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OPTIMIZATION OF POLYHYDROXYALKANOTES PRODUCTION USING TWO LEVEL FACTORIAL DESIGN

Ahmad Idi[a*], Abdulkarim Ali Deba[b], Zakari Bawa Gambo[c], Haruna Saidu[d]
[a] Department of Biotechnology, Faculty of Life Sciences, Modibbo Adama University, Yola
[b] Department of Plant Science, Faculty of Life Sciences, Modibbo Adama University, Yola
[c] Department of Science Education, Faculty of Technology Education, ATBU, Bauchi,
[d] Department of Biological Sciences, Faculty of Science, Gombe State University, Gombe
[*]**Corresponding author:** ahmadidy@mautech.edu.ng

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ABSTRACT The composition and type of polyhydroxyalkanoates (PHA) monomers are related to bacterial strain and media composition. This study evaluate the optimum media composition of PHA accumulation by photosynthetic bacterium, *Rhodobacter sphaeroides* ADZ101. Two level factorial design of response surface methodology was used to determine the optimum media composition for the production of PHA. The results showed that acetate and NH_4Cl were the best carbon and nitrogen sources good for the study. The optimum PHA accumulation were found to consist the combination of 3.00 g/L acetate, 0.06 g/L NH_4Cl and pH 7. The predicted combination was found to be in agreement with experimental results with R-squared value of 0.9962. The diagnostic plots for response were also in conformity with the results of the experimental runs in the study. Hence accumulation of PHA by *Rhodobacter sphareoides* ADZ101 occurs under stress conditions of excess carbon and limited nitrogen.
Keywords: Polyhydroxyalkanoates, *Rhodobacter sphaeroides* ADZ101, Plastic, Two level factorial

INTRODUCTION

Plastic has a variety of uses in our daily lives due to its durability, lightweight, inexpensive and a good conductor. However this material has a serious impact in the environment due to its non-biodegradability. The chemical bonds that make up plastic are not only resilient but also nonresponsive to biodegradation (Shaw and Sahni 2014). Furthermore, the chemicals that are used in plastic production are hazardous and detrimental to living organisms (Stanton et al. 2021). Chemicals such as mercury, cadmium and lead that are used in plastic production can caused cancer, immune system problems, congenital disabilities and other childhood development issues (Stanton et al. 2021). Hence the search for an alternative for the replacement of petroleum-based plastic become necessary.

PHA has shown to be an ideal replacement for the fossil derived plastic due to their close similarity in features and structure. PHA is accumulated as an inherent response to stress condition faced by microorganisms. The type and composition of PHA monomers produced varies with bacterial specie and the composition of the media. Since PHA accumulation is related to the composition and concentrations of the fermentation media, manipulating the media can be a good strategy that may influence the yield. This study therefore investigated the effect of three significant parameters (carbon, nitrogen and pH) reported in the literature to have an influence in PHA accumulation using 2-level factorial design. The conventional one factor at time method of statistical analysis is time consuming involving so many sets of experimental runs and unable to reveal the interactions between different parameters. On the contrary, the 2-level factorial design reveals significant interactions between experimental parameters and reduces the number of experimental runs. Thus a picture of the response surface can be obtained with minimum experimental runs. The study also examined the influence of these three significant parameters in PHA accumulation and revealed how interactions between them can influence the PHA yield.

MATERIALS AND METHODS

Fermentation Medium and Experimental Set up

The isolation *Rhodobacter spharoeides* ADZ101 was reported elsewhere (Idi et al. 2015). During the fermentation, *Rhodobacter sp.* ADZ101 was first grown in basal medium containing 1.00g disodium succinate, 0.50gNH_4Cl, 0.05g $CaCl_2.2H_2O$, 0.33g KH_2PO_4, $MgSO_4.7H_2O$ and NaCl. Then 10% (v/v) of the inoculum was transferred into 250 ml conical flask containing 100 mL of the fermentation medium and incubated at 37^{o}C with shaking speed of 150 rpm for 48 h. The fermentation medium contained the same components and proportion of the basal medium except for carbon, nitrogen and pH, which were prepared based on the experimental design provided by the software.

