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Research article

Batch and fed-batch production of polyhydroxyalkanoates from sugarcane molasses by *Bacillus flexus* Azu-A2

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Key words: Bioplastic; PHA-production, Fed-batch fermentation, *Bacillus flexus.*

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Abstract

Production of bioplastic has recently attracted great interest as an environmentally friendly alternative to petrochemical plastics. The present study aimed at production of polyhydroxyalkanoates (PHAs), a biodegradable thermoplastic, from agro-industrial wastes by potent bacterial isolates. Ninety-six isolates were obtained from different localities in Egypt and assayed for qualitative and quantitative production of PHA using sugarcane molasses. Bacterial isolate AZU-A2 showed the highest production of PHA. This strain was identified as *Bacillus flexus* strain AZU-A2 by 16S rRNA gene sequence and biochemical characterization. Physiological and nutritional factors affecting PHA production were optimized in batch fermentations. Agitation rate, supplementation of acetic acid as an auxiliary carbon source, and ammonium chloride as nitrogen source were critical factors affecting fermentation of PHA production. Maximum production of 3.97 g/L PHA with recovery yield of 88.0 (%, *w/w)* was achieved after 24h in batch fermentation in optimized medium. A maximal PHA production of 6.13 g/L at recovery yield of 92.1 (%, *w/w*) was obtained in fed-batch fermentation. The characteristics of extracted PHA were analyzed using ^{1}H-NMR, ^{13}C-NMR and FTIR spectroscopy techniques.

Introduction

The accumulation of synthetic plastics has become a major environmental problem due to their low biodegradation, therefore, production of biodegradable plastic materials as an alternative to synthetic plastics has become urgent in recent years. Polyhydroxy-alkanoates (PHAs), bio-based polymers, have received a great attention due to their inherent biodegradability, sustainability and environment-friendly properties beside its physical and mechanical properties that resemble those of petrochemical thermoplastics [1-4]. It has several applications including packaging, moulded goods, paper coatings, nonwoven fabrics, adhesives, films and performance additives [5].

PHAs are biogenic polyesters that can be naturally accumulated in bacterial cultures as hydrophobic inclusions of carbon and energy storage compounds under limiting nutrients in the presence of excess carbon source [6]. The property of PHA depends on their chemical composition (homo-or co-polyester, contained hydroxyl fatty acids) [5]. Structurally, PHAs are consisting of different monomers with straight, branched, saturated, unsaturated and also aromatic structures. According to monomer size, PHA can be classified into short chain length with 5 or less carbon atom in a monomer, and medium chain length with 3-14 carbon atom in monomer [1,7].

The market consumption of PHA is estimated to be 34,000 MT in 2018. Currently, the market for PHA is projected to grow annually at a CAGR of 4.88% that is valued to reach USD 93.5 Million by 2021 (http://www.marketsandmarkets.com). These rising demands are attracting to increase the production capacities to be competitive with synthetic plastics. To meet the worldwide requirements for PHA and to be commercially viable, overall production costs should be reduced. This can be achieved by using less expensive substrate or waste materials because the primary cost is mainly depending on the huge amount of carbon source required for the fermentative production. Another method that reduces the PHA cost is to improve the production, productivity and yield of PHA fermentation.

Seventy percent of global sugar comes from sugarcane where its current annual production of 1.89 billion tones as fresh cane [8]. It is estimated that from everyone hundred tons of sugarcane crushed in a factory, about 11 tons of sugars are produced and 3 tons of molasses can be recovered as non-food by-product raw waste of sugar industry [9]. Molasses contains plenty of sugars, up to 50% sucrose, and other nutrients including organic acids, vitamins and minerals [10]. The nitrogen content of cane molasses is very low at about 0.5–0.9% (*w/v*). These characteristics make these wastes materials are suitable for PHA-producing

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bacteria achieving high cell density with high PHA production [11].

Therefore, the present study aimed to isolate and screen novel PHA-producing bacteria from environmental sources having ability to produce PHA from sugarcane molasses. We also aimed to optimize the PHA production in different fermentation modes. Finally, the characterization of the extracted PHA will be analyzed.

Experimental

Materials and Methods

Isolation sources and medium

Isolation sources were collected from different localities in Egypt as follows: solid samples include soils collected from field, gas station, factory of leather production, paint factory, and wastes from vegetable processing company. Liquid samples include, oil contaminated water, sewage contaminated water and sea water and molasses. Enrichment medium for bacterial isolation consist of (g/L)[12]; Na_2HPO_4, 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 1ml 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. Glucose was supplemented at 20 g/L as sole carbon source. The pH of the medium was adjusted to 7.0 using 1 N NaOH and 1 N HCl.

Screening tests for PHA-producing isolates

Primary screening of PHA-producing microorganisms was done by viable-colony staining method using Nile Red stain as follows: 0.5 µg Nile Red /mL of solid medium was prepared, sterilized and poured into Petri plates. The obtained isolates were streaked on agar plates and incubated at 37°C for 3 days then examined under UV light. The lighted colonies were recorded as positive [13].

For secondary screening, selected bacterial isolates were cultivated in mineral salt medium supplemented with 2% glucose or molasses separately. The media were incubated for 72 h at 37°C and 150 rpm. The polymers were extracted and quantified.

Identification of selected isolate

Morphological, physiological and biochemical properties of the most potent isolate AZU-A2 were examined as described by Abdel-Rahman [14]. Then the identity of bacterial isolate was confirmed based on 16s rDNA sequence analysis. Genomic DNA was extracted, then 16S rRNA was amplified in PCR using the genomic DNA as template and two bacterial universal primers, 27f (5-GAGTTTGATCACTGGCTCAG-3) and 1492r (5-TACGGCTACCTTGTTACGACTT-3) as described by Abdel-Rahamn,[14]. The PCR products were sequenced by Sigma Company (Egypt) using ABI 3730xl DNA sequencer with the two primers. The 16S rRNA sequence was compared with 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.

Factors affecting PHA production

The following factors were examined for their effect on PHA production by the most potent bacterial isolate. The deduced optimal conditions resulted from each experiment was taken in consideration in the next experiment.

For optimal molasses concentration, different concentrations of molasses were applied (*viz*., 0.5, 1, 2, 3, 4, 5 and 10 %) to production media. Strain AZU-A2 was inoculated at 10 % (*v/v*) in 250 mL Erlenmeyer flask containing 50 ml mineral salt medium (MSM), and incubated at 37°C and 150 rpm. Samples were withdrawn periodically and analyzed for DCW, PHA production and residual sugar.

To study the optimal nitrogen source; ammonium chloride in the basal MSM-molasses 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. Fermentation media was supplemented with 3% molasses as sole carbon sources. The strain was inoculated at 10 % (*v/v*) as described previously and incubated at 37°C and 150 rpm. All the previous optimal conditions were applied. Samples were withdrawn periodically and analyzed for DCW, PHA production and residual sugar as mentioned before.

