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Production medium optimization for enhancement the exotoxin secretion by *Pseudomonas aeruginosa*

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Abstract

Media variations including eight variables were optimized for improvement of exotoxin production by *Pseudomonas aeruginosa*, using the statistical design Taguchi system. Eighteen experimental trials with final layout of L 18 (1^2 x 7^3) were used. The bacterial toxin production varied significantly among media within range of 1.0-31.0 µg ml^{-1}. This indicated the validity of optimization design in bioprocesses. The statistical design showed little variations between the actual experimental values and the Taguchi software predicted values. The (ANOVA) analysis implies "P-value" of 10.4 which revealed that the design model is significant. The F-value (less than 0.05) revealed that temperature, agitation and interaction between incubation period and beef extract are the most significant model terms. The regression values of the classical sum of squares indicated that predicted-R^2 (0.6208) and the adjusted-R^2 (0.8341) were in reasonable agreement which indicates the accuracy of experimentation. The predicted optimum medium from the Taguchi software database was beef extract 4 g l^{-1}, NaCl 7 g l^{-1} and peptone 3 g l^{-1}, incubation period 36 h, pH 6.5, temperature 27°C, inoculum size 3% and agitation 150 rpm.

Key words**:** *Pseudomonas aeruginosa*, exotoxins, statistical design, production medium.

Introduction

The pathogenic bacterium *Pseudomonas aeruginosa* has the ability to cause severe acute and chronic infections in humans. *P. aeruginosa* exotoxin (PE) is the most toxic virulence factor of this bacterium. It has ADP-ribosylation activity and affects the protein synthesis of the host cells. Interestingly, a medicinal benefit from this exotoxin has also been ascertained in recent years and several *P. aeruginosa* exotoxin-based immunotoxins have been constructed and tested for preclinical and clinical trials against different cancers [1]. Constructions of immunotoxins against cancers are designed whereby the enzymatic active domain of PE is specifically targeted to tumor-associated antigens [2, 3]. The toxin PE has been shown to be lethal for mice [4], and to be capable of eliciting hypotensive shock in cats and dogs[5]. Furthermore, it has been shown that the toxin inhibits uridine and amino acid uptake by tissue culture cells, so it inhibits protein synthesis [6].

PE is a potent toxin which inhibits protein synthesis in eukaryotic cells by catalyzing ADP ribosylation of elongation factor 2 [7, 8]. PE consists of three distinct domains which carry different functions. Domain I is the cell recognition domain which enables the toxin to bind to the cell receptor. Domain II facilitates the translocation of the toxin across the membrane of target cells into cytosol [3], and is important for expression and secretion from *E. coli* [9]. Domain III possesses the catalytic site for ADP ribosylation [10]. Strategies such as altering route of delivery, changing the dose, eliminating supporting or synergizing molecules and modifying structure of the molecule may convert a dangerous toxin into valuable therapeutic drug [4].

Optimization studies, involving the one-factor at a time approach, tend to overlook the effect of interaction among factors and might lead to misinterpretations of results. In contrast, statistical methodologies are generally preferred due to their advantages in minimizing the error in determining the effect of parameters in an economical manner [11-13]. In any bioprocess, the improvement in productivity of any metabolite, such as enzymes, toxins and hormones, would be achieved through manipulation of nutritional and physical parameters [14]. Statistical experimental design minimizes the error in determining the effect of parameters and allows more simultaneous, systematic and efficient variation of all parameters than the classical method [15-18].

The objective of this work was to optimize the cultural conditions for production of *P. aeruginosa* exotoxin in enough quantities for further molecular and antitumor studies using statistical Taguchi design.

Materials and Methods

Microorganism and culture conditions: *Pseudomonas aeruginosa* (TMN01) was kindly supplied by Prof. Dr. Tarek A. A. Moussa, Botany Department, Faculty of Science, Cairo University. The organism was maintained on nutrient agar at 4°C till use.

Experimental Taguich design: Experimental Taguchi design [19] was applied. It includes establishment of large number of variables described as orthogonal arrays to reduce experimental errors and to enhance their efficiency and reproducibility in laboratory experiments. Eight variables [(A), incubation period, (B) beef extract, (C) NaCl, (D) pH, (E) temperature, (F) inoculum size, (G) agitation rate and (H) peptone] were studied (Table 1).

Table 1. Cultural variables and assigned levels.

Code	Variable	Level 1	Level 2	Level 3
(A)	Incubation period (h)	24	36	-
(B)	Beef extract (gl^{-1})	1.5	2	4
(C)	NaCl (gl^{-1})	3	5	7
(D)	pH	5.5	6.5	8
(E)	Temperature (^oC)	27	37	40
(F)	Inoculum size (%)	1	3	6
(G)	Agitation rate (rpm)	Static	100	150
(H)	Peptone (gl^{-1})	3	5	7

The size of experimentation was represented by the symbolic arrays L_{18} which indicated 18 experimental trails. Seven factors have been assigned with three levels (7^3) except (A) incubation period with 2 levels (1^2) with final layout of L_{18} (1^2 x 7^3) as shown in Table 2.

