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Research Article

HYPER PRODUCTION OF CHOLESTEROL OXIDASE VIA TAGUCHI'S ORTHOGONAL ARRAY DESIGN

Florentyna Rodrigues[a], and Palani Perumal[b]*
[a]Saveetha School of Engineering, Saveetha Institute of Medical and Technical Sciences, Thandalam, Chennai-602105, India.
[b] Center of Advanced Studies, University of Madras, Guindy, Chennai-600025, India.
Email: Palanii.7@yahoo.com

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Abstract

This study focused on optimizing media components enhance the production of cholesterol oxidase by Taguchi orthogonal array design. One factor-at-a-time method was used to initially to investigate the effect of fermentation time, inoculum age, inoculum concentration, carbon sources, nitrogen sources and initial pH on biomass growth and enzyme production. The orthogonal array design 1 (OAD1) was used to identify the components required by the bacterium while the orthogonal array design 2 (OAD2) was used for determining the concentration of the identified components for hyper production of cholesterol oxidase. Based on the results obtained a production medium was formulated with the following components, cholesterol (2.0g/L), Tween-80 (4.0g/L), yeast extract (2.0g/L), magnesium sulphate (0.01g/L), zinc sulphate (0.01g/L), mono potassium phosphate (0.1g/L), sodium phosphate dibasic (0.5g/L), calcium chloride (0.01g/L) and ferrous sulphate (1.5g/L). The amount of cholesterol oxidase produced in the optimized medium by the bacterium was three times higher when compared to the amount of enzyme obtained in unoptimized medium.

Keywords: Cholesterol oxidase, Taguchi orthogonal array design, Production Optimization.

1. Introduction

Microorganisms capable of producing cholesterol oxidase constitutively at relatively lower concentrations have wider industrial and pharmaceutical applications. In order to enhance the production of cholesterol oxidase, researchers have introduced variations in the production methodology [1]. Generally, when the cellnumber of a microorganism is increased by altering the growth medium there is a corresponding increase in enzyme

production as well. Considering the importance of cholesterol oxidase many workers have attempted to enhance its production [2–6].

The conventional method of medium optimization involves the tedious process of altering a single factor at a time, while all other factors are maintained at a constant concentration. This method enables researchers to assess the impact of the single factor, however fails to provide data pertaining to the interaction that occurs between factors. These procedures are time consuming, cumbersome, require more experimental data sets and do not provide information about the mutual interactions of the parameters [7]. Computational software such as design of experiments (DOE) aids researchers in gaining comprehensive knowledge on the optimization of conditions in a short span of time with fewer trials. Statistical experimental design methods provide a systematic and efficient plan for bioprocess optimization considering the interactive effects among the control factors. Many factors can be studied simultaneously and optimized using statistical experimental designs [8,9]. Among various statistical experimental designs, Taguchi's experimental design offers a distinct advantage because many factors can be examined simultaneously and more quantitative information can be extracted with fewer experimental trials [10,11]. The basic principle of this method serves as screening filters which examine the effects of many process variables and identify those factors which have significant effect on the process using a few experiments [12]. Taguchi's method of DOE involves establishment of large number of experimental situations described as orthogonal arrays to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments [13].

In this study, One-factor-at-a-time method was used to initially determine the components required for optimization of a production medium. The effect of other parameters such as fermentation time, inoculum age, inoculum concentration and pH on cholesterol oxidase production was studied initially so that these results would be helpful in selecting other parameters for single factor optimization experiments. The process of single factor optimization enables in selecting the main factors that could be used in constructing orthogonal array designs.

2. Materials and methods

2.1. Chemicals

All the analytical grade chemicals used in this study were purchased from Sigma-Aldrich Chemicals Co. (USA). Components for bacteriological media were obtained from Hi-media (India). The inorganic and organic

chemicals, and solvents used were of analytical grade, obtained from Qualigens or SD-Fine chemicals (India). All the solutions were prepared with double distilled water just before use, unless mentioned otherwise.

2.2. Microorganism and culture conditions

E.cloacae FH68 (PPLB83-FH68) [14],was obtained from the Bacterial Culture Collection and Research Center, CAS in Botany, Chennai, and used in this study it was maintained in nutrient agar (Hi-media, India) at 4ºC and sub-cultured every 4 weeks.