To determine optimal ammonium chloride concentration, different concentrations of ammonium chloride *viz*., 0.5, 1, 1.5, 2, 2.5, 3 and 4g/L were supplemented to basal MSM-molasses and sterilized. Strain was inoculated at 10 % (*v/v*) and incubated at 37°C and 150 rpm for 36h. Samples were withdrawn periodically and analyzed for DCW, PHA production and residual sugar as mentioned before.

To determine optimal pH value, different fermentation media were prepared at various initial pH values viz., 5, 6, 6.5, 7, 7.5, 8, and 9 were used without pH control during fermentation. These media were filter sterilized. After inoculation of strain AZU-A2 at 6 % (*v/v*), the media were incubated at 37°C and 150 rpm for 36h. Samples were withdrawn periodically and analyzed for DCW, PHA production and residual sugar as mentioned before.

To investigate the effect of phosphorus, Na_2HPO_4 and KH_2PO_4 were supplemented to the fermentation media at various ratio of 12.0:2.0, 9.0:1.5, 6.0:1.0, 3.0:0.5, 1.0:0.25 g/l. Strain AZU-A2 was inoculated at 6 % (*v/v*), the media were incubated at 37°C and 150 rpm for 36 h. Samples were withdrawn at different time intervals and analyzed for DCW, PHA content and residual sugars as mentioned above.

To investigate the optimal agitation speed, fermentation media were prepared with the optimal nutritional factors

optimized before. Then inoculated and incubated at different agitationrates *viz*., 0 (static), 50,150, 200 and 250 rpm. After incubations, DCW, PHA and sugar were analyzed as previously mentioned.

To study the effect of addition of co-substrates (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-molasse (3%) media. Media were inoculated at 6 % and incubated at 37°C and 200 rpm. At the end of each incubation period DCW, PHA, and the residual sugar were estimated.

To study the optimal concentration of acetic acid, different concentrations at 0.25, 0.5, 0.75, 1, 2, 3, 5 % were supplemented to the optimized cultivation MSM-molasses media. Inoculation of strain and incubation was performed as previous experiment.

Fed-batch fermentation

Fed batch fermentations were carried out in 250 ml Erlenmeyer flask with optimized MSM- molasses (3%, *w/v*), with acetic acid (1%, *v/v*) and ammonium chloride at concentration (0.2%, *w/v*) and incubated at 37°C and 200 rpm. pH was adjusted to 7.5 during fermentation. Feedings were supplemented with different strategies (different feeding at various times). First, feeding was performed after 24 h either by addition of sugarcane molasses at 3% (*w/v*) [fed batch A] or sugarcane molasses at 3% (*w/v*) plus ammonium chloride at 0.15 % (*w/v*) [fed batch B]. Second, feeding was carried out after 12h with sugarcane molasses at 1.5 % (*w/v*) plus ammonium chloride at 0.075 % (*w/v*) [fed batch C] or sugarcane molasses at 1.5 % (*w/v*) plus ammonium chloride at 0.075 % (*w/v*) plus phosphorus at 0.35% (*w/v*) [fed batch D]. The feeding was done as the residual sugar concentration decreased below 8 g/L.

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.

PHA extraction: After incubation period, 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 supernatant was discarded and the pellet was then washed with distilled water, acetone for washing and extraction, respectively. After washing, the pellet was dissolved in 5 mL of boiling chloroform and allowed to evaporate.

PHA Assay

After evaporation, PHB extracted was subjected to spectrophotometric analysis by Dekwer *et al*., [41] method. Sample containing PHB 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.

Sugar concentration was assayed using the phenol sulphuric acid methods as described by Dubois, *et al*., [42].

Fermentation kinetics

Production kinetics of PHA and CDW were studied by calculating the product yield of PHA with respect to sugar consumption $Y_{P/S}$ (g/g), the product yield of PHA with respect to biomass $Y_{P/X}$ (g/g), biomass yield related to sugar consumption $Y_{X/S}$ (g/g) and productivity of PHA per h (g/L/h) of the culture media.

Characterization of PHA

Transform-Infrared Spectroscopy (FTIR):

The chemical structure of the extracted PHA was analyzed by Fourier transform infrared spectroscopy at Faculty of pharmacy, Al-Azhar University, Cairo, Egypt. The biopolymer was dissolved in chloroform and added to 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 Fourier transform infrared (FTIR) spectrophotometer (NICOLET-IR 200) using KBr disc [15].

NMR spectroscopy for PHA analysis

Mercury 400 NMR spectrometer was used for 400-MHz ^1H NMR, and ^{13}C NMR. NMR spectra were recorded from a $CDCl_3$ solution of the PHA (30 mg/ml) at 20°C, 1.30809856s acquisition times and 12.5250501 kHz spectral width.^{13}C-NMR spectra were recorded from a $CDCl_3$ solution of the samples, using ^1H decoupling, 0.83361792s acquisition time and 39.3081761 KHz spectral width. ^1H-NMR and ^{13}C-NMR chemical shifts were referred to $CHCl_3$ and $CDCl_3$ with $c = 5.0$ ppm and $c = 100.0$ ppm, respectively.

Results and Discussion

Results

Isolation and screening of PHA-producers from different sources

Several solid and liquid samples collected from different localities in Egypt were used for isolation of PHA-producing

isolates using enrichment media containing glucose as described in materials and methods. Ninety-six bacterial isolates were obtained and purified. These isolates were preliminary screened for evaluation PHA production through fluorescence using Nile red staining assay technique. Out of these isolates, 14 isolate showed bright fluorescence on agar plates upon exposing to UV light at wavelength at 312 nm. This indicate that these isolates are primarily able to produce PHA from glucose, therefore, selected for further quantification screening.

The selected isolates (14 isolates) were cultivated in mineral salt media supplemented with glucose and incubated for 72 h at 37°C. Cell growth was examined. Then the cells were collected for PHA extraction. PHA content was estimated and compared. Eight isolates showed PHA production ranged 0.2-0.48 g/L, 5 isolates produced PHA at range 0.570-0.930 g/L, whereas only one isolate produced 1.18 g/L of PHA as shown in Table (1).

Those isolates were also investigated for PHA production from molasses. As shown in Table (1), six isolates couldn't utilize molasses, whereas 3 isolates produced less PHA at 0.220-0.460 g/L. On the other hand, 5 strains showed increased production with a maximum PHA concentration of 0.790 g/L by isolate AZU-A2. This isolate also produced high PHA concentration from glucose at 0.880 g/L. Therefore, this strain was considered as the most potent isolate and used in further studies for identification and enhancement of PHA production.

