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OPTIMISATION OF PRESERVATIVE SYSTEM FOR LIQUID DOSAGE FORM USING BOX BEHNKEN DESIGN

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ABSTRACT

In this study Box Behnken design was used to determine antimicrobial activity. The effect of independent variables (concentration of methyl and propyl paraben and pH) on the preservative efficacy was evaluated. The log reduction of different species (*E.coli, S.aureus, Ps.aeruginosa, C.albicans and A.niger) at* 14 day was used as response. For optimization, the effects of independent variables upon the responses were modelled using the polynomial equations involving main effects and their interactions for various responses. A graphical optimization technique was carried out to achieve optimized preservative system. The use of a design of experiment can be successful in optimisation of preservative system to meet regulatory criteria.

KEYWORDS: *Box Behnken design, log reduction, Preservative efficacy, Paraben, Optimization.*

INTRODUCTION

Preservatives have been commonly used as excipients in pharmaceutical, cosmetic and food products. Liquid preparations are particularly sensitive to microbial growth due to the nature of their excipients. Preservatives are primarily effective in controlling mold, inhibiting yeast growth and protecting against bacterial growth.[1] Their antimicrobial and antifungal properties make them an integral part of the product formulation. The preservation of the liquid product is necessary for two main reasons. The first reason is the health risks. When microorganisms contaminate a drug product, it can be harmful to consumers. The second concern is the deterioration of the products. The proliferation of microorganisms can cause problems of stability, changes in pH, color or odor.

Among the various preservatives, parabens (p-hydroxybenzoic acid esters) are the most commonly used for liquid pharmaceutical products because of their low toxicity to humans and their effective antimicrobial activity, especially against mold and mildew yeasts.[2] Combinations of parabens are more effective as preservatives than individual esters. Optimizing a preservation system for a preparation requires determining the antimicrobial efficacy of various combinations of preservatives. The antimicrobial activity of the preservatives is influenced by several factors, such as pH, tonicity and temperature of the solution, the concentration of the preservative, the presence of EDTA, etc.[3,4] Several studies on the effect of these factors have been reported. These studies were based on a "one-factor-at-a-time" procedure that was often tedious and did not reveal the interactions between different variables. Factorial design provides a means of simultaneously assessing the influence of individual variables and their interactions at different levels with a minimum of experience, and subsequently in a shorter time and at lower cost. However, in the field of efficacy of microbial preservatives, factorial design has been little used until now. Bart De Spiegeleer et al. studied effect of the concentration of the co-solvent propylene glycol on the preservative efficacy of a complex pharmaceutical suspension-emulsion formulation containing methyl- and propyl paraben.[5] Karabit et al. evaluated the influence of pH, tonicity and EDTA on the antimicrobial activity of a phenolic preservative in a solution using a factorial design with two test organisms.[6] The effect of antimicrobial preservatives (benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol) on the stability and potency of a protein, as well as on the preservative efficacy of the formulation was assessed using an I-optimal experimental design after an initial screening.[7] Five factors (pH, sucrose, propylene glycol, glycerin and EDTA) were tested to determine the stability and storage capacity of lamivudine oral solution using a central composite model.[8]

The aim of this work was to demonstrate the usefulness of a Box-Behnken experimental design for determining the optimal preservative system used in a liquid pharmaceutical product. In this research, three independent variables (Methyl paraben, Propyl paraben and pH) were investigated and system selected as optimal was the one, which met the requirements of the European Pharmacopeia (EP) antimicrobial preservative efficacy test[9] (table I).

Table I: Acceptance criteria for preservation efficacy test as per European Pharmacopeia.

Log reduction				
		7 Day	**14 Day**	**28 Day**
Bacteria	A	3	-	NI
	B	-	3	NI
Fungi	A	-	2	NI
	B	-	1	NI

A recommended, B mandatory, NI no increase in number of viable micro-organisms compared to the 14 day.

