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APPLICATION OF RESPONSE SURFACE METHODOLOGY (RSM) TO ENHANCE MCL-PHA COPOLYMER PRODUCTION BY *MASSILIA* SP USING CANE MOLASSES AS A CARBON SOURCE

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ABSTRACT

Cane molasses is an inexpensive, sustainable by-product of the sugar industry, which supports the growth of microorganisms as it contains several growth factors. Response Surface Methodology (RSM) employing central composite design (CCD) was applied to enhance PHA production by *Massilia* sp at shake flask level by using molasses as a carbon source. Isolate produced maximum DCW (54.0 g/l) and PHA (3.0 g/l) when medium was optimized with the help of CCD. Optimized concentration of medium components to maximize PHA production was found to be as follows: molasses: 100.0 g/l, NH_4HPO_4: 1.67g/l, Na_2HPO_4: 5.86g/l, K_2HPO_4: 5.74g/l, $MgSO_4$: 0.25g/l, trace element solution: 0.1ml/l. The point optimization method reported a small difference of 0.02g/l, indicating the validity of mathematical models developed after applying CCD.

Keywords: Cane molasses, Central Composite Design, *Massilia* sp, DCW, PHA.

1. INTRODUCTION

Natural polymers (polysaccharides, polyamides, proteins, polyhydroxyalkanoates) are biodegradable in nature. Thus, they have attracted much attention in recent years as replacement for petroleum-based polymers. Polyhydroxyalkanoates (PHAs) have gained major attention because, apart from being completely biosynthetic and biodegradable, they are also biocompatible. They are intracellular storage compounds accumulated as energy reserve under excess of carbon and nutrient limiting conditions (nitrogen and/or phosphorous)[1].

The genus *Massilia*, member of the family Oxalobacteraceae is a common inhabitant of rhizosphere soil and reported to produce PHA [2]. Cerrone *et al.* (2011) showed that *Massilia lutea* produced maximum PHA when medium was supplemented with starch as a carbon source [3]. Bassas-Galia *et al.*(2012) reported that among all eleven strains of *Massilia* isolated from rhizosphere soil, strain 4D6 produced maximum PHA when grown on glycerol containing medium [4].All these studies have been restricted to use of pure carbon source for PHA production. Very few studies have shown utilization of agro-waste for PHA production by *Massilia* sp.

The use of inexpensive and renewable carbon substrates can contribute to as much as 40-50% reduction in the overall production cost. One such carbon source is molasses which is an inexpensive, sustainable by-product of sugar industry. Molasses contains approximately 30% of sucrose, vitamins, minerals, amino acids and proteins. Therefore, molasses can strongly support the growth of microorganisms. Molasses normally sells for about 33 to 50% of the cost of glucose and found to be most commonly used carbon source for PHA production. Gomaa *et al.*(2013) have used acid treated cane molasses as a carbon source to maximize PHA (5.30 g/l) production by *Bacillus subtilis* [5]. Similarly, Naheed *et al.* (2014) also reported use of sugarcane molasses as a carbon source to maximize PHA production by *Enterobacter* sp [6].

The potential of polyhydroxyalkanoates to replace conventional plastic on industrial scale requires process/medium optimization at shake flask level, which can be scaled up thereafter. The non- statistical one factor at a time (OFAT) approach optimizes conditions systematically, by adding or deleting medium components, without considering any interactions. On the other hand, use of statistical approach for optimization not only allows quick screening, but also studies the role of each component [7]. The primary goal of statistical designs are to perform experiments at shake flask level, collect and analyse the data, so as to obtain valid results with minimum efforts, time and resources (Design Expert Software Version 11).

