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Fermentative Production of Polyhydroxyalkanoates (PHAs) from Glycerol by *Zobellella taiwanensis* Azu-IN1

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ABSTRACT

Polyhydroxyalkalonates (PHAs) are biodegradable thermoplastics that are receiving immense attention as an alternative to petroleum derived plastics. The present study aimed to produce PHA using cheap and ecofriendly biodiesel waste glycerol as a substrate. Several PHA–producing bacterial isolates were isolated from different environmental samples and their efficacy for PHA production was assessed. Isolate Azu-IN1 showed the highest PHA production and was identified as *Zobellella taiwanensis* Azu-IN1 using 16S rRNA gene sequence and biochemical characterization. Factors affecting PHA production were optimized in batch fermentations. Supplementation of ammonium chloride as nitrogen source, methanol as an auxiliary carbon source, and agitation rate are the main limiting factors affecting PHA production. Maximum PHA production of 2.65 g/L at recovery yield of 50.3 (%), *w/w)* was achieved after 36 h in batch fermentation using optimized medium. Enhanced PHA production of 3.73 g/L with recovery yield of 61.7 % (*w/w*) was obtained in fed batch fermentation. The characteristics of extracted PHA were analyzed using Fourier Transform Infrared Spectroscopy (FTIR) ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy. This is the first report on accumulation of PHA by *Zobellella taiwanensis* using glycerol as the sole carbon source.

1. INTRODUCTION

A wide variety of petroleum-based synthetic polymers of approximately 187 million tons are annually produced globally and remarkable amounts of these polymers are deposited into the environment as non-biodegradable waste materials [1]. Besides, their incineration generates noxious compounds and elevates the atmospheric carbon dioxide level, causing environmental problems such as global warming [2].

Thus, replacement of conventional petroleum-derived polymers with biopolymers such as polylactide, aliphatic polyesters and PHAs that possess similar physicochemical properties as conventional plastics is of utmost importance [3].

Amongst biopolymer, PHAs are more promising bio-degradable plastics due to their inherent biodegradability, sustainability and environment-friendly properties [4]. It has several applications including packaging, paper coatings, nonwoven fabrics, adhesives, moulded goods, films and performance additives [5].

PHAs are biogenic polyesters that can be accumulated in microbial cultures under unbalanced growth conditions. These polymers are formed as intracellular hydrophobic inclusions of carbon and energy storage compounds in the cytoplasm [6]. PHAs are generally divided into two groups, short-chain-length (SCL) 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} [7]. The increasing use of PHA polymers, instead of conventional plastics, requires their cost should be competitive. Thus, potent microbial producer, cheap substrates, improved cultivation strategies, and easier downstream processing methods are quintessential [8].

Glycerol is the main by-product for the biodiesel production of about 10% (*v/v*) of the volume of biodiesel. The global biodiesel market is expected to produce 45,291 million liters by 2020, representing a CAGR of 10.1% during 2009 to 2020 (http://www.altenergymag.com/). Each ton of biodiesel produced generates co-production of approximately 100 kg crude

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glycerol. Due to this large production, the price of glycerol has sharply dropped, which makes it an interesting cheap source for microbial production of PHAs [9,10]. In addition, to gain sustainability of biodiesel industry, it is necessary to find an alternative feasible solution and effective approaches for utilization of waste glycerol into value-added products.

Therefore, the present study aimed to isolate, screen and characterize PHA-producing bacteria from different environmental sources for their ability to produce PHA from glycerol as low cost carbon source. We identified novel PHA producing bacterial strain and optimized its production in different fermentation modes. Furthermore, the characterization of the extracted PHA was performed using different spectroscopy techniques (NMR, FTIR).

2. MATERIALS AND METHODS

2.1 Isolation sources and medium

Different sites were selected from different localities of Egypt for bacterial isolation. The solid samples collected include soils collected from field, gas station, factory of leather production, chemical paint factory, and wastes from vegetable processing company. Additionally, liquid samples were also collected including oil contaminated water, sewage contaminated water, sea water and molasses. One gram or 1 ml of isolation samples was inoculated into enrichment culture and incubated for 72 h at 37^{o}C for bacterial isolation. Enrichment medium (mineral salt medium [MSM]) consist of (g/L); $Na_2HPO_412H_2O$, 9.0; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.2; NH_4Cl, 1.0; $CaCl_2.2H_2O$, 0.02; Fe(III)NH_4-Citrate, 0.0012; and 1 ml of trace elements solution containing (g/L): EDTA, 50.0; $FeCl_3$, 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. Glycerol was supplemented at 2% (*v/v*) as sole carbon source. The pH of the medium was adjusted to 7.0 using 1N NaOH and 1N HCl.

2.2 Screening tests of PHA-producing isolates

Primary screening of PHA-producing microorganisms was done by viable-colony staining method using fluorescent dye (Nile red) at 0.5 µg/mL of solid medium as described by Spiekermann et al., [11]. Pure isolates were streaked onto agar plates and incubated at 37^{o}C for 72 h. Then, the bacterial colonies were analyzed by exposing to UV light at 312 nm to detect PHA-accumulation that emit fluorescent. For secondary screening, selected bacterial isolates were cultivated in mineral salt liquid medium supplemented with 2% (*v/v*) glycerol then incubated for 72 h at 37^{o}C and 150 rpm. The polymers were extracted and quantified.

2.3 Identification of selected isolate

Morphological, physiological and biochemical properties of the most potent isolate (Azu-IN1) were examined as described by Abdel-Rahman [12]. Then the identity of bacterial isolate was confirmed based on 16S rDNA sequence analysis using universal primers, 27f (5-GAGTTTGATCACTGGCTCAG-3) and 1492r (5-TACGGCTACCTTGTTACGACTT-3). The PCR products were sequenced by Sigma Company (Egypt) using ABI 3730xl DNA sequencer. The 16S rRNA gene sequence was compared against the GenBank database using the NCBI BLAST program. Multiple sequence alignment was done using ClustalX 1.8 software package and a phylogenetic tree was constructed by the neighbor joining method using MEGA (Version 6.1) software. The confidence level of each branch (1,000 repeats) was tested by bootstrap analysis.