Preliminary PHA Detection

For the preliminary screening for PHA biosynthesis, the bacterium was grown in the fermentation

medium (agar) under the same growth conditions described in Section 2.1and later stained with Sudan Black B (Wong et al. 2004); (Anjum et al. 2016). The bacterial colony was heat fixed on a slide at 50^0C and the smears were later stained with Sudan Black B for 10 min. A solution of xylene was applied for decolourization before Safranin was added as counter stain for 10 seconds. The slide was later washed with distilled water and air dried before viewing under the light microscope.

Selection of Carbon and Nitrogen Source

Basal medium with different carbon sources comprising of glucose, sucrose, succinate, acetate and DL-Malic acid were prepared separately in the form of agar and broth. *Rhodobacter sp.* ADZ101 was first streaked onto each of the different agar plates and incubated under light at room (ambient) temperature. A colony from each plate was transferred into the respective broth and cultivated at 37 ^{0}C with shaking speed of 150 rpm. When the culture became turbid, 10% (v/v) was transferred into the fermentation medium comprising of the same carbon sources stated earlier and incubated under the same culture condition described above for 48 h. The fermentation medium was similar to the one described in Section 2.1 except for carbon and nitrogen, which were adjusted to 2.0 g/L and 0.5 g/L respectively. Excess carbon and low nitrogen has been reported as a prerequisite for PHA production (Ojumu, Yu, and Solomon 2004). Similar procedure was followed for the selection of nitrogen source with acetate as a carbon source. The selected nitrogen sources used in this study included $NaNO_3$, yeast extract, NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$.

Experimental Design

The experiment was designed using Design Expert software 6.04 and consisted of 24 different sets of experimental runs with 6 centre points. The selected parameters were varied as described in Table 1. The variations of these parameters were based on the various literature reports. For instance, excess carbon and limited nitrogen have been shown to be a prerequisite for PHA accumulation. Variations in pH have also been reported to influence PHA accumulation.

Table 1. Low and high values of the parameters

Factor	Name	unit	Low level (-)	High level (+)
A	Acetate	g/L	1.00	3.00
B	NH_4Cl	g/L	0.06	1.00
C	pH	-	7.00	9.00

PHA Extraction

The protocol reported by (Galia 2010) was adopted for PHA extraction. The PHA containing biomass grown in the fermentation medium was first harvested by centrifugation at 15,000 rpm for 15 min at 4^0C. The cell biomass was lysed by suspending in distilled water and maintained at 4^{o}C for 24 h. These conditions create an osmotic pressure which ensure the lyses of the cell as reported by (Fernandez-Castillo et al. 1986). The cells were then washed twice with double distilled water and lyophilised. The PHA was extracted by addition of 10 mL of chloroform to the lyophilised biomass in a 50 mL centrifuge tube and heated for 3 h at 80 ^{o}C in a water bath. The biomass was pipetted out and the suspension (solution) filtered through a 1 µm filter syringe and concentrated using rotary evaporator. The solution was filtered to remove minor cell debris as suggested by (Galia 2010).

PHA Quantification

PHA was quantified in form of percentage as reported by (Chaudhry et al. 2011) using the formula below.

$$PHA(\%) = \frac{Dry\ cell\ weight\ of extracted\ PHA}{Dry\ cell\ weight\ of\ the\ biomass} \times 100\%$$

RESULTS AND DISCUSSION

Preliminary detection of PHA

Rhodobacter sp. ADZ101 cells grown in the fermentation medium were stained with Sudan Black B after culturing for 48 h to detect the presence of PHA. The PHA when viewed under the light microscope appeared as black granules with light pink background as seen in Figure 1a Similar appearance of PHA stained with Sudan Black B was also reported by (Kumari and Dhingra 2013).