Response Surface Methodology (RSM) is the most commonly used statistical technique which helps to determine design factor settings, to improve or optimize the performance or response of a process or product. It combines design of experiments, regression analysis and optimization methods as a general-purpose strategy to optimize the expected value of a stochastic response. RSM employs experimental factorial design such as Central Composite Design (CCD) to optimize process parameters which helps to study role of each component as well as interactions among them [8, 9]. Singh *et al.* (2009) employed a five-level-four factor central composite rotary design (CCRD) to optimize the concentration of process variables for copolymer production by *P. aeruginosa* (MTCC 7925) [10]. Penkhrue *et al.* (2020) also used CCRD for optimizing fermentation medium to enhance PHA production by *Bacillus drentensis* (BP17) [11].

In this study, RSM was used for optimization of fermentation medium at shake flask level using molasses as a carbon source to maximize PHA production by *Massilia* sp.

2. MATERIAL AND METHODS

All medium components were purchased from Hi-Media laboratories, Mumbai. Analytical grade chemicals were purchased from LOBA- Chemie Pvt Ltd (India).

2.1. Microorganism used

Massilia sp isolated from rhizosphere soil (GenBank Accession number- *Massilia sp* 01PN MH730065.1) was used in the study. The isolate cultivated in quarter strength nutrient broth 34(b) for 24hrs at 28°C (O.D 530nm-1.0 i. e.10^8cell/ml) was used as seed for inoculation purpose in Mineral salt medium (MSM).

2.2. 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), $CoSO_4.7H_2O$-2.81(g/l), $ZnSO_4.2H_2O$-0.29(g/l) and $CuCl_2.2H_2O$-0.17(g/l) dissolved in 0.1M HCl [12].
3. Molasses (10 g/l) was hydrolyzed, sterilized separately and used as a carbon source.

2.3. Acid hydrolysis of molasses

The present *Massilia* sp was unable to utilize sucrose as a carbon source, thus molasses solution was further acid hydrolysed in order to convert it into reducing sugar. Molasses was diluted with distilled water in order to obtain 10% v/v total molasses solution. pH of molasses solution was adjusted to 3.0 with the help of 1N H_2SO_4. The liquid was allowed to stand for 24 hours at RT and then centrifuged at 5000 rpm for 25 mins. The pH of collected supernatant was adjusted to 7.5 with 1N NaOH and sterilized at 121°C for 10 mins.

The dinitro-salicylic acid (DNSA) method (Miller 1959) was used to measure the reducing sugar concentration in molasses [13].

2.4. 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. Earlier screening studies showed that out of total six factors maltose, NH_4HPO_4, Na_2HPO_4, K$_2$HPO4 were significantly affecting DCW (dry cell weight) and PHA production ($P<0.05$) [14]. In present study, molasses was replaced as a carbon source in place of maltose.

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. Table 1 depicts concentration ranges for all four variables which were included in design.

Table 1: Range of different variables included in CCD

Variables	Name	Level	
		Low (-1)	High (+1)
A(g/l)	Molasses	20	180
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

An experimental design of 30 experiments was formulated and performed into three blocks namely I, II and III. 50 ml of MSM was prepared according to the experimental design and following optimized conditions were maintained in MSM: Inoculum size -5%, temp-

28°C, pH-7.5, agitation - 150 rpm, incubation time - 72 hours. Responses were studied at the end of 72 hrs in terms of DCW (X) and PHA (Y).

The experimental results were analysed and a regression equation was obtained for DCW(X) and PHA(Y). 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. 3D 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 (maximum PHA) production were tested experimentally using a special feature of software called 'point prediction' to check the validity of model [10].

2.5. Extraction of PHA using sodium hypochlorite digestion method

Ten ml of MSM broth grown cells were harvested by centrifugation at 8000 rpm for 15 minutes at the end of the incubation period (72 hours). 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 room temperature for 90 minutes and subsequently centrifuged at 8000 rpm 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 film [15].

3. RESULT AND DISCUSSION

Utilization of pure carbon source for PHA production is costly. Therefore, when alternatively, cheaper carbon source is used, it reduces cost of PHA production substantially. Previous OFAT study showed that, when medium was supplemented with hydrolysed molasses along with NH_4HPO_4, Na_2HPO_4 & K_2HPO_4, isolate produced 2.04 g/l of PHA [16].These findings suggested that isolate was able to utilize hydrolysed molasses as a carbon source.

