight of extracted PHB (g/L) / DCW (g/L) × 100%.

2.6. Analysis of polymer

Obtained PHB was analyzed using both FT-IR and GC-MS in comparison with PHB standard obtained from commercial source (Sigma cat no: 29435-48-1).

2.6.1. FT-IR (Fourier transform infrared spectroscopy)

FT-IR analysis of the polymer sample was studied using NICOLET 380 FT-IR, Thermo scientific, made in china [17].

2.6.2. Gas chromatography–mass spectrometry (GC-MS)

Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) was used to determine chemical composition of samples [18].

2.7. Statistical analysis

All experiments were applied in triplicates. The significance of the main effects was determined by analysis of variance (ANOVA). The significance of variance treatments was evaluated by Duncans multiple range tests ($P < 0.05$). All analysis was made using a software package Costate, a product of Cohort Software Inc., Berkeley, California. All data were recorded as means of three replicates.

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of bacteria for PHB production

A total number of 35 bacterial isolates obtained from the new Suez Canal, the city of Qantara, Ismailia, Egypt were screened for PHB production using both rapid screening on the plates and slides stained with Sudan Black B.

Among the examined isolates 20 isolates were found to be PHB producers with different relative PHB accumulation ability (Fig 1). The isolates were purified; Gram stained and examined using the light microscope. Most isolates were belonged to the genus *Bacillus* (15) and only one isolate was Gram negative short rods.

The bacterial cells were further purified and stained by Sudan Black B staining and observed microscopically 100 X. The microscopic observation showed the presence of lipophilic black granules (Fig 2).

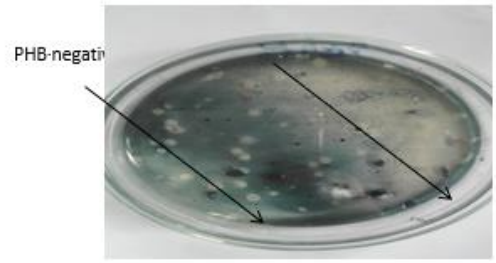

Fig 1. Rapid screening of PHB producers on Sucrose/Yeast extract media supplemented with 3% Nacl using Sudan Black B.

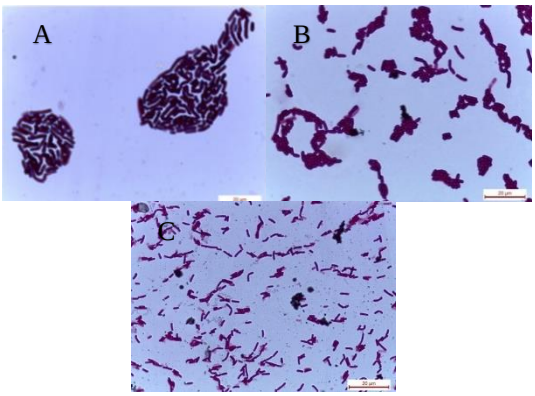

Fig.2. Photomicrograph of isolates showing the PHB granules produced in the form of dark granules in the bacterial cells under light microscope 100x oil immersion lens.
A: Strongly Sudan Black B: medium stained colonies, C: poorly stained colonies.

The first report of using SBB for staining bacterial fats was stated in 1940 [19], after that 1942 modified procedure for detection the intracellular fatty material of bacteria was studied using microscopic slides

stained with alcoholic solution of SBB and safranin as counter stained [20]. In this context the rapid detection and isolation of *Rhizobium meliloti* strains producing PHB were studied using viable colony screening method (0.02% alcoholic solution). PHB producer colonies appeared bluish black, while white colonies hadn't the ability to produce PHB [8].

A vast array of authors used the Sudan Black B Plate assay and staining method [21, 22, 23, 24]. Also several authors used stained the slides with Sudan Black B [1, 25].

To confirm the ability of isolates to produce PHB, Slides were prepared for each, stained with Nile blue A and examined with fluorescence microscope at wavelength 490 nm. The PHB producing isolates showed fluoresced bright orange (Fig 3). In this context Pierce *et al*. [26] screened *Pseudomonas* colonies which accumulated poly-β- hydroxyl butyrate on Nile blue A incorporated medium. Also Nile blue A staining method was used by many authors to confirm PHB production [27, 28].

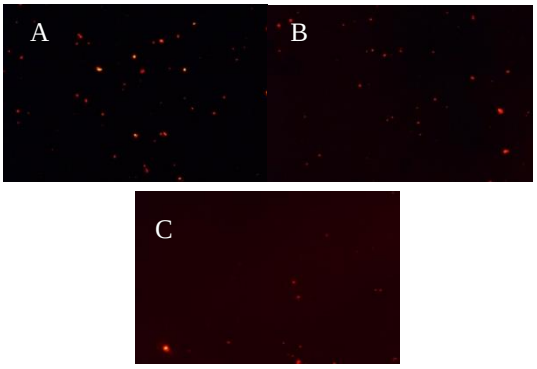

Fig.3. Fluorescence of PHB granules using Nile blue staining. Orange fluorescence under fluorescence microscope by PHB producer (A: High fluorescence, B: Moderate fluorescence and C: Weak fluorescence).