The control media used for cholesterol oxidase production was as follows [15] : Cholesterol 1.0 (g/L); Yeast extract 5.0 (g/L); $FeSO_4.7H_20$ 0.25 (g/L); NH_4NO_3 1.0 (g/L); $KH_2PO_4$0.25 (g/L); Distilled water to make up to1000 m pH 7.0. Initially, Optimization of medium for cholesterol oxidase production using one factor-at-a-time method was used to determine the optimum effects of incubation time, inoculum age , inoculum concentration, pH, carbon sources, nitrogen sources and mineral salts on cholesterol oxidase production was carried out.

2.2. Cholesterol oxidase Assay

Cholesterol oxidase activity was measured at 240 nm by the method of Richmond (1976) [16]. The reaction mixture composed of 3 ml of 0.1 M sodium phosphate buffer (pH 7) with 0.05% Triton X-100, 0.05 ml 6 mM cholesterol in 2-propanol and 0.05 ml culture supernatant. The enzyme reaction was carried out at 30ºC for 30 min followed by the measurement of the increase of absorbance at 240 nm. The molar absorption Δ of 4-cholesten-3-one was 12.2 × 103 l M^{-1} cm^{-1}. Cholesterol oxidase activity was calculated as (ΔA × reaction volume × 0.082)/volume of enzyme used) = ΔA × 5.1 Uml^{-1}. One unit of cholesterol oxidase was defined as the amount of enzyme which oxidizes 1 μ mole of cholesterol to 4-cholesten-3-one per min at 30ºC. Protein estimation was performed by following the method of Bradford (1976) [17]. Cell density was determined by measuring the culture absorbance at 600 nm using a spectrophotometer.

2.3. Optimization of medium for cholesterol oxidase production using Taguchi's orthogonal array design

This method of statistical testing was developed by Genichi Taguchi in 1960[10]. This method employs orthogonal arrays (a table with a fixed number of rows and columns) to maximize the production of a specific product. In this method, factors (media components) were specified as columns and their levels were tested at a high and a low concentration i.e., at 2 levels. This method can be used to test up to 15 different factors; however in the present study only 9 factors were tested in each of these experimental designs. The notation of

each orthogonal array was expressed by its number of rows and columns, since there were 16 different media formulations; the orthogonal array was denoted as L_{16}. Each of the media components was tested at two levels with 9 columns, since only 9 factors were tested in each experiment. As only 15 factors can be tested using this model, it is universally denoted as (2^{15}). Thus, the L_{16} (2^{15}) orthogonal array design (OAD) has 16 rows and 15 two-level columns [18]. In the design matrix, there were nine variables or factors to be studied and all the experiments were carried out according to a design matrix. Each column depicts a different variable (medium component) and each row depicts one trial (culture medium variant). The independent variables were selected from a classical single factor optimization process and in this experiment they were tested at two levels of nutrient concentrations i.e., a high level (coded as 2) and a low level (coded as 1). All the experiments were performed under uniform conditions according to the matrix, and the specific activity of the enzyme was recorded as the response for each experiment.

2.4. Optimization of media by orthogonal array design 1 (OAD1)

Orthogonal Array Design 1 (OAD1) was used to study the effect of nine media components (Table 1), and was made up of 16 experimental runs (Table 3). The main effects were the columns and these were named from A to J. The specific cholesterol oxidase activity was determined for each experimental run and the mean of squares for each treatment was calculated. This allowed the significant level of each component using Fisher's F-Test. The ANOVA values for the experimental results obtained by OAD1 were calculated. The purpose of OAD1 was to identify significant components with a positive and negative effect on the production medium.

2.5. Optimization of media by orthogonal array design 2 (OAD2).

Table 1: Media components for OAD1.

Constituent	Symbol	Concentration (g/L)	
		Level 1	Level 2
Cholesterol	A	1.0	2.0
Yeast Extract	B	4.0	5.0
Tween 80	C	1.0	2.0
NH_4Cl	D	1.0	2.0
$MgSO_4$	E	0.25	0.50
$FeSO_4$	F	0.10	0.50
NaCl	G	0.1	1.0
KH_2PO_4	H	0.25	0.50
Na_2HPO_4	J	0.25	0.50

Table 3: Experimental Field for OAD1.

