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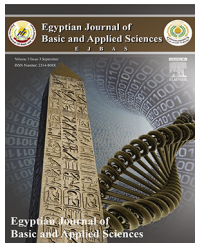

Full Length Article

Microbial biopolymer production by *Microbacterium* WA81 in batch fermentation

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ARTICLE INFO

Article history:
Received 21 January 2016
Received in revised form 26 April 2016
Accepted 27 May 2016
Available online 4 June 2016

Keywords:
Microbacterium sp
Design of experiment
Medium optimization
Bioreactor
Microbial biopolymer

ABSTRACT

Microbial Poly-β-hydroxybutyrate (PHB) was the first and the most widespread polymer to be used in the biodegradable polymer industry. The optimization of the Egyptian local bacterial strain *Microbacterium* sp. WA81 productivity was performed using statistical design of experiment tools. A Plackett–Burman design was carried out to screen the effect of different carbon and different nitrogen sources, each in a separate experiment. Moreover, a subsequent Response Surface Methodology was applied to achieve the maximum PHB productivity at shake flask level. Upon scaling up to a bench top bioreactor run scale (5 L), using the generated medium, the amount of the polymer significantly increased (1.42 g/L), which represented a 78-fold increase of that prior optimization. During the bioreactor run, the preliminary effect of Dissolved Oxygen (DO) was explored, and the levels of carbon and nitrogen uptakes were monitored. In conclusion, the statistical design of experiment tools enabled us to optimize and engineer a growth medium and conditions to enhance PHB productivity by the Egyptian isolate *Microbacterium* sp. WA81.

1. Introduction

The higher demand of plastics for manufacturing of millions of applications such as packaging, building materials and commodities, as well as hygiene products can lead to waste disposal problems. These traditional petroleum-derived plastics are made from crude oil which is considered as a nonrenewable resource. Although the used plastic could be recycled, the amount of solid waste generated by plastic became a serious problem as its accumulation and degradation resistance lead to serious hazards on the environment. These considerations had a great effect on focusing on the industrial scale production of biodegradable polymers. The major difference between oil based polymers and biodegradable polymers is the ability of the latter to be biodegraded by soil microorganisms. Biopolymers contain carbon and nitrogen, which permit the microbial growth leading to a final conversion to carbon dioxide and water vapor via enzymatic action [1,2].

One of the most interested biopolymers is polyhydroxyalkanoates (PHAs), which could be synthesized by numerous microorganisms as an energy reserve material when an essential nutrient such as nitrogen or phosphorus is available only in limited concentrations in the presence of excess carbon

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http://dx.doi.org/10.1016/j.ejbas.2016.05.001

source. As they possess properties similar to various synthetic thermoplastics, PHAs could replace synthetic thermoplastics in the plastic industry [3]. They are also completely degraded in water and carbon dioxide under aerobic conditions and in methane under anaerobic conditions. PHAs are divided into two groups depending on the number of carbon atoms in the chain: 1) short-chain length (SCL), which consists of 3–5 carbon atoms; and 2) medium-chain length (MCL), which consists of 6–14 carbon atoms. This difference is mainly due to the substrate specificity of the PHA synthases that can accept 3-hydroxyalkanoic acid of a certain range of carbon length [4].

SCL-PHAs are thermoplastics with a high degree of crystallinity, while MCL-PHAs are elastic or tacky materials with a low degree of crystallinity and a low melting temperature [5]. One of the most attracting SCL-PHA is polyhydroxybutyrate (PHB), which is polyester with crystallinity above 50% and its melting temperature is 180 °C with glass transition temperature to be approximately 2 °C [6]. PHB synthesis proceeds in the facultative chemolithoautotrophic hydrogen-oxidizing bacterium *Ralstonia eutropha* in three steps starting with acetyl coenzyme A (acetyl-CoA) as a central intermediate of metabolism. Two molecules of acetyl-CoA are condensed to acetoacetyl-CoA, catalyzed by a *β*-ketothiolase (*PhbA*), which is subsequently reduced by a stereospecific acetoacetyl-CoA reductase (*PhbB*) to *R*-(–)-3-hydroxybutyryl-CoA. The final step is the polymerization of 3-hydroxybutyryl-CoA to PHB with concomitant release of CoA which is catalyzed by the PHB synthase (*PhbC*) [7].

According to the culture conditions required to synthesize PHB, bacteria could be classified into two main groups: the first bacterial group that needs essential nutrient limitation such as phosphorous, magnesium, nitrogen or sulfur for PHB synthesis in presence of excess carbon source including *Alcaligenes eutrophus*, *Protomonas extorquens*, and *Protomonas oleovorans*; and the second bacterial group that does not need a nutrient limitation environment to accumulate PHB polymer including a mutant strain of *Alcaligenes latus*, *Azotobacter vinelandii* and recombinant strain of *Escherichia coli*. These characteristics have to be taken into consideration while producing PHB [4].

The use of bio-plastics as an alternative solution to petroleum-based plastics is limited due to their high cost for large scale production [8]. To overcome such problem, a number of strategies could be applied including applying less expensive substrates, improving cultivation strategies and developing an easier downstream processing method [9]. The PHB production cost evaluation reported that the cost of carbon substrate (up to 50%) is the major contributor to the overall cost. Hence, it is important to exploit cheap carbon sources to increase the PHB content and productivity. The production cost of any biotechnological process could be considerably reduced by optimization of the process [10]. Bioprocess optimization is one of the major factors to reduce the production cost of all biotechnological commercial products. The classical optimization process that always utilizes the 'one variable at a time' approach produces non-reliable results, and interactive effects of different variables for the production also cannot be resolved by this approach [11].

One of the statistical designs for the screening of the independent variables is the Plackett–Burman design of experiment. This design offers the screening of a large number of independent factors (N) in a small number of experiments (N + 1) [12]. Then, a subsequent response surface methodology (RSM) usually has to be applied to detect the optimum concentration values of each studied factor [13]. RSM was found to be effective in processes optimization for a vast number of industrial products including paints and coatings, foods and beverages, and pharmaceuticals [14]. RSM is able to capture the main effect of each factor and the interactions between them.

In the present study, a local isolated bacterial strain previously identified and screened for PHB production was optimized to develop a suitable fermentation process for large scale production. Media engineering methodologies were carried out to develop a potential chemically defined production medium. The generated medium was used for a bioreactor run to test its feasibility for large scale production.

2. Materials and methods

2.1. *Strain and cultivation conditions*

The Egyptian local isolate *Microbacterium* sp. WA81, previously identified by the *16S rRNA* gene sequences and deposited in the GenBank database under accession number KM191355, was used in all the fermentation processes. The culture was maintained on nutrient agar slants at 4 °C and subcultured monthly. For PHB production, the isolate was inoculated to the mineral salt medium which consisted of 2.0 g/L $(NH_4)_2SO_4$, 2.0 g/L KH_2PO_4, 0.6 g/L Na_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$, 20 mg/L $CaCl_2$, 10 mL/L trace metal solution, and 0.1 g/L yeast extract [13]. The trace metal solution consists of 1.3 mg/L $ZnSO_4.7H_2O$, 0.2 mg/L $FeSO_4.7H_2O$, 0.6 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$ and 0.6 mg/L H_3BO_3. Fructose was used as a carbon source in a concentration of 40 g/L for PHB production media and 10 g/L for inoculum development. Fructose was sterilized separately via filtration and then aseptically reconstituted at room temperature prior to inoculation. The pH of the resulting broth was adjusted to 7.0 with 2N NaOH/2N HCl.