2.4 Factors affecting PHA production by strain Azu-IN1

The following factors were investigated for optimization of PHA production. The deduced optimal conditions resulted from each experiment was taken in consideration in the next experiment. Samples were withdrawn periodically and analyzed for DCW, PHA production and residual glycerol.

For optimal glycerol concentration, different concentrations of glycerol were applied (*viz*., 0.5, 1, 2, 3, 4, 5 and 10 %) to production media. Strain Azu-IN1 was inoculated at 10 % (*v/v*) and incubated at 37^{o}C and 150 rpm. To determine the best nitrogen source; ammonium chloride in the basal MSM-glycerol medium was replaced at equimolecular weight with organic (peptone and yeast), or inorganic (ammonium chloride, ammonium sulphate, sodium nitrate and di-ammonium hydrogen orthophosphate) nitrogen sources, separately. Strain was inoculated at 10 % (*v/v*) and incubated at 37^{o}C and 150 rpm. To determine optimal ammonium chloride concentration, different concentrations of ammonium chloride (*viz*., 0.5, 1, 1.5, 2, 2.5, 3 and 4 g/L) were supplemented to basal MSM-glycerol and sterilized. Strain was inoculated at 10% (*v/v*) and incubated at 37^{o}C and 150 rpm for 36 h.

To determine optimal pH value, buffer solutions (citrate phosphate, phosphate and borax buffer) were used for medium preparations. Different pH values (viz., 5, 6, 6.5, 7, 7.5, 8, and 9) were used without pH control during fermentation. The media were filter sterilized. After inoculation of strain Azu-IN1 at 8% (*v/v*), the media were incubated at 37 ^{o}C and 150 *rpm* for 72 h. To investigate the optimal agitation rate, fermentation media were prepared with the optimal nutritional factors optimized previously. Then inoculated and incubated at different agitation rates (viz., 0 [static], 50, 150, 200 and 250 rpm). To study the effect of auxiliary carbon sources; methanol, ethanol, sodium acetate, acetic acid, sodium citrate, olive oil, corn oil, paraffin oil, sesame oil, and soy oil were supplemented separately at 0.5% to the previously optimized MSM-glycerol (1%) media. Media were inoculated at 8% (*v/v*) and incubated at 37 ^{o}C and 200 rpm.

2.5 Fed-batch fermentation

Fed batch fermentation was carried out in 250 ml Erlenmeyer flask with optimized MSM containing glycerol (1%, *v/v*), methanol (0.5%, *v/v*) and ammonium chloride (0.2%, *w/v*) and incubated at 37 ^{o}C and 200 rpm. pH was adjusted to 6.5 during fermentation. Feedings were supplemented with different strategies (different feeding at various times). First, feeding was performed after 36 h either by addition of glycerol at 1% (*v/v*) [fed batch A] or glycerol at 1 % (*v/v*) plus ammonium chloride at 0.1 % (*w/v*) [fed batch B]. Second, feeding was carried out after 24 h

with glycerol at 0.5 % (*v/v*) plus ammonium chloride at 0.05 % (*w/v*) [fed batch C] or glycerol at 0.5 % (*v/v*) plus ammonium chloride at 0.05 % (*w/v*) plus phosphorus at (0.075%, *w/v*) [fed batch D].

2.6 Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm using spectrophotometer (M-ETKAL-721 Spectrophotometer). Culture medium was centrifuged at 10,000 rpm, at 4°C for 5 min and the cell pellet was washed with distilled water. The cell pellet was harvested by centrifugation and dried at 105°C overnight till constant weight was obtained**.** Cell mass concentration was determined by the standard calibration curve between OD_{600} and cell dry weight. Glycerol concentration was assayed using glycerol detection method combining the Malaprade reaction and the Hantzsch reaction as described by Kuhn et al., [13].

PHA extraction: bacterial cells were harvested by centrifugation at 10000 rpm for 10 minutes. The pellet was then treated with sodium hypochlorite solution (4% *w/v*) and incubated at 37°C for 1 h and again centrifuged at 5000 rpm for 15 min. The pellet was then washed with distilled water, and acetone for washing and extraction, respectively. After washing, the pellet was dissolved in 5 mL of boiling chloroform and allowed to evaporate [14].

PHA assay: Sample containing PHA polymer in chloroform was transferred to a clean test tube, allowing chloroform to evaporate and 10 ml of concentrated H_2SO_4 was added. The tube is capped with a glass marble and heated for 10 min at 100°C in a water bath. After cooling, the solution was transferred to a silica cuvette and the absorbance at 235 nm was measured against a sulfuric acid blank using UV Spectrophotometric (JENWAY 6305 Spectrophotometer). Standard curve was established with PHB concentrations ranging from 0.5–3.5 mg /ml.

2.7 Production kinetics

Fermentation kinetic parameters were studied by calculating the biomass yield related to substrate (glycerol) consumption $Y_{X/S}$ (g/g), product recovery yield with respect to cell biomass $Y_{P/X}$ (%, g/g), product yield with respect to substrate consumption $Y_{P/S}$ (g/g), and volumetric productivity P_{PHA} (g/L/h), product yield to the fermentation time (h).

2.8 Characterization of PHA

2.8.1 Transform-infrared Spectroscopy (FTIR):

The chemical structure of the extracted PHA was analyzed by Fourier Transform Infrared (FTIR) spectroscopy at Faculty of pharmacy, Al-Azhar University, Cairo, Egypt. The biopolymer was dissolved in chloroform and added to potassium bromide (KBr) pellets and then the solvent was evaporated. The infrared spectra of the samples were recorded in the wave number range from 500 to 4000 cm^{-1} using a Perkin Elmer FTIR spectro-photometer (NICOLET-IR 200).

2.8.2 NMR spectroscopy for PHA analysis

The ^{1}H- NMR and ^{13}C- NMR spectra were recorded at 25 °C with Mercury 400MHz Spectrometer equipped with ^{1}H and ^{13}C dual probe to study the structural elucidation. 20 mg of the extracted PHB was dissolved in $CDCl_3$ and subjected to analysis.