Table 1. Quantitative screening test by 14 bacterial isolates for PHA production using 20 g/L of glucose and sugarcane molasses

No.	Isolates code	PHA(g/L) ±SD from:	
		Glucose	Molasse
1	WV1	0.430±0.007	0.0
2	BS 2	1.18±0.016	0.220±0.011
3	A1	0.400±0.014	0.660±0.012
4	AZU-A2	0.880±0.005	0.790±0.015
5	AKS 1	0.900±0.004	0.0
6	AKS 5	0.570±0.004	0.460±0.009
7	SH5	0.930±0.011	0.0
8	SH7	0.770±0.004	0.0
9	AD 3	0.380±0.003	0.400±0.007
10	FS1	0.400±0.007	0.0
11	FS5	0.420±0.005	0.0
12	Azu-IN1	0.280±0.009	0.510±0.004
13	InN3	0.200±0.003	0.500±0.005
14	AZS3	0.220±0.004	0.520±0.009

Identification of isolate AZU-A2

Isolate AZU-A2 was isolated from soil of leather production factory. Identification of isolate AZU-A2 was performed by studying its morphological (Gram's reaction, shape), biochemical and genetic characteristics (16S rRNA gene sequence) as shown in Table (2). The strain AZU-A2 is Gram-positive with rod shape, and was catalase-positive, which indicated the classification in the family Bacillaceae. It can grow in media with high salt concentration [up to 10 % sodium chloride]. The isolate showed capability of cellulose, gelatin and starch hydrolysis. However, it could not utilize citrate, pectin, or urea. This isolate could ferment different sugars including glucose, xylose, galactose, maltose, fructose, mannitol, sucrose, and lactose but not able to ferment inositol, myo-inositol, cellobiose, or inulin.

The16S rRNA gene sequence of the AZU-A2 isolate showed the highest similarity at 99. %with *Bacillus flexus* strain AUCAB18 [GenBank accession NO.: GU297608.1], and *Bacillus flexus* strain S2 [GenBank accession NO. KX242400.1] The phylogenetic tree constructed using strain AZU-A2 and other closely related type strains is depicted in Figure (1). The phylogenetic tree showed that strain AZU-A2 clustered with *Bacillus flexus* with 100 % bootstrap support. Therefore, we described the newly isolated strain as *Bacillus flexus* strain AZU-A2.

Table 2. A summary of the morphological, physiological and biochemical characteristics of the most potent bacterial isolate AZU-A2

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%	±		

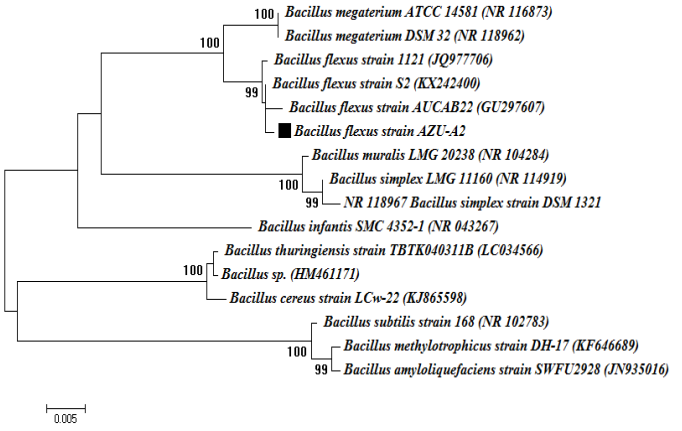


Figure 1. Phylogenetic analysis of 16S rRNA sequences of the bacterial isolates (AZU-A2) with the sequences from NCBI. Symbol ■ refers to 16S rRNA gene fragments retrieved from this study. The analysis was conducted with MEGA 6 using neighbor joining method.

Factors affecting PHA production

Effect of molasses concentrations

In order to investigate the influence of molasses concentrations on DCW and PHA production, strain AZU-A2 was cultivated in MSM medium at 37°C and pH7 with initial concentration of molasses range from (0.5-10%).As shown in Figure (2A), DCW was increased with increasing molasses concentration from 0.5 % up to 4% at 1.67 g/L and 3.87 g/L, respectively. Same DCW of 3.87 g/L was obtained at molasses concentration of 4.0-10.0 %. The highest PHA recovery yield of 36.1 (*w/w*) % was obtained with 3% molasses achieving the highest PHA production of 1.38 g/L with yield of 0.126 g/g-consumed sugar and low PHA productivity of 0.038 g/L/h (Figure 2B). From the above results, concentration 3% of molasses were considered as the best concentration for further studies.

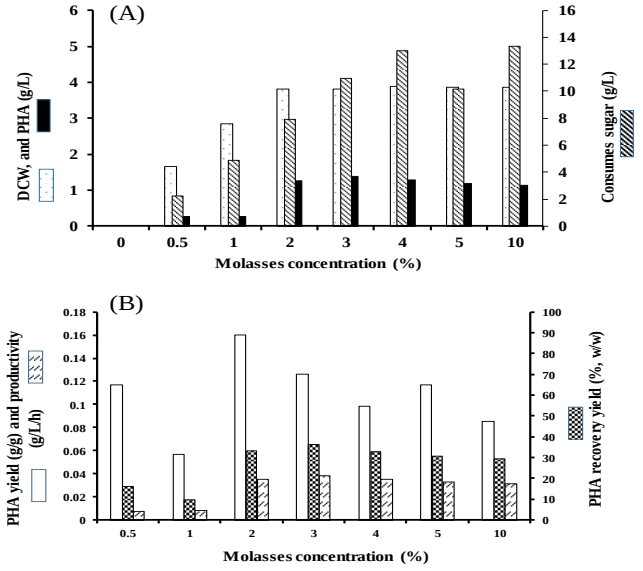


Figure 2. Effect of different concentrations of molasses on (A) cell growth (Dry cell weight), sugar consumption, PHA production; (B) PHA yield, productivity and recovery by strains AZU-A2 in batch fermentations.

Optimization of nitrogen source

Limited nitrogen condition is one of the critical factors affecting cell growth and PHA synthesis. The effect of some organic and inorganic nitrogen sources were investigated in PHA fermentation by strain AZU-A2 in MSM medium with 3 % molasses at 37°C as shown in Table (3). Cell growth was increased with fermentation time achieving maximum value of 3.75 g/L at 36 h using ammonium chloride. Although comparable DCW (ranged 3.02-4.64 g/L) was obtained from other nitrogen sources, the highest recovery yield at 42.40 (%, *w/w*) was obtained using ammonium chloride. The highest biomass yield based on substrate ($Y_{x/s}$) of 0.824 g/g was obtained after 12 h in a medium supplemented with sodium nitrate but a very low recovery yield and PHA concentration were obtained at 11.49 % and 0.35 g/L, respectively. Peptone, yeast extract, and ammonium dihydrogen orthophosphate achieved lower PHA recovery, titer and productivity than obtained using ammonium chloride (Table 3). A maximum PHA production of 1.59 g/L at yield of 0.144 g/g-consumed sugar was obtained using ammonium chloride as nitrogen source.