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Response
Expt Run No.	A: Chol-esterol	B: Yeast Extract	C: Tween 80	D: NH_4Cl	E: $MgSO_4$	F: $FeSO_4$	G: Nacl	H: KH_2PO_4	J: Na_2HPO_4	Specific Activity (U/mg)
1	1	1	1	1	1	1	1	1	1	1.124
2	1	1	1	1	2	2	2	2	2	2.322
3	1	1	2	2	1	1	1	1	2	3.533
4	1	1	2	2	2	2	2	2	1	2.161
5	1	2	1	2	1	1	2	2	2	1.057
6	1	2	1	2	2	2	1	1	1	1.230
7	1	2	2	1	1	1	2	2	1	1.097
8	1	2	2	1	2	2	1	1	2	4.000
9	2	1	1	2	1	2	1	2	2	4.160
10	2	1	1	2	2	1	2	1	1	4.706
11	2	1	2	1	1	2	1	2	1	4.333
12	2	1	2	1	2	1	2	1	2	7.470
13	2	2	1	1	1	2	2	1	1	5.406
14	2	2	1	1	2	1	1	2	2	2.364
15	2	2	2	2	1	2	2	1	2	9.976
16	2	2	2	2	2	1	1	2	1	0.870

A similar experiment as OAD1 was performed with 9 media components (Table 2), whose effects were studied. The main aim of OAD2 was to optimize the levels of individual components that have a significant positive effect on cholesterol oxidase production in OAD1. Apart from the components which showed significant effect in OAD1, a few other components namely, manganese sulphate ($MnSO_4$), zinc sulphate ($ZnSO_4$) and calcium chloride ($CaCl_2$) were also assessed in this design for their optimum concentrations and significance.

Table 2: Media components for OAD2:

Constituent	Symbol	Concentration (g/L)	
		Level 1	Level 2
$MnSO_4$	A	0.01	0.02
$ZnSO_4$	B	0.01	0.02
Cholesterol	C	2.0	4.0
KH_2PO_4	D	0.1	0.2
Na_2HPO_4	E	0.5	1.5
$CaCl_2$	F	0.01	0.02
NaCl	G	1.0	2.0
$FeSO_4$	H	0.5	1.5
Tween 80	J	2.0	4.0

2.6. Data analysis

All the statistical analyses were carried out using the procedure of Taguchi orthogonal array designs provided in the Design-Expert 8.0 Program (DOE, 2013). The medium components which had a positive effect on the production of cholesterol oxidase were identified. The analysis of variance (ANOVA) for the experimental designs was obtained, and the significant levels of each medium variable was analyzed using Fisher's F-test and accepted only at the 95% confidence level.

3. Results and discussion

One-factor-at-a-time method was used to initially determine the components required for optimization of a production medium. The effect of other parameters such as fermentation time, inoculum age, inoculum concentration and pH on cholesterol oxidase production was studied initially so that these results would be helpful in selecting other parameters for the optimization experiments. In the preliminary study, the maximum cholesterol oxidase production by *E. cloacae* was recorded at 36^{th} hour with 12.5% of 48 h grown inocula at a pH 7.0 .The bacterium grown with 1.0% (w/v) cholesterol emulsified with 0.05% (v/v) Tween-80 resulted in highest cholesterol oxidase production. In addition to this, yeast extract (2.013 U/mg) was proved to be the best of nitrogen source. This data was utilized in considering the parameters for the Taguchi's orthogonal array design.

3.1. Optimization of media using Orthogonal Array Designs

Taguchi's orthogonal array design was performed in two steps: the first was used to determine the component which has significant impact on cholesterol oxidase production by *Enterobacter cloacae*, and the second (OAD2) was used to optimize their concentrations and to test the influence of few other media components such that optimum levels of enzyme was produced in the present L_{16} (2^{15}).

3.2. Optimization of media using Orthogonal Array Design 1(OAD1)

The orthogonal array design was used to study the effect of nine media components, with 16 experimental runs. The main effects were exerted by the components in the columns and they were named A to J. The specific activity of the enzyme was used as the basis for the determination of components in each experimental design (Table 3). The specific activity of cholesterol oxidase was determined for each experimental design run (Table 3), and the mean of squares for each treatment was calculated. This allowed the significant contribution of each component as determined using Fisher's F-Test. The ANOVA for the experimental results obtained by OAD1

were calculated. The effects of yeast extract (B), NH_4Cl (D) and $MgSO_4$ (E) were not significant. However, cholesterol (A), Tween-80 (C), $FeSO_4$ (F), NaCl (G), KH_2PO_4 (H) and Na_2HPO_4 (J) showed significant effects on enzyme production (Table 4).

