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Utilization of palm oil mill effluent and clindamycin for optimization of polyhydroxy [r] alkanoates production

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ABSTRACT

Polyhydroxyalkanoates (PHA) are storage granules of most bacteria which can be used as biodegradable plastics but the production cost of PHA is twice than petrochemical based synthetic polymers because of substrate cost. The use of alternative renewable and cheap carbon sources are the best option, one such is palm oil mill effluent (POME). POME contains carbon source like volatile fatty acids and other organic components which can be utilised by microorganisms to accumulate PHA. The use of subinhibitory concentration of antibiotics like clindamycin may have an influence on PHA accumulation. In this study, 31 organisms were isolated from POME spillage area and subjected to PHA production. Seven organisms were found to accumulate PHA, which was confirmed by Nile blue staining method, the accumulated PHA was extracted and characterized using HPLC. All the organisms were found to produced poly hydroxy butyrate (PHB). Amongst all the seven isolates, two organisms namely *Bacillus* sp and *Pseudomonas aeruginosa* were found to accumulate more PHA. Both the organisms were subjected to produce PHA in POME and clindamycin containing media. PHA production condition was optimized using RSM.

Keywords: *POME (palm oil mill effluent); PHA (Polyhydroxyalkanoates); Optimization.*

1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polyesters of most microorganism as storage material [1-3] when excess amount of carbon source is available [4], mostly at stationary phase [5]. Polyhydroxyalkanoates (PHAs) can be manipulated into flexible structures and can be used as thermoplastics and various other fields like drug delivery [6-10]. Carbon source plays a major role in accumulation pattern and type [3, 11]. Maximal PHA production depend on growth, cell type, cell density etc. even the physiochemical factors like incubation time, carbon source, temperature, pH have much influence in PHA production ([12,13]. Cost for the production of PHA using chemicals is much expensive [14]. There are reports that any carbon source could influence the storage of PHA, even the carbon sources from waste water were used for PHA accumulation [15]. Polyhydroxybutyrate (PHB) is a type of short chained PHA (scl-PHA) accumulated by most bacteria [10,16] and it can be used for producing biomaterials and nanocarriers [8,9]. Malaysia is one amongst the highest producer of palm oil [17], simultaneously 85 to 100 million tonnes of palm oil mill effluent (POME) is also produced [18], these effluents are rich in lipid, where it can be used for as carbon source for PHA production. Thus, in this study, organisms were isolated from the POME spillage area. Organisms were isolated, PHA producing organisms were identified and their PHA was characterized. The higher PHA accumulating organism was subjected to optimization of PHA production using RSM.

2. MATERIALS AND METHODS

2.1. Sample Collection and Isolation of PHA Producing Heterotrophic Bacteria.

2.1.1. Palm oil mill effluent and soil samples collection

Soil samples were obtained from POME (palm oil mill effluent) spillage area in Penang, Johor, and Selangor states of Malaysia. Samples were stored in air-tight zip-lock bags and transported to the laboratory for bacterial isolation and screening of potential PHB producing strains. Prior to use, the samples were stored in 4 ^{0}C refrigerators.

2.1.2. Isolation of heterotrophic native microflora from POME spilled soil.

1 g soil sample was serially diluted and spread plate was performed on nutrient agar media. The plates were incubated for 24 h at 37 ^{0}C. The grown colonies were streaked on sterile nutrient agar plates to obtain a pure culture. Pure culture was transferred to a sterile nutrient agar slant and stored at 4 ^{0}C until further use.

2.2. Screening and Selection of Suitable PHA Producing Strains.

Organisms were streaked on mineral salt agar media (MSM) containing (g L^{-1}) K_2HPO_4 - 0.8, KH_2P0_4 - 0.2, $CaCl_2$ - 0.05, $MgCl_2$ - 0.5, $FeCl_2$ - 0.01, NaCl - 5.0, $(NH_4)_2SO_4$ - 1.0, Glucose - 10.0, Agar - 20. Plates were incubated at 37 ^{0}C. After incubation, plates were flooded with Nile Blue, which was prepared as mentioned in earlier studies [19]. Plates were allowed to stand for 20 min with intermittent gentle shaking, allowing complete staining of colonies. Once completed, excess stain was discarded and all plates were left to air dry at room temperature. Each plate was then observed for fluorescence colony using a UV-

transilluminator. Organisms fluorescing bright orange was recorded as positive. Those positive organisms were proceeded for further studies.