Table 2. L18 (1^2 x 7^3) orthogonal array of the designed experiment.

Media	Variable levels							
	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)
1	1	1	1	1	1	1	1	1
2	1	1	2	2	2	2	2	2
3	1	1	3	3	3	3	3	3
4	1	2	1	1	2	2	3	3
5	1	2	2	2	3	3	1	1
6	1	2	3	3	1	1	2	2
7	1	3	1	2	1	3	2	3
8	1	3	2	3	2	1	3	1
9	1	3	3	1	3	2	1	2
10	2	1	3	3	3	2	2	1
11	2	1	1	1	1	3	3	2
12	2	1	2	2	2	1	1	3
13	2	2	1	2	3	1	3	2
14	2	2	2	3	1	2	1	3
15	2	2	3	1	2	3	2	1
16	2	3	1	3	2	3	1	2
17	2	3	2	1	3	1	2	3
18	2	3	3	2	1	2	3	1

Subsequently, eighteen media were designed and assayed for the exotoxin production by *P. aeruginosa*. The compositions of those media are included in Table 3.

Table 3. Designed eighteen media composition by L18 (1^2 x 7^3) layout.

Media	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)
1	24	1.5	3	5.5	27	1	Static	3
2	24	1.5	5	6.5	37	3	100	5
3	24	1.5	7	8	40	6	150	7
4	24	2	3	5.5	37	3	150	7
5	24	2	5	6.5	40	6	Static	3
6	24	2	5	8	27	1	100	5
7	24	4	3	6.5	27	6	100	7
8	24	4	5	8	37	1	150	3
9	24	4	7	5.5	40	3	Static	5
10	36	1.5	7	8	40	3	100	3
11	36	1.5	3	5.5	27	6	150	5
12	36	1.5	5	6.5	37	1	Static	7
13	36	2	3	6.5	40	1	150	5
14	36	2	5	8	27	3	Static	7
15	36	2	7	5.5	37	6	100	3
16	36	4	3	8	37	6	Static	5
17	36	4	5	5.5	40	1	100	7
18	36	4	7	6.5	27	3	150	3

Extraction and determination of exotoxin protein: Exotoxin protein from *P. aeruginosa* extracted by inoculating 2.5 litre of the best medium out of 18 media with 2.5 ml of the broth culture and incubated at 37°C for 18 to 24 h. After incubation, the culture was centrifuged at 16,000 *xg* for 30 min. The supernatant liquid was passed through a prewashed 0.45 µm membrane filter and stored at -20°C till used [20]. Protein concentrations were determined by Lowry *et al.* [21], using egg albumin as standard.

Cytotoxicity assay: The mice morbidity bioassay [20] was used to measure toxicity of PE after growing *P. aeruginosa* on the optimized medium. Crude PE sample (0.2 ml) was filter sterilized, and serially diluted in phosphate buffer saline (PBs) to give 15, 30, 45 and 60 µg ml^{-1}. The PE dilutions were injected intravenously into female Swiss white mice weighing 20 g (three mice per dilution). Successive injection repeated for 3 days. Lethality and toxicity symptoms were recorded allover 1, 2, 3, 4 and 5 days post-injection.

Results and Discussion

The combination of standard experimental design technique and statistical analysis in the Taguchi approach produces consistency and reproducibility rarely found in any other statistical methods [22].

From the design, it was found that the actual values of the produced exotoxin protein revealed high variation among the different 18 media ranging 1.0-31.0 µg ml^{-1}. This indicated the importance of cultural optimization in toxin production from *P. aeruginosa* (Table 4). The optimization diagnostic case statistics for exotoxin production by *P. aeruginosa* indicated little variation between Taguchi software predicted values and the actual experimental values in 14 culture media (Table 4). However, Student's t-value indicated that the difference between the predicted and actual values of exotoxin exceeded limits in four media which were 1, 3, 6 and 18 with t-value higher than four.

Table 4. Diagnostic case statistics for exotoxin production by *P. aeruginosa*.

Media	Actual value (µgml^{-1})	Predicted value (µgml^{-1})	Student's t-value
1	4.0	8.38	*4.62
2	15.0	12.09	2.91
3	1.5	6.02	*-4.52
4	4.0	0.30	3.7
5	15.0	14.51	0.49
6	15.0	19.19	*-4.19
7	22.0	22.61	-0.61
8	2.5	3.72	-1.22
9	15.0	17.93	-2.93
10	30.0	31.46	-1.46
11	15.0	13.54	1.46
12	1.5	0.19	1.31
13	5.0	2.55	2.45
14	2.5	6.26	-3.76
15	1.5	3.12	-1.62
16	22.0	19.65	2.35
17	1.0	1.73	-0.73
18	31.0	26.24	*4.76

*Exceed limit.

