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EFFECT OF NUTRITIONAL AND PROCESS PARAMETERS ON PRODUCTION OF COPOLYMER, POLY (HYDROXYBUTYRATE-CO-HYDROXYVALERATE) BY *HALOMONAS CAMPISALIS* MCM B-1027 AS ASSESSED USING TAGUCHI DESIGN OF EXPERIMENTS

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are a group of microbial biodegradable polymers having potential to replace synthetic plastics. Several factors are known to influence PHA biosynthesis e.g., bacterial strain used, carbon and nitrogen sources provided, culture conditions, etc. These factors interact with each other during productionand their interaction can be assessed using statistical methods. In the present study, Taguchi Design of Experiments is performed to find out the effect of six different factors viz. incubation period, medium: flask volume ratio, pH of production medium, concentration of maltose, yeast extract and sodium chloride at three levels on production of PHA with 18 well defined experiments (L18 Orthogonal Array). The data obtained was analyzed statistically and it was found that incubation period was the most significant factor contributing 28.84% to PHA production while concentration of maltose was found to be the least significant (1.81%) in the PHA production.

KEYWORDS: **Poly(3-hydroxybutyrate-co-hydroxyvalerate), Taguchi Design of experiments, *Halomonas campisalis*,Process parameters, Interation of factors.**

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INTRODUCTION

Polyhydroxyalkanoates (PHAs), a group of biodegradable polymers of biological origin have gained tremendous impetus in recent years because of material properties similar to polypropylene and a biodegradable nature. Besides its biodegradable nature, it exhibits interesting properties as biocompatibility, insolubility in water and oxygen barrier capacity. It is a thermoplastic and piezoelectric material. These features make them suitable for several applications in packaging, coating, agriculture, food industry, pharmacy and most importantly in biomedicine[1]. Optimization of fermentation conditions has long been used to enhance yield and productivities of many bioprocesses. Conventionally, fermentation conditions were optimized by implementing the variation of one component at a time, but this approach is time consuming and process variables do not interact and therefore the process response is function of single parameter, which is varied. In the recent years, this approach has been replaced by statistical optimization designs like Plackett Burman, response surface methodology, Box-Behnken, Modified distance etc. which take into account the interaction of variables[2]. A few studies have been documented on statistical optimization of PHA production by microorganisms like *Cupriavidus necator*[2,3], *Rhodobacter sphaeroides*[4], *Bacillus megaterium*[5] etc. However to date, meager data is available on statistical optimization of production of copolymer, PHB-co-PHV using halophilic, alkaliphilic and haloalkaliphilic microorganisms. Extremely halophilic archeon *Haloferax mediterannie* is reported for production of copolymer PHB-co-PHV using extruded starch without addition of any precursor[6]. Moderate halophile, *Halomonas boliviensis* is reported for production of PHB using Factorial design using 2^3 levels[7]. Taguchi Design of Experiments (DOE) has been used successfully in many fermentation processes like production of xylitol [8], alkaline protease [9], ethanol, acid amylase [10], lacasse, xylanase [11,12]and cyclodextrin glycosyl transferase[13]. The effect of individual factors and the relationship between the variables and operational conditions is determined by Taguchi design of experiments (DOE) which in turn establishes the performance at the optimum levels. For finding out the optimum levels, analysis of the experimental data is carried out using Analysis of variance (ANOVA)and the factor effects studied for their statistically significant output. In our earlier studies on production of PHB-co-PHV copolymer by moderately haloalkalitolerant *H. campisalis*, conventional 'one factor at a time' approach was followed[14].In the present paper, the contribution of nutritional and process parameters that play a significant role in the production of PHB-co-PHV using Taguchi Design of experiments is presented.