For inoculum development, mineral salt medium containing 10 g/L fructose was used. The organism was cultivated at an agitation speed of 150 rpm and 30 °C for 24 h in a 250 mL Erlenmeyer flask containing 100 mL of the medium described above. For production of PHB, 1 L of media (containing 40 g/L fructose) was inoculated with 100 mL of inoculum and kept under shaking conditions for 48 h at 150 rpm and 30 °C. Bacterial cells were harvested by centrifugation at 5000 rpm for 20 min at 10 °C and the pellets were washed twice with distilled water. The cells were then dried in the freeze dryer (Heto PowerDry LL3000) [15].

2.2. *Plackett–Burman initial screening for carbon and nitrogen sources*

Nine different carbon sources in addition to one dummy variable and ten different nitrogen sources in addition to the dummy variable were investigated in two separate designs. The dummy variable was used to evaluate the standard error of the experiments. Each variable was investigated at a high (+) level and a low (–) level by using presence/absence

Table 1 – The nine screened carbon sources and their levels to be screened via Plackett–Burman design for the isolate under investigation.

Variable	Unit	Minimum level (–)	Maximum level (+)
Sorbitol	g/L	0	10
Lactose	g/L	0	10
Maltose	g/L	0	10
Sucrose	g/L	0	10
Fructose	g/L	0	10
Arabinose	g/L	0	10
Dextrose	g/L	0	10
Glycerol	g/L	0	10
Sodium acetate	g/L	0	10
Dummy	–	Distilled water	Deionized water

principle (Tables 1 and 2), where the low level represented the absence of the investigated variable.

The maximum level was selected depending on a preliminary experiment (data not shown). The main effect of each factor was determined using Eq. (1).

$$E(X_i) = 2\left[\sum Y_i^+ - Y_i^-\right]/N \quad (1)$$

where $E(X_i)$ is the effect of the tested variable and Y_i^+ and Y_i^- are the calculated responses, while the significance level (p value) of each variable was determined using the Student's t-test (Eq. 2):

$$t(xi) = E(xi)/SE \quad (2)$$

where (SE), the standard error of variables, is calculated as the square root of the variance of an effect. Any variable with P-value <0.1 was considered to be significant at 90% level of confidence. The relationship between the response and the significant variables within 2-level factorial design is based on first order polynomial order (Eq. 3), as no interactions could be calculated by this screening design.

$$Y = \beta_0 + \sum \beta_i X_i \quad (i = 1, 2, \ldots\ldots, k) \quad (3)$$

Table 2 – The ten screened nitrogen sources and their levels to be screened via Plackett–Burman design for the isolate under investigation.

Variable	Unit	Minimum level (–)	Maximum level (+)
Urea	g/L	0	1
Ammonium acetate	g/L	0	1
Threonine	g/L	0	1
Ammonium sulfate	g/L	0	1
Glycine	g/L	0	1
Ammonium chloride	g/L	0	1
Ammonium nitrate	g/L	0	1
Proline	g/L	0	1
Arginine	g/L	0	1
Cysteine	g/L	0	1
Dummy		Distilled water	Deionized water

Table 3 – Variables and their levels used for the CCD experiment.

Variable	Levels				
	–2	–1	0	+1	+2
Maltose (g/L)	0	10	20	30	40
Ammonium sulfate (g/L)	0	1	2	3	4
Fructose (g/L)	0	10	20	30	40
Ammonium chloride (g/L)	0	1	2	3	4

where Y is the calculated response, β_0 is model intercept, β_i is the regression coefficient for each corresponding variable, X_i is the corresponding variable and k is the number of variables [16].

Two separate designs each with 12 trials were formulated for nine different carbon sources screening and ten different nitrogen sources screening using Minitab 16 software. The experiments were done in Erlenmeyer flasks containing 100 mL of basal media at 150 rpm for 72 h. Responses were measured in terms of OD_{600} and PHB production (mg/L).

2.3. *Central Composite Design experiment to optimize the most potential carbon and nitrogen sources*

After identifying the most significant variables via the previously performed Plackett–Burman experiment in two designs, a subsequent Central Composite Design (CCD) algorithm was applied to determine the optimum level of each variable. The CCD matrix included five levels for each variable, six center points and star points to estimate the curvature. The CCD provided an indication of the main effect of each factor in addition to the interaction among them. A second-order polynomial model was generated for the prediction of the optimum production medium composition for PHB production (Eq. 4):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_{ii} + \sum \beta_{ij} X_{ij} \quad (4)$$

where β_i is the regression coefficient for each factor, β_{ii} is the regression coefficient for square effects and β_{ij} is the regression coefficient for interaction. The Analysis of variance (ANOVA) was carried out using Design-Expert 8.0 statistical package (StatEase, Inc, Minneapolis, MN, USA).

The potential production medium components were maltose, ammonium sulfate, fructose and ammonium chloride. The values used to generate the CCD matrix are shown in Table 3.

Thirty-one experiments (Table 8) were conducted in 250 mL Erlenmeyer flasks containing 100 mL of the tested media combination (initial pH for each was 6.5) prepared according to the design matrix.

The flasks were incubated at shaker incubator at 31.5 °C and 150 rpm. The investigated responses were optical density (OD_{600}) and PHB (mg/L). All flasks were harvested after 72 h from inoculation. 3D response surface plots were generated to understand the interaction among the tested variables and used to reveal the optimum concentration of each of the media components that majorly affect the targeted response. To check the validity of the optimum media proposed by the generated

mathematical model, a validation experiment was carried out by applying the suggested optimum media.

2.4. *Effect of C/N ratio*

Twenty six different Carbon/Nitrogen values were tested to determine the optimum C/N ratio to attain the maximum growth and Biopolymer production. The relation between the ratio values and each response was plotted, and a best fit line was estimated and the P-values for each response were calculated in order to test the effect of the C/N values on growth and PHB productivity.

2.5. *Batch growth in bioreactor*

A batch run was carried out using Eppendorf – New Brunswick 5 L Rushton turbine Stirred Tank Bioreactor (STR) with a working volume of 3 L. After sterilization, the bioreactor was set up according to the manufacturer's instructions.