Microscopic Characterization of PHA

Scanning electron microscopy (SEM)

SEM detailed the physiological and morphological state of the bacterial cell. However, the accumulation of PHA granules distorts the morphology of bacterial cell as seen in Figure 1b. The bacterial sample used for the SEM was the same sample that was used for TEM and other characterisation of PHA.

Transmission electron microscopy (TEM)

The biosynthesis of PHA usually occurs under stressful condition imposed unto the bacterial cells by the limitation of nitrogen and the presence of excess carbon. During this condition, the cells divert their metabolic activity from growth toward the biosynthesis of PHA. PHA granules begin to form gradually due to their insolubility in water and fill up as well as enlarge the cells. The micrographs of *Rhodobacter sp.* ADZ101 grown in the optimised condition clearly showed the accumulated PHA granules in the cells (Figure 1c. The micrographs

also showed that the entire bacterial cells are almost filled up with the PHA granules in all the captured images with averagely four granular inclusions per cell. The number and size of PHA granules formation varies between different bacteria. For instance, *Halomonas boliviensis* LC1accumulated 1 to 2 granules and up to 8 in elongated cells with various sizes (Quillaguaman et al. 2006) while *Ralstonia eutropha* accumulated 2-8 granules per cell (Brigham et al. 2012). Formation of large PHA granules is shown to be advantageous in terms of quality and purification over smaller PHA granules(Van-Thuoc et al. 2012).

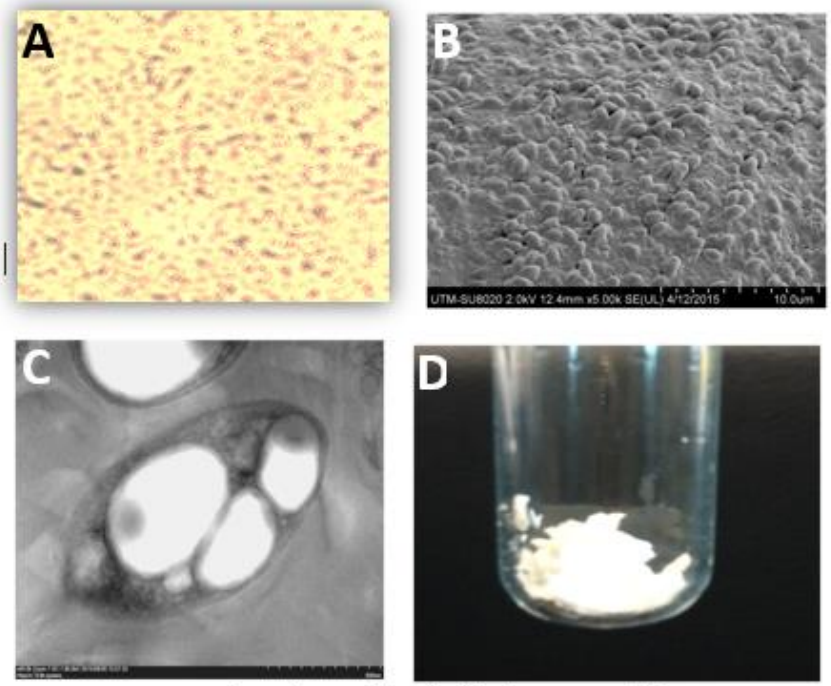

Fig. 1. Microscopic analysis of PHA, (a) Sudan black B staining, (b) SEM image, (c) TEM micrograph (d) collection of extracted PHA

Selection of carbon and nitrogen sources

Five different types of carbon sources comprising of glucose, succinate, acetate, sucrose and DL-Malic acid were screened to obtain the best for PHA accumulation. Acetate had the highest PHA accumulation of 30% while succinate had lowest PHA accumulation of 18%. Similarly, five different types of nitrogen sources comprising $NaNO_3$, yeast extract, NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$ were also screened. NH_4Cl had the highest PHA accumulation of 35% while $NaNO_3$ had the lowest PHA accumulation of 14%. Table 2 described the influence of carbon and nitrogen sources on PHA accumulation in comparison with other studies. The variation in PHA yield with other studies using different carbon and nitrogen sources may be attributed to different experimental set up, type of the bacteria and the medium composition, particularly the C:N ratio. For instance, carbon sources have been shown to influence not only the yield but also the type and monomeric composition of the accumulated PHA(Gumel, Annuar, and Heidelberg 2012b).