MATERIAL AND METHODS

Chemicals

All chemicals and reagents used were of analytical reagent grade. Methylparaben, propylparaben and propylene glycol were obtained from Sigma-Aldrich, while citric acid and ethylenediamine tetraacetate were from SD Fine Chemicals Ltd. The natural gums were from Signet Chemical Corporation, while butylated hydroxytoluene and monoglyceride citrate were from Yarrow Chem Products. Sodium hydroxide was from Colorcon Asia Pvt.Ltd and albendazole from Micro labs. The water used was deionized laboratory water.

Preparation of the Test Formulations

The test formulations were independently prepared by adding weighed quantities of the methyl-and propylparaben under investigation to obtain a solution of both parabens in propylene glycol. The obtained solution of parabens in propylene glycol was slowly added to a previously prepared aqueous phase, which consisted of the natural gums (15% mass/mass or m/m) in a citrate buffer containing citric acid, sodium hydroxide, ethylenediamine tetraacetate and monoglyceride citrate. This aqueous phase was vigorously mixed and homogenized with a previously prepared oily suspension of albendazole (10% m/m) in vegetable oil (35% m/m), containing butylhydroxytoluene. The density of the formulation was 1.0 g.mL−1. Sodium citrate, citric acid and dibasic sodium phosphate, heptahydrate were used to adjust the solutions to the desired pH. Table II shows the final composition of suspension emulsion formulations under investigation.

Table II: Box Behnken Design Matrix.

S.No	A:Conc.MP (% w/v)	B:Conc. PP (% w/v)	C:pH	European Pharmacopeia Compliance			
				Bacteria		Fungi	
				A	B	A	B
1	0.2	0.055	6.5	-	+	-	-
2	0.1	0.055	8	-	-	-	+
3	0.1	0.01	6.5	-	-	-	-
4	0.1	0.1	6.5	-	-	-	-
5	0.2	0.01	5	+	+	-	-
6	0.2	0.055	6.5	-	+	-	-
7	0.1	0.055	5	+	+	-	+
8	0.3	0.1	6.5	+	+	-	-
9	0.3	0.01	6.5	-	+	+	+
10	0.2	0.1	8	+	+	+	+
11	0.3	0.055	8	+	+	+	+
12	0.2	0.1	5	+	+	-	+
13	0.2	0.055	6.5	-	+	-	-
14	0.3	0.055	5	-	+	-	-
15	0.2	0.01	8	-	-	-	-

+ Compliance, - Non compliance.

Preservative efficacy test (PET) methodology

The preservative efficacy was assessed by the PhEur microbial challenge test method[14] concerning oral preparations. The formulations (samples of 20 g) were placed in sterile containers and separately inoculated with bacterial and fungal suspensions to give a final level of approximately 106 CFU.g−1. The preparations were well mixed to ensure a homogeneous microorganism distribution and incubated at 20–25°C. After a contact time of 0, 7, 14, 21 and 28 days, samples (1.0 g) were removed and placed into 99.0 mL of neutralizing medium MLEB (Difco). Cell viability was determined by the pour-plate count method in TSA or SDA plates, and colony forming units (CFUs) were counted after a 3- and 5-day incubation at 37°C and 30°C for bacteria and fungi, respectively. All counts were performed in duplicate. A growth control with the medium alone at day 0 was included for each organism (Control column of Table III), assuring consistent and adequate number of viable microorganisms were added to the formulations. Moreover, days 7 and 21 were included, although these are not evaluation points according to the PhEur, to allow a weekly data evaluation.[3,10]

RESULTS AND DISCUSSIONS

The study design including three factors under investigation and conclusions of compliance towards the pharmacopoeial prerequisites, are shown in Table II. Table III shows the detail

quantitative analysis, *i.e.* the log reduction (D). Logarithmic reduction results by species and time, expressed in log CFU mL^{-1}, were calculated from the difference between log CFU mL^{-1} at D0, D7, D14 and D28. A growth control with the medium alone at day 0 was included for each organism (Control column of Table III), assuring consistent and adequate number of viable microorganisms were added to the formulations. According to the European pharmacopeia mandatory criteria,[14] an oral preparation is effectively preserved if the log reduction of bacteria and fungi, not less than 3.0 and 1 respectively from the initial count within 14 days of challenge, with no subsequent increase in count from 14 day at the twenty-eighth day. Results of the preservative screening tests showed that the formulation 7,9,10,11 and 12 satisfied EP criteria.