The Half-Normal plot obtained depicts standardized effects in the same units of measure as the response, on the x axis. This type of a plot is the best choice for two-level factorial designs. The Half-Normal plot is used for choosing the statistically significant factors. Factors with a positive effect are colored orange and those with a negative effect are colored blue (Figure 1). Five factors viz. cholesterol (A), Tween-80 (C), $FeSO_4$ (F), NaCl (G) and Na_2HPO_4 (J) possessed significant positive effect on the production of the enzyme (Figure 1). One factor namely KH_2PO_4 had a significant negative effect on enzyme production. The addition of Tween-80 into the culture medium was necessitated to dissolve cholesterol, and has resulted in induction of cholesterol oxidase[19]. It has been suggested that the presence of a surfactant might change the permeability of the cell membrane and enhance the secretion of intracellular products into the culture medium [20]. The results obtained in the present investigation agrees with reports of Liu et al., (1988) and Kreit et al., (1992) for cholesterol oxidase production by *A.simplex* and *Rhodococcus* sp. GK1, respectively [21,22]. Lee et al., (1997) have reported that an initial pH of 7.0 played a vital role in increasing the production of the enzyme [23]. This fact was confirmed by our initial single factor optimization hence buffering salts Na_2HPO_4 and KH_2PO_4 were added to the design model to maintain the pH of the medium at 7.0.Watanabe, Kreit and Aihara [19,22,24] have also reported using phosphates in the production media.

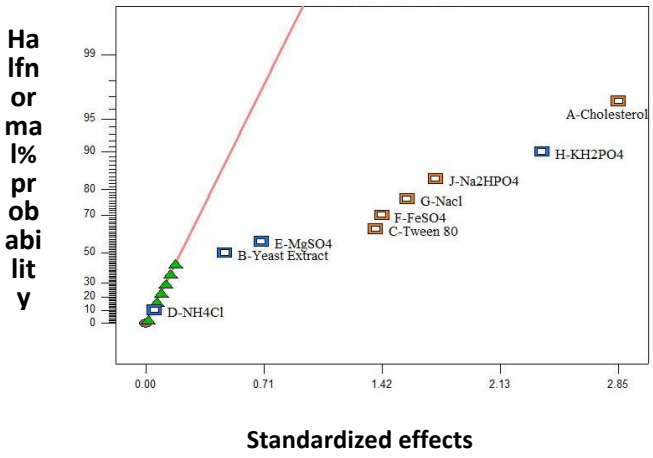

Figure-1: Half normal plot of effects of media components in OAD1

Error Estimates; A: Cholesterol; B: Yeast extract; C: Tween-80; D: NH_4Cl; E: $MgSO_4$; F: $FeSO_4$; G: NaCl; H: KH_2PO_4; J: NA_2HPO_4; Positive effect; Negative effect

Once the half-normal plot has been used to choose the relevant factors, the Pareto chart (Figure 2) was obtained; it was used for checking the significance of the selected factors and is best used after effects have been selected from the half-normal plot. Factors above the Bonferroni limit are almost certainly significant. Factors above the t-value limit are possibly significant and should be considered. Factors below the t-value limit are not likely to be significant. The ANOVA analysis confirmed that only statistically significant effects were in the model.

Six factors which lay above the Bonferroni limit were certainly significant (Figure 2). Of these six factors, five possessed a positive effect and one factor had a negative effect on the production of the enzyme. The remaining three factors possessed a negative effect on cholesterol oxidase enzyme production. However since the effect of these factors was quite small and their values lay beneath the t-value limit, these three factors had no significance in the model (Figure 2).