The C: N ratio had also been shown to affect the physiological condition of the bacteria in terms of cell growth and PHA production (Wei et al. 2011). Hence low PHA accumulation obtained during the selection of carbon and nitrogen source for the optimization process was probably attributed to low C: N ratio which was evident by low biomass production. It had been reported that increase in C: N ratio led to increase in PHA accumulation from 29% to 52% with the corresponding increase in C: N ratio of 1 to 20 times using gluconic acid and ammonium chloride as carbon and nitrogen sources respectively by *Cupriavidus taiwanensis* (Wei et al. 2011). Similarly, increased in C: N ratio also led to increase in PHA production and decrease in specific growth rate of mixed culture bacteria in activated sludge containing food waste (Wang et al. 2007). This result showed that nitrogen deficiency affected the normal bacterial growth and supported PHA production. On the contrary, decrease in C: N ratio had been shown to lead to an increase in polymer production by *Ralstonia eutropha strain* A-04 (Chanprateep et al. 2008). Hence, the effect of C: N ratio on the both growth and polymer production is an inherent characteristic of each microorganism and culture condition.

Table 2. Influence of carbon and nitrogen sources on PHA accumulation

Selection of Carbon source						
		This study			(Lorrungruang et al. 2006)	
Carbon source	**Nitrogen source**	**C: N ratio**	**DCW (g/L)**	**PHA (%)**	**Carbon**	**PHA (%)**
Glucose	NH_4Cl	1.20	0.425	21.0	Fructose	22.2
Succinate	NH_4Cl	1.30	0.225	18.0	Succinate	15.5
Acetate	NH_4Cl	2.60	0.363	30.0	Gluconate	15.3
Sucrose	NH_4Cl	0.63	0.132	20.0	Acetate	12.9
Malic acid	NH_4Cl	1.60	0.465	26.0	glucose	12.0
					Lactate	11.3
Selection of Nitrogen source						
		This study			(Gumel, Annuar, and Heidelberg 2012a)	
Nitrogen sources	**Carbon source**	**C: N ratio**	**DCW (g/L)**	**PHA (%)**	**Nitrogen**	**PHA (%)**
$NaNO_3$	Acetate	4.10	0.421	14.0	$(NH_4)_2SO_4$	58.8
Yeast extract	Acetate	1.70	0.324	20.0	NH_4NO_3	45.0
NH_4Cl	Acetate	2.60	0.358	35.0	$NaNH_4HPO_4.4H_2O$	53.9
NH_4NO_3	Acetate	3.90	0.532	18.0	Urea	11.1
$(NH_4)_2SO_4$	Acetate	6.40	0.451	16.0	Peptone	56.0

Two level factorial design

The 2-level factorial design reveals significant interactions between experimental parameters and reduces the number of experimental runs. Thus a picture of response surface can be obtained from few experimental runs. On contrary, the conventional one factor at time method of statistical analysis is time consuming involving so many sets of experimental runs and unable to reveal the interactions between different parameters. As such the 2-level factorial design was selected in this study.