To determine the optimal concentration of ammonium chloride, batch fermentations were conducted using various concentrations (0.5-4 g/L) as recorded in Table (4). Maximum DCW of 4.35g/L was obtained when ammonium chloride raised up to 4 g/L at 36h. On the other hand, the maximum PHA production 1.84 g/L, recovery yield of 44.86 g/L, and productivity of 0.069 g/L/h were obtained with 1.5 g/L of ammonium chloride after 36h. At this concentration, the PHA yield based on substrate consumed was 0.137 g/g-consumed sugar that is little lower than those obtained with higher ammonium chloride > 1.5 g/L. As a result, 1.5 g/L was selected as the optimal concentration for achieving high efficiency PHA production.

Table 3. Effect of different nitrogen sources on PHA production from sugar-cane molasses by *Bacillus felxus* AZU-A2

Nitrogen source	Max. DCW (g/L) ± SD at indicated time	Residual sugars (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]
Yeast extract	3.62 ± 0.013(36 h)	8.93±0.131	0.509	0.89±0.007	24.56	0.12 5	0.025
Peptone	3.21 ± 0.023 (36 h)	9.95±0.096	0.526	0.90±0.014	27.96	0.147	0.025
Ammonium sulfate	3.64 ± 0.041(36 h)	5.45±0.24	0.343	1.14±0.010	31.24	0.107	0.032
Ammonium chloride	3.75 ± 0.028 (36 h)	5.03±0.078	0.341	1.59±0.002	42.40	0.144	0.044
Diammonium hydrogen orthophosphate	3.55 ± 0.013 (36 h)	5.16±0.254	0.326	0.84±0.001	23.76	0.077	0.023
$NaNO_3$	3.02 ± 0.011 (12 h)	12.38±0.131	0.824	0.35±0.011	11.49	0.095	0.029

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

Table 4. Effect of ammonium chloride concentration on PHA production from sugar-cane molasses by *Bacillus felxus* AZU-A2

Ammonium chloride conc. (g/L)	Max. DCW (g/L) ± SD	Residual sugars (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	3.93±0.077	8.70±0.072	0.535	1.33±0.022	33.71	0.180	0.037
1.0	4.04±0.008	4.58±0.060	0.352	1.49±0.004	36.92	0.130	0.041
1.5	4.11±0.022	2.66±0.132	0.307	1.84±0.015	44.68	0.137	0.051
2.0	4.20±0.064	7.45±0.163	0.489	1.67±0.019	39.74	0.194	0.046
2.5	4.06±0.010	5.76±0.144	0.394	1.77±0.017	43.75	0.172	0.049
3.0	4.29±0.033	5.12±0.136	0.393	1.78±0.031	41.40	0.163	0.049
4.0	4.35±0.234	3.96±0.064	0.360	1.77±0.012	40.74	0.146	0.049

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

Effects of pH values

The influences of pH on growth and PHA accumulation by strains AZU-A2 was investigated by adjusting initial pH at different values (ranged pH 5.0-9.0) using the previously optimized conditions during fermentation.

As shown in Figure 3a, the DCW is almost comparable at a wide range of pH values (6.0-9.0) that ranged 4.02-4.49 g/L after 36 h with almost similar values obtained after 24 h (ranged 4.01-4.38 g/L). A drastic decrease in DCW was obtained at an initial pH 5 at 0.66 g/L. On the other hand, an obvious increase in the PHA production, recovery yield and productivity with an increase in initial pH value achieving their maximum values at pH 7.5 with 2.04 g/L and 46.4 (%, *w/w*), and 0.057 g/L/h, respectively (Figure 3 B and C). At this value, the PHA based on consumed substrate was 0.184 g/g (Figure 3C). pH controlled fermentations were also conducted, however, lower PHA production (Max. 1.71 g/L) was obtained compared to fermentations with uncontrolled pH (2.04 g/L)[Data not shown]. As result, initial pH 7.5 was considered as the best pH for achieving high efficiency fermentation of PHA by strain AZU-A2.

Effect of phosphorus sources

The influence of different concentrations of phosphorus sources, Na_2HPO_4 $12H_2O$ (range; 1.0-12.0 g/L): KH_2PO_4 (range; 0.16-1.0 g/L) at ration of 6:1, were investigated on the growth and PHA production by strain AZU-A2. An increased cell growth was obtained with high phosphorus content (total ≥ 3.5 g/L) that ranged 4.24-4.38 g/L compared to 3.81 g/L at phosphorus concentration of 1.16. PHA fermentation efficiency was improved with an increase of phosphorus concentration up to 7.0 g/L [Na_2HPO_4 $12H_2O$ (6.0 g/L):KH_2PO_4 (1.0 g/L)] (Table 5). At this concentration, a maximum biomass yield $Y_{x/s}$(0.377 g/g), PHA recovery yield (47.19 %, w/w), PHA production (2.02 g/L), PHA yield (0.178 g/g), and productivity (0.056 g/L/h) were obtained. Therefore, total phosphorus concentration of 7 g/L was considered as the best conditions for achieving high PHA fermentation efficiency within 36 h.

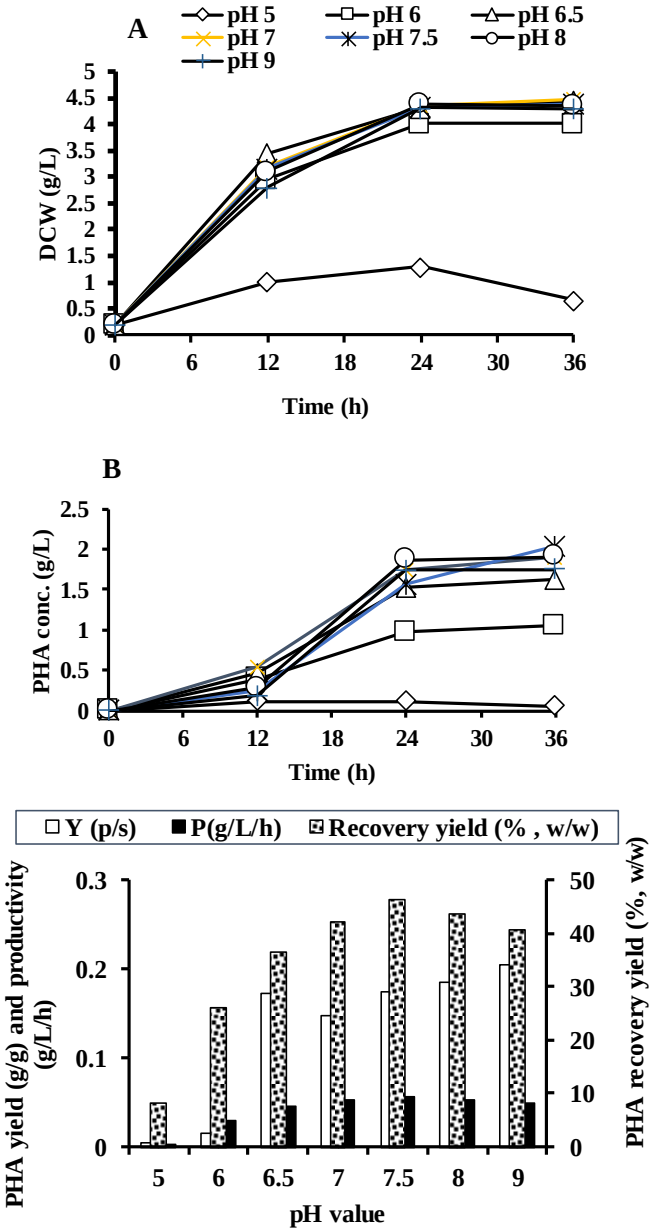


Figure 3. Effect of initial pH value on PHA fermentation from sugar-cane molasses by *Bacillus felxus* AZU-A2. (A) dry cell weight, (B) PHA production, g/L, and (C) PHA yield, productivity and recovery.

