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Mathematical Modelling as a Tool to Optimize PHA Production by *Massilia spp*

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors have designed the study. Author PN carried out experimental work and performed statistical analysis. Both authors have analyzed the statistical data and performed literature study. Both the authors have read and approved the final manuscript.

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ABSTRACT

Minimum Run Resolution IV screening design was employed to study important process factors to maximize Dry cell weight (DCW) and Polyhydroxyalkanoate (PHA) production by *Massilia spp*. From screening design, it was observed that maltose, NH_4HPO_4, Na_2HPO_4 and K_2HPO_4 were found to be significantly affecting DCW and PHA production. A three-level-four-factor central composite design (CCD) was employed in combination with response surface methodology (RSM) to optimize the concentration of screened factors. The statistical analysis of results showed that maltose and NH_4HPO_4 had a positive effect whereas Na_2HPO_4, K_2HPO_4 had a negative effect on DCW and PHA production. By using RSM, optimum concentration of significant factors was found to be as follows: maltose: 30.0 g/l, NH_4HPO_4: 3.0 g/l, Na_2HPO_4: 3.0 g/l, K_2HPO_4: 3.0 g/l. Verification of the predicted value resulted into a yield of 25.5 g/l of DCW and 6 g/l of PHA.

Keywords: DCW; PHA; minimum run resolution IV; central composite design; RSM.

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1. INTRODUCTION

Synthetic polymers (plastic) are an essential part of our day to day life due to their application in numerous fields (packaging, medical, agricultural, automobiles and many more). But their non-biodegradable and xenobiotic nature poses environmental problems. Consequently, these synthetic polymers urgently need to be substituted by biodegradable polymers.

Polyhydroxyalkanoates (PHAs) are bio-polyesters, accumulated under nutrient limiting conditions (nitrogen and/or phosphorous) and excess of carbon [1]. Apart from being biocompatible and biodegradable, they possess properties similar to conventional plastic [2]. They get completely degraded into carbon dioxide and water under aerobic conditions and to methane under anaerobic conditions by microorganisms [3]. Therefore, PHAs are currently being industrially produced and have been used in a broad spectrum of end products, ranging from packaging to medical applications.

Soil microbial communities are among the most complex, diverse agents able to produce these bio-polyesters. PHA are synthesized by a wide variety of plants (Alfalfa, Arabidopsis, Oil Palm, Potato, Sugar beet, Tobacco) and microorganisms (*Pseudomonas, Alcaligenes, Bacillus, Massilia* and to name a few) [4,5,6]. The genus *Massilia* belongs to the family Oxalobacteraceae and was first described by La Scola et al. (1998). Earlier studies revealed that, *Massilia aerilata, Massilia norwichensis*, *Massilia aurea*, *Massilia humi,Massilia armeniaca* and *Massilia flava* areknown to produce PHAs [4,7]. But studies have been restricted only to optimization and characterization of the polymer. Till date scant reports are available regrading statistical optimization of fermentation medium to enhance PHA accumulation by *Massilia spp.*

Although PHAs have advantages over petroleum-based plastics, their use is limited due to higher production cost [8,9]. This can be achieved either by using less expensive substrate or by optimizing medium components for higher metabolite production [10]. Considering a number of physio-chemical variables such as temperature, pH, carbon, nitrogen, trace element solution, one factor at a time (OFAT) approach is used for optimization. This approach is time consuming; besides it assumes that process variables do not interact with each other and the response is a function of a single parameter at a time [8]. Now a days, number of statistical methods are available which take into account all possible interactions of variables while generating a response.

There are a number of screening designs available such as two factorial, Plackett-Burman and Minimum Run Resolution IV. These screening designs offer screening of several independent variables which are actually influencing response in relatively smaller number of experiments. Screening step followed by optimization tool, such as Response surface methodology (RSM) [11,12].

RSM is a collection of mathematical and statistical techniques to design experiments, build models, evaluate the effect of factors and analyze optimum concentration of variables for desired response [13].

In the present study, Minimum Run Resolution IV screening design was used for preliminary screening of essential variables which might influence metabolite production. Further those essential variables were optimized by RSM to enhance PHA production by *Massilia spp* to locate real optimum levels.