3.1. Central Composite Design

Three level-four factor CCD was applied to find out optimized concentration of molasses along with other medium components.

Table 2 depicts CCD representing effect of molasses on DCW and PHA production. Following results were generated

3.1.1. Role of carbon source

Run no 29 showed that no growth was observed in the absence of molasses. Similarly, when medium was supplemented with low amount of molasses (20 g/l), it significantly affected DCW and PHA production (run 9 &11). This indicates that a carbon source is required as an energy source. When concentration of molasses increased in medium (180 g/l), decrease in DCW was observed which in turn decreased PHA production (run 6, 16 & 25). This is due to the fact that isolate does not have ability to tolerate such a high concentration of substrate. Molasses being a crude carbon source, once again it was needed to optimize molasses concentration, so the amount of carbon provided in the medium is equivalent to maltose in the well-defined MSM medium. Gomaa *et al.* (2014) reported that *B. subtilis* and *E. coli* produced maximum PHA when medium was supplemented with 60 g/l and 80 g/l of molasses respectively [5].

3.1.2. Role of nitrogen source

Run no. 24 showed that complete absence of nitrogen from medium led to decrease in DCW (4.8 g/l) and PHA (0.2 g/l) production. This could be due to the fact that complete absence of nitrogen does not support the growth of organism. Although some amount of nitrogen is provided by molasses, it is not enough to maximize growth and PHA production. Thus, additional nitrogen needs to be provided to increase biomass. Run 27 showed that when the amount of nitrogen increased in the medium due to added NH_4HPO_4 (3.0 g/l), it significantly decreased DCW and PHA production. Similar results were recorded by Dey *et al.* (2017) where he observed that *Cupriavidus necator* accumulated maximum PHA, when concentration of $(NH_4)_2SO_4$ decreased from 3.35 g/l to 2.25 g/l [17]. This indicates that nitrogen limitation was an important factor to be considered for PHA production during optimization. This was further done by optimizing C:N ratio.

In previous CCD, when maltose was used as carbon source, concentration of NH_4HPO_4 required in the medium was 3.0 g/l. Also, *Massilia* sp produced maximum amount of PHA when C:N ratio was maintained at 10:1 (14). However, when molasses was used as a carbon source, it was observed that isolate had produced maximum PHA (3.03g/l) when molasses was provided at a concentration of 100 g/l and NH_4HPO_4 at a concentration of 1.67 g/l. Here, molasses does provide some amount of nitrogen, thus required nitrogen concentration was reduced from 3.0 g/l to 1.67 g/l. Thus, C:N ratio of 60:1 needs to be maintained to maximize PHA production when molasses was used as a carbon source. The concentration of molasses in the medium is equivalent to pure carbon source to produce maximum PHA.