The seed culture was inoculated by a loopful of freshly activated bacterial plate of 48 h age and kept at 31.5 °C for 48 h in the incubator shaker at 150 rpm until the seed culture optical density (OD_{600}) reached 0.5. Batch cultivation was carried out at 31.5 °C, pH 6.5 and 200 rpm stirring. Culture pH was maintained by automatic addition of acid or base (4N) by pH–mV controller M 7832N. Samples were withdrawn at regular time intervals to be analyzed for growth (optical density at 600 nm) and PHB concentration (mg/L) and to perform a sterility test. The supernatant obtained by centrifugation of the culture broth at 10,000 rpm for 10 min was used for residual substrate analysis. Residual sugar was estimated by a dinitrosalicylic acid (DNS) method [17], while residual ammonia nitrogen was determined according to a Kjeldahl method [18]. Air flow was disabled all over the batch run.

Depending on the previously performed CCD experiment (section 2.3), the suggested production medium was found to be 6.06 g/L, 1.09 g/L, 12.12 g/L and 4 g/L for maltose, ammonium sulfate, fructose and ammonium chloride respectively. Seed culture was prepared as 300 mL medium with nutrient concentrations similar to that of the production medium in addition to the minimal medium components and trace metal solution which consisted of 0.2 mg/L $FeSO_4.7H_2O$, 0.3 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$ and 0.3 mg/L H_3BO_3 [19].

3. Results and discussion

3.1. *Plackett–Burman initial screening for carbon and nitrogen sources*

Microbacterium WA81 growth, expressed as optical density (600 nm), and its PHB production, expressed in mg/L, were optimized, using two separate Plackett–Burman screening experiments to determine the most potential carbon and nitrogen sources (Tables 4 and 5). The basic medium used was chemically defined minimal medium supplemented with any of the carbon and nitrogen sources, and the culture conditions were at pH 6.5, 31.5 °C, and 150 rpm for 72 h. Both statistical experimental designs were intended to determine the most potential carbon and nitrogen sources and not only the exact optimum combination of these sources, but the development of a predictable mathematical model for the whole process.

From Table 4 (screening the effect of different carbon sources), the obtained data indicated that the lowest responses were obtained at runs 3 (OD_{600} = 0.24) and 12 (PHB conc. = 0.095 mg/L) and the highest responses were obtained at runs 4 (OD_{600} = 0.65) and 5 (PHB conc. = 2.01 mg/L) for the bacterial growth and PHB accumulation.

From Table 5 (screening the effect of different nitrogen sources), the highest responses were 1.9 (run 10) and 6.05 mg/L (run 1) for bacterial growth (OD_{600}) and PHB accumulation (mg/L), whereas the lowest responses were 0.91 (run 3) and 1.56 mg/L (run 10).

Statistical analysis of the responses (Minitab 16 environment) determined the most significant factors affecting growth and PHB production at 90% level of confidence and $\alpha = 0.1$. A P-value equal or less than α was considered to be significant (bold values in Tables 6 and 7). Fructose and arabinose showed the most statistically significant effect on growth (P-values = 0.042 and 0.066), whereas maltose was the most statistically significant (P-value = 0.079) for the PHB production (Table 6). As for nitrogen sources, it was found that five

Table 4 – The matrix applied and the calculated responses for the performed design of experiment to screen the effect of different carbon sources on the bacterial growth and PHB production.

Run	Variables										Responses	
	Sorbitol	Lactose	Maltose	Sucrose	Fructose	Arabinose	Dextrose	Glycerol	Sod. acetate	Dummy	OD_{600}	PHB (mg/L)
1	–	–	–	–	–	–	–	–	–	–	0.57	0.68
2	–	+	+	–	+	–	–	–	+	+	0.52	0.508
3	+	+	–	+	–	–	–	+	+	+	0.24	0.2
4	–	–	+	+	+	–	+	+	–	+	0.65	1.47
5	+	+	+	–	+	+	–	+	–	–	0.52	2.01
6	+	–	+	+	–	+	–	–	–	+	0.48	1.06
7	–	–	–	+	+	+	–	+	+	–	0.51	1.06
8	–	+	–	–	–	+	+	+	–	+	0.58	0.41
9	+	+	–	+	+	–	+	–	–	–	0.52	1.48
10	+	–	+	–	–	–	+	+	+	–	0.25	0.88
11	–	+	+	+	–	+	+	–	+	–	0.57	0.93
12	+	–	–	–	+	+	+	–	+	+	0.45	0.095

Table 5 – The matrix applied and the calculated responses for the performed design of experiment to screen the effect of different nitrogen sources on the bacterial growth and PHB production.

Run	Variables											Responses	
	Urea	Amm. acetate	Threonine	Amm. sulfate	Glycine	Amm. chloride	Amm. nitrate	Proline	Arginine	Cysteine	Dummy	OD_{600}	PHB (mg/L)
1	–	+	+	+	–	+	+	–	+	–	–	1.78	6.05
2	+	–	+	–	–	–	+	+	+	–	+	1.47	1.96
3	–	–	–	–	–	–	–	–	–	–	–	0.91	2.61
4	–	+	+	–	+	–	–	–	+	+	+	1.1	3.7
5	–	–	+	+	+	–	+	+	–	+	–	1.07	2.2
6	+	+	–	+	–	–	–	+	+	+	–	1.1	2.1
7	+	–	–	–	+	+	+	–	+	+	–	1.31	3.5
8	+	–	+	+	–	+	–	–	–	+	+	1.1	2.07
9	–	+	–	–	–	+	+	+	–	+	+	1.07	2.2
10	+	+	+	–	+	+	–	+	–	–	–	1.9	1.56
11	–	–	–	+	+	+	–	+	+	–	+	1.74	6.2
12	+	+	–	+	+	–	+	–	–	–	+	1.24	3.29

variables were found to have a statistically significant effect on the growth including ammonium chloride, arginine, threonine, glycine and proline with P-values of 0.014, 0.012, 0.046, 0.059 and 0.062 respectively. Further, arginine, ammonium sulfate, ammonium chloride and glycine showed statistically significant effects on the PHB production with P-values of 0.012, 0.027, 0.033 and 0.079 respectively.

The relation between the tested variables and the calculated responses could be mathematically modeled as shown in Equations (5) and (6). Response according to the carbon sources for PHB concentration (mg/L) would be as follows:

$$Y = 0.9 + 0.24 * \text{maltose} - 0.28 * \text{sodium acetate} \quad (5)$$

Table 6 – Statistical analysis of design of experiment showing the effect, regression coefficient, T value, and p value for each variable on the growth and PHB production.

Variable	Optical density (OD_{600})				PHB (mg/L)			
	Effect	Coefficient	T-value	P-value	Effect	Coefficient	T-value	P-value
Sorbitol	–0.15	–0.079	–9.42	**0.011**	0.109	0.054	0.28	0.8
Lactose	0.007	0.003	0.47	0.687	0.049	0.024	0.13	0.91
Maltose	0.018	0.009	1.08	0.392	0.491	0.24	2.11	**0.079**
Sucrose	0.015	0.007	0.92	0.453	0.27	0.13	1.17	0.28
Fructose	0.078	0.039	4.70	**0.042**	0.41	0.20	1.75	0.13
Arabinose	0.061	0.03	3.68	**0.066**	0.06	0.03	0.15	0.89
Dextrose	0.029	0.014	1.74	0.224	–0.04	–0.021	–0.11	0.92
Glycerol	–0.059	–0.029	–3.55	**0.071**	0.21	0.106	0.92	0.39
Sodium acetate	–0.129	–0.064	–7.72	**0.016**	–0.57	–0.28	–2.46	**0.049**

Table 7 – Statistical analysis of design of experiment showing the effect, regression coefficient, T-value, and p-value for each variable on the growth (OD_{600}) and PHB production (mg/L).