MATERIALS AND METHODS

(i) Microorganism and production medium

Halomonas campisalis MCM B-1027 was employed for production of PHA using the production medium consisting of (g/l) maltose 10, yeast extract 1, sodium chloride 45, calcium chloride 0.13, magnesium sulphate 0.38, potassium chloride 0.75 and sodium bromide 0.2. pH of the medium was adjusted to 9.5 by 1 N NaOH solution[14]. All chemicals used for fermentation studies were of analytical grade. The culture having cell density of 10^7 cell/ml of medium was inoculated (4 % v/v) in 100 ml of production medium and incubated at 37 ºC for 24 h in orbital shaker incubator at 150 rev/min. Growth of the organism was checked by measuring its optical density (OD) at 600 nm and dry cell weight. The production of PHA was checked qualitatively by Nile blue sulphate staining of cells and observing under phase contrast microscope equipped with UV lamp (Nikon, Japan) [15]. For extraction of PHA from cells of *H. campisalis,* after 24 h incubation, the cells containing PHA granules were harvested and PHA was extracted from dry cell mass in chloroform using soxhlet apparatus for 40 h. After extraction, chloroform was allowed to evaporate and the pellet of PHA remained at the bottom of the Petri plate was collected, weighed and

expressed as % PHA produced on dry cell weight basis[14].

(ii) Taguchi Design of experiments for production of PHB-co-PHV

Six factors viz. incubation period, medium: flask volume ratio, pH of production medium, concentration of maltose, yeast extract and sodium chloride were selected to evaluate their role in the production of polyhydroxyalkanoates (PHA) by using Taguchi design of experiments. The experiment was run in 18 well defined sets with these six variables at three levels (L 18 orthogonal array). The factors and their levels are described in Table 1, while Table 2 shows outlay of the L18 orthogonal array. Production and extraction of PHA was carried out as described in (i). All 18 experiments were performed in duplicate with appropriate medium control. The experimental data was processed according to Phadke (1989)[16]. "Bigger- the- better" quality characteristic was considered. Individual influence of factors at assigned levels and ANOVA were calculated. Optimized process conditions were calculated based on pooled ANOVA. Finally prediction under optimized culture conditions was made and the validation experiment was conducted under the optimized process conditions.

Table 1
Factors and their levels used for L 18 orthogonal arrays

Sr. No.	Factors	Levels		
		1	2	3
A.	Incubation period, h	18	24	30
B.	Medium : Flask volume ratio	1:3.33	1:2.5	1:1.66
C.	pH of the production medium	7	8	9
D.	Concentration of maltose , g/L	5	10	20
E.	Concentration of yeast extract, g/L	0.5	1	2
F.	Concentration of sodium chloride, g/L	5	20	45

Table 2
Design of the experiment by L18 orthogonal array for production of PHA

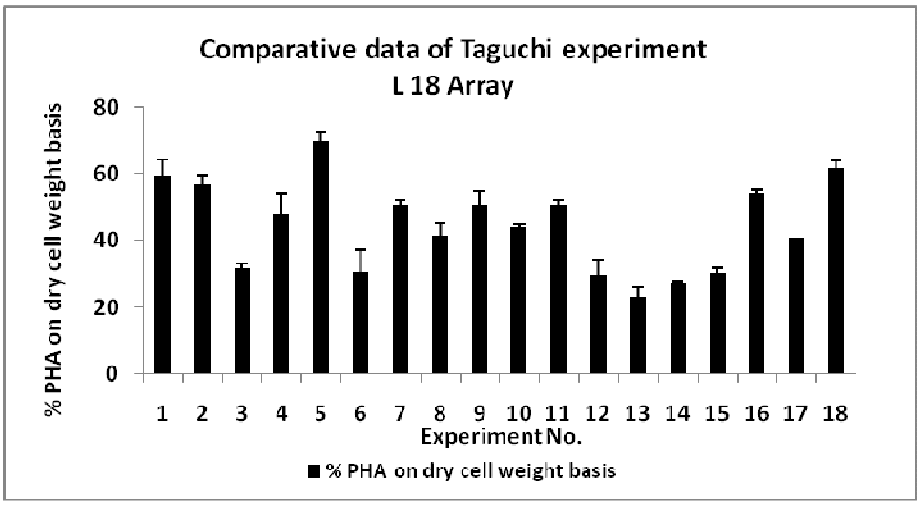
Experiment No.	Factors at their levels					
	A	B	C	D	E	F
1.	1	1	1	1	1	1
2.	1	2	2	2	2	2
3.	1	3	3	3	3	3
4.	2	1	1	2	2	3
5.	2	2	2	3	3	1
6.	2	3	3	1	1	2
7.	3	1	2	1	3	3
8.	3	2	3	2	1	1
9.	3	3	1	3	2	2
10.	1	1	3	3	2	1
11.	1	2	1	1	3	2
12.	1	3	2	2	1	3
13.	2	1	2	3	1	2
14.	2	2	3	1	2	3
15.	2	3	1	2	3	1
16.	3	1	3	2	3	2
17.	3	2	1	3	1	3
18.	3	3	2	1	2	1