2.3. Screening for highest PHA Producing Strains.

All the PHA producing organisms were inoculated into 100 mL media containing (g in 100 mL^{-1}) - K_2HPO_4 - 0.08, KH_2P0_4 - 0.02, $CaCl_2$ - 0.005, $MgCl_2$ - 0.05, $FeCl_2$ - 0.001, NaCl – 0.5, $(NH_4)_2SO_4$ - 0.1, POME – 2 mL and incubated for 48 h at 37 ^{0}C. After 48 h, each bacterial biomass was centrifuged at 10000 rpm for 5 min at 4 ^{0}C. Bacterial biomass was subjected to centrifugal washing with acetone and kept at - 20 ^{0}C for 16 h. PHA was extracted following the modified method of Samrot et al (2011). Washed mass was dried in a hot air oven at 60 ^{0}C until reaching a constant weight. The dried biomass was added with 5 mL 4% sodium hypochlorite and 5 mL chloroform, incubated in a 60 ^{0}C water bath with intermittent vortexing until the solution become clear and subjected to centrifugation at 5000 rpm for 5 min. The lower organic phase containing the PHB was transferred into separate tubes for estimation. PHB was estimated by recording OD value of obtained PHB at 235 nm after digestion with Con. H_2SO_4 in boiling water bath and correlating it with the standard graph plotted using commercial PHB [21].

2.4. Characterization of PHA.

Characterization of PHA was done as described earlier [22]. 1 g acetone dried PHA containing biomass added with 3.0 mL of 2.5 N NaOH. Tubes were sealed with Teflon membranelined caps and heated at 100 ^{0}C in in silicone water bath for an hour with intermittent vortexing. After that, the tubes were cooled to bring it to room temperature. 1 mL 0.8M Na_2HPO_4 – KH_2PO_4 buffer (pH 6.9) and 0.5 mL of 10 N HCl were then added and centrifuged at 6800 g for 12 min. The supernatant was filtered using a 0.45µm pore size PTFE Millipore membrane filters. Hydrolysed PHA was filtered and subjected to HPLC. The HPLC was equipped with a 4.6 x 250 mm column coated with polystyrene divinyl benzene sulfonic acid resin with 0.005 M sulphuric acid as eluent. Flow rate of 0.6 mL/min was maintained. Detection of repeating units derivatives of PHA was done by detecting the absorption at 210 nm and identified by comparing with purified standards of PHB and the co-polymer, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate).

2.5. Optimization using Clindamycin.

Optimization condition for PHA accumulation was done using central composite design (CCD) in Response Surface Methodology of STAT-EASE Design Expert (Ver. 9.0.4.1, Stat-Ease Corp., USA) software. Four factors and five variables were chosen, with POME as a carbon source and Clindamycin (subinhibitory concentration) as the metabolic inhibitor. The parameters were entered into the system and it constructed an experiment design with different media composition, by forming an interaction among each of the parameters, giving the best possible composition. A total of 30 sets of experiments were obtained with each sets having various composition of POME, clindamycin, nitrogen and phosphorus with the response that was performed experimentally for the concentration of POME, clindamycin, carbon and nitrogen etc. All the substance was mixed together according to each composition for the 30 sets of experiment and inoculated with the selected strains. This was then incubated for 48 h and harvested.

3. RESULTS

31 organisms were isolated from the samples obtained from various locations.

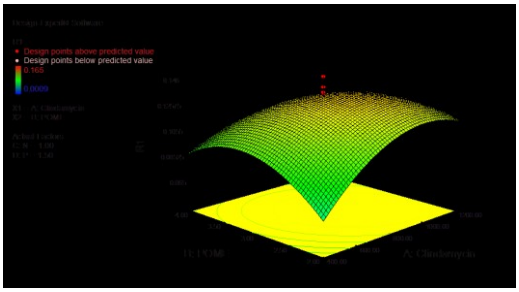

Figure 1. Graph showing the interaction of various concentrations of clindamycin and POME in response to PHB accumulation by strain PHA-11.