Variable	Optical density (OD_{600})				PHB (mg/L)			
	Effect	Coefficient	T-value	P-value	Effect	Coefficient	T-value	P-value
Urea	0.076	0.038	1.92	0.19	–1.42	–0.71	–8.10	**0.015**
Amm. acetate	0.1	0.05	2.53	0.12	0.061	0.03	0.26	0.84
Threonine	0.17	0.089	4.49	**0.046**	–0.38	–0.19	–2.21	0.15
Amm. sulfate	0.04	0.021	1.09	0.39	1.05	0.52	6	**0.027**
Glycine	0.15	0.078	3.92	**0.059**	0.58	0.29	3.3	**0.079**
Amm. chloride	0.33	0.16	8.36	**0.014**	0.95	0.47	5.4	**0.033**
Amm. nitrate	0.011	0.005	0.21	0.86	0.16	0.08	0.91	0.45
Proline	0.15	0.076	3.82	**0.062**	–0.83	–0.41	–4.76	**0.041**
Arginine	0.2	0.1	5.14	**0.036**	1.6	0.8	9.11	**0.012**
Cysteine	–0.37	–0.18	–9.52	**0.011**	–0.97	–0.48	–5.55	**0.031**

while response according to the nitrogen sources for PHB concentration (mg/L) would be:

$$Y = 3.12 - 0.71 * \text{urea} + 0.52 * \text{ammoniumsulfate} + 0.29 * \text{glycine} + 0.47 * \text{ammoniumchloride} - 0.41 * \text{proline} + 0.8 * \text{argnine} - 0.48 * \text{cysteine} \quad (6)$$

The normal plots of the standardized effects are presented in Figs. (1a and b) and (2a and b), where the variables on the right side of the line have positive effects and those on the left side have negative effects.

The analysis of the normal plot of these figures showed that fructose and maltose were the most significant carbon sources for the bacterial growth and the biopolymer production, respectively. The preference by this local isolate is in agreement with Singh et al. [20] and Aslam et al. [21]; both have reported that maltose was the best carbon source that enhanced PHB yield by *Bacillus subtilis* NG220 and *Enterobacter aerogenes*, respectively.

Contrary to that, several other bacteria showed different inclination to different sugars for the highest production of PHB. For example, the highest PHB productivity was achieved by

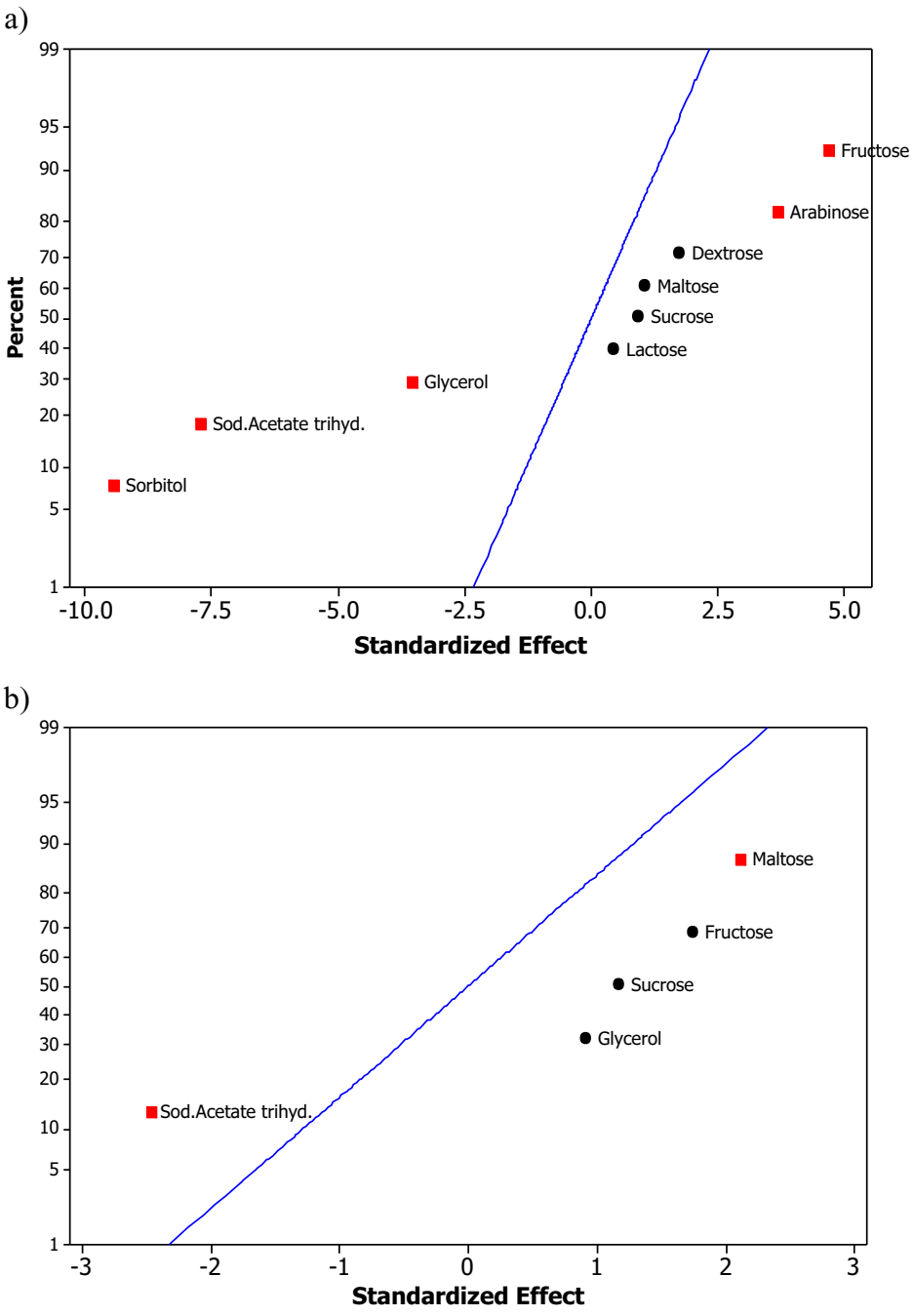

Fig. 1 – Normal plot of the standardized effect showing the significant effect of each factor of different carbon sources on (a) optical density with fructose and arabinose as the most potential carbon sources; (b) PHB concentration (mg/L) with maltose as the most potential carbon source.