2. MATERIALS AND METHODS

2.1 Micro-organism and Growth Conditions

A rhizosphere soil isolate identified as *Massilia spp* (GenBank Accession: MH730065.1) was used in this study.

All medium components were purchased from HI-MEDIA and Chemicals were purchased from LOBA- CHEMIE.

The isolate cultivated in quarter strength nutrient broth 34(b) for 24hrs at 150 RPM at 28°C (O.D$_{530nm}$–1.0 i. e.10^8cell/ml) was used as seed for inoculation purpose in MSM.

Mineral salt medium (MSM):

1. Na_2HPO_4 – 3.32(g/l), K_2HPO_4- 2.8(g/l), NH_4HPO_4- 0.5(g/l), $MgSO_4$- 0.25(g/l), Trace element solution – 0.1 ml/l.
2. Trace element solution: $FeSO_4.7H_2O$-2.78(g/l), $MnCl_2$.4H2O- 1.98 (g/l), $CaCl_2.2H_2O$-1.67(g/l), CoSO4.$7H_2O$-2.81(g/l), ZnSO4.$2H_2O$-0.29(g/l) and $CuCl_2$.2H2O-0.17(g/l) dissolved in 0.1M HCl [14].

3. Maltose (10 g/l) used as carbon source was sterilized separately at 121°C.

Cultural conditions: Culture was grown at 28°C for 72 hrs at 150 RPM in MSM.

2.2 Statistical Design

2.2.1 Minimum run resolution IV screening design

Two level factorial experiments are factorial designs in which each factor is investigated at only two levels. It usually involves the investigation of a large number of potential factors to discover the “vital few” factors. However, in the presence of two factor interactions Minimum Resolution IV option offers accurate screening.

A total of six variables were studied: Maltose (A), Na_2HPO_4 (B), K_2HPO_4 (C), NH_4HPO_4 (D), $MgSO_4$ (E), trace element solution (F). A design of 12 experiments was formulated using Design Expert Software Version 11. Each parameter was tested at two levels, high (+1) and low (-1). Concentration range for the variables was decided on the basis of previous OFAT studies with respect to growth of the microorganism. Response was measured in terms of dry cell weight (DCW) and PHA production. Significance of the variables were identified by confidence level above 95% (i.e. $P<0.05$). The variables which gave $P < 0.05$ were selected for further optimization studies [3,15].

2.2.2 Response Surface Methodology (RSM)

RSM was used to optimize concentration of most influential variables, to maximize the response by keeping rest of the variables at a constant level. A 2^n factorial Central Composite Design (CCD) developed using Design Expert software Version 11 (Stat-Ease Corporation, USA) was used to optimize concentration of the selected variables from the screening studies. An experimental design of 30 experiments was formulated. 50 ml of MSM was prepared according to the experimental design and inoculated with 5% inoculum. All flasks were incubated at 28°C at 150 rpm. Responses were studied at the end of 72 hrs in terms of DCW (X) and PHA (Y).

The experimental results were analyzed and a regression equation was obtained for DCW and PHA. This multiple regression equation was obtained after the elimination of insignificant variables. Lack of fit obtained after analysis would determine the significance of the model. Counter plots were generated to understand the interaction of various factors. To maximize the response, the above was used to optimize the concentrations of significant variables. The combination of different optimized factors, which gave maximum response i.e maximum PHA production were tested experimentally to see the validity of model.

2.3 Extraction of PHA using Sodium Hypochlorite Digestion Method

10 ml of MSM broth grown cells were harvested by centrifugation at 8000 rpm for 15 minutes at the end of the incubation period (72 hrs). The pellet obtained after centrifugation was dried at 60°C to remove moisture. PHA extraction from dried pellet was carried out by sodium hypochlorite digestion method. Dried pellet was treated with 10 ml of sodium hypochlorite (4%) at RT for 90 minutes and subsequently centrifuged at 8000g for 15 minutes. The pellet obtained was then sequentially washed twice with distilled water and acetone: methanol (1:1). Washed pellet was then dissolved in 10 ml of hot chloroform, filtered through Whatman filter paper no-1 and filtrate was then evaporated to dryness to obtain PHA powder [16].