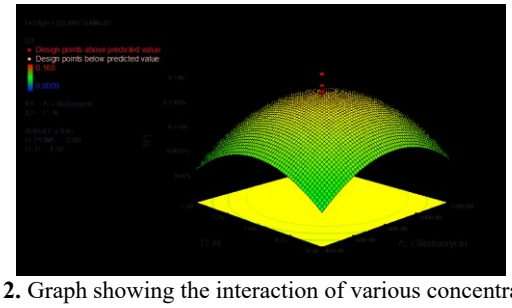

Figure 2. Graph showing the interaction of various concentrations of clindamycin and N in response to PHB accumulation by strain PHA-11.

Sample number PHA - 4, PHA - 6, PHA - 9, PHA - 11, PHA - 15, PHA - 17 and PHA - 25 were found to accumulate more PHA as they fluoresced under UV light(Table 1), for screening this is a better faster method [20]. PHA accumulated by these organisms were isolated and found to be polyhydroxy butyrate (PHB) (Results not shown).

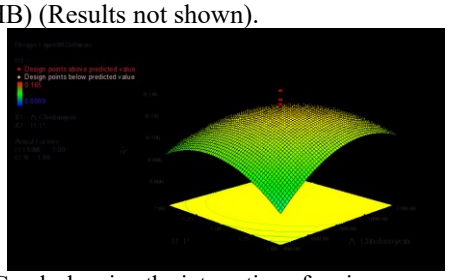

Figure 3. Graph showing the interaction of various concentrations of clindamycin and P in response to PHB accumulation by strain PHA-11.

PHB accumulation by the sample number 11 and 17 were found to be higher than the other organisms (results not shown). Sample number 11 was identified as *Pseudomonas aeruginosa* (GENBANK accession number - MK742718) and Sample number 17 was identified as *Bacillus* sp (GENBANK accession number - MK720102). Both the organisms were subjected for optimization studies using RSM.

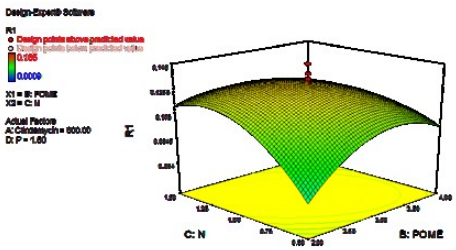

Figure 4. Graph showing the interaction of various concentrations of N and POME in response to PHB accumulation by strain PHA-11.

Table 1. Identification of PHA accumulating organisms in MSM with different C:N ratio.

Strain No.	C:N Ratio					Scoring for Positive PHA producers
	100:100	100:50	100:25	100:10	100:0	
PHA-1	-	-	-	-	-	-
PHA-2	+	+	-	-	-	+
PHA-3	-	-	-	-	-	-
PHA-4	+	+	+	+	+	+++++
PHA-5	-	-	-	-	-	-
PHA-6	+	+	+	+	+	+++++
PHA-7	-	-	-	-	-	-
PHA-8	-	-	-	-	-	-
PHA-9	+	+	+	+	+	+++++
PHA-11	+	+	+	+	+	+++++
PHA-12	-	-	-	-	-	-
PHA-14	-	-	-	-	-	-
PHA-15	+	+	+	+	+	+++++
PHA-16	-	-	-	-	-	-
PHA-17	+	+	+	+	+	+++++
PHA-19	-	-	-	-	-	-
PHA-21	-	-	-	-	-	-
PHA-22	+	-	+	-	+	+++
PHA-24	-	-	-	-	-	-
PHA-25	+	+	+	+	+	+++++
PHA-26	-	-	-	-	-	-
PHA-28	+	+	+	-	-	+++
PHA-29	-	-	-	-	-	-
PHA-30	-	-	-	-	-	-
PHA-31	-	-	+	-	+	++

Table 2. Experimental design to investigate the effect of clindamycin for optimal PHA accumulation using CCD in RSM.