3.2. Molecular identification of efficient isolates in PHB production

a. Bacterial fingerprints and genotypic diversity

BOX-PCR fingerprints were generated for 20 bacterial isolates obtained from sea water The fingerprint profiles (Fig.4) show the genotypic diversity of tested isolates, the only identical fingerprint profiles were detected between the isolates (S1 and YSBA1) while the rest of isolates showed a unique finger print profiles. One representative isolate from each different fingerprint profile was identified based on the sequence of 16S rRNA gene.

a. Identification of bacterial isolates by 16S rRNA gene sequencing
b. 16S rRNA gene sequence analysis of 19 bacterial isolate representing different BOX-PCR fingerprint profiles (Fig.4). The 16S rRNA sequence of 9 bacterial isolates (YSBA1, YSBM1,YSBM2 ,YSBM3, YSBM4,YSBZ, YSBM5,YSBM6,YSBM7) showed 99-100% similarity to *Bacillus megaterium*, Isolate YSBA2, YSBA3 was 100% similar to *Bacillus aryabhattai*, while isolate YSBL1, YSBL2 and YSBL3 showed 100% similarity to *Bacillus amyloliquefaciens.* Isolates YSBS and YSBS1 was 100% similar to *Bacillus subtilis.*
c. Isolate YSBT was 99% similar to *Bacillus altitudinis*, isolate YSBP was 100% similar to *Bacillus pumitus.* only isolate YSS was not belonging to Bacillus sp. and was 100% similar to *Salinicola zeshunii*. 16S rRNA sequences were deposited in the Gene Bank under the accession numbers from MT903313 to MT903330 and MZ411429 (Fig 5 and Table 1).

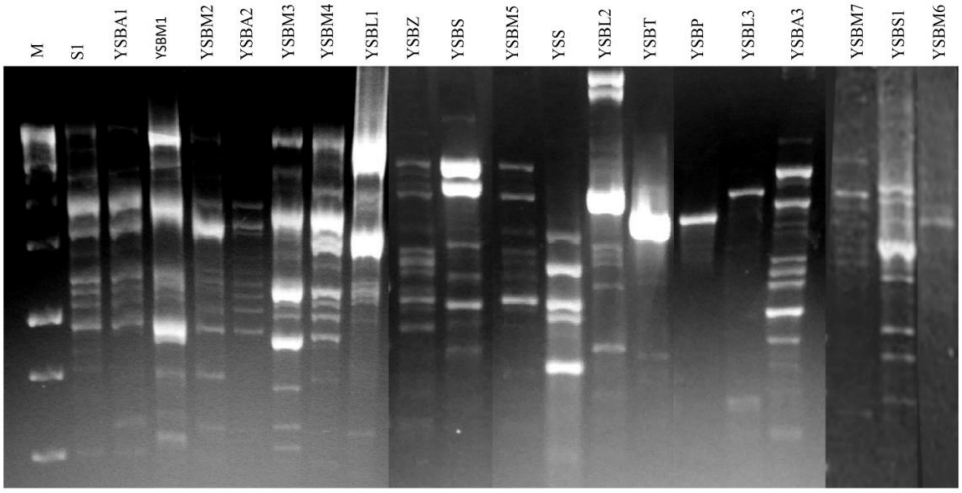

Fig.4. BOX-PCR fingerprints of 20 bacterial isolates obtained from sea water; M, 1Kb ladder