3. RESULTS AND DISCUSSION

3.1 Minimum Run Resolution IV Screening Design

Screening designs are often used as a starting point to understand if variables are important in the process prior to optimization study. Screening also characterizes how ‘Vital few” variables interact and individually affect the process. A total of six variables were studied, whose concentration ranges are given in Table 1.

Table 1. Range of different variables screened for minimum run resolution Factor – IV

Variables	Name	Level	
		-1	+1
A(g/l)	Maltose	5.0	30.0
B(g/l)	NH_4HPO_4	0.3	3.0
C(g/l)	Na_2HPO_4	3.0	9.0
D(g/l)	K_2HPO_4	3.0	9.0
E(g/l)	$MgSO_4$	0.1	1.0
F(ml/l)	Trace elements	0.1	1.0

Table 2. Experimental design and response of minimum run resolution Factor – IV

Run no.	A(g/l)	B(g/l)	C(g/l)	D(g/l)	E(g/l)	F(ml/l)	Observed response	
							(X) (g/l)	(Y)(g/l)
1	5	3	9	3	0.1	0.1	6.45	0.7
2	5	3	3	9	0.1	1	6.35	0.8
3	30	0.3	9	3	0.1	1	4.95	0.25
4	30	3	3	3	0.1	0.1	20.1	6.0
5	5	3	3	9	1	0.1	6.7	0.3
6	5	3	3	3	1	1	6.75	0.8
7	30	0.3	9	9	0.1	0.1	4.85	0.2
8	30	3	9	9	1	1	3.7	0.35
9	30	0.3	9	3	1	0.1	3.1	0.4
10	30	0.3	3	9	1	1	7.8	0.4
11	5	0.3	3	3	0.1	0.1	3.9	1.3
12	5	0.3	9	9	1	1	3.6	0.5

Preliminary screening design for the selected variables and response obtained in terms of X and Y shown in Table 2 and following results were obtained:

Positive effect: Maximum DCW (20.1 g/l) and PHA (6.0 g/l) production was achieved in run no 4. It was also observed that when maltose concentration decreased there was decrease in DCW (6.75 g/l) and PHA (0.8 g/l) production (Run 6). This indicates that maltose had a positive effect on growth as well as PHA production. According to Khanna et al.[3], *Ralstonia eutropha* produces highest amount of PHA (6.44 g/l) when medium was supplemented with high fructose concentration (40 g/l). These results suggest that fructose had positive effect on growth and PHA production [3].

Negative effect: Run no 11 shows that decrease in NH_4HPO_4 concentration further decreases DCW (3.9 g/l) but increases PHA (1.3 g/l) production. This can be explained by the fact that excess carbon or limiting nitrogen/phosphorous source is required for maximum PHA production. When either of the phosphate concentration i.eNa_2HPO_4or K_2HPO_4increased in the medium, a decrease in X and Y production was observed (run nos.1, 2,3,5,7, 8, 9, 10, 12).This indicated that Na_2HPO_4 or K_2HPO_4 had a negative effect on DCW and PHA production. According to Johar et al. [15], PHA production by *Comamonassp* EB172 was influenced by $(NH_4)_2SO_4$concentration. Increase in PHA content (13.21 %) was reported when medium was supplemented with low concentration of $(NH_4)_2SO_4$ (0.5 g/l) [15]. B. A. Zahra et al., [17] observed increase in PHA production by *Methylobacterium extorquens* DSMZ 1340, when medium was deficient in nitrogen (NH_4NO_3) and phosphorus (Na_2HPO_4 or K_2HPO_4) [17]. These studies suggest that complete absence of nitrogen led to reduction in DCW as well as PHA content.

Table 3. ANOVA results (P-Value) for the effect of medium component on DCW and PHA production

Factors	Name	P Value	
		X	Y
A(g/l)	Maltose	0.0111	0.0284
B(g/l)	NH_4HPO_4	0.0178	0.0379
C(g/l)	Na_2HPO_4	0.0108	0.0291
D(g/l)	K_2HPO_4	0.1224	0.0499
E(g/l)	$MgSO_4$	0.0676	0.0570
F(ml/l)	Trace Element	0.1358	0.0756

When considering PHA production, maltose, NH_4HPO_4, Na_2HPO_4 and K_2HPO_4 had p-value < 0.05 indicating that the variables are significant.