Factor	Name	Units	Variables			
			Low	High	-alpha	+alpha
A	Clindamycin	ng/mL	400	1200	0	1600
B	POME	ml/10ml media	2	4	1	5
C	N	g/L	0.5	1.5	0	2
D	P	g/L	1	2	1	1

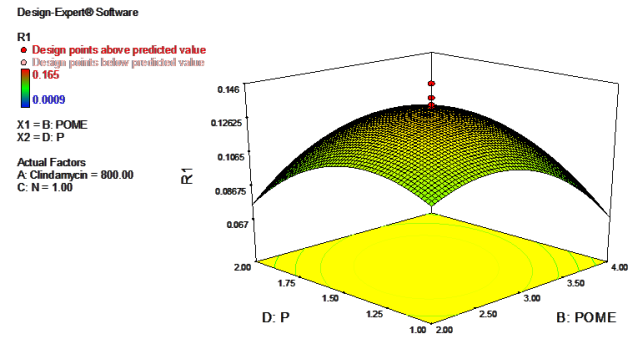

Figure 5. Graph showing the interaction of various concentrations of P and POME in response to PHB accumulation by strain PHA-11.

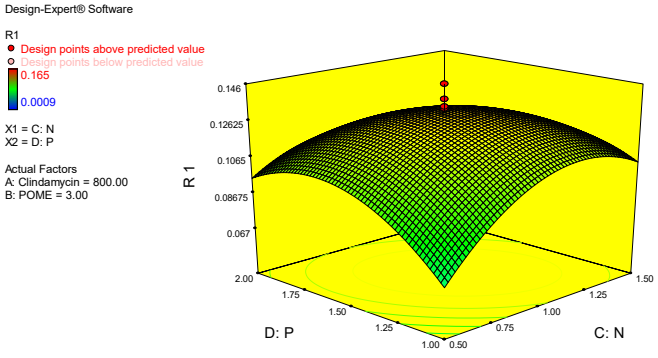

Figure 6. Graph showing the interaction of various concentrations of P and N in response to PHB accumulation by strain PHA-11.

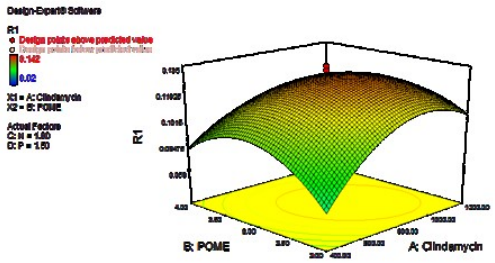

Figure 7. Graph showing the interaction of various concentrations of POME and clindamycin in response to PHB accumulation by strain PHA-17.

The Experimental design and results of the central composite design were as shown in Table 2 & 3. Strain PHA-11 was able to accumulate the highest PHA concentration of **0.165** g/L under media composition of 1200 ng/mL of Clindamycin, 2 mL / 10mL media of POME, 1.5g/L of $(NH_4)_2SO_4$ (N) and 1.0g/L of K_2HPO_4-KH_2PO_4 mixture (P/K). The statistical significance of the model equation was evaluated by F-test for analysis of variance (ANOVA), as shown in Table 4. The Model F-value of 15.76 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case linear term A, interactions AB, AD, BC, BD, CD, quadratic A^2, B^2, C^2, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 2.65 implies the Lack of Fit is not significant relative to the pure error. There is a 14.65%

chance that a "Lack of Fit F-value" this large could occur due to noise.

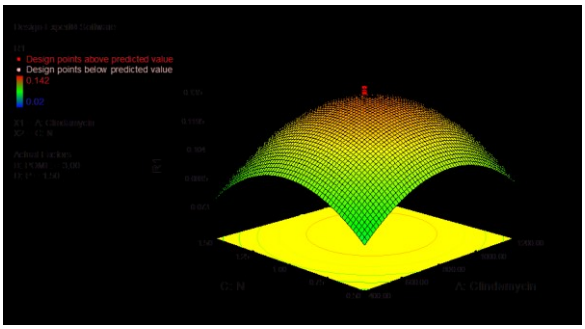

Figure 8. Graph showing the interaction of various concentrations of N and clindamycin in response to PHB accumulation by strain PHA-17.