3. RESULTS

3.1 Isolation and screening of PHA-producers

Ninety-six bacterial isolates were obtained from the different environmental sampling sites. Out of these isolates, 14 isolates showed bright fluorescence on agar plates upon exposing to UV light at wavelength of 312 nm. This indicated that these isolates have ability to produce PHA, therefore, further these isolates were subjected to PHA quantification screening by cultivating on MSM supplemented with glycerol. PHA content was estimated and compared. As shown in Table (1), 12 isolates showed PHA production ranged lower than 1.0 g/L. On the other hand, isolate In-N3 produced 1.11 g/L of PHA while PHA concentration of 1.37 g/L was produced by isolate Azu-IN1. The Azu-IN1strain was observed to be highest PHA producer among all other isolates and thus one of the potent candidate for further studies.

Table 1: Quantitative screening test by 14 selected isolates for PHA production using 2% (*v/v*) glycerol.

Isolates code	PHA (g/L) ±SD
WV1	0.180 ± 0.002
BS 2	0.160 ± 0.002
A1	0.110 ± 0.009
AZU-A2	0.190 ± 0.007
AKS 1	0.210 ± 0.002
AKS 5	0.220 ± 0.003
SH5	0.390 ± 0.002
SH7	0.410 ± 0.011
AD 3	0.300 ± 0.004
FS1	0.160 ± 0.004
FS5	0.170 ± 0.006
Azu-IN1	1.37 ± 0.008
In-N3	1.11 ± 0.010
AZS3	0.190 ± 0.008

Table 2: A summary of the morphological, physiological and biochemical characteristics of the most potent bacterial isolate Azu-IN1.

Test	Result	Test	Result
Gram stain	-	Sugar fermentations:	
Shape	rod	Glucose	+
KOH	+	Xylose	+
Catalase	+	Galactose	-
Citrate utilization	+	Maltose	+
Urea hydrolysis	+	Fructose	+
Pectin hydrolysis	-	Mannitol	+
Cellulose hydrolysis	-	Sucrose	-
Gelatin hydrolysis	-	Lactose	-
Starch hydrolysis	±	Inositol	-
Growth at different NaCl conc.:		Myo-inositol	-
2%	+	Cellobiose	-
5%	±	Inulin	+
10%	-		

3.2 Identification of isolate Azu-IN1

Identification of isolate Azu-IN1 was performed by studying its morphological (Gram's reaction, shape), and biochemical characteristics (Table 2). The strain Azu-IN1 was Gram-negative with rod shape and was catalase-positive. It can grow at high salt concentration [up to 5 % sodium chloride]. The isolate showed capability of urea hydrolysis and citrate utilization. However, it could not utilize cellulose, gelatin or pectin. This isolate could ferment different sugars including glucose, xylose, maltose, fructose, mannitol, and inulin but was not able to ferment galactose, sucrose, lactose, inositol, myo-inositol, and cellobiose.

The 16S rRNA gene sequence of the Azu-IN1 isolate showed the highest similarity at 99.0 % with *Zobellella taiwanensis* strain DN-7 [accession number, KM361042], strain ZT1 [accession numbers, NR_043630.1 and DQ195676.1]. The phylogenetic tree was constructed using strain Azu-IN1 and other closely related type strains as depicted in Fig. (1). Therefore, the strain was identified as *Zobellella taiwanensis* Azu-IN1. 16S rRNA gene sequence was deposited in GenBank with the accession number MF422186.

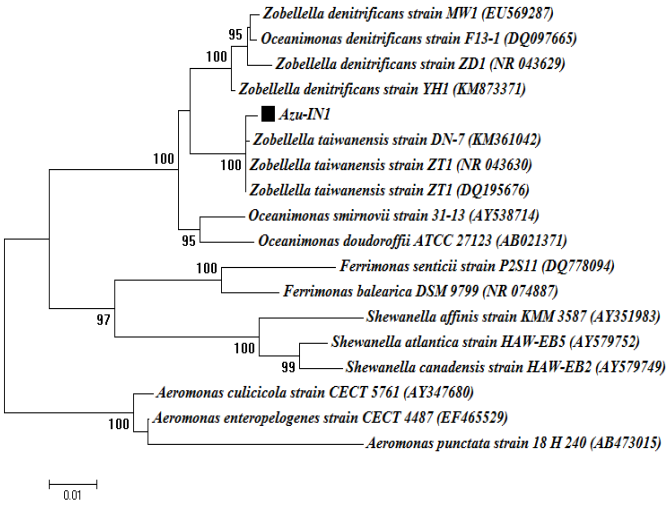

Fig. 1: Phylogenetic analysis of 16S rRNA sequences of the bacterial isolates (Azu-IN1) with the sequences from NCBI. Symbol ■ refers to 16S rRNA gene fragments retrieved from this study. The analysis was conducted with MEGA 6 using neighbor joining method. Bar 0.1 nucleotide substitutions per position.

3.3 Factors affecting PHA production

3.3.1 Effect of glycerol concentrations

Strain Azu-IN1 was cultivated in MSM medium containing different glycerol concentrations (0.5–10 %, *v/v*). Almost comparable DCW was obtained at all tested glycerol concentrations (Fig. 2). The highest PHA production of 1.96 g/L with yield of 0.341 g/g-consumed glycerol and recovery yield of 46.30 % (*w/w*) was obtained using 1.0 % glycerol after 72 h that resulted in low productivity at 0.027 g/L/h (Fig. 2B). Beyond this concentration (> 1%), decline in PHA accumulation was obtained. This shows that glycerol does not inhibit cell growth but inhibits product formation. From the above results, concentration 1.0 % of glycerol was considered as the best concentration for accumulation PHA by strain Azu-IN1.