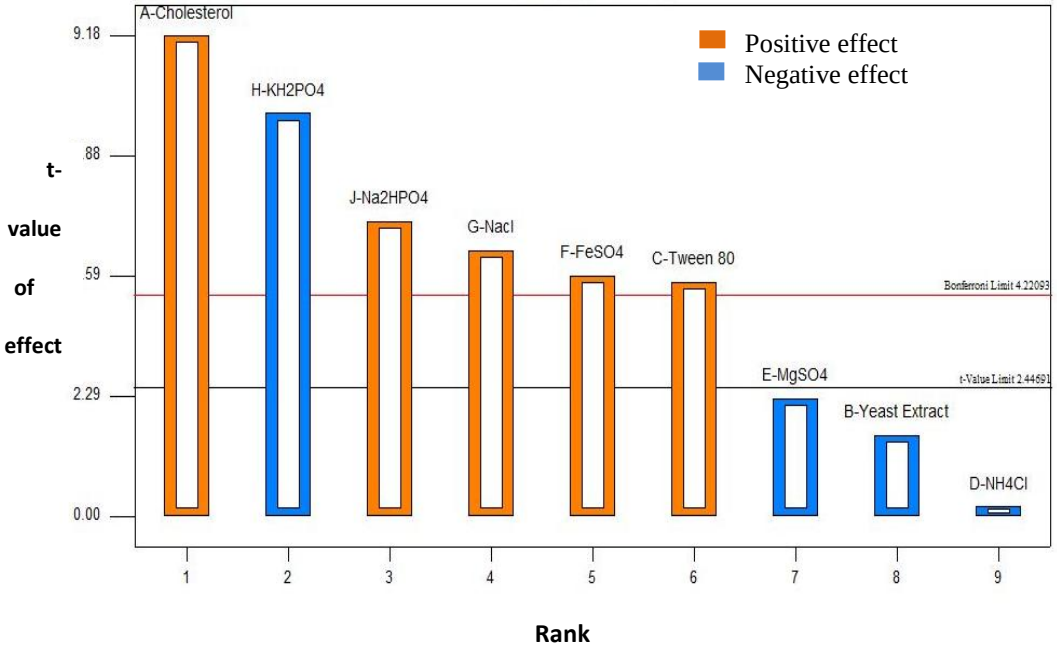

Figure-2: Pareto chart of effects of media components in OAD1

Apart from the confirmation from the ANOVA and the Fishers F-test, the Half- normal and Pareto chart were constructed and the significance of cholesterol (A), Tween 80 (C), $FeSO_4$ (F), NaCl (G), and Na_2HPO_4 (J) were confirmed. Thus their initial concentrations were increased in the next improvement step. In contrast, KH_2PO_4 (H) exhibited a negative effect on enzyme production and therefore its concentration was decreased. The main effects of yeast extract (B), NH_4Cl (D) and $MgSO_4$ (E) were found to be of no significance on enzyme production as shown in the ANOVA (Table 4) and therefore their concentrations were not modified.

Table 4: ANOVA for OAD1.

Analysis of variance table						
	Sum of		Mean	F	p-value	
Source	Squares	Df	Square	Value	Prob > F	
Model	95.786	9	10.643	27.672	0.000328	Significant
A-Cholesterol	32.379	1	32.379	84.187	9.44E-05	
B-Yeast Extract	0.907	1	0.907	2.358	0.176	
C-Tween 80	7.661	1	7.661	19.918	0.005	
D-NH_4Cl	0.0112	1	0.012	0.0291	0.871	
E-$MgSO_4$	1.935	1	1.934	5.029	0.066	
F-$FeSO_4$	8.076	1	8.076	20.996	0.004	
G-Nacl	9.893	1	9.893	25.722	0.003	
H-KH_2PO_4	22.756	1	22.755	59.165	0.0002	
J-Na_2HPO_4	12.172	1	12.171	31.647	0.001	
Residual	2.308	6	0.385			
Cor Total	98.094	15				

df- Degree of freedom

Table 5: Statistical analysis of OAD1.

Std. Dev.	0.620	R-Squared	0.976
Mean	3.488	Adj R-Squared	0.941
C.V. %	17.780	Pred R-Squared	0.833
PRESS	16.410	Adeq Precision	17.210

Std.Dev. – Standard deviation; C.V – Coefficient of variation; PRESS – Predicted residual error sum of squares; Adj R – Squared - Adjusted R- Squared; Pred R- Squared – Predicted R- Squared; Adeq Precision – Adequate Precision.

3.3. Optimization of media using orthogonal array design 2 (OAD2)

The corollary of OAD1 aided in the construction of the second orthogonal array design, in which three additional components namely: Magnesium sulphate ($MnSO_4$), zinc sulphate ($ZnSO_4$) and calcium chloride ($CaCl_2$), were studied and the levels of components used in OAD1 were regulated. Magnesium sulphate ($MnSO_4$), zinc sulphate ($ZnSO_4$) and calcium chloride ($CaCl_2$) have also been used as medium components for enzyme production [22]. Therefore, a second $L_{16}(2^{15})$ experimental design (OAD2) was used and the concentration of each constituent was optimized with nine parameters in 16 runs (Table 6). The second L_{16}

(2^{15}) experimental design (OAD2) was used to study nine parameters in 16 runs (Table 6) Six parameters (components) cholesterol (A), tween 80 (C), $FeSO_4$ (F), NaCl (G), KH_2PO_4 (H) and Na_2HPO_4 (J) showed significant effect, as observed in OADl, and the three new variables such as manganese sulphate, zinc sulphate and calcium chloride were used in OAD2.