When considering DCW, p-value of K_2HPO_4 was found to be >0.05 indicating that these are insignificant. $MgSO_4$ and trace element solution had p-value >0.05 for both responses i.e X and Y, making them insignificant. Hence lower limit of these factors was considered as their optimum value.

Although $K_2HPO_4$was insignificant w.r.t DCW production it was found to be significant for PHA production. Hence maltose, NH_4HPO_4, Na_2HPO_4 and K_2HPO_4 were considered for further optimization studies (Table 3).

3.2 Response Surface Methodology (RSM)

3.2.1 Central composite design

From previous screening design, four variables (maltose, NH_4HPO_4, Na_2HPO_4, K_2HPO_4) were selected to determine optimum concentration and to maximize DCW and PHA production for Central composite design (CCD).

Thehighest amount of DCW (25.5-g/l) and PHA (6- g/l) was observed (run no 17) when highest amount of carbon (maltose) and lowest amount of phosphate (K_2HPO_4 and Na_2HPO_4) present into the medium. Run no 21 shows that in the absence of nitrogen source lowest amount PHA (0.2 g/l) production was observed. This could be due to the fact that complete absence of nitrogen is inhibitory to PHA production. Run no 25 shows that no PHA production was observed in the absence of maltose, indicating that a carbon source is required as an energy source. Run no 24 and 28 shows inhibition of DCW as well as PHA production when highest amount of phosphate present in the medium (Table 4).This result indicates that increase in PHA accumulation is favored under phosphate limiting conditions. These results are in agreement with Singh et al. [13], where he reported enhanced production of co-polymer when growth was restricted due to unavailability of phosphorous [13]. Studies carried out by Lee et al. [18] and Panda et al. [19] also suggest that rather than complete nitrogen or phosphorous deficiencies, limited concentration of nitrogen or phosphorous was found to be essential for enhanced PHA accumulation [18,19].

Table 4. Central Composite Design (CCD) representing effect of significant variables on DCW and PHA production

Run. No	A(g/l)	B(g/l)	C(g/l)	D(g/l)	Response	
					X (g/l)	Y (g/l)
1	30	0.3	9	9	4.2	0.1
2	30	3	9	3	8.6	0.1
3	5	3	3	3	5.3	0.5
4	5	0.3	9	3	4.9	0.4
5	17.5	1.65	6	6	15	1.9
6	30	3	3	9	20	2.4
7	5	0.3	3	9	5.2	0.8
8	17.5	1.65	6	6	7.3	1.6
9	30	0.3	3	3	10.8	0.8
10	5	3	9	9	3.8	0.1
11	17.5	1.65	6	6	11.8	1.8
12	30	0.3	3	9	7.1	0.6
13	5	0.3	9	9	2.9	0.6
14	5	3	9	3	6.2	0.6
15	5	0.3	3	3	3.9	1.4
16	5	3	3	9	8	0.3
17	**30**	**3**	**3**	**3**	**25.5**	**6**
18	17.5	1.65	6	6	17.8	2.2
19	30	0.3	9	3	4.8	0.8
20	30	3	9	9	8	0.6
21	**17.5**	**-1.05**	**6**	**6**	**3.8**	**0.2**
22	17.5	1.65	0	6	14	2.4
23	17.5	1.65	6	6	11.7	1.9
24	**17.5**	**1.65**	**6**	**12**	**8.3**	**0.3**
25	**-7.5**	**1.65**	**6**	**6**	**1.4**	**0**
26	17.5	1.65	6	6	12	1.9
27	42.5	1.65	6	6	12.1	1.7
28	**17.5**	**1.65**	**12**	**6**	**4.2**	**0.4**
29	17.5	1.65	6	0	12.1	1.8
30	17.5	4.35	6	6	12.3	1.7

3.2.2 Regression analysis

Regression analysis of experimental design demonstrated that linear model terms (A, B, C), quadratic model terms (A^2, B^2, C^2) and the interactive model term (AC) were highly significant (i.e. $P< 0.05$) for DCW production (Table 5A). However, other terms were found to be insignificant for DCW production (i.e. $P > 0.05$). After applying multiple regression analysis, the results fitted to a second order polynomial equation.