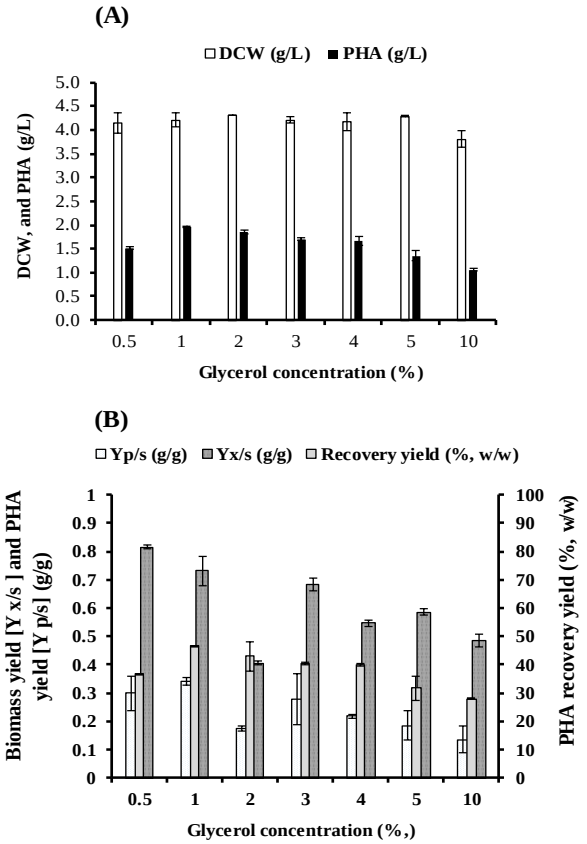

Fig. 2: Effect of different concentrations of glycerol on (A) cell growth (Dry cell weight), PHA production, and (B) Biomass yield, PHA yield and PHA recovery by *Zobellella taiwanensis* Azu-IN1 in batch fermentations.

Table 3: Effect of different nitrogen sources on PHA production from glycerol by *Zobellella taiwanensis* Azu-IN1 after 72 h.

Nitrogen sources	Max. DCW (g/L) ± SD	Residual glycerol (g/L) ± SD	$Y_{(x/s)}$ [a] (g/g)	PHA (g/L) ± SD	Recovery yield % (*w/w*)	$Y_{(p/s)}$ [b] (g/g)	$P_{(g/L/h)}$ [c]
Ammonium sulfate	3.22±0.040	2.19±0.170	0.320	1.57±0.036	48.88	0.156	0.022
Ammonium chloride	4.33±0.072	1.38±0.048	0.398	2.01±0.014	46.52	0.185	0.028
Diammonium hydrogen ortho phosphate	3.34±0.024	5.87±0.195	0.524	1.45±0.014	43.28	0.227	0.020
Yeast extract	2.60±0.108	5.80±0.030	0.403	0.86±0.012	33.00	0.133	0.012
Peptone	2.62±0.024	1.95±0.047	0.254	0.88±0.036	33.76	0.086	0.012
Sodium Nitrate	1.73±0.018	1.05±0.022	0.154	0.48±0.019	27.63	0.043	0.007

[a] Yield of biomass based on substrate consumed; [b] Yield of PHA based on substrate consumed; [c]Productivity of PHA.

Different organic and inorganic nitrogen sources were investigated as shown in Table 3. Maximum DCW of 4.33 g/L with highest PHA production of 2.01 g/L was obtained with ammonium chloride after 72 h compared to other nitrogen sources tested. With ammonium chloride, the PHA recovery yield at 46.5 % (*w/w*), PHA yield of 0.185 g/g-consumed sugar and productivity of 0.028 g/L/h were achieved. Ammonium sulfate and diammonium hydrogen ortho-phosphate attained high PHA production at 1.57 and 1.45 g/L, respectively. On the other hand, the supplementation of organic nitrogen sources (peptone, yeast extract) or sodium nitrate showed a negative effect on PHA accumulation by strain Azu-IN1.

High DCW (ranging from 4.81-4.85 g/L) was achieved after 72 h using 2-4.0 g/L of ammonium chloride in the fermentation medium. However, the maximal PHA production of 2.30 g/L and the highest recovery yield of 49.76 %, (*w/w*) was obtained using 1 g/L ammonium chloride after 72 h (Table 4). As a results, ammonium chloride at concentration (1.0 g/L) was considered as the best concentration for PHA production by strain Azu-IN1.

Table 4: Effect of different concentrations of ammonium chloride on PHA production from glycerol by *Zobellella taiwanensis* Azu-IN1.

Ammonium chloride conc. (g/L)	Max. DCW (g/L) ± SD	Residual glycerol (g/L) ± SD	$Y_{(x/s)}$[a] (g/g)	PHA (g/L) ± SD	Recovery yield % (*w/w*)	$Y_{(p/s)}$[b] (g/g)	$P_{(g/L/h)}$[c]
0.5	3.92±0.027	7.23±0.599	0.744	1.35±0.007	34.55	0.257	0.019
1.0	4.62±0.030	1.59±0.374	0.424	2.30±0.009	49.76	0.211	0.032
1.5	4.76±0.075	2.64±0.427	0.621	2.27±0.010	47.73	0.296	0.032
2.0	4.81±0.055	2.92±0.015	0.502	2.00±0.018	41.56	0.209	0.028
2.5	4.81±0.028	1.12±0.034	0.416	2.00±0.016	41.55	0.173	0.028
3.0	4.83±0.056	3.77±0.153	0.554	1.84±0.053	38.13	0.211	0.026
4.0	4.85±0.025	5.60±0.429	0.703	1.48±0.039	30.40	0.214	0.020

[a] Yield of biomass based on substrate consumed; [b] Yield of PHA based on substrate consumed; [c]Productivity of PHA

3.3.3 Effect of pH values

The influence of pH (5.0–9.0) on PHA accumulation was investigated using the previously optimized conditions during fermentation. As shown in Fig. 3 A, the DCW is almost comparable at a wide range of pH values (6.0–8.0) that ranged 3.41-5.27 g/L after 72 h that is similar to that obtained after 48 h (3.01–5.26 g/L). On the other hand, PHA production, recovery yield and $Y_{p/s}$ value had maximum values at pH 6.5 with 2.36 g/L, 44.78 % (*w/w*) 0.232 g/g, respectively (Fig. 3 B and C). As result, initial pH 6.5 was selected for further investigations.