Table 5. Effect of different concentrations of phosphorus sources on PHA production from sugar-cane molasses by *Bacillus felxus* AZU-A2.

$Na_2HPO_412H_2O$ / KH_2PO_4 Concentration (g/L)	Total phosphorus (g/L)	Max. DCW (g/L) ± SD	Residual sugars (g/L) ± SD	$Y_{(x/s)}$[a](g/g)	PHA (g/L) ± SD	Recovery yield % (*w/w*)	$Y_{(p/s)}$[b] (g/g)	$P._{PHA}$ (*g/L/h*)[c]
1.0/0.16	1.16	3.81±0.083	4.75±0.320	0.337	0.86±0.003	22.65	0.076	0.024
3.0/0.5	3.5	4.33±0.002	3.26±0.240	0.338	1.80±0.004	41.51	0.140	0.050
6.0/1.0	7.0	4.29±0.015	4.68±0.052	0.377	2.02±0.020	47.19	0.178	0.056
9.0/1.5	10.5	4.38±0.007	3.67±0.064	0.353	2.01±0.014	45.91	0.162	0.056
12.0/2.0	14.0	4.24±0.000	3.03±0.055	0.326	1.63±0.019	38.30	0.125	0.045

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

Effect of agitation rate

Agitation is important for mixing, mass transfer and heat transfer. The influence of varies agitation rates (0.0 [static], 50,150, 200 and 250 *rpm*) on the growth and PHA production by strain AZU-A2 at the previously optimized conditions were investigated as shown in Figure (4).

It was found that, there is a positive correlation between agitation rate and efficiency of PHA fermentation by strain AZU-A2. Static condition achieved the lowest cell biomass (1.41 g/L) and fermentation kinetic parameters (PHA conc., 0.2 g/L; recovery yield, 14.1 % (*w/w*); $Y_{p/s}$, 0.013 g/g; and productivity, 0.003 g/L/h). However, cell biomass, sugar consumption, and PHA production were increased with an increase of agitation speed up to 200 rpm. The highest DCW of 4.39 g/L with highest PHA concentration of 3.0 g/L were obtained after 24 h at an agitation rate of 200 rpm. Also, the highest recovery of 68.4 % (*w/w*), $Y_{p/s}$ of 0.226 (g/g), and productivity of 0.125 g/L/h were achieved. Higher agitation speed (250 rpm) achieved the highest growth, but less PHA was produced (Fig 4). As result, an agitation rate of 200 rpm was considered as the optimal speed for achieving highly efficient PHA fermentation that resulted in an increased PHA production fold by 50 % compared to control experiment.

Effect of auxiliary carbon sources (co-substrates)

To improve PHA production by AZU-A2, different additional carbon sources substrates were supplemented separately at concentration of 0.5% to MSM media containing molasses (3%). PHA production was investigated at different time intervals at 37°C and agitation rate of 200 rpm as shown in Table (6).

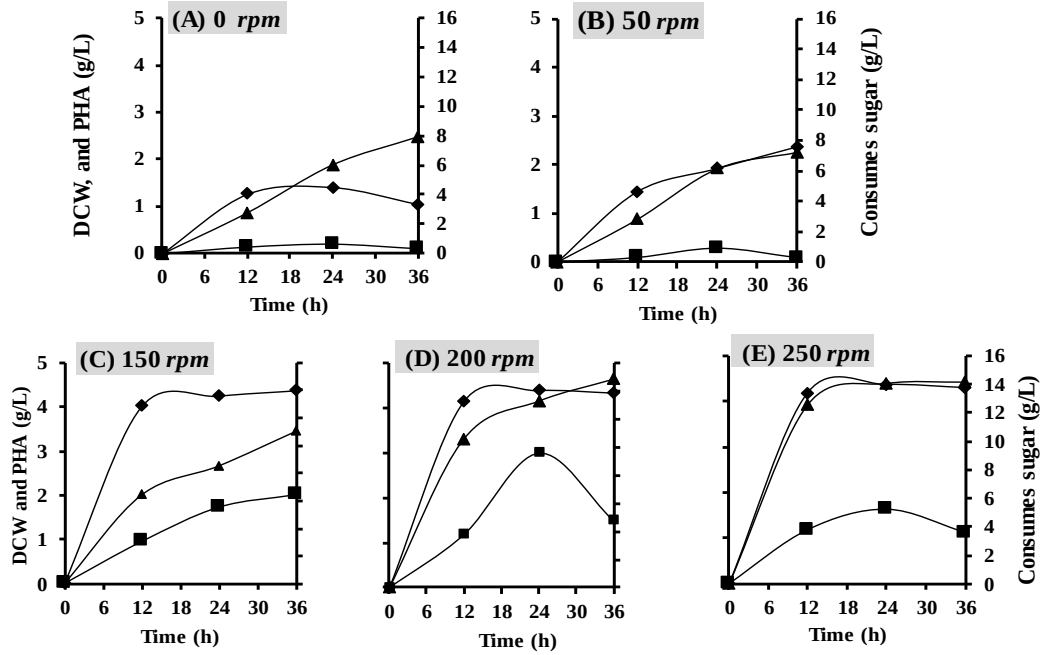


Figure 4. Effect of different agitation rates on cell growth (dry cell weight), sugar consumption and PHA production from sugar-cane molasses by *Bacillus felxus* AZU-A2. (a) fermentation at static condition, 0 rpm; (b) fermentation at 50 *rpm*; (c) fermentation at 150 *rpm* ; (d) fermentation at 200 *rpm* ; (e) fermentation at 250 *rpm. Symbols;*▲, Consumed sugar (g/L); ■, PHA concentration (g/L); ♦, DCW (g/L).