Thus, mathematical model for DCW production (X) fitted in terms of coded factors as follows:

$$X=+2.50+0.3980A+0.2798B-0.2788C-0.0807D+0.1174AB-0.1497AC-0.0346AD-0.00562BC+0.0248BD-0.0786CD-0.2743A^2-0.1474B^2-0.1187C^2-0.0518D^2 \quad (1)$$

The regression analysis of experimental design demonstrated that linear model terms (A,B,C,D), quadratic model terms (A^2,B^2,C^2,D^2) and interactive model term (AB, AC,BC,AD,BD,CD,ABC,ACD,BCD) were highly significant (i.e. $P < 0.05$) for PHA production (Table 5A). After applying multiple regression analysis, the results were fitted to a third order polynomial equation i.e. cubic model. High level of interaction was found among all four variables indicating aliasing. Thus, mathematical model for PHA production (Y) fitted in terms of coded factors as follows:

$$Y=+1.88+0.4208A+0.3375B-0.5625C-0.3375D+0.5312AB-0.4312AC-0.1812AD-0.3812BC-0.1563BD+0.2562CD-0.2677A^2-0.2427B^2-0.1302C^2-0.2177D^2-0.5187ABC+0.1937ACD+0.2187BCD \quad (2)$$

The goodness of fit of model was checked by determination of coefficient (R^2).

- DCW production: R^2 value was found to be 0.9170. This indicates that 91.70% of total variability in the response could be explained by this model. Adjusted R^2 was found to be 0.8277.
- PHA production, R^2 value was found to be 0.9846. This indicates that 98.46% of total variability in the response could be explained by this model. Adjusted R^2 was found to be 0.9585.

Table 5. A) Summary of model terms B) ANOVA analysis for DCW and PHA production by *Massilia spp*

Variables	**P value**	
	X	**Y**
Model	< 0.0001	< 0.0001
A	< 0.0001	< 0.0001
B	0.0003	< 0.0001
C	0.0003	< 0.0001
D	0.1762	< 0.0001
AB	0.1130	< 0.0001
AC	0.0494	< 0.0001
AD	0.6250	0.0145
BC	0.4307	0.0001
BD	0.7255	0.0292
CD	0.2761	0.0019
A^2	0.0002	0.0002
B^2	0.0152	0.0004
C^2	0.0424	0.0196
D^2	0.3442	0.0009
ABC	-*	< 0.0001
ACD	-*	0.0103
BCD	-*	0.0052

** Interaction with p-value > than 0.05 were eliminated in CCD to fit model*

	X	Y
Lack of Fit	0.7833 **(not significant)**	0.3701 **(not significant)**
Coefficient of determination (R^2)	0.9170	0.9846
(Adjusted R^2)	0.8277	0.9585

For both responses (X & Y), large difference between R^2 and adjusted R^2 may be due to large block difference while performing the experiment. However still it confirms the satisfactory adjustment of model with the given data (Table 5B).These results are further correlated with contour plots and 3D response plot generated to confirm the fitness of model with the given data.

3.2.3 Contour plots

It was observed that when Na_2HPO_4 and K_2HPO_4 were varied from higher to lower value, they were found to be meeting at a point. Above and below that there was decrease in DCW as well as PHA production(Fig 1 A & B).