RESULTS

(i) Taguchi Design of experiments for production of PHB-co-PHV

To study the effect of various factors individually and in combination on production of PHB-co-PHV copolymer, Taguchi Design of Experiment was carried out using six factors at three levels using L18 array. The results showed significant variation in the production of PHA by *H. campisalis*. Thus production levels were found to be very much dependent on the culture conditions. From Fig. 1, it was observed that trial 5 showed highest PHA production i.e. 69.82 % on dry cell weight basis, followed by trial 18 and trial 1 with 62.07 % and 59.61% respectively, while lowest PHA production was observed in trial 13 with 23.33 % (Fig. 1).

Figure.1
Production of PHA by H. campisalis in 18 experimental sets according to Taguchi orthogonal array



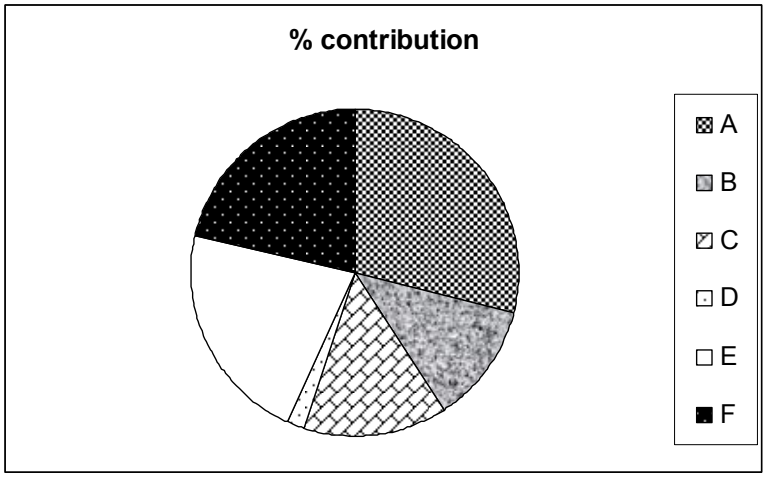
The effect of individual factors on PHA production and growth of the culture at assigned levels were studied. While studying the effect of incubation period at assigned levels, it was observed that growth of the culture as well as PHA production was highest at level 3 i.e. at 30 hrs of incubation. In case of concentration of yeast extract, it was observed that growth as well as PHA production was optimum at level 2 i.e. at 1 g/L concentration. As regards concentration of NaCl at assigned levels, level 1 i.e. 5g/L NaCl concentration was found to be the optimum with highest PHA production. While studying the concentration of maltose at the assigned levels, it was observed that at level 1 (5 g/L) , the cell mass and % PHA was highest as compared to level 2 and level 3, i.e. at 10 g/L and 20 g/L respectively. Studies on pH of production medium at assigned levels on growth of the culture and production of PHA showed that pH 7 i.e. level 1 was optimum. While studying the effect of medium: flask volume ratio, it was observed that level 2 with standard 2:5 medium: flask volume ratio was optimum with the highest PHA and cell mass concentration. (Table 3). The average effect of factors at the assigned levels on the production of PHA is depicted in Table 3.