Table 6: Experimental Field for OAD2 –results.

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Response
Expt. Run No.	A: $MnSO_4$	B: $ZnSO_4$	C: Cholesterol	D: KH_2PO_4	E: Na_2HPO_4	F: $CaCl_2$	G: NaCl	H: $FeSO_4$	J:Tween 80	Specific Activity
1	1	1	1	1	1	1	1	1	1	1.7
2	1	1	1	1	2	2	2	2	2	8.3
3	1	1	2	2	1	1	1	1	2	6.03
4	1	1	2	2	2	2	2	2	1	8.2
5	1	2	1	2	1	1	2	2	2	6.4
6	1	2	1	2	2	2	1	1	1	1.2
7	1	2	2	1	1	1	2	2	1	5.7
8	1	2	2	1	2	2	1	1	2	6.2
9	2	1	1	2	1	2	1	2	2	6.1
10	2	1	1	2	2	1	2	1	1	2.82
11	2	1	2	1	1	2	1	2	1	6.09
12	2	1	2	1	2	1	2	1	2	7.6
13	2	2	1	1	1	2	2	1	1	2.15
14	2	2	1	1	2	1	1	2	2	5.24
15	2	2	2	2	1	2	2	1	2	7.29
16	2	2	2	2	2	1	1	2	1	5.44

The average specific activity of cholesterol oxidase for each experimental run was calculated. The ANOVA for the experimental results obtained by OAD2 was also calculated. The components $MnSO_4$ (A) and KH_2PO_4 (D) showed no significant effect, while $ZnSO_4$ (B), cholesterol (C), Na_2HPO_4 (E) $CaCl_2$ (F), NaCl (G) $FeSO_4$ (H) and Tween 80 (J), showed significant effect on the cholesterol oxidase production (Table 6). Thus the concentrations were adjusted according to the results obtained from all the statistical data, such that optimum enzyme production was achieved.

To determine the significant level of each constituent, the specific activity of the enzyme was calculated for each experimental run of the design (Table 6), the mean of squares for each treatment and error (residual) were calculated using Fisher's F-test (Table 7). The data analyzed by ANOVA have indicated that $ZnSO_4$ (B), cholesterol (C), Na_2HPO_4 (E) $CaCl_2$(F), NaCl (G) $FeSO_4$ (H) and tween-80 (J), have shown significant positive effects on the response. The effect of $MnSO_4$ (A) and KH_2PO_4 (D) found to be insignificant (Table 7). Cells require specific metal ions and some organic growth factors and in many cases the need for these substances arises from their roles as cofactors of metabolic and biosynthetic enzymes.

Table 7: ANOVA for OAD2.

Analysis of variance table						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	75.622	9	8.4024	71.0410	2.1025E-05	Significant
A-$MnSO_4$	0.0625	1	0.0625	0.5284	0.4947	
B-$ZnSO_4$	3.258	1	3.2580	27.5462	0.0020	
C-Cholesterol	21.716	1	21.7156	183.6026	1.0023E-05	
D-KH_2PO_4	0.0156	1	0.0157	0.1321	0.7287	
E-Na_2HPO_4	0.783	1	0.7832	6.6220	0.04215	
F-$CaCl_4$	1.323	1	1.3225	11.1816	0.01554	
G-NaCl	6.838	1	6.8383	57.8164	0.00027	
H-$FeSO_4$	16.975	1	16.9744	143.5163	2.0506E-05	
J-Tween-80	24.652	1	24.6512	208.4230	6.9199E-06	
Residual	0.710	6	0.1183			
Cor Total	76.330	15				

df- Degree of freedom

The Half-Normal plot indicates the standardized effects of the factors on the x axis (Figure-3). Six factors viz., cholesterol (C), Na_2HPO_4 (E) $CaCl_2$ (F), NaCl (G) $FeSO_4$ (H) and tween-80 (J), had a positive impact on the production of the enzyme. However, one factor, $ZnSO_4$ (B) had a negative effect on enzyme production (Figure-3). These results were in agreement with those found on the Pareto chart (Figure 4). Lee *et al.*, (1997), observed that $ZnSO_4$ had significant positive effect on cholesterol oxidase production, since it serves as an enzyme cofactor and also required for protein synthesis and cell division [20]. The same metal ions were also

added to the medium for the production of cholesterol oxidase by *Rhodococcus* sp. GK1 [22]. However, Aihara et al., (1986) used only Fe^{2+} and Mg^{2+} ions in the cholesterol oxidase production[24]. However, Watanabe et al., (1989) used only Fe^{2+} for *R. equi* No. 23, while used Mg^{2+} for *A. simplex* [19,21]. Thus the concentrations were adjusted according to the results obtained from all the statistical data, such that optimum enzyme production was achieved.