Table III: Preservative efficacy (logarithmic reduction) towards different strains at zero hour (D0), 7 day (D7), 14 day (D14), 21 day (D21) and 28 day (D 28).

S.No	Strain	Control (CFU/ml)	Log reduction				
			D0	D7	D14	D21	D28
1	*E.coli*	1.8E + 05	0.4	4.8	4.8	4.8	4.8
	S.aureus	2.0E + 05	0	5.3	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	0.9	1.34	4.7	5.3	6.2
	C.albicans	9.8E + 04	0.0	0.83	4.8	5.0	5.0
	A.niger	5.2E + 05	0.4	0.31	0.39	0.73	0.90
2	*E.coli*	1.8E + 05	0.3	4.8	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.22	1.83	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	1.1	1.38	1.59	6.2	6.2
	C.albicans	9.8E + 04	0.18	1.32	2.78	4.9	4.9
	A.niger	5.2E + 05	0.0	0.17	0.46	1.11	1.41
3	*E.coli*	1.8E + 05	0.47	4.8	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.60	5.3	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	0.84	1.39	1.75	6.2	6.2
	C.albicans	9.8E + 04	0.29	1.45	2.9	3.9	4.9
	A.niger	5.2E + 05	0.39	0.24	0.37	1.17	1.24
4	*E.coli*	1.8E + 05	0.57	2.12	3.6	4.8	4.8
	S.aureus	2.0E + 05	0.30	5.3	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	0.90	0.92	0.90	1.33	1.79
	C.albicans	9.8E + 04	0.13	1.83	3.21	4.9	4.9
	A.niger	5.2E + 05	0.33	0.45	0.56	1.17	1.23
5	*E.coli*	1.8E + 05	0.73	4.8	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.47	5.3	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	1.2	6.2	6.2	6.2	6.2
	C.albicans	9.8E + 04	0.14	1.07	2.65	4.9	4.9
	A.niger	5.2E + 05	0.0	0.0	0.0	0.63	0.87
6	*E.coli*	1.8E + 05	0.50	2.32	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.35	1.63	3.86	5.3	5.3
	Ps.aeruginosa	1.6E + 06	0.84	6.2	6.2	6.2	6.2
	C.albicans	9.8E + 04	0.46	4.9	4.9	4.9	4.9

	A.niger	5.2E + 05	0.0	0.0	0.0	0.71	0.41
7	*E.coli*	1.8E + 05	0.35	4.8	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.82	5.3	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	1.68	6.2	6.2	6.2	6.2
	C.albicans	9.8E + 04	0.14	1.74	4.75	4.9	4.9
	A.niger	5.2E + 05	0.0	0.0	0.0	0.63	0.87
8	*E.coli*	1.8E + 05	0.82	2.04	4.8	4.8	4.8
	S.aureus	2.0E + 05	1.0	3.82	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	1.90	6.2	6.2	6.2	6.2
	C.albicans	9.8E + 04	0.32	2.49	4.29	4.9	4.9
	A.niger	5.2E + 05	0.31	0.47	0.64	0.78	1.51
9	*E.coli*	1.8E + 05	0.84	2.63	3.26	4.8	4.8
	S.aureus	2.0E + 05	0.82	1.84	3.52	5.3	5.3
	Ps.aeruginosa	1.6E + 06	0.76	1.82	3.45	6.2	6.2

Table III: (Continued).