The result of the experimental runs and PHA accumulation using 2-level factorial design is presented in Table 3. From Table 3 the highest PHA accumulation of 46.5% was obtained at run 18 with the combination of 3.00 g/L acetate, 0.06 g/L NH_4Cl and pH 7. This result is supported by runs 5 and 11 with similar combinations having 45.5 and 46% respectively. Treatment combination of 1.00 g/L acetate, 0.06 g/L NH_4Cl and pH of 7 in run 1 accumulated 36.5% of PHA. The combination was supported by the replicates having the same combination in run 4 and 6 with 35.4 and 35.0% respectively. The lowest PHA accumulation of 10.0% was obtained at run 23 with treatment combination of 1.0 g/L acetate, 1.0 g/L NH_4Cl and pH 9. The result was supported with similar combination in run 12 and 16 with the accumulation of 11.0 and 12.0% respectively. Following to the lowest PHA accumulation was obtained at run 25 with 12.3% having the combination of 1.0g/L acetate, 1.0 NH_4Cl and pH 7. The result was supported by runs 15 and 22 with similar treatment combinations having 13.0 and 12.0% respectively.

It can be deduced from the results that unbalance media composition of excess carbon and limited nitrogen influence the PHA yield. These results therefore support the assertion of (Gumel, Annuar, and Heidelberg 2012b) that PHA are usually produced under stressful condition of limited nitrogen and excess carbon. The results further showed low amount of PHA accumulation in experimental runs with balance amount of carbon and nitrogen content. PHA accumulation under normal growth condition of balance C:N content has been reported to produced low amount of PHA between 2-10% DCW depending on the bacterial strain (Du et al. 2001). Thus this assertion is in line with the results obtained from this study. *Rhodobacter sp.* ADZ101 can therefore be classified under PHA producers that require limitation of essential nutrients such as nitrogen. Similarly, the influence of initial pH on PHA accumulation can also be deduced from the results. Neutral pH (7) had the highest influence compared to alkaline. This can be seen in the experimental runs 1, 2, 4, 5, 6, 11 and 18 where neutral pH, excess carbon and limited nitrogen produced the best conditions for PHA accumulation. This result is in conformity with PHA accumulation by *Comamonas* sp. EB172 in one-step fermentation process where neutral pH influence not only PHA yield but also biomass accumulation (Zakaria et al. 2010).

Table 3. Experimental runs and PHA accumulation

Std	Run	Acetate (g/L)	NH_4Cl (g/L)	pH	PHA (%)
2	1	1.00	0.06	7.00	36.5
10	2	3.00	1.00	7.00	32.0
15	3	1.00	0.06	9.00	21.0
3	4	1.00	0.06	7.00	35.4
4	5	3.00	0.06	7.00	45.5
1	6	1.00	0.06	7.00	35.0
30	7	2.00	0.53	8.00	28.0
14	8	1.00	0.06	9.00	22.0
24	9	3.00	1.00	9.00	24.5
18	10	3.00	0.06	9.00	28.5
5	11	3.00	0.06	7.00	46.0
19	12	1.00	1.00	9.00	11.0
27	13	2.00	0.53	8.00	26.5
23	14	3.00	1.00	9.00	24.0
8	15	1.00	1.00	7.00	13.0
21	16	1.00	1.00	9.00	12.0
11	17	3.00	1.00	7.00	33.0
6	18	3.00	0.06	7.00	46.5
12	19	3.00	1.00	7.00	31.5

17	20	3.00	0.06	9.00	28.5
13	21	1.00	0.06	9.00	20.0
9	22	1.00	1.00	7.00	12.0
20	23	1.00	1.00	9.00	10.0
26	24	2.00	0.53	8.00	27.0
7	25	1.00	1.00	7.00	12.3
22	26	3.00	1.00	9.00	23.0
28	27	2.00	0.53	8.00	28.0
25	28	2.00	0.53	8.00	28.0
29	29	2.00	0.53	8.00	27.5
16	30	3.00	0.06	9.00	29.0

Analysis of variance

The ANOVA obtained for the accumulation of PHA is described in Table 4. The probability less than 0.0500 showed how significant the model is. The most significant model terms are A, B, C, AB, AC, BC and ABC where A is acetate, B is NH_4Cl and C is pH. AB described the interaction between acetate and NH_4Cl, AC described the interaction between acetate and pH, BC described the interaction between NH_4Cl and pH, while ABC described the interaction between all the investigated parameters (acetate, NH_4Cl and pH). The model F-value of 792.93 indicated that the model is significant and the curvature F-value of 12.660 also indicated that the design space has a significant curvature.