3.3.4 Effect of agitation rate

The influence of agitation rate (0.0–250 *rpm*) on DCW/PHA production by strain Azu-IN1 were investigated as shown in Fig. (4). It was found that, there is a positive correlation between agitation rate and DCW /PHA production by strain Azu-IN1. The best PHA production was obtained at agitation rate of 200 rpm after 36 h at 2.34 g/L and 46.07 % (*w/w*), respectively. Therefore, an agitation rate of 200 rpm was considered as the optimal agitation rate PHA fermentation by Azu-IN1.

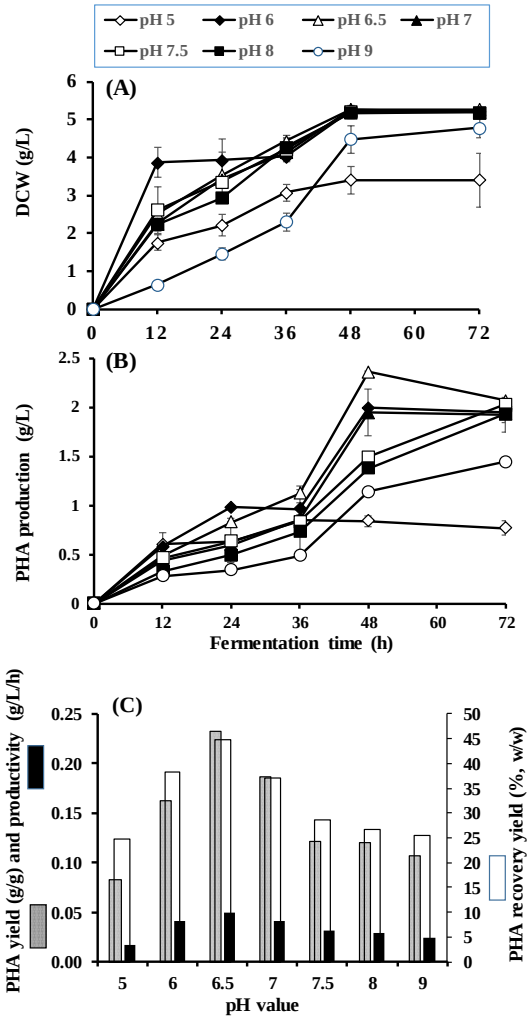

Fig. 3: Effect of pH value on PHA fermentation from glycerol by *Zobellella taiwanensis* Azu-IN1 (A) dry cell weight, (B) PHA production, g/L, and (C) PHA yield, productivity and recovery. Data points represent the means and standard deviations of results from 3 independent experiments. The standard deviation is less than that corresponding to the size of the symbol if no error bars are seen.

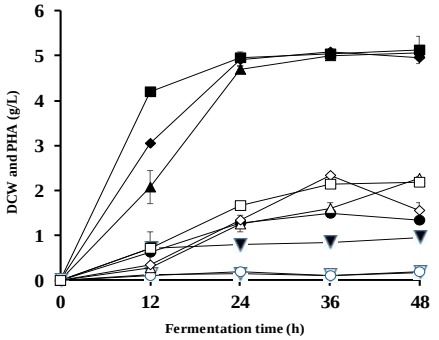

Fig. 4: Effect of different agitation rates on cell growth (dry cell weight and PHA production from glycerol by *Zobellella taiwanensis* Azu-IN1. Symbols; inverted triangle, 0 rpm; circle, 50 rpm; triangle, 150 rpm; diamond, 200 rpm; square, 250 rpm. Closed symbols show DCW and open symbols show PHA production (g/L). Data points represent the means and standard deviations of results from 3 independent experiments. The standard deviation is less than that corresponding to the size of the symbol if no error bars are seen.

3.3.5 Effect of auxiliary carbon sources (co-substrates)

Different additional carbon sources were supplemented separately at 0.5% to optimized MSM media containing 1% glycerol (1.0 %) at 37°C and 200 rpm. As shown in Table 5, sodium acetate and acetic acid resulted in decreased biomass at 0.75 g/L and 1.44 g/L, respectively after 36h. Other tested co-substrates resulted in high cell growth that was ranged 5.07–5.29 g/L. There is a significant increase in PHA production and recovery yield by supplementations of methanol at 2.66 g/L, and 50.06 %, respectively that is almost 13% and 4 % increase compared to that produced without any supplementation at 46.07 %, and 2.34 g/L, respectively. As result, methanol considered as the best auxiliary carbon source for achieving high efficiency fermentation of PHA.

Table 5: Effect of different auxiliary carbon sources on PHA production from glycerol by *Zobellella taiwanensis* Azu-IN1 after 36 h.

Different carbon sources	Max. DCW (g/L) ± SD	Residual glycerol (g/L) ± SD	$Y_{(x/s)}$[a] (g/g)	PHA (g/L) ± SD	Recovery yield % (*w/w*)	$Y_{(p/s)}$[b] (g/g)	$P._{PHA}$[c] (g/L/h)
Methanol	5.31 ±0.007	0.84 ± 0.128	0.455	2.66 ±0.009	50.06	0.228	0.074
Ethanol	5.23 ±0.003	4.00 ± 0.397	0.615	2.20 ±0.019	42.10	0.259	0.061
Sodium citrate	5.13 ±0.020	2.45 ± 0.135	0.511	1.66 ±0.007	32.36	0.165	0.046
Sodium acetate	0.75 ±0.185	10.11 ± 0.482	0.312	0.15 ±0.002	20.63	0.064	0.004
Acetic acid	1.44 ±0.029	9.76 ± 0.382	0.525	0.19 ±0.002	13.23	0.070	0.005
Caster seed oil	5.29 ±0.027	1.96 ± 0.121	0.501	1.70 ±0.007	32.11	0.161	0.047
Corn oil	5.28 ±0.021	1.71 ± 0.086	0.490	0.82 ±0.007	15.50	0.076	0.023
Olive oil	5.29 ±0.020	1.97 ± 0.108	0.503	1.38 ±0.005	26.07	0.131	0.038
Soy bean oil	5.19 ±0.052	2.21 ± 0.075	0.504	1.85 ±0.010	35.71	0.180	0.051
Sesame oil	5.07 ±0.007	1.48 ± 0.047	0.460	1.64 ±0.012	32.44	0.149	0.046
Paraffin oil	5.28 ±0.018	1.52 ± 0.111	0.481	0.87 ±0.002	16.54	0.080	0.024