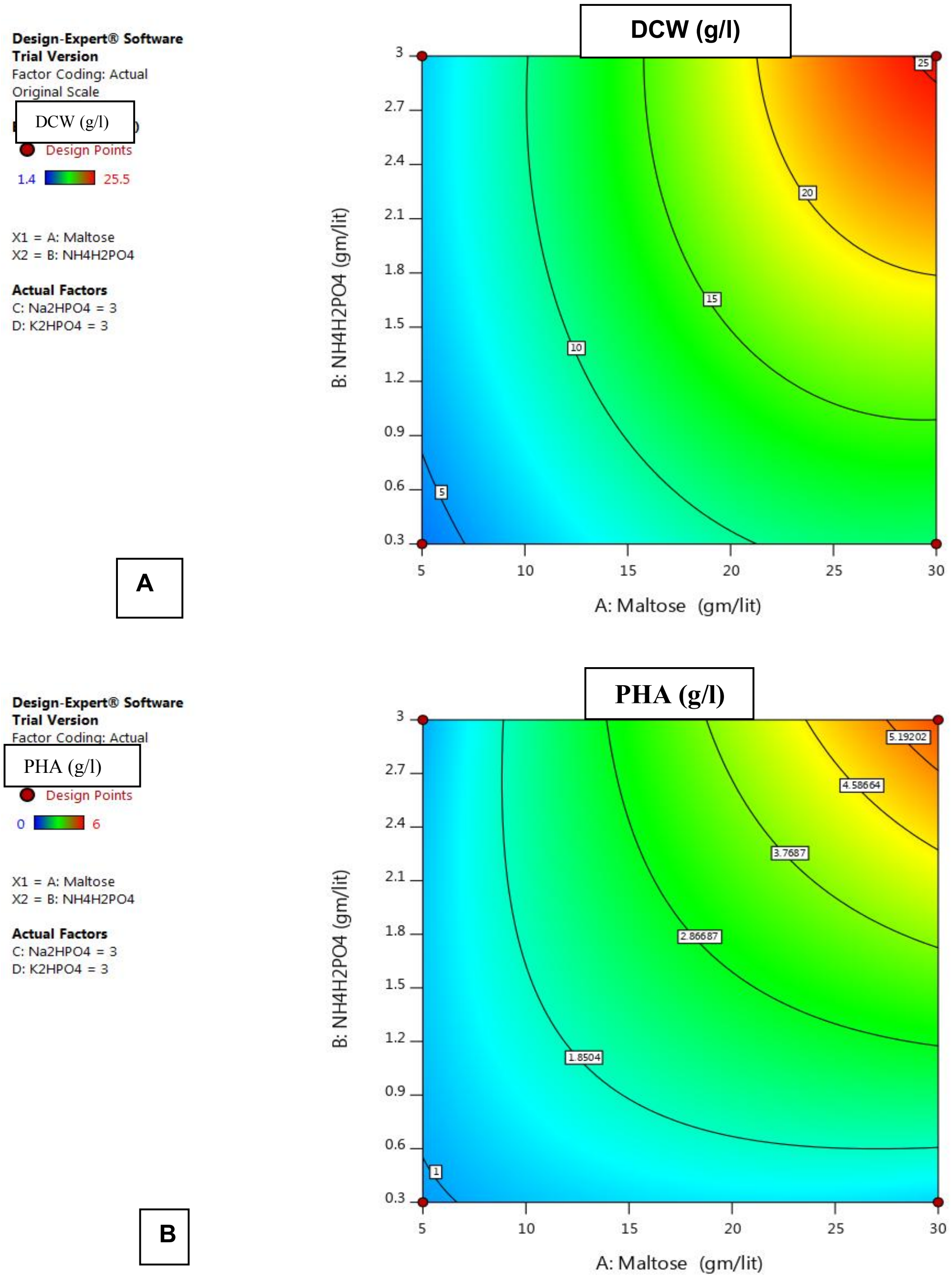
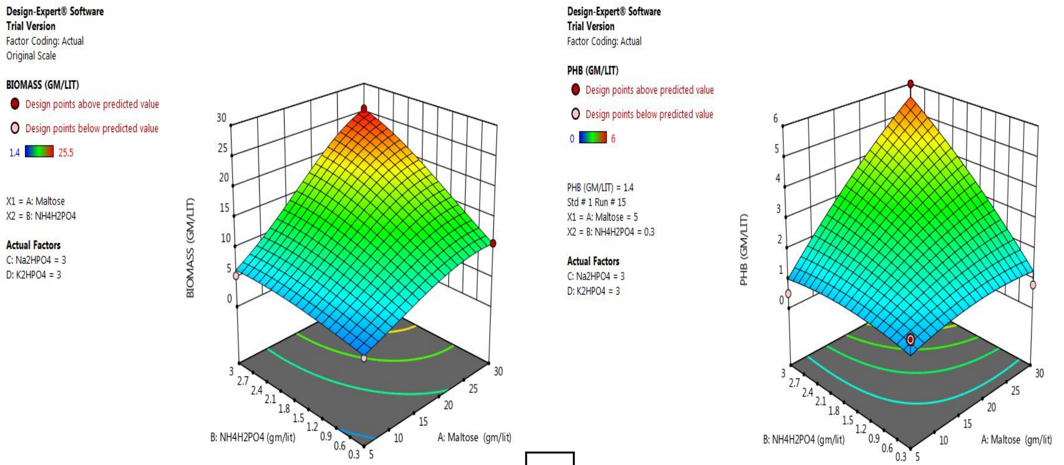