Table 2: CCD representing effect of variables on DCW and PHA

Block	Run No	A(g/l)	B(g/l)	C(g/l)	D(g/l)	OR		PR	
						X(g/l)	Y(g/l)	X (g/l)	Y (g/l)
I	1	100	1.65	6	6	54.8	3	52.26	2.86
	2	20	0.3	9	3	6.5	0.1	5.20	0.16
	3	20	0.3	3	9	10.8	0.3	6.97	0.28
	4	180	3	3	9	10.7	0	12.07	0.13
	5	180	0.3	9	9	8.9	0	4.49	0.13
	6	180	0.3	3	3	12.8	0.2	15.95	0.11
	7	100	1.65	6	6	50	2.9	52.26	2.86
	8	20	3	9	9	7.5	0.2	4.42	0.18
	9	20	3	3	3	5.9	0.1	10.39	0.16
	10	180	3	9	3	7.5	0.1	11.40	0.01
II	11	20	0.3	3	3	4.4	0.1	8.29	0.15
	12	20	0.3	9	9	5	0	2.78	-0.01
	13	180	0.3	3	9	6.8	0.6	8.33	0.48
	14	20	3	3	9	5.6	0.5	8.81	0.48
	15	100	1.65	6	6	54.8	3	51.56	3.30
	16	180	3	3	3	13.5	0.6	17.14	0.65
	17	180	3	9	9	7.7	0.5	5.23	0.48
	18	20	3	9	3	4.4	0.2	4.29	0.15
	19	180	0.3	9	3	12.2	0.1	10.41	0.15
	20	100	1.65	6	6	54	3	51.56	3.03
III	21	100	1.65	12	6	6	0.4	12.49	0.38
	22	100	1.65	6	6	53.2	2.9	56.23	3.02
	23	100	1.65	6	12	10.5	0.2	16.21	0.18
	24	100	-1.05	6	6	4.8	0.2	8.04	0.18
	25	260	1.65	6	6	10.4	0.7	8.69	0.68
	26	100	1.65	0	6	30.4	0.3	22.42	0.28
	27	100	4.35	6	6	15.6	0.2	10.87	0.18
	28	100	1.65	6	0	30.9	0.6	23.71	0.58
	29	-60	1.65	6	6	0	0	0.2217	-0.01
	30	100	1.65	6	6	53.3	3	56.23	3.02

OR-Observed response PR- Predicted response

3.1.3. Role of phosphate

Run no 21 and 23 showed inhibition of DCW as well as PHA production when high amount of phosphate (12 g/l) was present in the medium. This indicates excess of phosphate is inhibitory for growth. Run 26 & 28 showed that absence of either of phosphate in the medium significantly limit the growth of *Massilia*. This indicates importance of phosphate in various cellular mechanisms. Similarly, when concentration of each phosphate source was increased to 9 g/l, PHA production decreased drastically. Maximum growth (DCW) and PHA was observed when medium was supplemented with total of 12 g/l of phosphate. Studies carried out by Lee *et al*. and Panda *et al*. 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].

Massilia sp produced the maximum amount of DCW and PHA when median amount (100g/l) of carbon (molasses), nitrogen (1.65 g/l) and phosphate (6 g/l) (K_2HPO_4 and Na_2HPO_4) were present in the medium.

Evangeline *et al.* (2020) used CCD to optimize the concentration of cultural parameters to maximize PHA production by *Bacillus cereus* and PHA production was reported to increase up to 1.42 g/l [20]. Similarly, Bozorg *et al.* (2015) applied 2^n factorial central composite design to increase PHA production by *Ralstonia eutropha*. After optimizing, PHA concentration increased to 1.63 g/l [13]. As compared to previous studies, present isolate *Massilia* produced significantly good amount of PHA (3.0 g/l) when medium was optimized using CCD by molasses as a carbon source.

3.2. Regression analysis

According to Table 3, p values were found to be < 0.05 for linear model terms (A & C), quadratic model terms (A^2, B^2, C^2, D^2) indicating they are highly significant w.r.t DCW production. However, terms such as B and D were found to be having p-value > 0.05 indicating they are insignificant for DCW production.

After applying multiple regression analysis, the results fitted to a second order polynomial equation which was found be quadratic model. Thus, mathematical model for DCW production (X) was a fit in terms of coded factor in equation 1.

Table 3: Summary of model terms

Variables	X		Y	
	P- value	F-value	P- value	F-value
Model	< 0.0001	51.33	< 0.0001	226.54
A	0.0431	4.55	0.0004	23.12
B	0.4839	0.5097	1.0000	0.0000
C	0.0216	6.27	0.5052	0.4718
D	0.0741	3.57	0.0177	7.55
A^2	< 0.0001	194.53	< 0.0001	1164.77
B^2	< 0.0001	158.77	< 0.0001	1298.33
C^2	< 0.0001	109.11	< 0.0001	1164.77
D^2	< 0.0001	95.49	< 0.0001	1121.77
ABD	-*	-	0.0759	3.77
ACD	-*	-	0.0130	8.49
BCD	-*	-	0.0053	11.56
A^2B	-*	-	0.2639	1.26
A^2C	-*	-	0.0445	5.30
A^2D	-*	-	0.0095	9.52
AB^2	-*	-	0.0095	9.52