[a] Yield of biomass based on substrate consumed; [b] Yield of PHA based on substrate consumed; [c]Productivity of PHA

3.4 Fed-batch fermentation

In order to further enhance cell biomass and PHA production by *strain Azu-IN1*, fed batch fermentations were conducted using different feedings. As shown in Fig. S1, in fed batch A [see supplementary Fig. S1-A], where the feeding with only glycerol (1%), cell biomass reached 5.33 g/L after 36 h with a little increase after that and achieving its maximal value of 5.94 g/L after 60 h. On the other hand, the accumulation of PHA achieved its maximum value of 3.14 g/L with recovery yield of 53.17% (w/w) at 84 h.

Conducting similar fermentation with feeding solution of glycerol and ammonium chloride (Fed-batch B, see supplementary Fig. S1-B) have resulted in almost similar DCW of 6.03 g/L after 84 h, however, higher accumulation of PHA was obtained achieving its maximal value of 3.24 g/L with recovery yield of 54.60 % (*w/w*) after 60 h.

In another strategy using lower glycerol concentration in feeding solution, fed-Bach C and D were conducted. In those fermentations, feeding was intermitted supplied to maintain glycerol concentration at same level. In fed batch C (see supplementary Fig. S1-C), where the feeding with glycerol (0.5 %) and ammonium chloride (0.05%), maximum production of DCW 5.60 g/L after 48 h with PHA 2.14 g/L at 60 h is lower than obtained with other fed batches tested. In fed batch D (Fig. 5), where the feeding with glycerol (0.5 %), phosphorus (0.075%, w/v), and ammonium chloride (0.05%), high PHA was produced at 3.73 g/L with recovery yield of 61.65 % (*w/w*) after 72 h. These result, are higher than the amount of PHA of 2.65 g/L and recovery yield of PHA 50.27 % (*w/w*) obtained with batch process and other fed batch strategies.

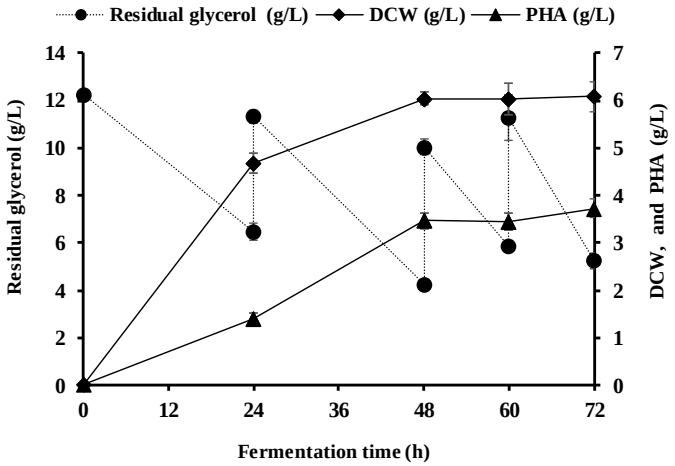

Fig. 5: Fed batches fermentation for PHA production from glycerol by *Zobellella taiwanensis* Azu-IN1 at 37 °C, initial pH 6.5 and agitation rate 200 rpm. Feeding solution consists of glycerol (0.5 %), phosphorus (0.075%, w/v), and ammonium chloride (0.05%). Data points represent the means and standard deviations of results from 3 independent experiments. The standard deviation is less than that corresponding to the size of the symbol if no error bars are seen

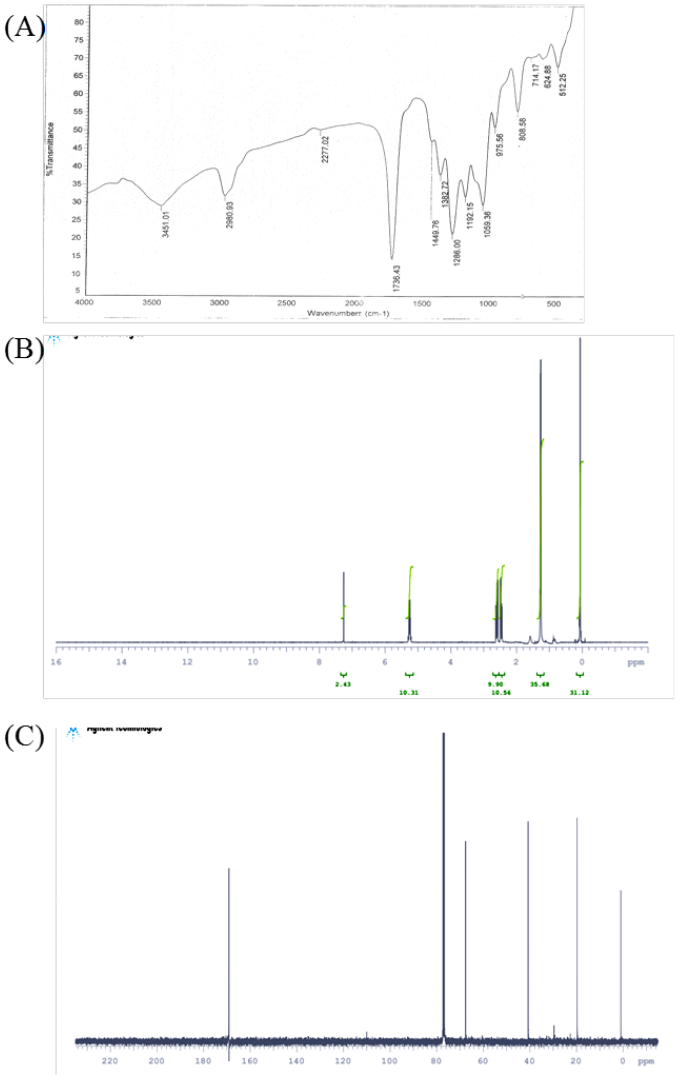

Fig. 6: (A) Fourier transform infrared spectra analysis (B) ^{1}H chemical shifts, (C) ^{13}C chemical shifts of constituent monomers of purified PHA produced by *Zobellella taiwanensis* Azu-IN1.