Table 6. Effect of different auxiliary carbon sources on PHA production from sugar-cane molasses by *Bacillus felxus* AZU-A2

Different carbon sources	Fermentation Time (h)	Max. DCW (g/L) ± SD	Residual sugars (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	12	3.86±0.009	6.32±0.098	0.397	1.11±0.010	28.78	0.114	0.093
	24	3.95±0.022	2.03±0.017	0.282	1.89±0.010	47.75	0.135	0.079
Ethanol	12	3.85±0.006	4.34±0.069	0.329	1.17±0.011	30.42	0.100	0.098
	24	3.97±0.005	2.23±0.069	0.287	1.69±0.026	42.62	0.123	0.071
Sodium citrate	12	4.10±0.024	5.29±0.017	0.381	1.05±0.008	25.51	0.097	0.087
	24	4.10±0.013	3.12±0.121	0.317	1.52±0.003	37.16	0.118	0.063
Sodium acetate	12	4.30±0.012	2.95±0.006	0.328	2.20±0.007	51.18	0.168	0.168
	24	4.42±0.033	1.51±0.003	0.304	3.56±0.065	80.51	0.245	0.148
Acetic acid	12	4.17±0.041	6.87±0.100	0.455	2.43±0.070	58.16	0.265	0.202
	24	4.23±0.039	3.38±0.095	0.334	3.60±0.067	85.13	0.284	0.150
Caster seed oil	12	3.98±0.022	4.69±0.017	0.351	1.49±0.016	37.42	0.131	0.124
	24	4.01±0.031	3.73±0.046	0.325	2.09±0.041	52.28	0.170	0.087
Corn oil	12	4.01±0.012	9.37±0.012	0.600	1.51±0.051	23.61	0.227	0.126
	24	4.05±0.007	3.18±0.023	0.314	2.03±0.002	27.89	0.158	0.085
Olive oil	12	4.02±0.023	6.03±0.058	0.397	1.31±0.051	32.46	0.129	0.109
	24	4.05±0.007	6.02±0.081	0.404	1.79±0.002	44.07	0.178	0.074
Soy bean oil	12	4.00±0.002	7.43±0.035	0.464	1.55±0.005	38.79	0.180	0.129
	24	4.04±0.024	1.79±0.000	0.283	1.99±0.019	49.26	0.139	0.083
Sesame oil	12	3.94±0.012	7.08±0.000	0.439	1.34±0.008	33.98	0.149	0.112
	24	4.02±0.013	2.88±0.023	0.305	1.96±0.017	48.73	0.149	0.082
Paraffin oil	12	4.03±0.056	5.75±0.058	0.391	1.42±0.002	35.27	0.138	0.118
	24	4.27±0.011	2.80±0.017	0.322	1.49±0.003	34.94	0.113	0.062

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

None of these co-substrates inhibited cell growth that was ranged 3.85-4.23 g/L, however, lower PHA production (ranged 1.49-2.25 g/l) with lower recovery yield (ranged 27.9-53.17 %, w/w) and productivity (ranged 0.062-0.094 g/L/h) were obtained after 24 h using methanol, ethanol, sodium citrate, caster seed oil, corn oil, olive oil, soybean oil, sesame oil, or paraffin oil, compared to parameters that obtained without any supplementation (Table 6). On the other hand, there is a significant increase in PHA production and recovery yield by supplementations of acetate containing substrates (sodium acetate or acetic acid). Supplementation of sodium acetate or acetic acid have enhanced recovery yield and PHA production at (80.51%, *w/w* and 3.56 g/L) and (85.13%, *w/w*, 3.6 g/L), respectively that is almost 18 % increase compared to that produced without any supplementation (68.4%, and 3.0 g/L). Using these co-substrates, an increase in PHA yield ($Y_{p/s}$) ranging 0.245-284 g/g-consumed sugars and productivity (P_{PHA}) ranging 0.148-0.150 g/L/h were also obtained as compared to control experiment of 0.226 (g/g) and 0.125 g/L/h, respectively. Supplementation of sodium acetate has resulted in almost complete consumption of all sugars (14.54 g/L) contained in molasses (30 g/L) with residual sugar of 1.51 g/L compared to residual sugar of 3.38 g/L using acetic acid as an auxiliary carbon source. As result, acetic acid and sodium acetate considered as the best auxiliary carbon sources for achieving high efficiency fermentation of PHA. However, it is of great interest to optimize the acetate concentration for PHA production by strain AZU-A2.

Optimization of acetic acid concentration

In order to determine the optimal acetic acid concentration for enhanced PHA production by strain AZU-A2, different concentrations of acetic acid (0.25-5.0%, *v/v*) were supplemented to optimized MSM fermentation media containing molasses (3%). As shown in Figure (5), cell biomass was ranged 4.12-4.51 g/L at acetic acid concentration 0.25-3%, while there is a drastic decrease in biomass at 0.63 g/L when acetic acid supplemented at 5% (*w/v*). Low concentration of acetic acid (0.25 %) resulted in little decrease of PHA production as compared to control, however high acetic acid supplementation (3-5%) led to a drastic decrease in PHA production achieving 0.4-0.7 g/L. On the other hand, the PHA recovery was greatly improved that ranged 83.02-88.01 % (*w/w*) when acetic acid was supplemented to fermentation medium at 0.5-2% (*w/v*). The highest PHA of 3.97 g/L with recovery yield of 88.01 % (*w/w*), $Y_{p/s}$ of 0.318 and productivity of 0.166 g/L/h was obtained at 1% acetic acid supplementation in batch fermentation mode.

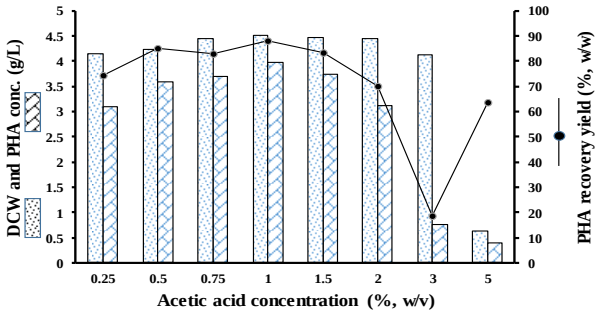


Figure 5. Effect of different acetic acid concentrations on cell growth (dry cell weight), PHA recovery yield, and PHA production from sugar-cane molasses by *Bacillus felxus* AZU-A2.

Fed-batch fermentation

In order to further enhance cell biomass and PHA production by strain AZU-A2, fed batch fermentation using different feedings were performed. The fermentations were initially started with components of the optimized medium. Different feeding solutions were applied at different time intervals based on its content [either molasses only or molasses mixed with nitrogen and/or phosphorus source; Fed-batch A, b, C, or D as indicated in Material and Methods].

As shown in Figure (6), in fed batch A, where the feeding with molasses only (3%), cell biomass reached 5.50 g/L after 24 with an increase after than achieving its maximal value of 7.07 g/L after 96 h. On the other hand, the accumulation of PHA was increased achieving its maximum value of 5.96 g/L with recovery yield of 84.3 % (*w/w*) at 96 h. Conducting similar fermentation with feeding solution of molasses and ammonium chloride (Fed-batch B) have resulted in almost similar DCW of 7.17 g/L after 96 h, however, higher accumulation of PHA was obtained achieving its maximal value of 6.13 g/L with recovery yield of 92.12 % (*w/w*) after 72 h. In another strategy using lower sugar concentration feeding, fed-bach C and D were conducted. In those fermentations, feeding was intermitted supplied to maintain sugar concentration at same level. However, none of those fermentations supported high PHA production than previous even with supplementations with extra nitrogen or phosphorus sources. The maximal PHA produced from fed-batch C and D were 5.05 g/L and 4.31 g/L after 36 h and 48 h, respectively that is lower than obtained in fed-batch A and B. Furthermore, although fed batch A and B achieved higher PHA production than obtained in batch fermentation, the high amount of molasses amount required and fermentation time retard this process.