Fig. 1. Contour plots between Maltose and NH_4HPO_4 for response A) DCW, B) PHA

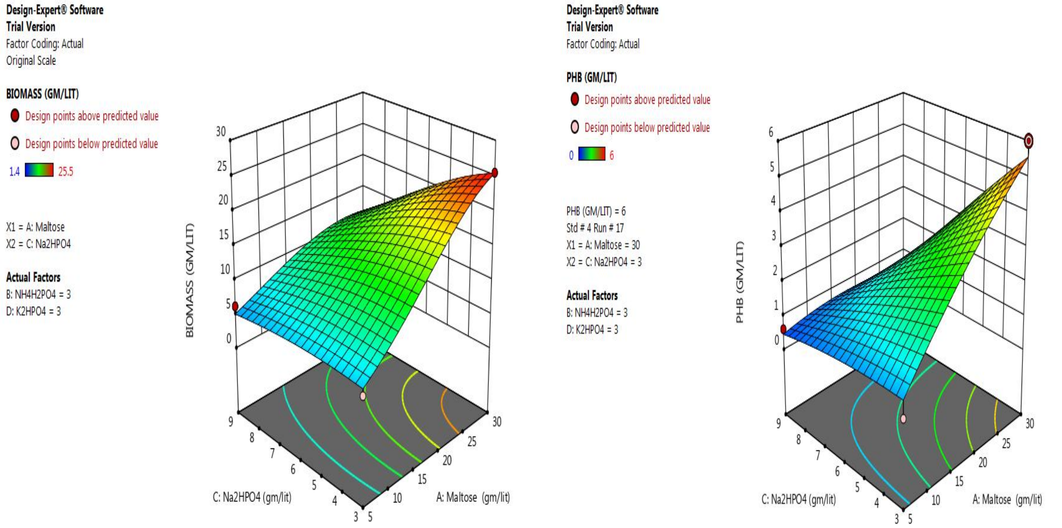
Therefore, converging values for Na_2HPO_4 and K_2HPO_4were found to be 3.0 g/l for both. The contour plots clearly indicate that if upper limit of maltose (30 g/l) and NH_4HPO_4 (3 g/l) would have increased, and then contour would have converged at a point.

The 3D plots between various factors were generated and optimum concentrations of medium components were found. Interactive effects of varied maltose and NH_4HPO_4concentrations at minimal level of Na_2HPO_4 (3.0 g/l) and K_2HPO_4 (3.0 g/l) demonstrated that both DCW and PHA production increased with increase in the level of maltose (30 g/l) and NH_4HPO_4 (3.0 g/l). This indicates that both the variables have positive effect on DCW as well as PHA production (Fig. 2A).

The interaction between maltose and Na_2HPO_4 was found to be significantly affecting DCW as well as PHA production. Although increasing concentration of maltose was found to be having positive effect on DCW as well PHA production, increasing concentration of Na_2HPO_4 was found to be having negative effect on DCW as well as PHA production. At highest concentration of Na_2HPO_4 (9.0 g/l) decrease in DCW as well as PHA production was observed (Fig. 2B). Similar results were obtained when interactive effect between maltose and K_2HPO_4 was studied. K_2HPO_4 (9.0 g/l) has shown negative effect on DCW as well as PHA production (Fig. 2C).