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

Table 4: ANOVA analysis for DCW and PHA

Statistical analysis	X	Y
Lack of Fit	0.0683[ns]	0.1448[ns]
Coefficient of determination (R^2)	0.9558	0.9965
(Adjusted R^2)	0.9372	0.9921
Predicted R^2	0.7774	0.7652
Std deviation	4.86	0.1029
Adequate precision	19.0303	38.1975

ns-Not significant

Equation 1

$$X=+53.35+2.12A+0.7083B-2.48C-1.88D-12.94A^2-11.69B^2-9.69C^2-9.07D^2$$

The p values were found to be <0.05 for linear model terms (A & D), quadratic model terms (A^2,B^2,C^2,D^2) and other model terms (AB^2, A^2C, A^2D) indicating that they are highly significant for PHA production. However model terms such as B, C, A^2B, ABD, ACD and BCD had p value >0.05 making them insignificant w.r.t PHA production. After applying multiple regression analysis, the results were fitted to a third order polynomial equation which was cubic model. Thus, mathematical model for PHA production (Y) was a fit in terms of coded factors in equation 2.

Equation 2

$$Y=+2.97+0.1750A+0.0000B+0.0250C-0.1000D-0.6708A^2-0.7083B^2-0.6708C^2-6583D^2-0.0500ABD+0.0750ACD+0.0500A^2B+0.0000AC^2+0.0000AD^2+0.0000B^2C+0.0000BC^2+0.0000BD^2+0.0000C^2D+0.0000CD^2+0.0000A^3+0.0000B^3+0.0000C^3+0.0000D^3.$$

Table 4 explains the significance of model on the basis of ANOVA analysis which is as follows:

1. Goodness of fit of model was checked by determination of coefficient (R^2).

- DCW production: R^2 value was found to be 0.9588. This indicates that 95.88% of total variability in the response could be explained by this model. Adjusted R^2 was found to be 0.9372.
- PHA production: R^2 value was found to be 0.9965. This indicates that 99.65% of total variability in the

response could be explained by this model. Adjusted R^2 was found to be 0.9921.

With the help of Design expert software 11 (stat-ease) it was deduced that the difference between adjusted R^2 and predicted R^2 was found to be less than 0.2. Thus, it confirms the satisfactory adjustment of model with the given data (X and Y) which is further confirmed by lack of fit and adequate precision analysis.

2. The "lack of fit "value for DCW and PHA production were found to be 0.0683 and 0.1448. It implies that lack of fit is not significant to relative pure error. Non-significant lack of fit is good and considered that model is fit [21].
3. "Adequate precision" measures signal to noise ratio. A ratio of greater than 4 is desirable. For the DCW (biomass) production ratio of 19.0303 indicates an adequate signal, thus this model can be used to navigate the design space. Similarly, for PHA production, the ratio obtained was 38.1975 indicating model can be used further. (Design expert software- 11)

These results are further correlated with 3D response plots generated to confirm the fitness of model with the given data.

3.3. Three dimensional (3-D) plots

The three-dimensional (3D) response surface curves showing interactive effect of various components on DCW and PHA production were generated & following results were obtained.