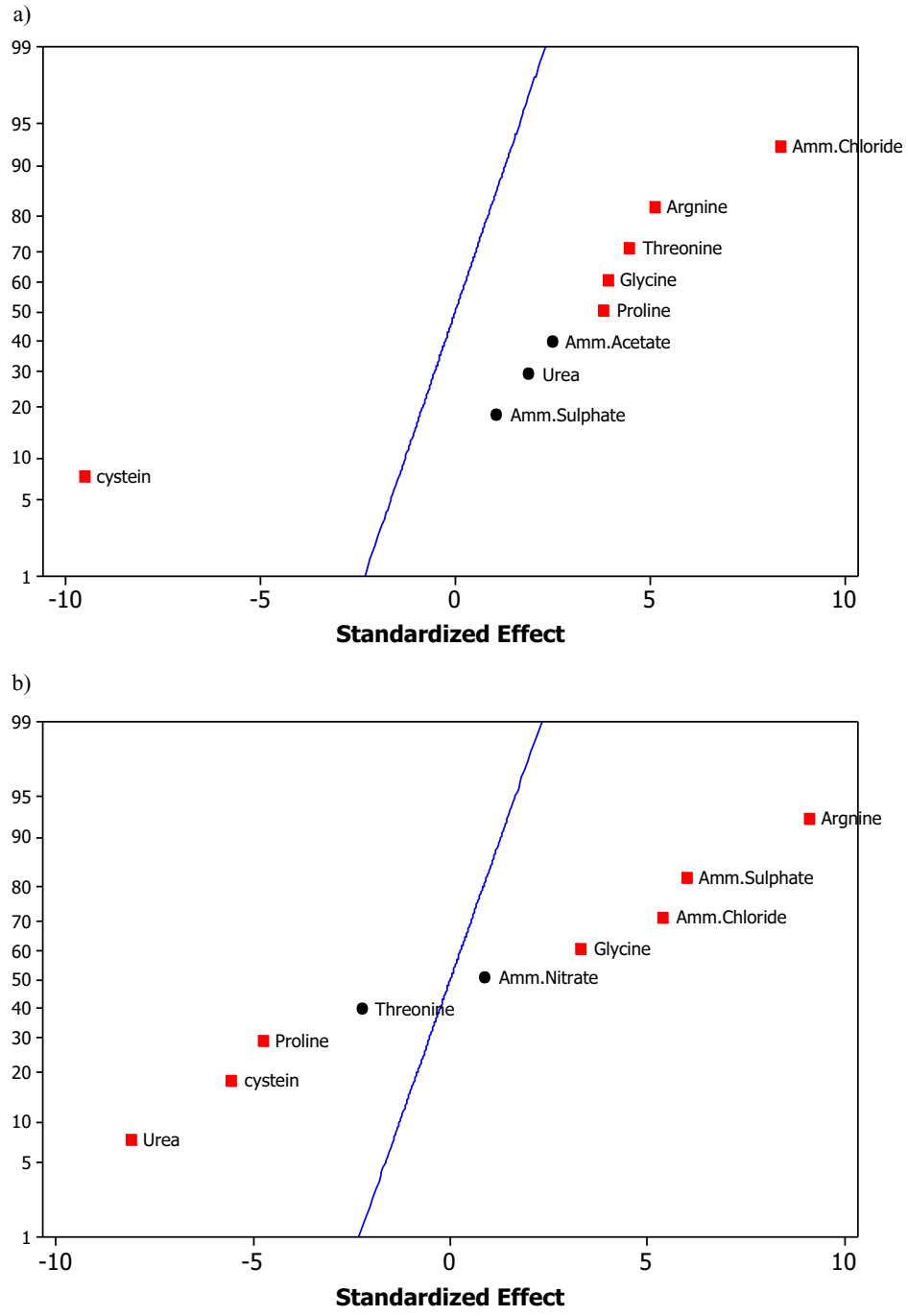

Fig. 2 – Normal plot of the standardized effect showing the significant effect of each factor of different nitrogen sources on (a) optical density with ammonium chloride and arginine as the most potential nitrogen sources; (b) PHB concentration (mg/L) with arginine and ammonium sulfate as the most potential nitrogen sources.

R. eutropha [13], *Bacillus megaterium* [22] and *Bacillus* sp. JMa5 [23] when fructose, glucose and sucrose were used during fermentation, respectively. These data emphasize the need to optimize the fermentation conditions for each organism separately.

Nitrogen source is one of the most important factors in the biopolymer accumulation by microbial cells and serves as precursor for vitamins, amino acids, growth factors etc. In some microbial strains PHA accumulation can appear in parallel to biomass production. This 'growth-associated' PHA accumulation is known for *A. latus*, *Methylobacterium* sp. ZP24 [24], *Bacillus mycoides* RLJ B-017 [25] and recombinant *E. coli* [26]. *Microbacterium* sp. WA81 was no exception; it did accumulate PHB as cell growth progressed and the final amount of polymer obtained depended on the maximum biomass produced.

When ten different nitrogen sources were subjected to Plackett–Burman screening design of experiment in culture of *Microbacterium* sp. WA81, it showed that ammonium chloride and arginine were the most significant sources that enhanced

Table 8 – Matrix and responses for the CCD experiment (the levels for each variable are shown in Table 4).

Run	Variables				Responses	
	Maltose	Ammonium sulfate	Fructose	Ammonium chloride	OD_{600}	PHB (mg/L)
1	0	0	0	0	2.89	0.14
2	0	0	2	0	2.036	0.76
3	0	0	0	0	3.087	0.14
4	1	1	–1	1	2.366	0.306
5	0	2	0	0	2.99	1.43
6	–1	–1	1	–1	1.437	0.75
7	0	0	0	0	2.89	0.14
8	0	0	0	–2	1.85	5.37
9	0	0	0	2	2.44	2.93
10	0	0	0	0	3.87	0.14
11	1	1	1	1	2.074	0.69
12	1	–1	1	–1	1.419	0.138
13	1	–1	1	1	2.62	1.1
14	–1	–1	–1	1	2.95	**5.51**
15	–1	1	1	1	2.92	1.48
16	–1	–1	1	1	2.331	1.56
17	–2	0	0	0	2.5	2.04
18	0	0	0	0	3.087	0.14
19	0	–2	0	0	3.168	0.46
20	–1	1	1	–1	1.938	1.99
21	1	1	1	–1	3.062	1.83
22	0	0	0	0	2.89	0.14
23	1	–1	–1	1	2.911	0.46
24	0	0	–2	0	2.263	0.016
25	1	–1	–1	–1	1.78	0.34
26	–1	–1	–1	–1	1.492	0.26
27	1	1	–1	–1	2.19	0.62
28	–1	1	–1	1	2.217	2.05
29	2	0	0	0	2	1.25
30	–1	1	–1	–1	1.7	1.66
31	0	0	0	0	2.89	0.14

The bold values represent the highest achieved yield.

the microbial growth, and arginine and ammonium sulfate were shown to be the best nitrogen sources for PHB production (Fig. 2a and b). Similar results were reported by Beaulieu et al. [27], where ammonia or ammonium salts were essential in maximizing the concentration of biomass and hence the accumulation of PHB.