Table 3
Average effect of the factors at the assigned levels on PHA production.

Factors	L1	L2	L3	L2-L1	L3-L1	L3-L2
Incubation period	32.855	30.993	33.885	-1.862	1.030	2.892
Medium: flask volume ratio	33.031	33.216	31.487	0.185	-1.543	-1.729
pH of production medium	33.219	33.112	31.402	-0.107	-1.817	-1.710
Concentration of maltose	32.982	32.487	32.264	-0.495	-0.718	-0.223
Concentration of Yeast extract	31.101	33.380	33.253	2.279	2.152	-0.127
Concentration of sodium chloride	33.869	32.509	31.355	-1.360	-2.515	-1.154

While studying the effect of each factor individually at the level stage, it was observed that incubation period has the highest average effect in level 3 followed by concentration of sodium chloride, yeast extract and maltose in the level 1, level 2 and level 1 respectively on production of PHA. The difference between the average value of each factor at higher level and lower level indicate the relative influence of the affect at their individual capacity. Larger the difference, stronger is the influence. The sign of the difference (+/-) indicates whether the changes from level 1 to level 2 or level 3 increased or decreased the yield of PHB-co-PHV (Table 3). Based on the data, it was observed that incubation period has stronger influence followed by concentration of yeast extract and sodium chloride, while the least influence was observed in case of concentration of maltose, pH of the medium and medium: flask volume ratio (aeration). The percent contribution of each factor is illustrated in Fig. 2.

Figure. 2
% contribution of factors on production of PHA by H. campisalis



A: Incubation period, B: Medium: flask volume ratio, C: pH of production medium, D: Concentration of maltose, E: Concentration of Yeast extract, F: Concentration of sodium chloride.Incubation period was found to be the most significant factor contributing 28.84 % to PHA production followed by concentration of yeast extract (22.02 %) and concentration of NaCl (21.27 %). Maltose concentration was found to be the least significant with 1.81 % contribution (Fig. 2). The signal-to-noise ratio (S/N ratio) is simply the ratio of mean to the standard deviation. S/N ratio analysis method was chosen to observe the significant effect of each factor on PHA production and for prediction. The average values of S/N ratios are shown in the Table 4.

Table 4
Analysis of pooled ANOVA

Factors	Average SN ratio (dB) by levels			DOF	sum of squares	Mean square	F ratio	
	1	2	3					
Incubation period	32.86	30.99	33.89	2	12.890	6.44491978	3.105164	significant
Medium: flask volume ratio	33.03	33.22	31.49	2	5.406	2.70277261	1.302196	
pH	33.22	33.11	31.40	2	6.237	3.11861013	1.502547	
Concentration of maltose	32.98	32.49	32.26	2	0.811	0.40526424	0.195256	
concentration of yeast extract	31.10	33.38	33.25	2	9.842	4.92091175	2.370896	Significant
Concentration of NaCl	33.87	32.51	31.35	2	9.505	4.7570598	2.289855	significant
Error				5				
Total				17	44.690			
(Error)				(6)	(12.453294)	2.07554899		

From F ratios given in the Table 4, it was observed that incubation period followed by concentration of yeast extract and sodium chloride is significant while concentration of maltose, pH of medium and medium: flask volume ratio is insignificant for production of PHA. Pooling was done until the degree of freedom (DOF) of the error term becomes close to half the total DOF. Thus, the least significant factors were pooled and pooled ANOVA is shown in Table 4. After pooling, the predictions were made on the basis of SN ratios and the optimum conditions are derived. It is predicted that incubation period 30 h, medium: flask volume ratio as 2:5, pH of production medium 7, concentration of maltose 5 g/l, yeast extract 1 g/l and sodium chloride 5 g/l would give production of PHA > 69 % on the basis of S/N ratio of 37.66. Confirmation and validation studies resulted in PHA yield of 70 % on dry cell weight basis at optimized environmental conditions.