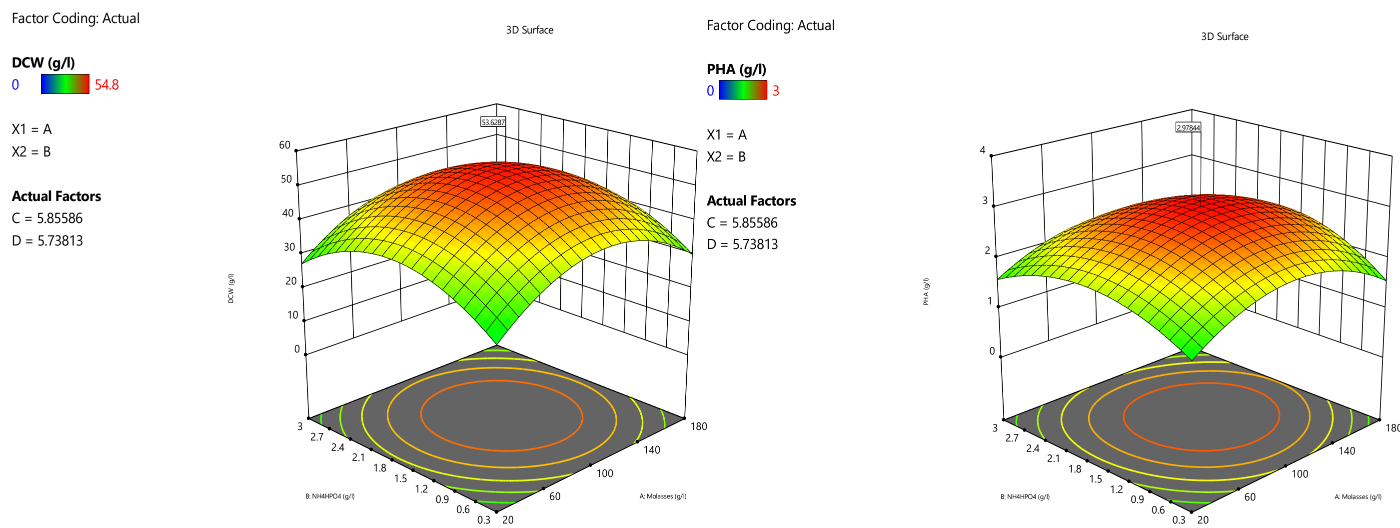

Fig. 1: Three-dimensional (3D) response surface curves showing interactive effect of molasses and NH_4HPO_4 on DCW and PHA production