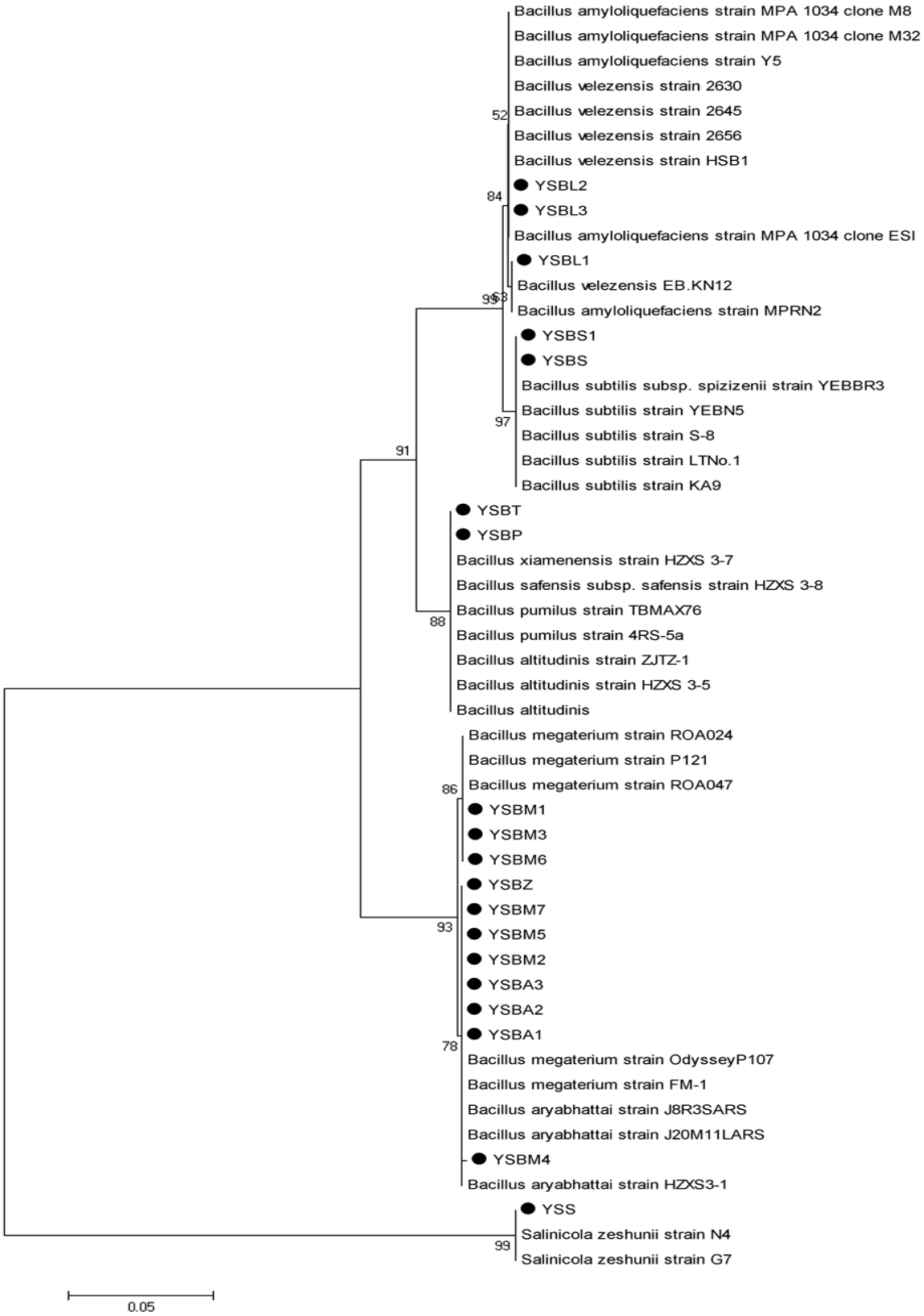

Fig.5. Aneighbor-joining phylogenetic tree based on 16S rRNA gene sequences of 19 bacterial strains (Darkcircles) with the closest hits obtained from the NCBI gene bank.

3.3. Production of PHB in Liquid Media

The selected isolates (16) were evaluated for PHB production using batch culture technique in 500 ml conical flasks containing 100 ml Sucrose/Yeast extract medium supplemented with 3% Nacl. All flasks were inoculated with 1% inoculum size and incubated at 25˚C and 30˚C in static conditions at 48 h. At the end of the experiment, PHB g/l, PHB cell dry weight and PHB yield were determined as shown in table (2). Based on the effect of temperature on PHB production, the obtained PHB (g/l) was increased as a result of rising the incubation temperature from 25°C to 30°C. The maximum increment in PHB production (165 %) was recorded by strain YSBL3 (Fig 5). The bacterial isolates YSBM1 and YSBP produced a higher amount of PHB 0.80 g/l and 0.72 g/l at 30°C and 25°C respectively. On the other hand the high PHB yield 54.63% and 44.11% were recorded with isolates YSBL3 and YSBA1 at 30˚C and 25˚C respectively. Regarding biomass production, it could be noted that 25˚C temperature was favorable to biomass production with the most examined strains, while 30˚C was more suitable for PHB production, consequently the yield with better at 30˚C (table 2).

In this context**,** many workers have reported production of PHB by various species of *Bacillus*. Thirumala *et al*. [29] reported PHB production by *Bacillus sp.*871 and *Bacillus sp*.112A. The author obtained high amount of PHB at 30°C (1.890 g/l and 1.579 g/l) and lower amount at 25C° (0.600 g/l and 0.712 g/l) respectively. Also Yüksekdağ *et al*. [30] reported PHB production by *Bacillus subtilis* and *B. megaterium*. The amount of PHB produced was 0.097, 0.071 g/L while the percentage yields were 7.98 % and 6.55 % after 48h using nutrient broth medium. Production and characterization of PHB produced by *Bacillus megaterium* NCIM 2475 were also stated [31]. Prasanna *et al*. [32] produced 0.93 g/l of PHB from *Bacillus megaterium* isolated from soil.