Characterization of polyhydroxyalkanoates

Fourier Transform-Infrared Spectroscopy (FTIR)

The IR spectrum of PHA compound (KBr, υ_{max} cm^{-1}), Figure 7, showed absorption bands at 3453 cm^{-1} (OH stretching), 2980 cm^{-1} (C-H stretching), 1736 cm^{-1} (C=O stretching), 1448 and 1285 cm^{-1} (methylene bending), [16].

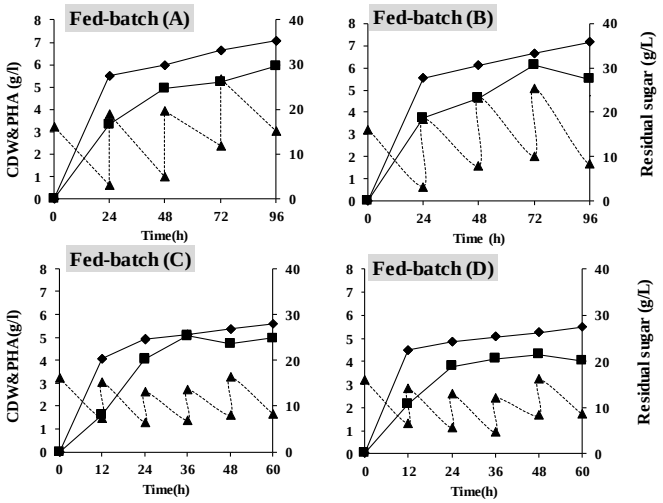


Figure 6. Fed batches fermentation with different feeding for PHA production from sugar-cane molasses by *Bacillus felxus* AZU-A2 at 37 ^{0}C, initial pH 7.5 and agitation rate 200 rpm. *Symbols;* ▲, Residual sugar (g/L); ■, PHA concentration (g/L); ♦, DCW (g/L).

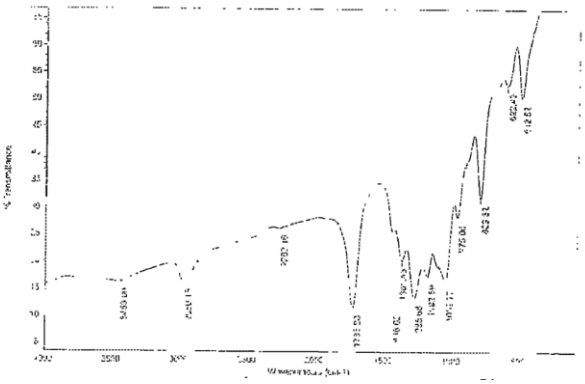


Figure 7. Fourier transform infrared spectra analysis of PHA produced by strain AZU-A2

Nuclear Magnetic Resonance (NMR)

^{1}H-NMR spectrum of our compound (Figure 8A) indicated characteristic signals of PHA, namely a doublet at 1.31 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. This data was matched with the published data for same compound [17]. The values of the chemical shifts as well as the assignments of the ^{1}H NMR signals, which appeared in the spectra are in agreement 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 (Figure 8 B).

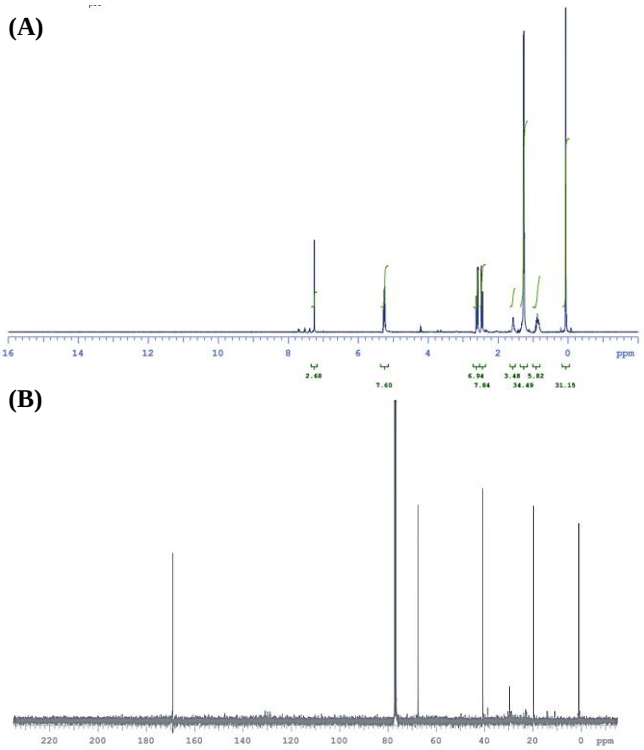


Figure 8. (A) ^{1}H chemical shifts, (B) ^{13}C chemical shifts of constituent monomers of purified PHA produced by strain AZU-A2.

Discussion

Production of biodegradable and biocompatible green thermoplastics has currently received much attention as an alternative to nonrenewable petroleum derived plastics. PHAs are bio-polyesters materials synthesized by bacteria as intracellular storage material under limited nitrogen condition and excess carbon source. Taking in consideration these conditions, this study aimed to select suitable bacterial strain accumulating high PHA content from inexpensive carbon sources and determining the environmental and nutritional factors that lead to the maximization of PHA production

To achieve this aim, 96 bacterial isolates were isolated from different samples collected from Egypt. Bacterial cultures grown on Nile red-containing medium were analyzed under UV to detect hydrophobic compounds inside the cells as previously reported [13, 18]. 14 isolates showed bright to red fluorescence color and used for quantitate analysis of PHA produced from glucose or sugarcane molasses as an inexpensive carbon source at 37°C for 72 h. Isolate AZU-A2 could accumulate the highest amount of PHA (0.79 g/l) using molasses as substrate and therefore was characterized using biochemical characteristics and genetic 16S rRNA analysis.

In the phylogenetic tree based on the neighbor-joining algorithm (Figure 1), strain AZU-A2 clustered with the members of the genus *Bacillus*, showing the highest 16S rDNA gene sequence similarity of 99.0% with *Bacillus flexus strain* AUCAB18, and strain S2, and was identified as *Bacillus flexus* strain AZU-A2. *Bacillus* spp. is a black horse in industry due to their advantages of low nutritional requirements, rapid growth, having machinery enzymes for utilization of several sugars and lacking LPS in Gram positive [19]. Interestingly our isolated strain AZU-A2 could accumulate the highest amount of PHA within 36 h that is very short time compared to previously reported *Bacillus* strains. Maximum PHB production from molasses was obtained after 72 h by *Bacillus flexus* ME-77 [19] and *Bacillus thuringiensis* [20]. Kalaivani and Sukumaran, [21] reported maximum production of PHA from molasses observed after 76 h by *Bacillus* sp. KSN5.