	C.albicans	1.6E + 06	0.41	2.5	3.3	4.9	4.9
	A.niger	9.8E + 04	0.0	1.04	2.69	4.21	4.56
10	*E.coli*	1.8E + 05	0.49	4.8	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.30	5.3	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	6.2	6.2	6.2	6.2	6.2
	C.albicans	9.8E + 04	0.40	4.9	4.9	4.9	4.9
	A.niger	5.2E + 05	0.35	1.25	2.7	5.7	5.7
11	*E.coli*	1.8E + 05	0.63	4.8	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.47	5.3	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	6.2	6.2	6.2	6.2	6.2
	C.albicans	9.8E + 04	0.56	4.9	4.9	4.9	4.9
	A.niger	5.2E + 05	0.30	2.1	3.1	3.41	5.7
12	*E.coli*	1.8E + 05	0.41	1.36	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.35	5.3	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	2.46	3.83	6.2	6.2	6.2
	C.albicans	9.8E + 04	0.54	0.74	4.9	4.9	4.9
	A.niger	5.2E + 05	0.35	0.85	1.11	3.41	3.71
13	*E.coli*	1.8E + 05	0.77	1.39	4.65	4.8	4.8
	S.aureus	2.0E + 05	0.12	5.3	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	0.72	1.27	6.2	6.2	6.2
	C.albicans	9.8E + 04	0.08	1.64	3.33	4.9	4.9
	A.niger	5.2E + 05	0.27	0.36	0.42	0.68	0.79
14	*E.coli*	1.8E + 05	0.0	4.8	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.30	1.72	4.55	5.3	5.3
	Ps.aeruginosa	1.6E + 06	0.90	6.2	6.2	6.2	6.2
	C.albicans	9.8E + 04	0.16	4.9	4.9	4.9	4.9
	A.niger	5.2E + 05	0.21	0.28	0.36	0.59	0.32
15	*E.coli*	1.8E + 05	0.0	0.83	4.8	4.8	4.8
	S.aureus	2.0E + 05	0.45	1.73	5.3	5.3	5.3
	Ps.aeruginosa	1.6E + 06	0.50	1.42	2.72	6.2	6.2
	C.albicans	9.8E + 04	0.82	1.39	2.26	4.9	4.9
	A.niger	5.2E + 05	0.03	0.21	0.36	0.59	0.32

A Box Behnken Design was used to found the optimum level of methyl paraben, propyl paraben and pH to achieve a preservative system which is complying with EP criteria. A total of 15 trial formulations were proposed by Box Behnken design for three independent variables at three levels. The reduction in bacterial and fungal counts following incubation days 14 at room temperature was taken as the measured response and modelled by polynomial equations. The result of the analysis of variance (ANOVA) for responses ($P > 0.05$) shown in table IV.

Table IV: Analysis of variance (ANOVA) of the log reduction at 14 day for different strains.

Source	SS	df	MS	F	Significance(p-value)
E.coli					
Model	10.14	9	1.13	101.27	< 0.0001
A	0.0378	1	0.0378	3.40	0.1246
B	6.88	1	6.88	618.43	< 0.0001
C	0.0861	1	0.0861	7.74	0.0388
AB	0.0400	1	0.0400	3.59	0.1165
AC	0.0056	1	0.0056	0.5055	0.5089
BC	0.1156	1	0.1156	10.39	0.0234
A^2	0.0024	1	0.0024	0.2143	0.6628
B^2	2.94	1	2.94	264.05	< 0.0001
C^2	0.0032	1	0.0032	0.2904	0.6131
Residual	0.0439	5			
Core Total	3.28	14			
S.aureus					
Model	1.27	3	0.4222	1.33	0.0314
A	0.8001	1	0.8001	2.52	0.1404
B	0.3960	1	0.3960	1.25	0.2875
C	0.0703	1	0.0703	0.2218	0.6469
Residual	3.49	11			
Core Total	4.75	14			
Ps.aeruginosa					
Model	55.87	9	6.21	11.64	0.0073
A	16.85	1	16.85	31.60	0.0025
B	3.62	1	3.62	6.79	0.0480
C-pH	8.18	1	8.18	15.34	0.0112
AB	3.24	1	3.24	6.08	0.0569
AC	5.31	1	5.31	9.97	0.0252
BC	3.03	1	3.03	5.68	0.0629
A^2	7.82	1	7.82	14.67	0.0122
B^2	5.08	1	5.08	9.53	0.0273
C^2	2.36	1	2.36	4.43	0.0893
Residual	2.67	5			
Core Total	58.54	14			

Table IV: (Continued).