Several studies were carried out to investigate the most significant amino acids as nitrogen sources that enhanced the microbial biomass accumulation and the microbial biopolymer production. Hamieh et al. have reported for *Lactobacillus acidophilus* that PHB production increased with high levels of glycine [28]. Mercan et al. was reported in two strains of *Rhizobium sp.*; the highest level of PHB accumulation was observed in the medium with L-cysteine and L-glycine [29]. By using COBRA toolbox analysis for metabolic modeling and simulation, L-arginine was found to be the most significant nitrogen source on PHB production by recombinant *E. coli* K-12 MG1655 as reported by Heshiki [30], which showed an agreement with the result of our experiment.

Overall the importance of ammonium ion for maximization of the concentration of biomass and the accumulation of PHB was widely circulated in the literature [27].

The maximum biomass and PHB yields by *A. latus* were achieved at 1.5 g/L ammonium chloride and 1.4 g/L ammonium sulfate, respectively, and the yields were relatively sensitive to the concentration changes [31]. Moreover, the halotolerant photosynthetic bacterium *Rhodobacter sphaeroides* grown under aerobic and dark conditions showed the highest PHB accumulation in the presence of ammonium sulfate [32]. Nutrient limitation is necessary to achieve the highest PHB production, and generally, ammonia is used as the critical regulator for uncoupling of the microbial growth and PHA production [33].

In conclusion, fructose, ammonium chloride, maltose and arginine were the most significant factors for the bacterial growth and microbial biopolymer accumulation. To reduce the production cost, arginine was substituted by ammonium sulfate as potential nitrogen source for PHB production. These factors were chosen to generate a chemically defined production medium.

3.2. *Central Composite Design experiment to optimize the most potential carbon and nitrogen sources*

To determine the optimum medium composition, a CCD matrix was performed as previously mentioned in section 2.3. The matrix and responses of the design were summarized in Table 8.

From Table 9, the main effect of ammonium chloride and quadratic effect of fructose have a significant effect on the bacterial growth, while the main effect of maltose, ammonium

Table 9 – Analysis of variance (ANOVA) of the performed CCD experiment for the calculated responses at 90% confidence level.

Variable	Responses					
	Optical Density (OD_{600})			PHB (mg/L)		
	Sum of squares	F value	P-value Prob > F	Sum of squares	F value	P-value Prob > F
A: Maltose	0.054	0.39	0.54	3.37	6.24	**0.02**
B: Ammonium sulfate	0.24	1.75	0.2	1.68	3.11	**0.09**
C: Fructose	0.040	0.29	0.59	3.54	6.55	**0.021**
D: Ammonium chloride	1.17	8.41	**0.011**	0.42	0.78	0.39
AB	0.042	0.30	0.58	0.016	0.029	0.86
AC	0.15	1.09	0.31	0.3	0.56	0.46
AD	0.078	0.56	0.46	0.57	1.06	0.32
BC	0.28	1.98	0.18	0.25	0.46	0.5
BD	0.67	4.77	**0.045**	3.93	7.28	**0.016**
CD	0.014	0.098	0.75	0.11	0.2	0.65
A^2	1.38	9.87	**0.0067**	10.08	18.67	**0.0006**
B^2	6.768e-003	0.048	0.82	5.23	9.69	**0.0071**
C^2	1.71	12.25	**0.0032**	0.11	0.20	0.661
D^2	1.72	12.33	**0.0031**	19.06	35.31	**<0.0001**

The bold values represent the most significant factors.

sulfate, fructose and quadratic effect of ammonium sulfate have a significant effect on PHB accumulation. However, the interaction between ammonium sulfate and ammonium chloride, the quadratic effect of maltose and ammonium chloride has a significant effect on both bacterial growth and PHB production.

The interaction among the variables could be modeled (in terms of coded units) mathematically as follows (Eq. 7):

$$PHB = -1.96 - 0.37 * A + 0.26 * B + 0.38 * C - 0.5 * B * D + 0.61 * A^2 + 0.44 * B^2.83 * D^2 \quad (7)$$

Using the generated mathematical models, the optimum condition for PHB production was estimated. According to the model predictions, the maximum PHB production and optical density could be achieved by using different combinations of different concentrations of maltose, ammonium sulfate, fructose and ammonium chloride. This prediction was verified experimentally and the maximum PHB production was achieved when maltose, ammonium sulfate, fructose and ammonium chloride are 6.06 g/L, 1.09 g/L, 12.12 g/L and 4 g/L, respectively. The predicted value was 6 mg/L of PHB but the actual concentration obtained was slightly less than the expected value (actual value was 5.4 mg/L).

After determining the most significant carbon and nitrogen sources that enhanced the bacterial biomass and biopolymer accumulation, a CCD matrix was performed to determine the exact values for optimizing the productivity and reveal the interaction among the tested variables.

The results of ANOVA of the main effects of each factor, interactions among them and the quadratic effects are shown in Table 8. The model *F*-values for each of the response variables and the model *P*-values all imply that the models are significant with very low chance that a model *F*-value is large owing to noise for optical density (4.6%) and PHB (0.21%) production respectively. At the 90% level of confidence, any factor with a *P*-value less than 0.1 could be considered as statistically significant. The smaller the *P*-value, the higher the significance of the corresponding variable [34]. The *P*-values of each variable shown in Table 8 clearly showed that the main effect of ammonium chloride was the most significant factor affecting the growth, whereas the main effect of maltose, fructose and ammonium sulfate were found to have a significant effect on PHB yield concentration. Also, it indicated that the two way interaction between ammonium chloride and ammonium sulfate had a significant effect on both growth and PHB production by the isolate under investigation. In addition, the quadratic effect of maltose and ammonium chloride had a significant influence on the growth and PHB production, whereas the quadratic effect of ammonium sulfate was significant only on PHB concentration. Unlike one-factor-at-a-time experiments, statistically designed experiments are able to test the effect of the interactions among the factors in addition to the square terms which evaluate the quadratic effect of the variables. The significant interaction between ammonium sulfate and ammonium chloride in relation to the PHB concentration could be attributed to the adequate concentration of NH_3, allowing most of the NADPH to be utilized for amino acids biosynthesis, and cell growth was activated. NADPH was used as a coenzyme of acetoacetyl-CoA reductase for the conversion of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA under nitrogen-limiting conditions. From these results, it could be concluded that PHB production can be enhanced by providing a condition in which NADPH is in excess. Supplementation of complex nitrogen sources, oleic acid, or amino acids to the chemically defined medium significantly enhanced PHB production by recombinant *E. coli* due to the increase of the availability of acetyl-CoA [35]. These experimental findings were supported by studies on the effects of acetyl-CoA and NADPH on the intracellular metabolic flux distribution of recombinant *E. coli*. The results of Metabolic Flux Analysis suggested that in order to achieve the maximum PHB yield, about one-half of the carbon flux should be directed to the pentose phosphate (PP) pathway,

and flux to the TCA cycle should be shut down. These two pathways affect the availability of two substrates for poly(3HB) synthesis, NADPH and acetyl-CoA [36]. Depending on the generated mathematical model, the optimum combination of factors for maximum production was estimated as 6.06 g/L, 1.09 g/L, 12.12 g/L and 4 g/L for maltose, ammonium sulfate, fructose and ammonium chloride respectively. The maximum productivity achieved under these optimum conditions was 5.4 mg of PHB per liter at the shake flask level.