Another set of batches was incubated under static conditions and shaking conditions (110 rpm) at 30°C for 48 h. As shown in table (3), the PHB g/l, PHB cell dry weight and PHB yield were determined. Based on the effect of aeration on PHB production, the obtained results show that the increment in PHB production was ranged between 0 % to 283 %, respectively. The maximum increment in PHB production was recorded by strain YSBM6 (Fig.7). The isolates YSBM1 and YSBM6 showed maximum PHB production of 0.80 g/l and 2.30 g/l at 30°C in static state and shaking condition (110 rpm) respectively. The isolate YSBM2 showed maximum PHB yield 63.70 % with polymer concentration of 2.14 g/L at 30°C using shaking (110rpm). Using aeration was more suitable for PHB production with all examined. In this regard 55.6 % PHB yield was obtained using *Bacillus* sp with incubation temperature 37°C for 48 h at 150 rpm [26]. Also a high amount of PHB *i.e.* 1.8 g/L from 3.2 g/L of biomass with 57.20 % yield was recorded using *Bacillus mycoides* DFC1 incubated at 37°C at 120 rpm for 48 h [33]. DCW of the PHB was 2.7g/l and the production was 2.1g/l as reported by Sharma. [34]. Almost the same amount of PHB (2.5 g/l) was produced by Werlang *et al*. [35].

Table1. Bacterial identification and accession numbers of isolates

Isolates	GenBank cbsest hit	% similarity	Accession number
YSBA1	*Bacillus megaterium* strain AK19	99.79	MT903314
YSBM1	*Bacillus megaterium* strain B69	100	MT903315
YSBM2	*Bacillus megaterium* strain AK19	99.89	MT903316
YSBA2	*Bacillus aryabhattai* strain ZJJH-2	99.64	MT903317
YSBM3	*Bacillus megaterium* strain ROA047	99.42	MT903318
YSBM4	*Bacillus megaterium strain E71CS3*	99.72	MT903319
YSBL1	*Bacillus amyloliquefaciens* strain DE-4	99.87	MT903320
YSBZ	*Bacillus megaterium* strain 11w6FMR12	99.75	MT903313
YSBS	*Bacillus subtilis* strain BD77	100	MT903321
YSBM5	*Bacillus megaterium* strain AK19	99.77	MT903322
YSS	*Salinicola zeshunii* strain N4	100	MZ411429
YSBL2	*Bacillus amyloliquefaciens* Strain MPA 1034	99.87	MT903323
YSBT	*Bacillus altitudinis* strain 4RS-5a	99.76	MT903324
YSBP	*Bacillus pumitus* strain TBMAX76	100	MT903326
YSBL3	*Bacillus amyloliquefaciens strain* BV2007	100	MT903328
YSBA3	*Bacillus aryabhattai strain ZDX*	100	MT903330
YSBS1	*Bacillus subtilis* strain S-8	100	MT903329
YSBM6	*Bacillus megaterium strain AK4*	100	MT903325
YSBM7	*Bacillus megaterium* strain 5A1-13	100	MT903327

Table (2) Effect of incubation temperature on PHB production

strains	25°C temperature			30°C temperature		
	BHP (g/l)	DCW (g/l)	BHP yield%	BHP (g/l)	DCW (g/l)	BHP yield%
YSBM5	0.50	6.17	8.16	0.66	2.12	31.24
YSBM2	0.47	1.31	35.63	0.76	1.93	39.20
YSBM4	0.22	2.21	10.09	0.40	1.18	33.71
YSBL2	0.46	2.48	18.53	0.51	1.48	34.23
YSBM7	0.40	3.39	11.80	0.50	3.42	14.63
YSBM6	0.40	4.65	8.60	0.60	4.01	14.97
YSBA1	0.40	0.91	44.11	0.52	1.14	45.63
YSBA2	0.70	9.21	7.60	0.70	6.15	11.40
YSBM1	0.70	3.22	21.77	0.80	3.12	25.60
YSBL1	0.57	4.44	12.77	0.73	2.83	25.91
YSBP	0.72	4.50	16.01	0.78	5.00	15.53
YSS	0.66	3.87	16.98	0.75	2.80	28.01
YSBT	0.66	2.60	22.67	0.67	3.40	19.58
YSBS	0.58	4.20	13.82	0.64	2.21	28.96
YSBS1	0.44	1.25	35.20	0.56	2.13	26.33
YSBL3	0.23	1.11	20.42	0.61	1.12	54.63

LSD 0.05= 0.0399 for BHP g/l, LSD 0.05=0.2167 for biomass, LSD 0.05=0.7364 for BHP yield %

Table (3) Effect of Shaking (110rpm) on PHB production

strains	30°C in static			30°C in shaker		
	BHP (g/l)	DCW (g/l)	BHP yield%	BHP (g/l)	DCW (g/l)	BHP yield%
YSBM5	0.66	2.12	31.24	2.12	5.51	38.56
YSBM2	0.76	1.93	39.2	2.14	3.36	63.7
YSBM4	0.4	1.18	33.71	0.61	1.4	43.67
YSBL2	0.51	1.48	34.23	0.66	1.21	54.12
YSBM7	0.5	3.42	14.63	1.61	5.04	31.93
YSBM6	0.6	4.01	14.97	2.3	6.35	36.23
YSBA1	0.52	1.14	45.63	0.99	4.29	23.07
YSBA2	0.7	6.15	11.4	1.07	4.67	22.87
YSBM1	0.8	3.12	25.6	0.93	3.31	28.2
YSBL1	0.73	2.83	25.91	0.73	5.21	14.07
YSBP	0.78	5	15.53	0.83	2.6	31.92
YSS	0.75	2.8	28.01	0.79	1.61	49.07
YSBT	0.67	3.4	19.58	0.85	3.5	24.19
YSBS	0.64	2.21	28.96	0.72	1.92	37.39
YSBS1	0.56	2.13	26.33	0.67	2.61	25.16
YSBL3	0.61	1.12	54.63	0.68	1.55	43.75