Since R-squared represents the variation in any response variable. The R-squared value of 0.9962 obtained from this study illustrated that the model described the variability of the response data around its mean. The R-square further revealed that the model fitted the data in this study. Table 6.5 gives the values from ANOVA for the model. The "Pred R-Squared" of 0.9921 is in reasonable agreement with the "Adj R-Squared" of 0.9950. The predicted combination of 3.00 g/L of acetate, 0.06 g/L of NH_4Cl and pH 7 to produced 45.9991 PHA was in agreement with experimental results which produced 46% of PHA.

Table 4. ANOVA table [Partial sum of squares]

Source	Sum of squares	DF	Mean square	F value	Prob>F
Model	2925.01	7	417.86	792.93	<0.0001
A	953.82	1	953.82	1809.96	<0.0001
B	1002.33	1	1002.33	1902.02	<0.0001
C	658.35	1	658.35	1249.29	<0.0001
AB	81.03	1	81.03	153.77	<0.0001
AC	35.77	1	35.77	67.88	<0.0001
BC	187.60	1	187.60	355.99	<0.0001
ABC	6.10	1	6.10	11.58	<0.0027
Curvature	6.67	1	6.67	12.66	0.0019
Pure Error	11.07	21	0.53		
Cor Total	2942.75	29			

Table 5 Values from ANOVA for the model

R-Squared	0.9962
Adj R-Squared	0.9950
Pred R-Squared	0.9921
Adeq Precision	88.026
Std. Dev.	0.73
Mean	26.56
C.V.	2.73
PRESS	23.28

Diagnostic plots for response (PHA)

The diagnostic plots of response of PHA is presented in Figure 2.The normal plot of residuals (NPR) shows how the data are normally distributed graphically. The graph of NPR shows not only the normality of data distribution but also the deviation of any data from the normal distribution. The experimental data from this study showed that the data are normally distributed by aligning approximately on a straight line with little deviation arising from human error. (Thus the data support the assumption of normality hence no need for further response transformation). The Outlier described how abnormal data or runs are scattered far away from other values. From the plot it can be clearly seen that the data are scattered within the border limit of ± 3.5 indicating that the all the experimental runs

are not far apart from the predicted values. The Cook's distance described the effect of each experimental runs on the model. The closer the distance of the experimental runs to other values or zero the more the effect and vice versa. From the generated cook's distance graph in this study, all the runs are situated close to zero and within the border limit of ± 0.25 as seen in figure 2. This further validated the model obtained from the study. The graph of predicted versus the actual plot illustrates that all experimental runs are scattered around the 45-degree straight line conforming a good prediction. Thus the model is well fitted to the experimental runs. Leverage plot described how the design points influence the fitness of the model. From Figure 2 the experimental runs are distributed within the stipulated borderline of 1 and 0 suggesting that none of the runs control the fitness of the model. The cook's distance and leverage plots suggested that the model developed included all the experimental responses. Figure 2 further shows the predicted values for optimum PHA accumulation