3.3. *Effect of C/N ratio*

One of the advantages of using a chemically defined medium is that the effect of C/N on PHB production could be easily tested. The C/N values were calculated theoretically from the performed CCD experiment performed. The design contained 31 different trials with 26 different C/N values. The relation between the ratio values and each response was plotted (Fig. 3a and b) and a best fit line was estimated and the P-values for each response were calculated in order to test the effect of the C/N values on growth and productivity of the isolate.

The effect of high C/N ratio was negative on growth and productivity of isolate. Relatively low C/N values were preferred, indicating that a higher nitrogen was required for better growth and PHB production. The P-values for the effect of C/N ratio on bacterial growth and PHB production were found to be 0.048 and 0.024 respectively. These results indicated that the effect of C/N could be significant at the 99.97% and 99.96% levels of confidence, which were statistically significant. The C/N ratio of 8.2/1 generated the highest values of PHB production (5.5 mg/L). These results suggest that a C/N ratio of 8/1 is optimal for the accumulation of PHB, and above which the polymer accumulation remarkably reduced.

In living organisms, carbon (C) requirements are generally larger than nitrogen (N) requirements. The balance of these elements (C/N) determines how bacteria use an organic material [37]. To enhance PHB production, different C/N ratios generated from the different combinations in the performed CCD trials were compared to determine the optimal ratio. C/N ratio of 8.2/1 resulted in the highest PHB productivity (5.5 mg/L). This result suggests that a C/N ratio of 8/1 is optimal for the accumulation of PHB, which agreed with the results reported by Wei et al. about *Cupriavidus taiwanensis* 184 [38]. Increasing the C/N ratio from (5.7/1 to 52/1) led to a significant reduction in PHB accumulation within the isolated strain; which could be due to the relatively high concentration of Carbon which was previously reported to inhibit PHB biosynthesis by the strain *Cupriavidus taiwanensis* 184 at relatively high concentrations [38]. Additionally, high C/N and low C/N ratios might affect the physiological conditions of the microorganisms, including cell proliferation and polymerization of PHB [37]. These data indicate that, although absolute concentration of carbon source is important, C/N ratio has also critical value since growth and PHB production decrease at very high or low concentrations of carbon and nitrogen sources. Suppressed PHB accumulation could be attributed to the high concentration of ammonium sulfate or the high C/N ratio [39].

3.4. *Batch growth in Bioreactor*

Upon optimizing the medium composition at shake flask level, a final batch run was carried out using 5 L Rushton turbine Stirred Tank Bioreactor (STR) with a working volume of 3 L. Fig. 4a and b shows the growth profile of *Microbacterium* sp. WA81 on the developed medium.

After a lag phase of approximately 20 h, the bacterial growth increased and OD_{600} reached 2.343 after 56 h during which the maximum PHB accumulation reached 1.42 g/L. In the same growth phase, 1.2 g/L of nitrogen was consumed out of an initial value of 1.6 g/L, whereas approximately 13.5 g/L of the supplemented sugars were metabolized. Then, the bacterial growth continued slowly by feeding on the residual sugar and the amount of the accumulated PHB was decreased. In most microorganisms, PHB is a food reserve that is degraded to provide carbon and energy when an external carbon source is exhausted [31,40].

Dissolved oxygen is one of the most probable cause of PHB accumulation during growth phase. The key feature of this control is the fate of acetyl-CoA, which may be oxidized via tricarboxylic acid (TCA) cycle or can serve as a substrate for PHB synthesis. Under oxygen limitation when NADH/NAD ratio increases, citrate synthase and isocitrate dehydrogenase are inhibited by NADH, and in consequence, acetyl-CoA no longer enters the TCA cycle at the same rate. Instead it is converted to acetoacetyl-CoA by 3-ketothiolase (the first enzyme of PHB biosynthesis). Therefore, under such conditions there is a greatly decreased flux of carbon through the TCA cycle [41]. The effect of different air flow rates were tested on the isolate *Microbacterium* sp. WA81 and it was found that sparging air into the vessel led to a significant inhibition of the isolate growth and its capability to accumulate PHB in addition to the formation of biofilm. As a result, the batch bioreactor runs were carried out in the complete absence of air.

The inhibition of PHB accumulation in presence of high oxygen could be due to the generated high shear force which was proven to inhibit the PHB biosynthesis by a number of microbes [42,43].

4. Conclusions

In the present study, the biodegradable polymer PHB was targeted for enhancing its productivity from the local Egyptian isolate *Microbacterium* sp. WA81 using DOE tools. Due to the importance of carbon and nitrogen sources on the bioaccumulation, this study was conducted by screening different sources via two separate PBD experiments, and it was found that maltose and arginine had the most positive significant effect on the PHB accumulation, and fructose and ammonium chloride on the bacterial growth.

A subsequent CCD was applied to detect the optimal concentration of each source and C/N ratio for the generation of a chemically defined medium and it was combined as follows: 6.06 g/L, 1.09 g/L, 12.12 g/L and 4 g/L for maltose, ammonium sulfate, fructose and ammonium chloride respectively. The