LSD 0.05= 0.0878 for BHP g/l, LSD 0.05=0.2684 for DCW, LSD 0.05=1.4415 for BHP yield %. Incubation temperature 30°C, Incubation period 48 h, Initial sugar: 2%, Initial salt: 3%

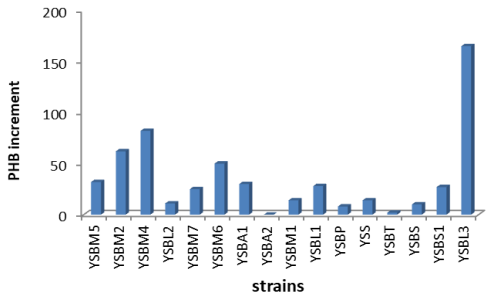

Fig.6. PHB increment % as affected by change in incubation temperature using all the isolated strains.

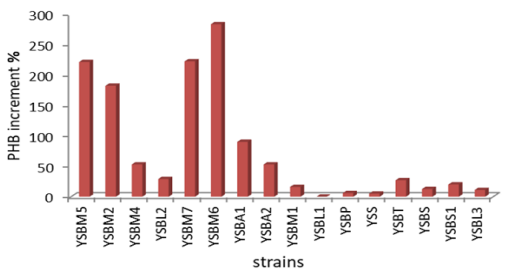

Fig.7. increment of PHB % as affected by change in aeration using all the isolated strains

3.4. Analysis of PHB polymer produced by YSBM6

3.4.1 FTIR analysis:

Polymer extracted from YSBM6 was used for recording IR spectra. The PHB FTIR spectrum (Fig.8) show significant peaks at several wavelengths, which represent Polyhydroxybutyrate features. FTIR spectroscopy of the polymer produced using sucrose as substrate was investigated along with standard of PHB. The polymer extracted showed peak at 3435, 2976 and 2934, 1724 as well as intense peaks located at 1101 and 1053 cm^{-1}, which indicated the presence of O-H stretching of alcohol, C-H stretching of alkanes, C=O and C-O stretching of ester, respectively**.** The FTIR spectrum of the PHB compared well with standard and the FTIR spectrum obtained by Brinda Devi *et al*. [36] and confirmed that the strain produced PHB.

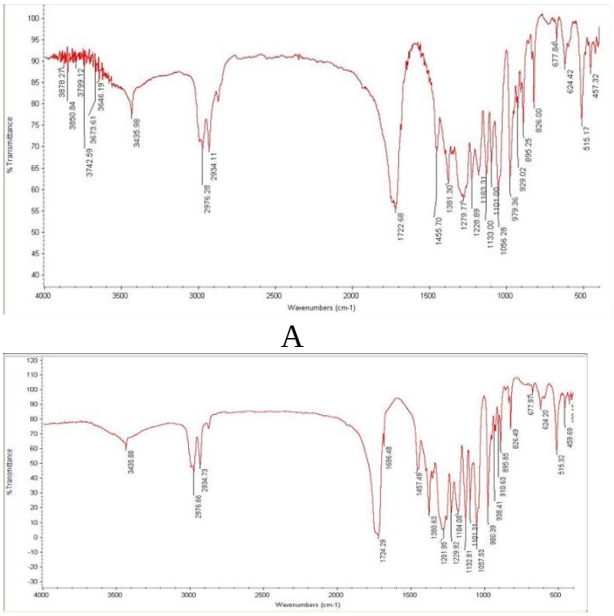

Fig.8. FTIR spectrum of PHB produced by bacterial strain (A: Standard, B: YSBM6)

3.4.2. GC- MASS analysis:

The results in Tables (4 and 5) of GC-MS analysis for PHB standard and YSBM6 show the biodegradable compounds obtained from the polymer. GC-MS analysis of PHB standard showed that twenty three different biodegradable compounds as shown in (Table 4). While fourteen biodegradable compounds were recorded with PHB obtained from YSBM6 (Table 5).