DISCUSSION

The present study was aimed for the optimization of production of copolymer PHB-co-PHV using moderately haloalkalitolerant *Halomonas campisalis* and employing Taguchi design of experiment. Meager data are available on optimization of production of PHA using Taguchi Design of Experiments. A few researchers have found out that production of PHA is influenced by various nutritional and environmental factors viz. concentration of carbon (glucose, fructose and volatile fatty acids [VFA], textile waste water, petrochemical waste water) nitrogen (typtone, yeast extract), phosphorus (potassium di hydrogen phosphate), micronutrients (Iron), C: N ratio, pH, temperature, agitation speed, incubation period, age of inoculum, rate of aeration, etc. Among the variables culture age and initial concentration of fructose were found to be the most significant factors with 92.36% PHB on dry cell weight basis by *R. eutropha*[17]. When petrochemical waste water was used for production of PHB by *Haloarcula* sp.IRU1, concentration of petrochemical waste water showed stronger influence on production of PHB with maximum 46.6 % PHB on dry cell weight[18]. Likewise concentration of textile waste water was found to be a significant (28.7%) parameter influencing production of PHB by *Haloarcula* sp.IRU1[19]. When the effect of process variables on production of PHA by activated sludge in sequencing batch reactor (SBR) was studied, concentration of VFA was found to be the effective variable which increased the PHA production up to 49% [20]. Micronutrient (iron) was found to be the significant factor contributing 81 % in the bioplastic production using mixed culture[21]. As compared to these reports, incubation period has been found to be the significant factor contributing 28.84 % in maximum (70 %) production PHA on dry cell weight basis by *Halomonas campisalis* in the present paper. Thus various factors are

found to influence production of PHA depending upon the microbial culture used.

The present study on the influence of six factors at three levels showed that incubation period has the most significant contribution to PHA production. From preliminary studies, production of PHA was found to be in the range of 60- 67% within 15-30 h of incubation[14]. From Taguchi design of experiment, the exact period for production of PHA was noted as 30 h. Thus, maximum accumulation occurred at stationary phase of growth. This observation was in complete agreement with the earlier report of *H. boliviensis*[7]. The second significant factor contributing 22.02 % to production of PHA was concentration of yeast extract. Limitation of nitrogen i.e. yeast extract (1g/L of medium) appeared to be a suitable stimulant for production of PHA. Under nitrogen deficient condition, a rise in production of PHA was observed. This could be explained in the light of earlier reports[22, 23, 24]. The similar results were obtained in case of the reports of PHB production by *H. boliviensis* using Factorial design 2^3, wherein 1.5 g/L yeast extract was found to be optimum with the yield of 69 % PHB on dry cell weight basis[7]. The third significant factor contributing 21.27 % to the production of PHA was concentration of sodium chloride. *H. campisalis* is a moderately haloalkalitolerant organism isolated from saline and alkaline Lonar Lake, India. Being moderately halotolerant, the organism tolerates NaCl concentration up to 3M[25]. To detect exact concentration of salt needed by the organism for production of PHA, Taguchi design of experiment was performed and from results, it was concluded that 5 g/L NaCl concentration was optimum. The high concentration of salt decreased growth as well as production of PHA. This finding is important since incorporation of 5 g/L NaCl in the production medium (instead of 45 g/L) would help in maintaining a low concentration of chlorides in the spent medium, thus avoiding the chloride contamination of the process water generated during the large scale production of PHA. The optimum salt concentration obtained in case of moderately halophile, *H. boliviensis* was 45 g/L [26].

CONCLUSION

Taguchi Design of experiments resulted in significant variation (23.3 % to 69.8 %) in the production of PHA by *H.campisalis*. Thus the production levels were found to be very much dependent on the culture conditions. Incubation period was found to be the significant factor contributing 28.84 % to production of PHA followed by concentration of yeast extract (22.02%) and NaCl (21.27%). Confirmation and validation studies resulted in PHA yield of 70% on dry cell weight basis under optimized environmental conditions. Taguchi Design of experiment facilitated understanding the influence and contribution of each factor on production of PHA.

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