A



B

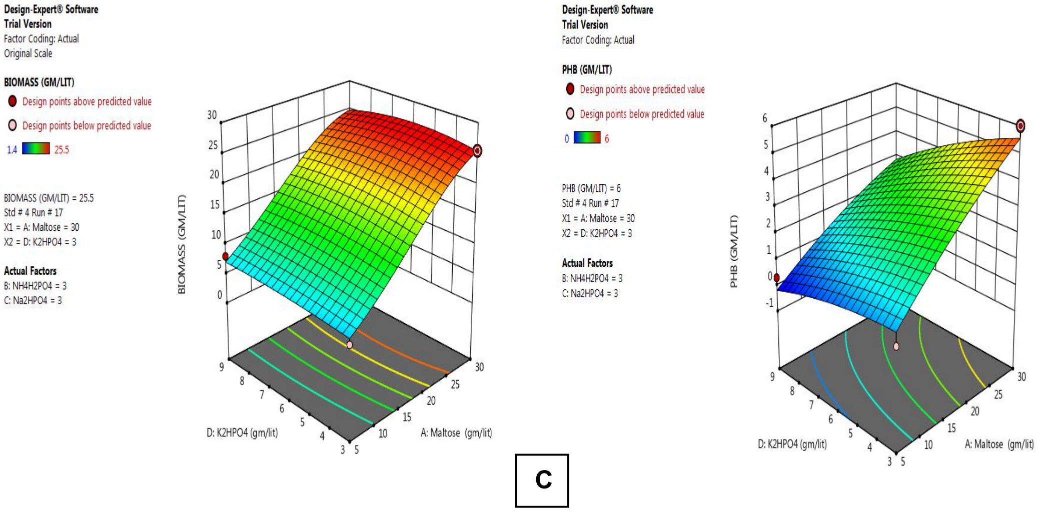


Fig. 2. Three dimensional (3D) response surface curves showing interactive effect of various components on DCW and PHA productionA) Maltose and NH_4HPO_4 B) Maltose and Na_2HPO_4 C) Maltose and K_2HPO_4

Table 6. PHA yield before and after optimization of nutritional parameters

	Maltose (g/l)	**NH_4HPO_4 (g/l)**	**Na_2HPO_4 (g/l)**	**K_2HPO_4 (g/l)**	**DCW**		**PHA**	
					A (g/l)	**B (g/l)**	**A (g/l)**	**B (g/l)**
Before optimization	10	0.5	3.32	2.8	20	-	2.0	-
After optimization	30	3.0	3.0	3.0	25.5	20.0	6.0	5.85

A – Experimental B- Predicted

Thus, optimum concentration of medium component to maximize response (X & Y) was found to be as follows: maltose: 30.0 g/l, NH_4HPO_4: 3.0 g/l, Na_2HPO_4: 3.0 g/l, K_2HPO_4: 3.0 g/l, $MgSO_4$: 0.25 g/l, trace element solution: 0.1 ml.

Experiments were performed in triplicate using optimized conditions to verify the model. It was observed that experimental (6.0g/l) and predicted (5.85g/l) values for maximum PHA production were in good agreement (Table 6). Zafar et al. [20] reported a difference of 1.3 g/l between the actual and predicted PHA concentrations using the RSM statistical tool. Using statistical tool, Jong et al. [21] reported a difference of 0.7% between actual and predicted PHA concentration by *Massilia spp* [20,21].

4. CONCLUSION

Minimum Run Resolution IV was found to be suitable screening design for preliminary screening of essential variables in relatively smaller number of experiments in order to maximize DCW and PHA production by *Massilia spp*. It also helped to study interactions between the process variables. Maltose, NH_4HPO_4, Na_2HPO_4 and K_2HPO_4 were found to be significantly affecting DCW and PHA production. Response Surface Methodology (RSM) proved to be a powerful statistical tool in optimizing the screened variables to maximize response. Optimized concentration of screened factors obtained by applying RSM, consisted of - Maltose: 30.0 g/l, NH_4HPO_4: 3.0g/l, Na_2HPO_4: 3.0g/l, K_2HPO_4: 3.0g/l, $MgSO_4$: 0.25g/l, trace element solution: 0.1ml. Optimizing medium components by applying Central Composite Design (CCD) has significantly increased PHA production from 2.0 g/l (before optimization) to 6.0 g/l (after optimization).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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