C.albicans					
Model	10.14	9	1.13	101.27	< 0.0001
A	0.0378	1	0.0378	3.40	0.1246
B	6.88	1	6.88	618.43	< 0.0001
C	0.0861	1	0.0861	7.74	0.0388
AB	0.0400	1	0.0400	3.59	0.1165
AC	0.0056	1	0.0056	0.5055	0.5089
BC	0.1156	1	0.1156	10.39	0.0234
A^2	0.0024	1	0.0024	0.2143	0.6628
B^2	2.94	1	2.94	264.05	< 0.0001
C^2	0.0032	1	0.0032	0.2904	0.6131
Residual	0.0556	5			
Core Total	10.20	14			
A.Niger					
Model	5.54	3	1.85	2.89	0.0435
A	2.37	1	2.37	3.70	0.0805
B	0.0084	1	0.0084	0.0132	0.9105
C	3.16	1	3.16	4.95	0.0479
Residual	7.02	11			
Core Total	12.56	14			

A=Conc. MP, B= Conc. PB, C=pH, SS= Sum of square, MS= Mean square

ANOVA result showed all responses response models were significant ($p<0.05$. The F value in the ANOVA table was the ratio of model mean square (MS) to the appropriate error (i.e. residual) mean square. The larger the F value and the more likely that the variance contributed by the model was significantly larger than random error. The model F-value and high R square values suggested that these models were significant. The log reduction of microbial counts could be presented by cube plots (Figs 1, 2, 3, 4 and 5).

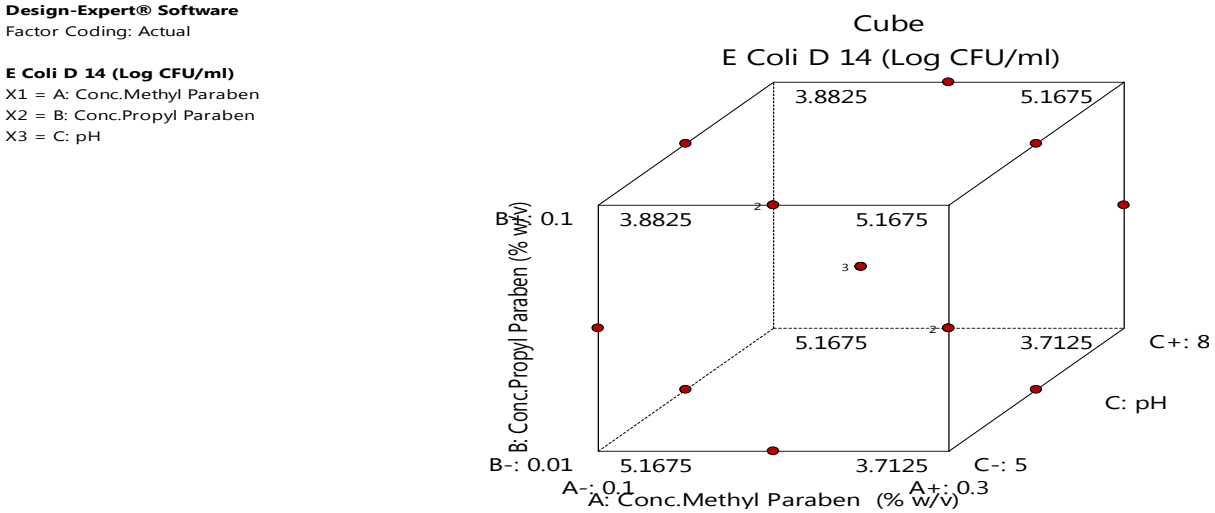

Figure 1: Cube plots of estimated response: *E.coli* (D14).