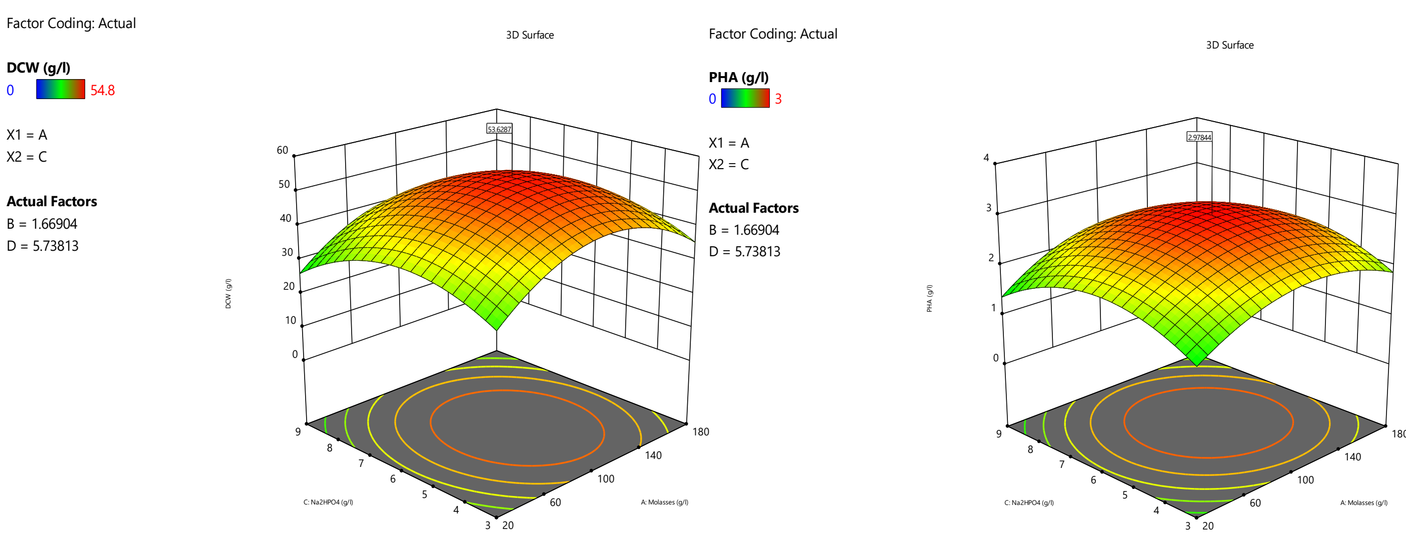

Fig 2: Three-dimensional (3D) response surface curves showing interactive effect of molasses and Na_2HPO_4 on DCW and PHA production

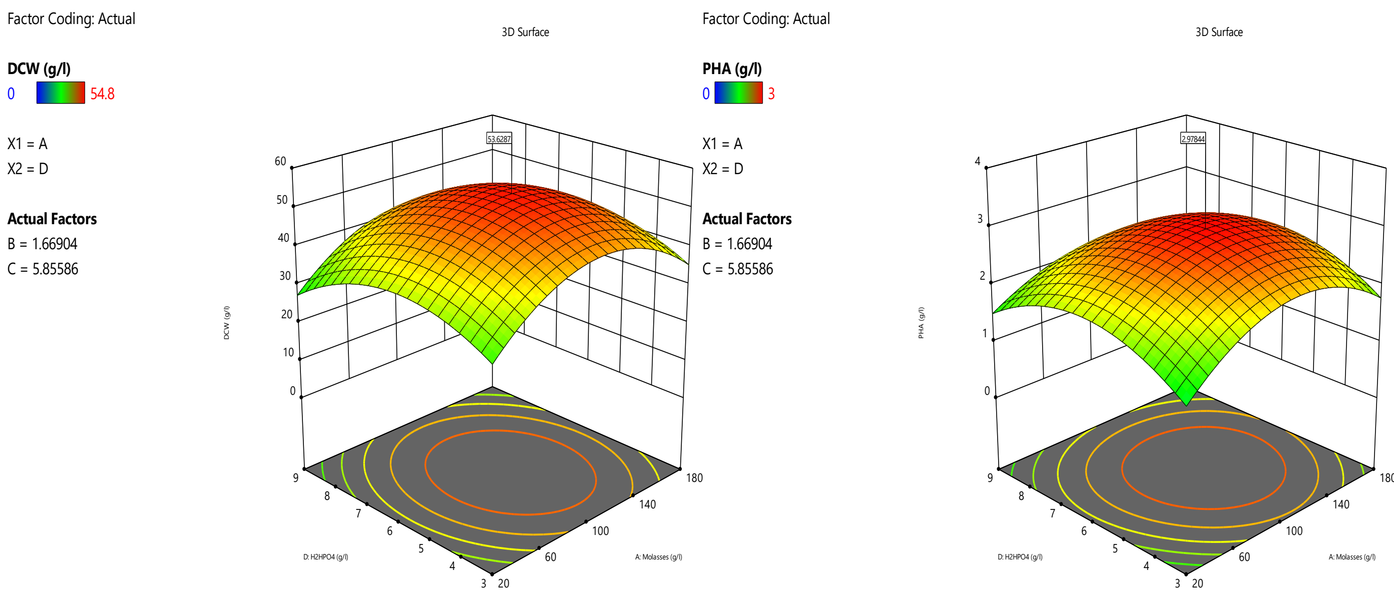

Fig. 3: Three-dimensional (3D) response surface curves showing interactive effect of Molasses and K_2HPO_4 on DCW and PHA production.