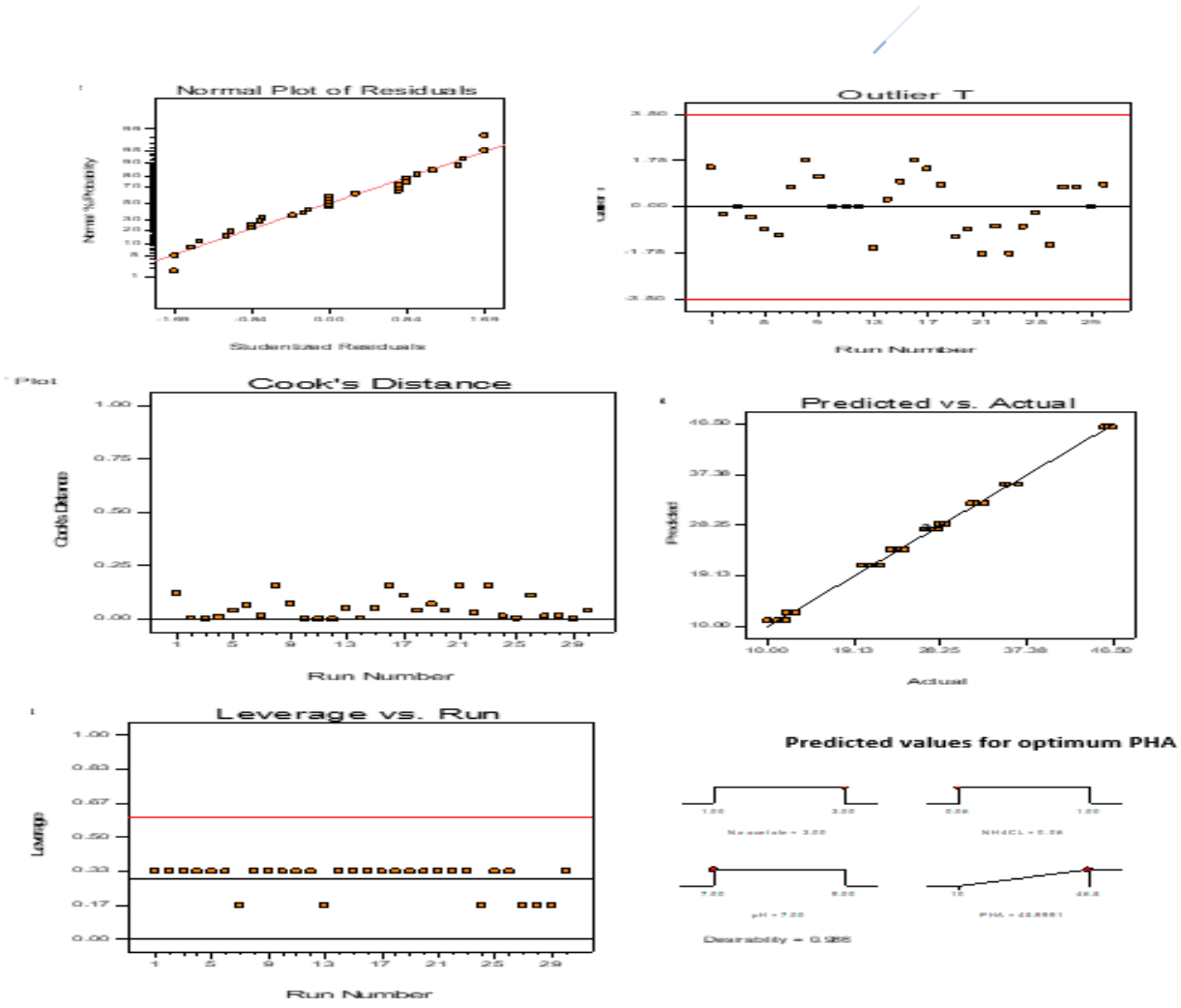

Figure 2. Diagnostic plots for response (PHA)

CONCLUSION

The study confirm the feasibility of utilizing *Rhodobacter sphaeroides* ADZ101 in producing PHA. It can accumulate high amount of PHA with different carbon and nitrogen sources. Acetate and NH_4Cl were found to accumulate higher amount of PHA compared to other carbon and nitrogen sources used in this study. The accumulation of PHA using this bacterium occurred under stress condition of excess carbon and limited nitrogen. The optimum PHA accumulation consists of the treatment combination of 3.00 g/L acetate, 0.06 g/L NH_4Cl and pH 7. Hence *Rhodobacter sphaeroides* ADZ101 is a good candidate for the production of PHA.

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