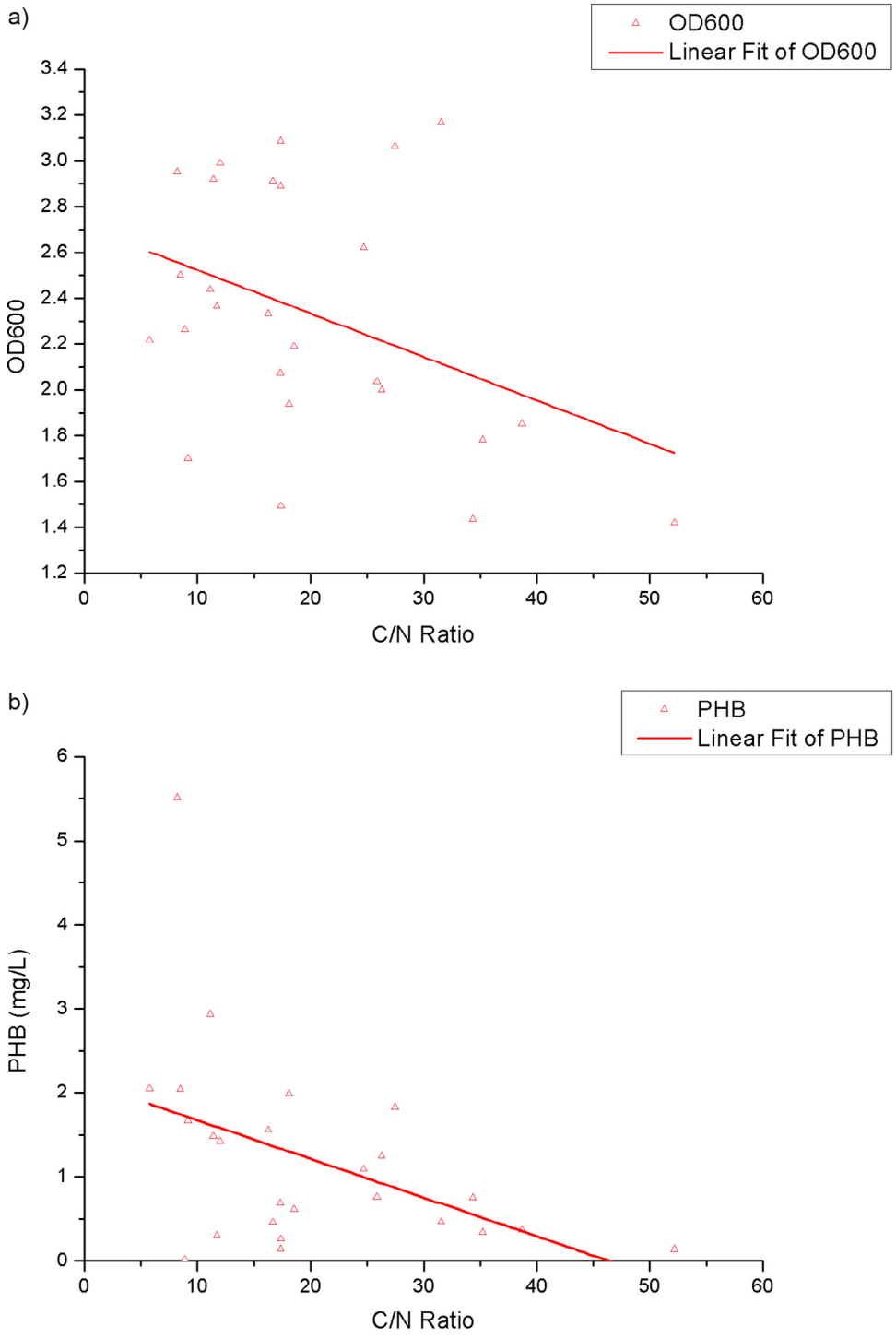

Fig. 3 – The relation between the C/N ratio and the (a) bacterial growth and (b) PHB production by the isolate with the best fit line.

highest yield of PHB was achieved at C/N ratio of 8.2/1. The optimized medium was eventually used to test the growth and PHB accumulation in a bioreactor (5 L) under pH-stat conditions to investigate the improvement in growth and production of PHB by the isolate under investigation. In the batch cultivation, the produced PHB amount was extraordinarily increased to be 1.5 g/L with a 78 fold increase of the production. This value was obtained even prior to optimizing the cultivation conditions at the bioreactor level, which indicates that optimizing the bioreactor condition could lead to increased PHB productivity making our isolate a potential high producer for industrial scale production of such valuable biopolymer.

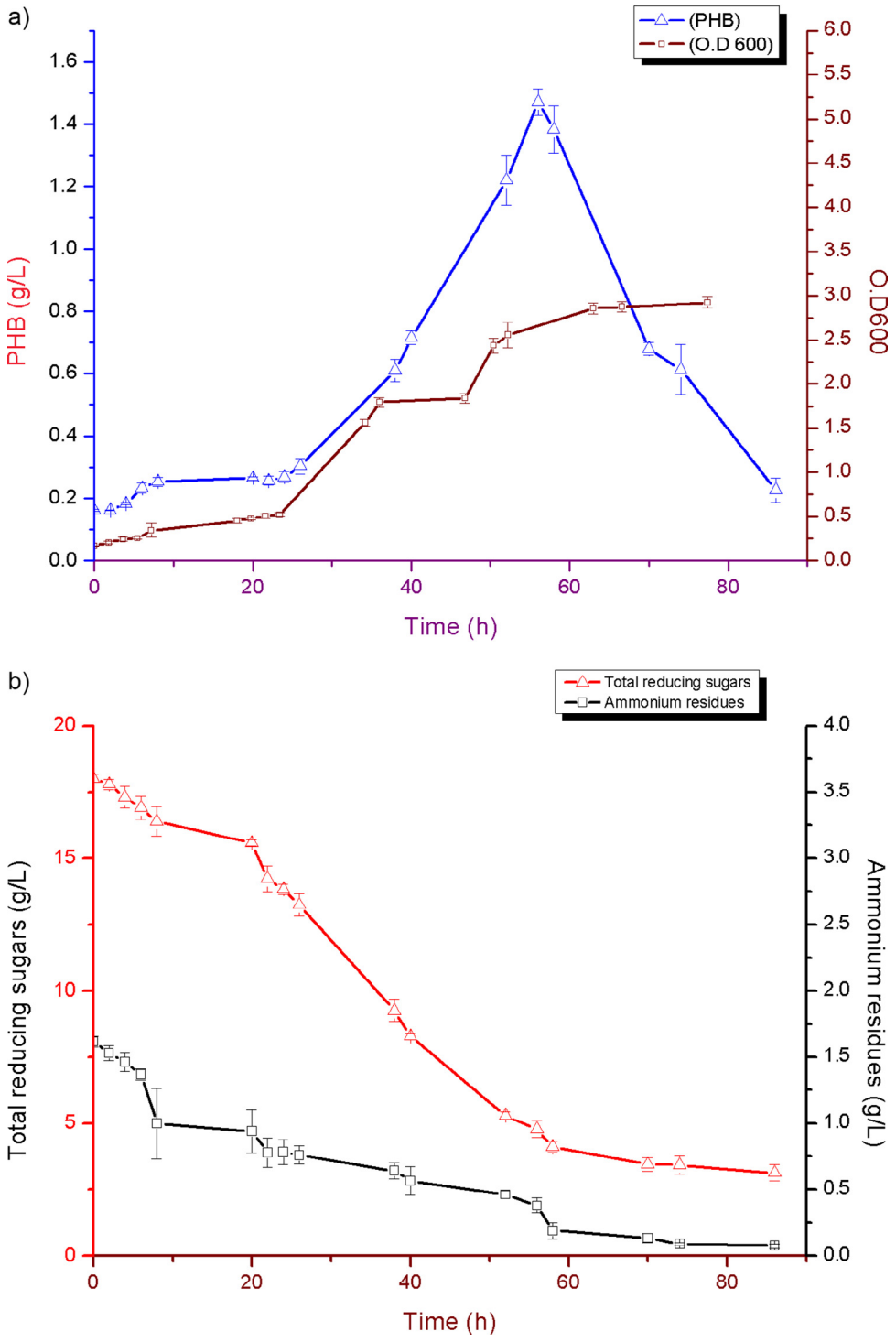

Fig. 4 – The figure shows the batch growth of *Microbacterium* sp. WA81 on the optimized medium components under controlled conditions: pH 6.5, temperature 31.5 °C and 200 rpm. (a) Growth and productivity of the isolate under investigation; (b) sugar and nitrogen utilization by the isolate.

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