Table (4): GCMS analysis of PHB standard showing chemical composition of biodegradable polymer

S/N	Retention time (minutes)	% Area	Compound Name	Molecular Weight	Molecular Formula
1	3.95	1.71	Diallyl disulphide	146	$C_6H_{10}S_2$
2	4.31	0.91	Diallyl disulphide	146	$C_6H_{10}S_2$
3	7.49	0.75	Tetradecane, 2,6,10-trimethyl-	240	$C_{17}H_{36}$
4	7.85	0.69	Trisulfide, di-2-propenyl	178	$C_6H_{10}S_3$
5	8.01	4.67	Trisulfide, di-2-propenyl	178	$C_6H_{10}S_3$
6	8.18	0.87	Phenol, 2-methyl-5-(1methyl ethyl	150	$C_{10}H_{14}O$
7	9.28	1.03	5-Methyl-1,2,3,4-tetrathiane	170	$C_3H_6S_4$
8	11.76	1.09	Decane, 2,3,5,8-tetramethyl	198	$C_{14}H_{30}$
9	11.96	20.26	1-Dodecanamine, N,N-di methyl	213	$C_{14}H_{31}N$
10	12.22	3.08	3,4-Di hydro-2H-1,5-(3"-T-butyl) benzo dioxepine	206	$C_{13}H_{18}O_2$
11	12.65	0.68	Pentacosane	352	$C_{25}H_{52}$
12	13.65	0.58	1-Nonadecene	266	$C_{19}H_{38}$
13	15.75	8.72	Nizatidine	331	$C_{12}H_{21}N_5O_2S_2$
14	16.62	1.24	Tetradecane, 2,6,10-trimethyl	240	$C_{17}H_{36}$
15	17.33	0.50	1-Nonadecene	266	$C_{19}H_{38}$
16	19.53	1.72	7,9-Di-tert-butyl-1-oxaspiro(4,5)dec a-6,9-diene-2,8-dione	276	$C_{17}H_{24}O_3$
17	19.61	23.13	Hexadecanoic acid, methyl ester	270	$C_{17}H_{34}O_2$
18	20.23	0.76	Tetradecane, 2,6,10-trimethyl-	240	$C_{17}H_{36}$
19	22.38	22.30	9-Octadecenoic acid (Z)-, methyl ester	296	$C_{19}H_{36}O_2$
20	22.55	3.38	2-Methyl enebrexane	134	$C_{10}H_{14}$
21	22.90	0.68	Docosane	310	$C_{22}H_{46}$
22	23.53	0.51	Dotriacntane	450	$C_{32}H_{66}$
23	25.60	0.75	N-Methyl-N-benzyl tetra decanamine	317	$C_{22}H_{39}N$

Table (5): GCMS analysis of biodegradable compounds produced by YSBM6 strain

S/N	Retention time (minutes)	% Area	Compound Name	Molecular Weight	Molecular Formula
1	5.09	0.50	2-Pentenoic acid, 5- (decahydro - 5, 5, 8 a-tri methyl-2- oxo -1 - naphthalenyl)-3-methyl-, methyl ester	320	$C_{20}H_{32}O_3$
2	5.18	0.36	4-Hydroxy benzoic acid, 2TMS derivative	282	$C_{13}H_{22}O_3Si_2$
3	5.63	1.55	Cyclotetra siloxane, octamethyl-	296	$C_8H_{24}O_4Si_4$
4	6.74	2.60	Propanedioic acid, [2-[(4-methyl phenyl) sulfonyl] ethyl idene]-, di methyl ester	312	$C_{14}H_{16}O_6S$
5	6.88	0.13	Benzenamine,4,5-difluoro-2- (1pyrenyl)-	329	$C_{22}H_{13}F_2N$
6	7.12	0.21	Spiro [acridine 9(10H),9'(10'H) anthracene],2,7-di methyl	373	$C_{28}H_{23}N$
7	7.18	0.22	2-Anthracene carboxylic acid, 9, 10- di hydro-3 - hydroxy- 6, 8 di methoxy-1-methyl-9,10-d ioxo-, ethyl ester	370	$C_{20}H_{18}O_7$
8	9.15	0.10	3-Methyl-1,3-bis(trimethylsilyloxy) butane	248	$C_{11}H_{28}O_2Si_2$
9	9.51	1.71	1,2:4,5-di-o –isopropyl idene-8-tetradecyne-D- glycerod- manno-1,2,3,4,5,6-hexol	370	$C_{20}H_{34}O_6$
10	10.52	0.15	Acetamide, N-(5,6,7,9-tetra hydro-1,2,3,10 -tetramethoxy-9-oxobenzo [A] heptalen-7-yl)-, (S)-	399	$C_{22}H_{25}NO_6$
11	16.84	4.17	Hexadecanoic acid methyl ester	270	$C_{17}H_{34}O_2$
12	18.92	3.02	9-Octadecanoic acid,methyl ester	296	$C_{19}H_{36}O_2$
13	19.94	2.44	Hexadecanoic,ethyl ester	284	$C_{18}H_{36}O_2$
14	20.06	0.48	5- hexa decamethyl octa siloxane	578	$C_{16}H_{50}O_7Si_8$

The major compounds obtained were Hexadecanoic acid methyl ester, molecular weight of 270 and 9-Octadecenoic acid (Z)-, methyl ester, molecular weight of 296 in both standard and YSBM6. The results compared with the standard and results obtained from Okwuobi *et al.* [37] and confirmed that the strain produced PHB.

3. CONCLUSIONS

Among the examine strains *B. megaterium* strain (YSBM6) was the superior in PHB production. The production increased at 30°C incubation temperature and aeration. The obtained PHB was confirmed by FTIR and GC-MS analysis. It is recommended to use YSBM6 in production of PHB using Agricultural waste like molasses and salted whey to decrease the processing cost.