Design-Expert® Software
Factor Coding: Actual

S.aureus D14 (Log CFU/ml)
X1 = A: Conc.Methyl Paraben
X2 = B: Conc.Propyl Paraben
X3 = C: pH

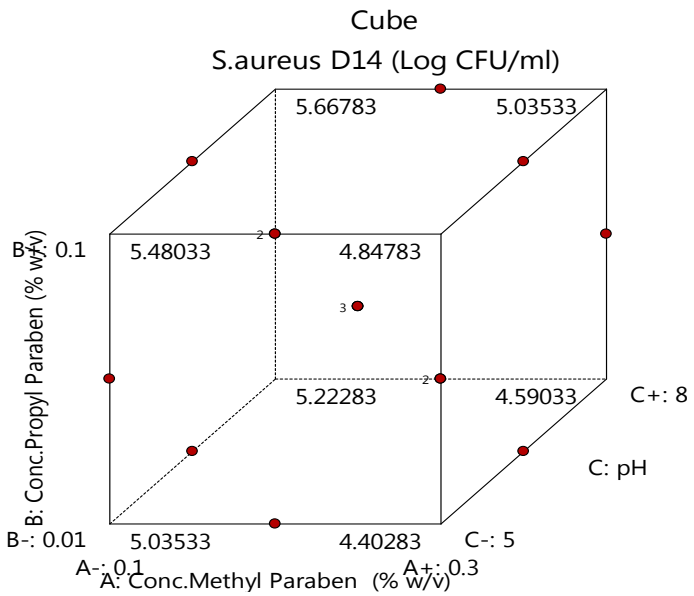

Figure 2: Cube plots of estimated response: *S.aureus* (D14).

Design-Expert® Software
Factor Coding: Actual

Ps.aeruginosa D14 (Log CFU/ml)
X1 = A: Conc.Methyl Paraben
X2 = B: Conc.Propyl Paraben
X3 = C: pH

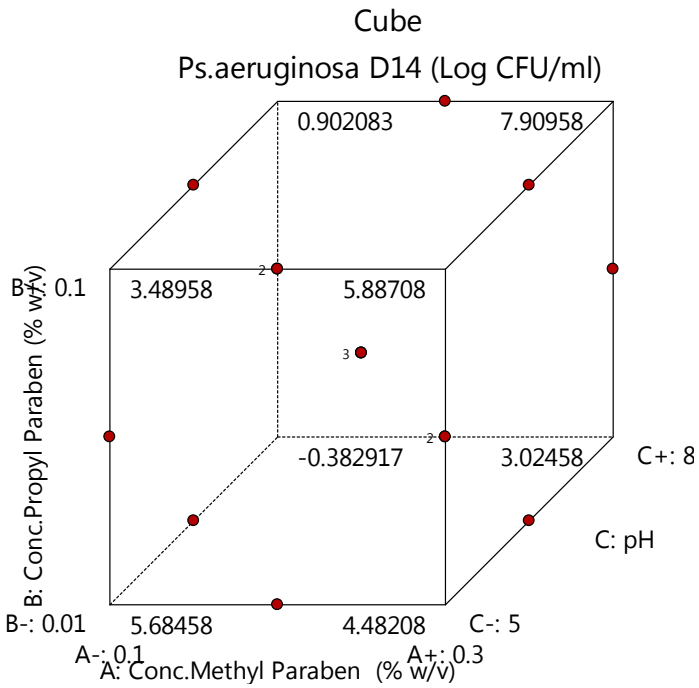

Figure 3: Cube plots of estimated response: *S.aureus* (D14).

Design-Expert® Software
Factor Coding: Actual

C.albicans D14 (Log CFU/ml)
X1 = A: Conc.Methyl Paraben
X2 = B: Conc.Propyl Paraben
X3 = C: pH