The "Pred R-Squared" of 0.6770 is in reasonable agreement with the "Adj R-Squared" of 0.8770. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 11.935 indicates an adequate signal. Response surface graphs (Figure 1- 7) clearly show the interaction of various concentrations of studied variables.

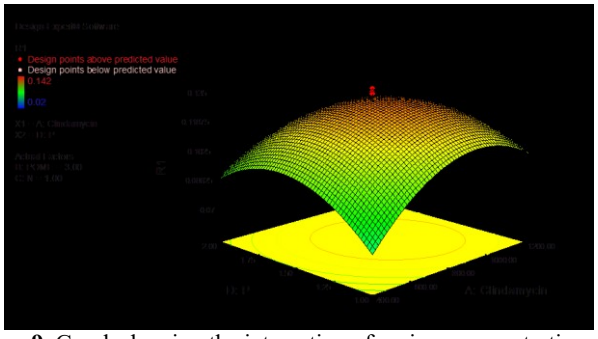

Figure 9. Graph showing the interaction of various concentrations of P and clindamycin in response to PHB accumulation by strain PHA-17.

Table 3. Concentration of PHB in response to various concentration of media composition in Strain PHA-11.

Std	Run	Factor A: Clindamycin (ng/ml)	Factor B: POME (ml/10ml media)	Factor C: N (g/L)	Factor D: P/K (g/L)	Response 1: PHB/48 h (g/L)
22	1	400	2	0.5	1	0.001
30	2	1200	2	0.5	1	0.052
11	3	400	4	0.5	1	0.021
12	4	1200	4	0.5	1	0.030
4	5	400	2	1.5	1	0.050
17	6	1200	2	1.5	1	0.165
27	7	400	4	1.5	1	0.020
14	8	1200	4	1.5	1	0.021
6	9	400	2	0.5	2	0.021
1	10	1200	2	0.5	2	0.033
28	11	400	4	0.5	2	0.082
19	12	1200	4	0.5	2	0.065
15	13	400	2	1.5	2	0.031
21	14	1200	2	1.5	2	0.056
24	15	400	4	1.5	2	0.029
13	16	1200	4	1.5	2	0.019
25	17	0	3	1	1.5	0.021
23	18	1600	3	1	1.5	0.036
8	19	800	1	1	1.5	0.021
29	20	800	5	1	1.5	0.035
16	21	800	3	0	1.5	0.021
7	22	800	3	2	1.5	0.033
20	23	800	3	1	0.5	0.029
3	24	800	3	1	2.5	0.042
2	25	800	3	1	1.5	0.133
5	26	800	3	1	1.5	0.112
26	27	800	3	1	1.5	0.146
18	28	800	3	1	1.5	0.138
9	29	800	3	1	1.5	0.127
10	30	800	3	1	1.5	0.134

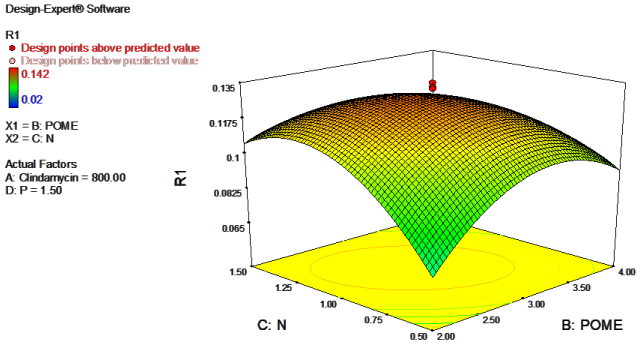

Figure 10. Graph showing the interaction of various concentrations of N and POME in response to PHB accumulation by strain PHA-17.