5. CONFLICTS OF INTEREST

Declared None

6. ACKNOWLEDGEMENT

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7. REFERENCES

[1] Bhuwal A. K.., Singh G., Aggarwal N. K., Goyal V., and Yadav A., Isolation and screening of polyhydroxyalkanoates producing bacteria from pulp, paper, and cardboard industry wastes, *Int. J. Biomater* **2013** (2013), doi: 10.1155/2013/752821.

[2] Bela E. B. l and Farid M. A., Production of Poly-β-hydroxybutyric acid (PHB) by Bacillus cereus, *Int.J.Curr.Microbiol.App.Sci*, **5**, 7, 442–460 (2016), doi: 10.20546/ijcmas.2016.507.048.

[3] Akdoğan M., and Çelik E., Purification and characterization of polyhydroxyalkanoate (PHA) from a Bacillus megaterium strain using various dehydration techniques, *J. Chem. Technol. Biotechnol.*, **93**, 8 2292–229 (2018).

[4] Gatea D. I. H., Bioplastic (Poly -3-Hydroxybutyrate) Production By Local Pseudomonas Aeruginosa Isolates Utilizing Waste Cooking Oil, *World J. Pharm. Res.*, **6**, 8 289–302, (2017), doi: 10.20959/wjpr20178-8631.

[5] Gao X., Chen J.-C., Wu Q., and Chen G.-Q., Polyhydroxyalkanoates as a source of chemicals, polymers, and biofuels, *Curr. Opin. Biotechnol.*, **22**, 6, 768–774 (2011).

[6] Hawas J. M. E., El-banna T. E., Belal E. B. A., and El-Aziz A. A., Production of Bioplastic from some selected Bacterial strains, *Int. J. Curr. Microbiol. Appl. Sci.*, **5**, 1, 10–22 (2016), [Online]. Available: http://www.ijcmas.com/vol-5-1/Jehan Mohamed El-Mohamedy Hawas, et al.pdf.

[7] Biosci I. J., Phanse N., Chincholikar A., Patel B., Rathore P., and Vyas P., Screening of PHA (poly hydroxyalkanoate) producing bacteria from diverse sources, *Int. J. Pharm. Chem. Sci.*, **1**, 6, 27–32 (2011).

[8] Bormann E. J., Leissner M., and Beer B., Growth-associated production of poly (hydroxybutyric acid) by Azotobacter beijerinckii from organic nitrogen substrates, *Appl. Microbiol. Biotechnol.*, **49**, 1, 84–88 (1998).

[9] Liu M., González J. E., Willis L. B., and Walker G. C., A novel screening method for isolating exopolysaccharide-deficient mutants, *Appl. Environ. Microbiol.*, **64**, 11, 4600–4602 (1998).

[10] Smibert R. M. and Krieg N. R., General characterization manual of method for general bacteriology. Edited by: Gerhardt Murray, RGE, Briggs Phillips, G., Costilow, RN, Nester …, (1981).

[11] Ostle A. G. and Holt J. G., Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate., *Appl. Environ. Microbiol.*, **44**, 1, 238–241 (1982).

[12] Boom R., Sol C. J., Salimans M. M., Jansen C. L., Wertheim-van Dillen P. M., and Van der Noordaa J., Rapid and simple method for purification of nucleic acids, *J. Clin. Microbiol.*, **28**, 3, 495–503 (1990).

[13] Rademaker J. L. W., Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis," *DNA markers Protoc. Appl. overviews*, 151–171 (1997).

[14] Williamson D. H. and Wilkinson J. F., "The isolation and estimation of the poly-β-hydroxy-butyrate inclusions of Bacillus species," *Microbiology*, 19, 1, 198–209 (1958).

[15] Hahn S. K., Chang Y. K., Kim B. S., and Chang H. N., "Optimization of microbial poly (3-hydroxybutyrate) recover using dispersions of sodium hypochlorite solution and chloroform," *Biotechnol. Bioeng.*,. **44**, 2, 256–261 (1994).

[16] Kunioka M and Nakamura Y., "New bacterial copolyesters produced in Alcaligenes entrophus from organic acids," *Polym. Commun.*, 29, 6, 174–176 (1988).

[17] Raveendran S., Parameswaran B., Soccol C., and Pandey A., Production and Characterization of Poly-3-hydroxybutyrate from Crude Glycerol by Bacillus sphaericus NII 0838 and Improving Its Thermal Properties by Blending with Other Polymers, August (2011, 2014), doi: 10.1590/S1516-89132011000400019.

[18] El-Kareem M. S. M. A., Rabbih M. A. E. F., Selim E. T. M., Elsherbiny E. A. E., and El-Khateeb A. Y., Application of GC/EIMS in Combination with Semi-Empirical Calculations for Identification and Investigation of Some Volatile Components in Basil Essential Oil, *Int. J. Anal. Mass Spectrom. Chromatogr.*, **04**, 01, 14–25 (2016), doi: 10.4236/ijamsc.2016.41002.