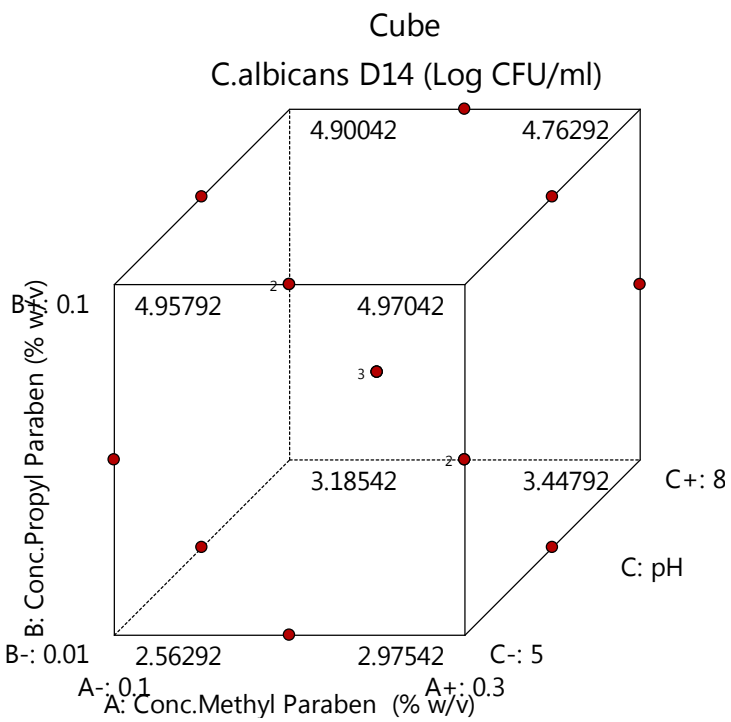

Figure 4: Cube plots of estimated response: *C.albicans* (D14).

Design-Expert® Software
Factor Coding: Actual

A.Niger D14 (Log CFU/ml)
X1 = A: Conc.Methyl Paraben
X2 = B: Conc.Propyl Paraben
X3 = C: pH

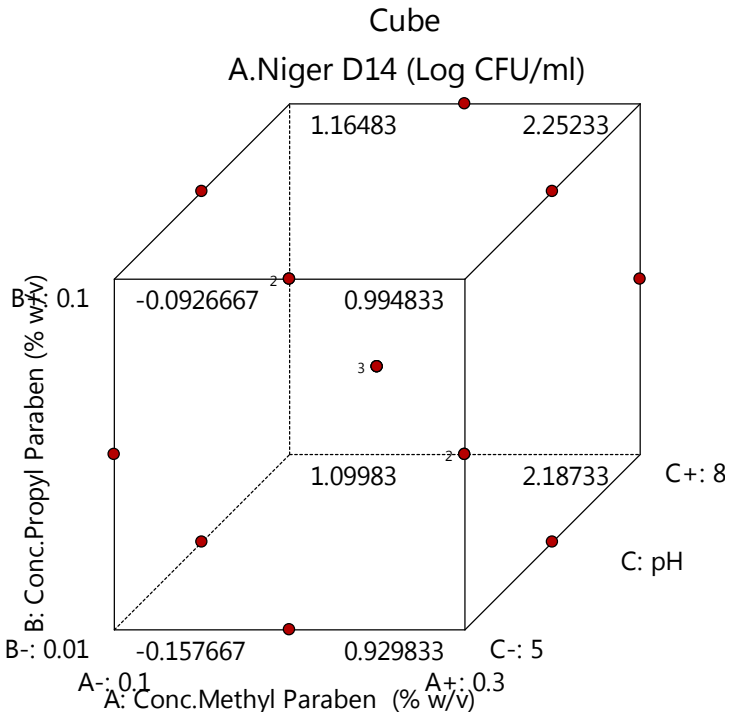

Figure 5: Cube plots of estimated response: *A.niger* (D14).

The polynomial equation of reduced model by backward elimination ($p>0.05$) for each response variable was as follow:

E.coli (D14) = $4.75 + 0.685 * AB - 0.3175 * A^2 - 0.3175 * B^2 + 0.3675 * C^2$

S.aureus (D14)= 5.03533 - 0.31625 * A + 0.2225 * B + 0.09375 * C

Ps.aeruginosa (D14) = 4.728 + 1.45125 * A

C.albicans (D14) = 4.86429 + 0.9275 * B + 0.10375 * C - 0.17 * BC - 0.891786 * B^2

A.niger (D14) = 1.04733 + 0.54375 * A + 0.0325 * B + 0.62875 * C

The response *E.coli* (D14) was not significantly affected by the main effects (*A,B* & *C*) but depend on quadratic contribution (A^2, B^2 and C^2) as well as cross-product contribution (interaction effects) of effects *A* and *B*. Result indicate that significant factors affecting the response *S.aureus* (D14) were synergistic effect of the linear contribution of main effects (*A,B* & *C*) without producing any interaction and response *Ps.aeruginosa* (D14) was significantly affected by factor C (pH). The response *C.albicans* (D14) was significantly affected by the main effects (*B* & *C*), quadratic contribution (B^2) and cross-product contribution (interaction effects) of effects *B* and *C*. Log reduction of *A.niger* at 14 day (D14) was significantly affected by main effects (A, B and C).