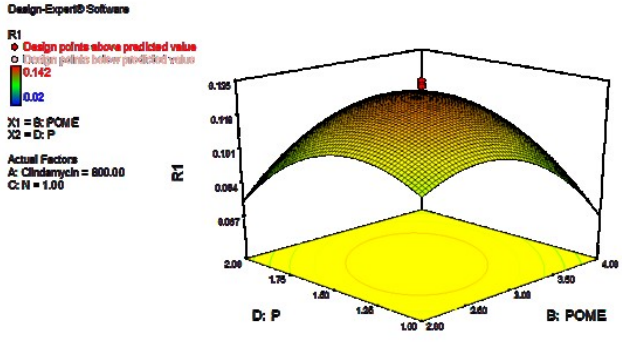

Figure 11. Graph showing the interaction of various concentrations of P and POME in response to PHB accumulation by strain PHA-17

Strain PHA-17 was able to accumulate the highest PHA concentration at 0.142 g/L under media composition of 1200 ng/mL of Clindamycin, 2 mL/10mL media of POME, 1.5g/L of $(NH_4)_2SO_4$ (N) and 1.0g/L of K_2HPO_4-KH_2PO_4 mixture (P/K) (Table 5). The statistical significance of the model equation was evaluated by F-test for analysis of variance (ANOVA), as shown in Table 6. The Model F-value of 17.74 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case linear term A, interactions AB, BC, BD, CD, quadratic A^2, B^2, C^2, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 4.64 implies there is a 5.21% chance that a "Lack of Fit F-value" this large could occur due to noise. The "Pred R-Squared" of 0.6959 is in reasonable agreement with the "Adj R-Squared" of 0.8899. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 11.964 indicates an adequate signal. Response surface graphs (Figure 8 - 12) clearly show the interaction of various concentrations of studied variables.

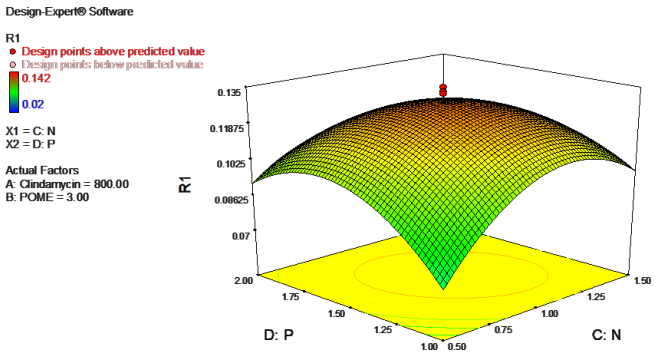

Figure 12. Graph showing the interaction of various concentrations of P and N in response to PHB accumulation by strain PHA-17.

Table 4. Analysis of variance (ANOVA) of quadratic model for PHB accumulation by Strain PHA-11.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.061373	14	0.004384	15.76329	< 0.0001 significant
A-Clindamycin	0.001946	1	0.001946	6.996716	0.0184
B-POME	0.000367	1	0.000367	1.32104	0.2684
C-N	0.000505	1	0.000505	1.816182	0.1978
D-P	1.84E-07	1	1.84E-07	0.000661	0.9798
AB	0.003028	1	0.003028	10.8872	0.0049
AC	0.00036	1	0.00036	1.294671	0.2730
AD	0.001724	1	0.001724	6.200336	0.0250
BC	0.00578	1	0.00578	20.78303	0.0004
BD	0.003303	1	0.003303	11.87829	0.0036
CD	0.002973	1	0.002973	10.69024	0.0052
A^2	0.015149	1	0.015149	54.47201	< 0.0001
B^2	0.01531	1	0.01531	55.05301	< 0.0001
C^2	0.015636	1	0.015636	56.22427	< 0.0001
D^2	0.012977	1	0.012977	46.66156	< 0.0001
Residual	0.004172	15	0.000278		
Lack of Fit	0.00351	10	0.000351	2.653878	0.1465 not significant
Pure Error	0.000661	5	0.000132		
Cor Total	0.065545	29			

Std. Dev.	0.005959		**R-Squared**	0.985533	
Mean	0.066733		**Adj R-Squared**	0.972031	
C.V. %	8.929052		**Pred R-Squared**	0.946418	
PRESS	0.001973		**Adeq Precision**	26.17638	

Table 5. Concentration of PHB in response to various concentration of media composition in strain PHA-17.