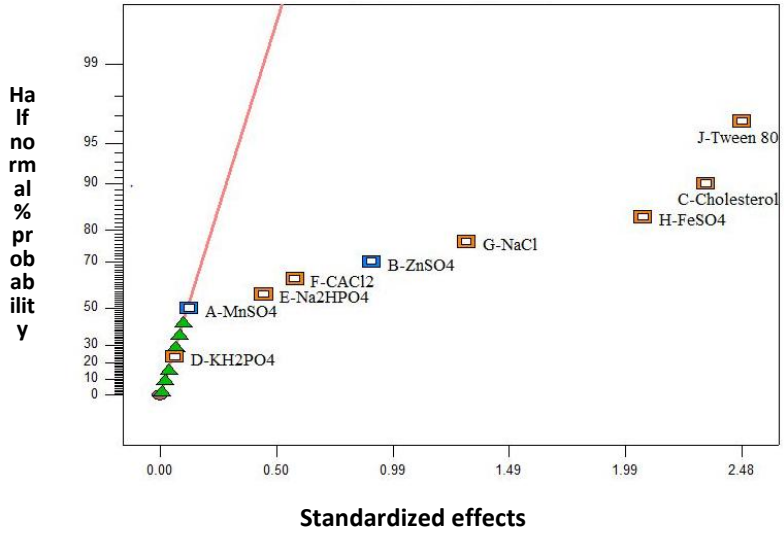

Figure-3: Half-normal plot of effects of media components in OAD2.

▲ Error Estimates; A: $MnSO_4$; B: $ZnSO_4$; C: Cholesterol; D: KH_2PO_4; E: NA_2HPO_4; F: $CaCl_2$; G: NaCl: H: $FeSO_4$; J: Tween-80: ■ Positive effect: ■ Negative effect

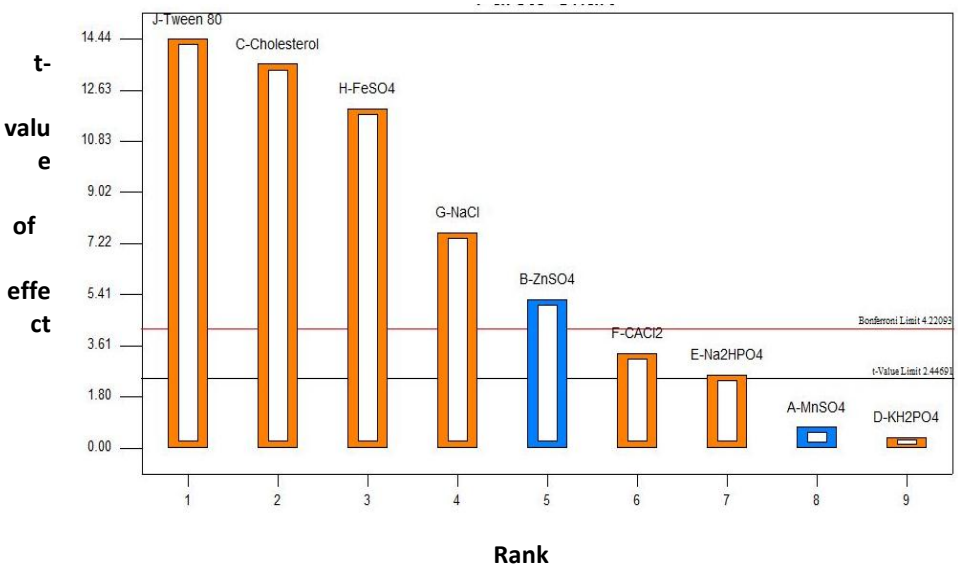

Figure-4: Pareto Chart of effect of media components in OAD2.