[19] Hartman T. L., The use of Sudan Black B as a bacterial fat stain, *Stain Technol.*, **15**, 1, 23–28 (1940).

[20] Burdon K. L., Stokes J. C., and Kimbrough C. E., Studies of the common aerobic spore-forming bacilli: I. Staining for fat with Sudan Black B-safranin, *J. Bacteriol.*, **43**, 6, 717–724 (1942).

[21] Phanse N., Chincholikar A., Patel B., Rathore P., Vyas P., and Patel M., Screening of PHA (poly hydroxyalkanoate) producing bacteria from diverse sources, *Int. J. Biosci.*, **1**,. January 2011, 27–32,(2014).

[22] Mascarenhas J and Aruna K., SCREENING OF POLYHYDROXYALKONATES (PHA) ACCUMULATING SCREENING OF POLYHYDROXYALKONATES (PHA) ACCUMULATING, January (2020, 2017), doi: 10.13140/RG.2.2.29966.72005.

[23] Upadhayay V., Verma S., and Kuila A., Plant Science Today Eichhornia crassipes through microbial fermentation, *Plant Sci. Today*, **6**, 1, 541–550 (2019).

[24] Mostafa Y. S., Alrumman S. A., Alamri S. A., Otaif K. A., Mostafa M. S., and Alfaify A. M., Bioplastic (poly-3-hydroxybutyrate) production by the marine bacterium Pseudodonghicola xiamenensis through date syrup valorization and structural assessment of the biopolymer, *Sci. Rep.*, **10**,. 1, 1–13 (2020), doi: 10.1038/s41598-020-65858-5.

[25] Getachew A and Woldesenbet F., Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material, *BMC Res. Notes*, **9**, 1, 1–9 (2016), doi: 10.1186/s13104-016-2321-y.

[26] Pierce L and Schroth M. N., Detection of Pseudomonas colonies that accumulate poly-beta-hydroxybutyrate on Nile blue medium, *Plant Disease*, **78**, 7. 683–685 (1994), doi: 10.1094/PD-78-0683.

[27] Bhagowati P., Bio-degradable plastic production by bacteria isolated from marine environment and organic-waste, *Thesis*, May,. 48 (2013), [Online]. Available: http://ethesis.nitrkl.ac.in/4721/.

[28] Chetia J., Isolation and Characterization of PHA Producing Bacteria from Sewage

Samples of Assam, *Int. J. Recent Technol. Eng.*, **8**, 4, 10686–10692 (2019), doi: 10.35940/ijrte.d4287.118419.

[29] Thirumala M., Reddy S. V., and Mahmood S. K., Production and characterization of PHB from two novel strains of Bacillus spp. isolated from soil and activated sludge, *J. Ind. Microbiol. Biotechnol.*, **37**, 3, 271–278 (2010), doi: 10.1007/s10295-009-0670-4.

[30] Yüksekdağ Z. N., Aslim B., Beyatli Y., and Mercan N., Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by Bacillus subtilis 25 and Bacillus megaterium 12, *African J. Biotechnol.*, **3**, 1, 63–66 (2004).

[31] Otari S. V and Ghosh J. S., Production and characterization of the polymer polyhydroxy butyrate-co-polyhydroxy valerate by Bacillus megaterium NCIM 2475, *Curr. Res. J. Biol. Sci.*, **1**, 2, 23–26 (2009).

[32] Prasanna T., Babu P. A., Lakshmi P. D., Chakrapani R and Rao C. Production of poly (3-hydroxybutyrates) by Bacillus species isolated form soil, *J Pharm Res Rev*, **1**, 15–18 (2011).

[33] Aarthi N and Ramana K. V, Identification and Characterization of Polyhydroxybutyrate producing Bacillus cereus and Bacillus mycoides strains, *Int. J. Environ. Sci.*, **1**, 5, 744–756 (2011).

[34] Sharma N., Polyhydroxybutyrate (PHB) Production by Bacteria and its Application as Biodegradable Plastic in Various Industries, *Acad. J. Polym. Sci.*, **2**, 3 (2019), doi: 10.19080/ajop.2019.02.555587.

[35] Werlang E. B.., Polyhydroxybutyrate (PHB) Production via Bioconversion Using Bacillus pumilus in Liquid Phase Cultivation of the Biomass of Arthrospira platensis Hydrolysate as a Carbon Source, *Waste and Biomass Valorization*, 0123456789 (2020), doi: 10.1007/s12649-020-01213-z.

[36] Brinda DevA. i, Valli Nachiyar C., Kaviyarasi T and A. Samrot V., Characterization of polyhydroxybutyrate synthesized by Bacillus Cereus, *Int. J. Pharm. Pharm. Sci.*, **7**, 3, 140–144 (2015).

[37] Okwuobi P. N and Ogunjobi A. A., Production and analysis of polyhydroxyalkanoate (PHA) by Bacillus megaterium using pure carbon substrates, *World Appl. Sci. J.*, **28**, 9, 1336–1340 (2013), doi: 10.5829/idosi.wasj.2013.28.09.2531.