PHA produced in this study was characterized as polyester by Fourier transform infrared (FTIR) spectroscopy to study the molecular structure [22], and NMR spectroscopy. Due to the Low PHA produced in MSM with molasses at 0.79 g/L, physical and nutritional factors affecting PHA production were optimized in batch fermentations. Sugarcane molasses concentration at 3%; ammonium chloride, 1.5 g/L; phosphorus concentration, ($Na_2HPO_4.12H_2O$, 6 g/L; KH_2PO_4, 1 g/L); acetic acid conc. 1 %; initial pH, 7.5; agitation speed, 200 rpm; and 37°C were the optimal fermentation conditions for PHA production by AZU-A2. Under these conditions, maximum PHA production of 3.97 g/L at recovery yield of 88.0 (%, *w/w)* were achieved after 24 h in batch fermentation.

The optimal molasses concentration of 3% (*w/v*) achieved better growth with high recovery yield. The decreased cell growth at higher molasses concentration (> 3%) might due to osmotic stress. Several authors also reported that 3% molasses concentration is an optimal for achieving high PHA production from *Bacillus* spp. [19–20, 23–24]. Sharma and Bajaj, [25] reported that maximum PHB yield was obtained at 2% of molasses by *Enterobacter* sp. SEL2.

Supplementation of 1.5 g/L ammonium chloride as nitrogen source was positively enhanced the accumulation of PHA by strain AZU-A2 amongst other tested sources. Higher accumulation of PHA inside that cells was obtained at 42 % recovery yield while other tested sources attained only 11–30 % PHA recovery. It is observed that supplementation of organic nitrogen sources (peptone or yeast extract) and sodium nitrate supported high growth (ranged 3.02-3.62 g/L) but not PHA accumulation that ranged 0.35–0.90 g/L. Ammonium chloride was also reported as the best nitrogen source for PHAs from molasses by *Bacillus megaterium* [23, 26–27]. Ammonium sulphate supported high growth and PHA production by *Rhizobium elnti* and *Pseudomonas stutzeri* [28].

pH is one of the critical factors that affects the cell growth and product formation. Initial pH 7.5 (uncontrolled) was found to be optimum for PHA production by AZU-A2. Most studies reported neutral to slightly acidic or slightly alkaline pH (pH 6.5–8.0) is the optimal for PHAs accumulation by several bacterial [19, 25, 29–30].

Phosphate limited condition is another important factor for induced PHA accumulation [31]. In our study, a maximum

production of PHA and maximum recovery yield of PHA was observed at phosphorus concentration (Na_2HPO_4 $12H_2O$ 6 g/L - KH_2PO_4 1 g/L) after 36 h that is higher requirement than reported by other reported strains [32-33]. Qiu *et al*., [33] achieved maximum production of PHA using Na_2HPO_4 $12H_2O$ at 3 g/L and KH_2PO_4 of 0.5 g/L by *A. hydrophila* 4AK4. Goma, [32] stated that, the maximum production of PHA by *B. subtilis* and *E. coli* was recorded in the presence of 2 g/L of KH_2PO_4 and $Na_2HPO_4.2H_2O$. Sangkharak and Prasertsan [34] reported a maximum PHB production at phosphorus concentration 0.1 g/L K_2HPO_4, 0.1 g/L KH_2PO_4 by *R. sphaeroides*.

Agitation rate is reported to be a critical factor for PHA accumulation by our strain, AZU-A2. 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 which might attributed to the increase in oxygen transfer and mass transfer rates and requirement of aerobic condition for *Bacillus* growth [35]. Beyond this level, higher growth was achieved but accumulation of PHA was significantly decreased that attributed to limitations of stress conditions required for PHA granules storage [36]. Tripathi *et al.,* [29] stated that maximum cell mass and PHB production occurred at an agitation speed of 175 rpm by *Pseudomonases aregunoisa* NCIM No. 2948.

Supplementation of 1 % acetic acid as an auxiliary carbon source maximized PHA recovery yield of PHA to 88.01% (*w/w*) by strain AZU-A2. This recovery yield is superior to that obtained by Venkates war *et al*., [37] who achieved maximum recovery at 63.0 5% using 20 g/L VFA (acetate 19 g/L - propionate 1g/L) by *Hydrogenopha gapalleronii***.** Combinations of acetate with valerate and acetate with propionate were reported to induce the accumulation of poly- β -hydroxybutyrate-co- β -hydroxyvalerate within the cell [38].

In order to avoid or reduce substrate associated osmotic stress and achieve high product concentration, fed-batch fermentation has been conducted. However, studies of fed-batch fermentation on molasses are limited, mainly because of difficulties to determine adequate feeding strategies for control of critical parameters. Therefore, different feeding strategies were applied as shown in result section. Feeding with 3% molasses plus ammonium chloride supported higher cell growth and PHA accumulation as compared to feeding of molasses only achieving maximum recovery of 92.12 % and 84.28%, respectively. While feeding with lower molasses concentrations of 1.5% with nitrogen and/or phosphorus attained lower fermentation efficiency. The maximum PHA production of 6.13 g/L was achieved by feeding 3% molasses plus ammonium chloride that is 54.4 % increase as compared to data obtained in batch fermentation. The results obtained in this study is superior to that obtained by Hamieh *et al.,* [39] who reported an increase in PHB to 27.5% in fed batch fermentation as compared with batch process. Van-Thuocet *et al.,* [40] reported that P(3HB-co-3HV) content reached maximum value of 39.8 wt, % after 54 h in fed-batch fermentation by *Yangia* sp. Nd199 using molasses.

Conclusion

In the present report, polyhydroxyalkanoates production and optimization using sugarcane molasses as cheap substrate was achieved by a newly isolated *Bacillus flexus* strain AZU-A2. Sugarcane molasses concentration at 3%; ammonium chloride, 1.5 g/L; phosphorus concentration, ($Na_2HPO_4.12H_2O$, 6 g/L; KH_2PO_4, 1 g/L); acetic acid conc. 1 %; initial pH, 7.5; agitation rate, 200 rpm; and 37°C were the optimal fermentation conditions for PHA production. Under these conditions, maximum PHA production of 3.97 g/L at recovery yield of 88.0 (%, *w/w)* were achieved after 24 h in batch fermentation. Fed batch fermentation resulted in improved PHA production (6.13 g/L) and recovery yield (92.1 % of DCW, *w/w*). PHA was characterized as polyester by Fourier transform infrared (FTIR) and NMR spectroscopy.

Conflict of interest

All the authors declare that they have no conflicts of interest regarding this paper.

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