To develop an optimized preservative system which comply the EP acceptance criteria A and B, a graphical optimization technique based on the desirability approach was adopted. The overlay plot (figure 6) represented graphical solution.

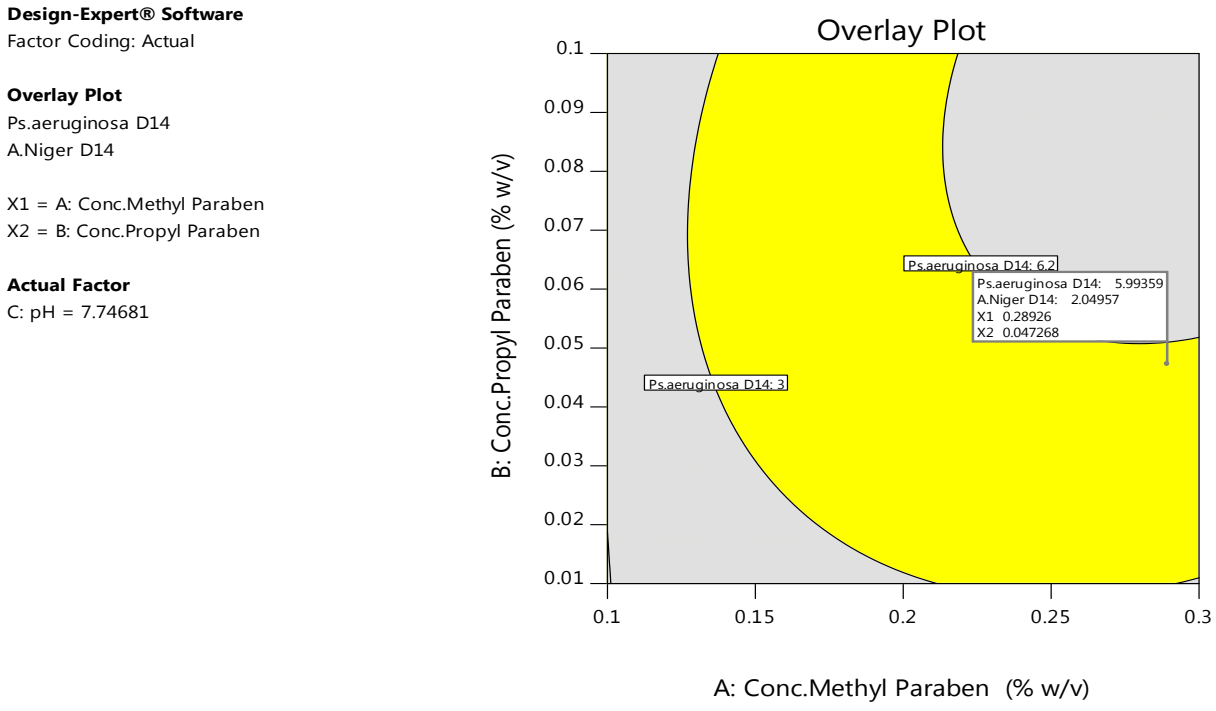

Figure 6: Graphical solution by overlay plot.

CONCLUSION

In this study concentration of preservatives and pH was optimised to comply with EP criteria by using a design of experiment approach. A Box Behnken Design was carried out to study the effect of independent variables (conc. of MB, conc. of PB and pH) on antimicrobial efficacy by a limited number of experiments. The model also indicated several effective preservatives systems that meet the antimicrobial efficacy constraints. The experimental design approach were found to be effective in determine optimal concentrations of paraben preservatives and pH.

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