3.4. Cholesterol oxidase production with optimized and unoptimized media

Using one-factor-at-a-time and orthogonal array methods, the most effective components were selected to design a simple yet very effective medium for increased production of cholesterol oxidase. The selected orthogonal array was L_{16} (2^{15}), along with the optimized components for cholesterol oxidase production have

resulted in a threefold increase, in comparison to the control medium (Figure 5). Yazdi *et al.*,(2001) was able to achieve a six-fold increase in cholesterol oxidase production from *Rhodococcus* sp., by altering the media components[1]. Similarly, Lee et al., (1997) demonstrated an increase in cholesterol oxidase by four fold through media improvement[23].

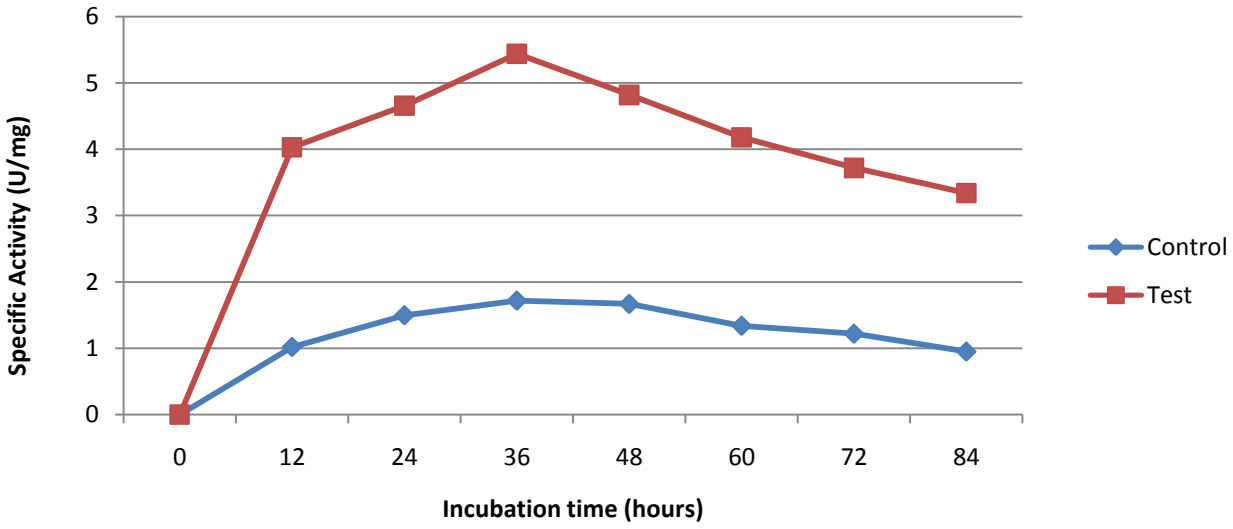

Figure-5: Cholesterol oxidase production in optimized and unoptimized media.

Table 8: Statistical Analysis of OAD2.

Std. Dev.	0.34392	R-Squared	0.99070
Mean	5.40375	Adj R-Squared	0.97675
C.V. %	6.36431	Pred R-Squared	0.93389
PRESS	5.0464	Adeq Precision	25.2588

Std.Dev: Standard deviation; C.V: Coefficient of variation; PRESS: Predicted residual error sum of squares; Adj R-Squared: Adjusted R- Squared; Pred R- Squared: Predicted R- Squared; Adeq Precision: Adequate Precision.

Table 9: Kinetics of cholesterol oxidase production

Duration of incubation	Cholesterol oxidase specific activity (U/mg)	
	Unoptimized medium	Optimized medium
0	0	0
12	1.02 ± 0.02	4.03 ± 0.04
24	1.5 ±0.04	4.66 ± 0.02
36	**1.72 ±0.02**	**5.44 ± 0.06**

48	1.67 ±0.03	4.82 ± 0.02
60	1.34 ±0.03	4.18 ± 0.3
72	1.22 ±0.02	3.72 ± 0.05
84	0.95 ± 0.04	3.34 ± 0.02

Each value represents the mean of independent experiments.

4. Conclusion

This OAD methodology can be used efficiently and successfully for the improvement of multi-variable biological systems. In the present study, the improved medium obtained by OAD in two steps showed a three-fold increase in cholesterol oxidase production and thus it can be used for industrial scale production.

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Conflict of Interest

The authors declare that they do not have any conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

Declarations of interest: none

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Corresponding Author:

Palani Perumal*

Email: *drppalani@yahoo.in*