3.5 Characterization of polyhydroxyalkanoates

The IR spectrum of PHA compound (KBr, υmax cm-1) by Fourier Transform-Infrared Spectroscopy (FTIR) shown in Fig. 6A, showed absorption bands at 3451 cm^{-1} (OH stretching), 2980 cm^{-1} (C-H stretching), 1736 cm^{-1} (C=O stretching), 1449 and 1286 cm^{-1} (methylene bending), [15].

^{1}H-NMR spectrum of PHA compound (Fig. 6B) indicated characteristic signals of PHA, namely a doublet at 1.25 ppm, which is attributed to the methyl (CH_3) group coupled to one proton while a doublet of quadruplet at 2.44 ppm due to the methylene (CH_2) group adjacent to an asymmetric carbon atom bearing a single proton. The third signal at 5.21 ppm, which was attributed to the methine (CH) group.

4. DISCUSSION

A biodegradable bioplastic such as aliphatic polyester belongs to the family of PHAs which shows material properties as petrochemical plastics (e.g. polyethylene or polypropylene) but having positive environmental impacts. The major objective of this study was to select potential microbial PHA producers using low cost substrates and optimize the production conditions in different fermentation modes.

In the present study 96 bacterial isolates were isolated from different localities in Egypt. These isolates were analyzed for hydrophobic compounds like PHA inside the cells as previously reported [11, 16]. Amongst these, 14 isolates showed bright to red fluorescence color and used for quantitate analysis of PHA produced from glycerol as an inexpensive carbon source. Isolate Azu-IN1 could accumulate the highest amount of PHA at 1.37 g/L and was selected for characterization. Based on 16S rRNA analysis (Fig. 1), strain Azu-IN1 clustered with the members of the genus *Zobellella*, showing the highest 16S rDNA gene sequence similarity of 99% with *Zobellella taiwanensis* and was identified as *Zobellella taiwanensis* Azu-IN1. The genus *Zobellella* is well known for being able to synthetize PHA using different carbon sources [17], but to the authors knowledge, there is no report on PHA production by *Zobellella taiwanensis*.

The monomer composition of the PHA was determined by FTIR, ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy. ^{1}H NMR spectra were collected with a Varian Mercury 400 MHz spectrometer. The data was matched with the published data for same compound [18]. The values of the chemical shifts as well as the assignments of the ^{1}H NMR signals, which appeared in the spectra agree with the authentic PHB sample produced from Aldrich Company, which clearly classified the extracted biopolymer from the isolated strain in the present study as poly-3-hydroxybutyric acid and confirmed by ^{13}C NMR spectrum (Fig. 6C).

Physical and nutritional factors affecting PHA production by strain Azu-IN1 were optimized in batch fermentations. In this study, maximum production of DCW and PHA and high recovery yield of PHA by strain Azu-IN1 achieved with 1% (*v/v*) of glycerol, beyond this concentration PHA accumulation was reduced. This might be attributed to substrate inhibition that cause osmotic stress and affect cellular metabolism that decreased enzymatic activities and PHA recovery [17]. The use of 1% (*v/v*) glycerol was reported as the optimal concentration for PHA production in *Bacillus sphaericus* [19], whereas 2% (*v/v*) glycerol was reported to be the optimal for PHA production by *Pseudomonas mendocina* PSU [20], *Halomonas* sp. SA8 [21], and *Pandoraea* sp. [22].

Limited nitrogen condition is one of the critical factors affecting PHA synthesis in different bacterial strains. In this study, 1.0 g/L of ammonium chloride as nitrogen source positively enhanced the accumulation of PHA amongst other tested sources. A maximum production of PHA 2.30 g/L with accumulation of PHA at 49.76 % (*w/w*) recovery yield after 72 h were attained. On the other hand, supplementation of organic nitrogen sources leads

to decreased PHA production. Inorganic chemicals such as ammonia, or ammonium salts are an important for maximizing biomass concentration and PHA accumulation [23]. Ammonium chloride was reported as the best nitrogen source for PHAs production by several researchers [17, 24-26]. Kalaiyezhini and Ramachandran [27] found that ammonium sulphate supported high growth and PHA production from glycerol in *Paracoccus denitrificans*.

pH is one of the important factors affecting cell growth, enzymatic activities and product formation. pH 6.5 was found to be optimum for PHA production by strain Azu-IN1 after 48h. Most studies reported neutral to slightly acidic or slightly alkaline pH (pH 6.0-8.0) is the optimal for PHAs accumulation by several bacterial strains [27-30].

In this study, agitation rate is reported to be limiting factor for PHA accumulation by strain Azu-IN1. The lowest growth rate was achieved at static condition, while a significant increase in growth rate accompanied by accumulation of PHA was gradually increased by increasing agitation rate up to 200 rpm after 36 h. This might be attributed to the increase in oxygen transfer and mass transfer rates due to increased surface area of contact among media components [26]. Beyond this level, higher growth was achieved but accumulation of PHA was decreased that attributed to limitations of stress conditions required for PHA granules storage [31]. Wei et al., [32] reported maximum production of DCW and PHB production at agitation rate 200 rpm by *Cupriavidus denitrificans* 184, while 175 rpm was the optimal for *Pseudomonases aregunoisa* NCIM No. 2948 [30].