Std	Run	Factor A: Clindamycin (ng/ml)	Factor B: POME (ml/10ml media)	Factor C: N (g/L)	Factor D: P/K (g/L)	Response 1: PHB/48 h (g/L)
22	1	400	2	0.5	1	0.020
30	2	1200	2	0.5	1	0.051
11	3	400	4	0.5	1	0.022
12	4	1200	4	0.5	1	0.030
4	5	400	2	1.5	1	0.050
17	6	1200	2	1.5	1	0.142
27	7	400	4	1.5	1	0.020
14	8	1200	4	1.5	1	0.021
6	9	400	2	0.5	2	0.021
1	10	1200	2	0.5	2	0.033
28	11	400	4	0.5	2	0.075
19	12	1200	4	0.5	2	0.068
15	13	400	2	1.5	2	0.031

Std	Run	Factor A: Clindamycin (ng/ml)	Factor B: POME (ml/10ml media)	Factor C: N (g/L)	Factor D: P/K (g/L)	Response 1: PHB/48 h (g/L)
21	14	1200	2	1.5	2	0.056
24	15	400	4	1.5	2	0.029
13	16	1200	4	1.5	2	0.020
25	17	0	3	1	1.5	0.021
23	18	1600	3	1	1.5	0.036
8	19	800	1	1	1.5	0.021
29	20	800	5	1	1.5	0.036
16	21	800	3	0	1.5	0.021
7	22	800	3	2	1.5	0.032
20	23	800	3	1	0.5	0.029
3	24	800	3	1	2.5	0.041
2	25	800	3	1	1.5	0.132
5	26	800	3	1	1.5	0.114
26	27	800	3	1	1.5	0.125
18	28	800	3	1	1.5	0.132
9	29	800	3	1	1.5	0.135
10	30	800	3	1	1.5	0.133

Table 6. Analysis of variance (ANOVA) of quadratic model for PHB accumulation by strain PHA-17.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.052676	14	0.003763	17.73907	< 0.0001 significant
A-Clindamycin	0.001395	1	0.001395	6.578682	0.0216
B-POME	0.00033	1	0.00033	1.556026	0.2314
C-N	0.00021	1	0.00021	0.99027	0.3355
D-P	4.17E-08	1	4.17E-08	0.000196	0.9890
AB	0.001743	1	0.001743	8.217901	0.0118
AC	0.000264	1	0.000264	1.244958	0.2821
AD	0.00077	1	0.00077	3.630563	0.0761
BC	0.004193	1	0.004193	19.7664	0.0005
BD	0.003053	1	0.003053	14.39171	0.0018
CD	0.001828	1	0.001828	8.616288	0.0102
A^2	0.014209	1	0.014209	66.99024	< 0.0001
B^2	0.014209	1	0.014209	66.99024	< 0.0001
C^2	0.01484	1	0.01484	69.96585	< 0.0001
D^2	0.012253	1	0.012253	57.76606	< 0.0001
Residual	0.003182	15	0.000212		
Lack of Fit	0.002872	10	0.000287	4.639876	0.0521 not significant
Pure Error	0.00031	5	6.19E-05		
Cor Total	0.055857	29			
Std. Dev.	0.014564		R-Squared	0.943041	
Mean	0.056567		Adj R-Squared	0.889879	
C.V. %	25.74634		Pred R-Squared	0.695852	
PRESS	0.016989		Adeq Precision	11.96407	

4. CONCLUSIONS

Totally 31 organisms were isolated from the POME effluent spillage area. Amongst the 31 organisms, 7 organisms were found to accumulate more PHA and from the seven, two potential PHA accumulating organisms were isolated. Both the organisms were allowed to accumulate PHA in the POME and clindamycin containing medium. Both the PHA- 11 and PHA -17 i.e. *Pseudomonas aeruginosa* and *Bacillus* sp respectively were found to accumulate more PHA in the medium containing 1200 ng/mL of Clindamycin, 2 mL / 10mL media of POME, 1.5g/L of $(NH_4)_2SO_4$ (N) and 1.0g/L of K_2HPO_4-KH_2PO_4 mixture (P/K).

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