- The 3D plots between various factors were generated and optimum concentrations of medium components were found. Fig. 1 depicts interactive effects of varied molasses and NH_4HPO_4 concentrations at a median level of Na_2HPO_4 (5.85 g/l) and K_2HPO_4 (5.73 g/l) demonstrated that both DCW and PHA production increased when molasses was present at the concentration of 100 g/l and NH_4HPO_4 at the concentration of 1.66g/l. Further increase in molasses concentration significantly affected DCW and in turn PHA production. When there is an increase in NH_4HPO_4 concentration, DCW was not significantly affected but there was a negative effect on PHA production. Possible explanation to this condition is that under nitrogen limiting condition, NADPH consumption is reduced which in turn prevents amino acid synthesis pathway resulting in build-up of excess NADPH in the cell. This excess NADPH might be accountable for the increased PHA synthesis in nitrogen deficient cells [22].
- The interaction between maltose and $Na_2HPO_4$was found to be significantly affecting DCW as well as PHA production. Both variables were found to be having negative effect on DCW as well as PHA production. At high concentration of molasses (180g/l) & Na_2HPO_4 (9.0 g/l) DCW as well as PHA production was decreased (Fig. 2).
- Similar results were obtained when interactive effect between maltose and K_2HPO_4 was studied. K_2HPO_4 (9.0 g/l) had shown negative effect on DCW as well as PHA production (Fig.3).

Thus, by using CCD, optimum concentration of medium components to maximize response (X & Y) was found to be as follows: molasses: 100.0 g/l, NH_4HPO_4: 1.67g/l, Na_2HPO_4: 5.86g/l, K_2HPO_4: 5.74g/l, $MgSO_4$: 0.25g/l, trace element solution: 0.1ml/l.

3.4. Validation of model

After knowing the possible concentrations of all the factors present in the medium, 'point optimization' technique was applied. To verify the model, experiments were performed in triplicate using optimized conditions provided by the Design Expert software version 11 (Stat-ease).

Previously, when conditions were optimized using OFAT method, *Massilia* was able to produce 2.04 g/l of PHA by using hydrolysed molasses [16]. When medium was optimized by performing proper designing of experiment using Design expert software 11 (Stat-Ease), PHA production increased to 3.0 g/l. The increase in PHA production was observed, but it was not as good as pure carbon sources. The other components present in the molasses may be affecting PHA production.

Table 4 represents PHA yield before and after optimization of medium components. Point optimization study revealed that, experimental (3.0g/l) and predicted (3.02g/l) values for PHA production are in good agreement. Penkhrue *et al.* (2020) also applied similar tool to verify the model obtained in CCD and reported a difference of 0.2 g/l between predicted and experimental values [11].

Bioanalytical study revealed that PHA produced by *Massilia* sp using molasses as a carbon source is an unsaturated mcl-copolymer of poly-9-hydroxy-hexadecenoic acid methyl ester (b & c-C:17), and octadecanoic acid 9,10,12 trimethoxy methyl ester [P(9-HHD-co-9,10,12-MOD)] with improved thermal properties. Thus, it can have application where higher temperature treatment is needed [16].

Table 4: PHA yield before and after optimization of medium components using molasses as carbon source

Optimization	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	100	0.5	3.32	2.8	12.0	-	2.0	-
After	100	1.65	6.0	6.0	54.0	53.6	3.0	3.02

A: Experimental B: Predicted - Not calculated from previous OFAT study

4. CONCLUSION

Response surface methodology (RSM) based statistical method (CCD) was successfully applied to increase PHA yield by *Massilia* sp at shake flask level by using molasses as a carbon source. Statistical experiments designed with the help of CCD helped to optimize concentration of medium components and study the role of each component to enhance growth and PHA production. It showed that carbon and nitrogen positively influenced PHA production whereas inorganic sources of phosphate showed negative impact on PHA production. The predictive models developed in the study appropriately predicted yield of PHA and found to be most suitable one. PHA production was increased from 2.04 g/l (OFAT) to 3.0 g/l when medium was optimized using CCD. Optimized concentration of medium components obtained by applying RSM is as follows: molasses: 100.0 g/l, NH_4HPO_4: 1.67g/l, Na_2HPO_4: 5.86g/l, K_2HPO_4: 5.74g/l, $MgSO_4$: 0.25g/l, trace element solution: 0.1ml/l.

Conflict of interest

None declared

Source of funding

None declared

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