Supplementation of 0.5 % methanol as an auxiliary carbon source to fermentation medium maximized PHA recovery yield of PHA to 50.72 % (*w/w*) by strain Azu-IN1. This indicated that methanol is the principal precursor for PHA synthesis by strain Azu-IN1 as previously reported [33-34]. Besides, the recovery yield is superior to that obtained to Yezza et al., [35] who reported that, after 66 h, a copolymer content of 30 % (*w/w*) was achieved when 1 % (*v/v*) methanol solution supplemented with 0.1 % (*v/v*) valeric acid used as substrate. In addition, the yield ($Y_{p/s}$) of 0.228 g/g obtained in this study is about 84.5 % of the maximum theoretical yield based on glycerol (0.47 g/g) *via* acetyl-CoA pathway [22].

To achieve high product concentration, fed-batch fermentation has been conducted to reduce the effect of high initial substrate concentration. Different feeding strategies were applied. Amongst all, feeding with glycerol (0.5 %), phosphorus (0.15-0.05%) and ammonium chloride (0.05%) achieved the highest production of DCW at 6.05 g/L, PHA at 3.73 g/L and recovery yield at 61.65 % (*w/w*) after 72 h. The obtained PHA production and PHA recovery yield are about 28 % and 22.6 % increase in when compared to that obtain by batch fermentation with 2.65 g/L and 50.27 %, respectively. This indicated that this feeding regimen supported both cell growth and PHA accumulation during growth by strain Azu-IN1. Zhu et al., [36] achieved 7.4 g/L of PHB with 31.4% recovery yield by *Burkholderia cepacia* ATCC 17759 in fed batch fermentation from glycerol after at 120 h. Cui et al., [37] reported maximum PHB of 11.5 g/L with recovery of 46.6 % of DCW, (*w/w)* after 30 h in fed-batch fermentation by *Chelatococcus daeguensis.* Although the results achieved in this study is superior to all previously reported studies, the concentration of PHA production is required to be further improved. Further investigations on repeated batch studies with cell recycle will be conducted to enhance cell growth and PHA accumulations. Up to the authors knowledge, this is the first study on PHA production by *Zobellella taiwanensis* by utilization of glycerol as the sole carbon source.

5. CONCLUSION

In the present report, PHAs production and optimization using glycerol as substrate was performed using a new isolate, *Zobellella taiwanensis* Azu-IN1 strain at 37^{o}C. Glycerol at 1% (*v/v*); ammonium chloride, 1 g/L; methanol. 0.5 %; pH, 6.5; and agitation rate, 200 rpm were the optimal fermentation conditions for PHA production by Azu-IN1 strain. Under these conditions, maximum PHA production of 2.65 g/L with recovery yield of 50.27 % (*w/w*) were achieved after 36 h in batch fermentation. Fed batch fermentation based on feeding solution containing glycerol, phosphorus and ammonium chloride resulted in improved production (3.73 g/L) and recovery yield (61.65 % of DCW, *w/w*).

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Conflict of Interests: There are no conflicts of interest.

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Supplementary Data

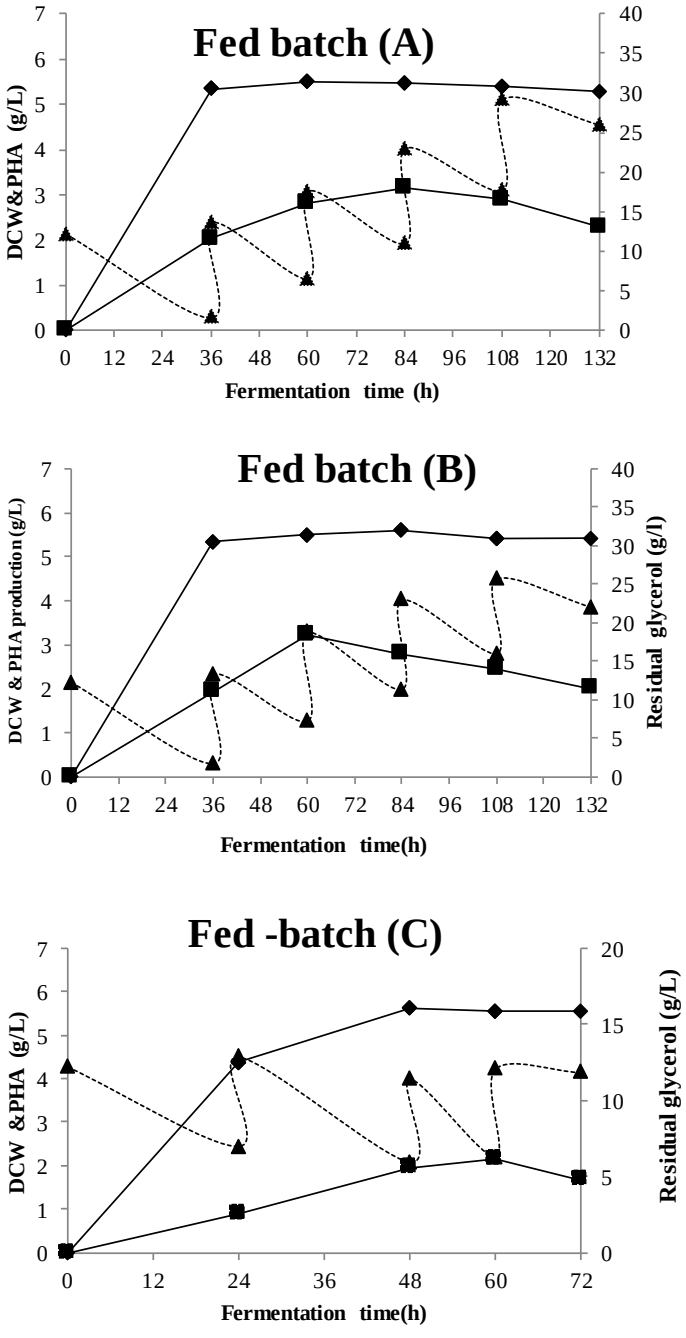

Fig. S1: Fed batch fermentation with different feeding strategies for PHA production from glycerol by *Zobellella taiwanensis* Azu-IN1 at 37^{o}C, initial pH 6.5 and agitation rate 200 rpm.