From "ANOVA" analysis (Table 5) the "F-value" of 10.4 implies that the source model in Taguchi experimental design is significant. On the other hand, P-value of less than 0.05 indicated that the model variables are significant. In this case, (E) temperature, (G) agitation and (AB) interaction between incubation period and

Table 5. ANOVA analysis of variance for selected factorial model.

ANOVA	Sum of squares	Degree of freedom (df)	Mean square	F-value	p-value
Source model (significant)	1545.96	9	171.77	10.49	0.0015
A-incubation period	28.81	1	28.81	1.76	0.2212
B-beef extract	91.39	2	45.69	2.79	0.1203
E-temperature	184.08	2	92.04	5.62	0.0299
G-agitation	657.17	2	328.59	20.08	0.0008
AB	279.64	2	139.82	8.54	0.0103

beef extract are the most significant model variables, in exotoxin production by *P. aeruginosa*.

The regression analysis reveled that the observed model error was very low which indicated the accuracy of experimentation (Table 6) with standard deviation equal 4.05 and regression square (R^2) of 0.9219. The predicted R-square (0.6208) was found to be in reasonable agreement with the adjusted R- square (0.8341). The adequate precision (10.370) indicated that an adequate signal, as a value greater than 4 is desirable in the Taguchi system.

Table 6. The regression analysis values.

Std. Dev	4.05	R-Square	0.9219
Mean	11.64	Adj R-Square	0.8341
C.V. (%)	34.76	Pred R-Square	0.6208
Precission	63.84	Adeq Precision	10.370

The estimate model coefficient and their associated statistics were reported in (Table 7). Low levels of variables (A) incubation period, (B) beef extract, and (E) temperature are desirable (with -ve sign), while high level of (G) agitation rate and (AB) was suitable (with +ve sign) for PE production. The final yield of exotoxin in terms of coded factors is:

Table 7. The coefficient estimate with standard error.

	Coefficient estimate	Standard error	95% CI low
Intercept	11.01	1.00	8.70
[A]	-0.63	0.99	-2.90
B[1]	3.30	-0.027	6.82
B[2]	-3.83	-7.03	-0.65
E[1]	0.32	-2.87	3.51
E[2]	-4.27	-7.69	-0.85
G[1]	-0.77	-4.19	2.65
G[2]	7.53	4.46	10.60
AB[1]	6.20	1.51	2.73
AB[2]	-3.54	1.37	6.70

Protein yield (Y) = + 11.01 - 0.63 A + 3.3 B[1] - 3.83 B[2] + 0.32 E[1] - 4.27 E[2] - 0.77 G[1] + 7.53 G[2] + 6.20 AB[1] - 3.34 AB[2]

The optimized medium for exotoxin production from *P. aeruginosa* by the Expert® software in Taguchi system is as follows: (A) incubation period, 36 h; (B) beef extract, 4 g l^{-1}; (C) NaCl, 7 g l^{-1}; (D) pH, 6.5; (E) temperature 27°C; (F) inoculum size 3.0%; (G) agitation rate, 150 rpm and (H) peptone, 3 g l^{-1}.

Many statistical experimental design methods have been employed in optimization bioprocesses [15, 19, 23]. These methods have been successfully applied to optimize different process parameters [24, 25]. These designs minimize the error in determining the effect of parameters than the classic time-by-time optimization experiments [18]. Heir *et al*. [26] studied the importance of recipe and process variables on verotoxigenic *E. coli* (VTEC) reductions in two types of dry fermented sausages through three satisfactory designed experiments. Linear regression and ANOVA analysis showed that no single variable had a dominant effect on VTEC reduction. High levels of NaCl, $NaNO_3$ and glucose, low pH and fermentation temperature reduced VTEC production, while high fat and large casing diameter gave the opposite effect.

The cytotoxicity assay of mice (Table 8) indicated that 15 µg ml^{-1} of *P. aeruginosa* exotoxin exerted no effect and no symptoms on experimental mice. Less active mice were detected after 4 days of post injection with 30 µg ml^{-1}. A typical exotoxin poisoning (crusted eyes and ruffled fur) was induced in mice after 5, 3 and 2 days of post injection with 30, 45 and 60 µg ml^{-1}, respectively. Mice death was observed after 4 and 5 days of post-injection with 45 and 60 µg ml^{-1}, respectively. These results indicated that the quantity of exotoxin in crude culture filtrate is sufficient to elicite severe signs of morbidity shortly after injection. Pavlovskis and Gordon [6] described a number of cytotoxic effects of crude exotoxin preparations from *P. aeruginosa*. They claimed that the exotoxin may be a single component or a family of closely related proteins in the crude preparations. PE is the virulence factor in *P. aeruginosa* [27]. Studies on mice have identified median lethal dose of PE as being about 200 ng [4].

Table 8. Cytotoxicity assay of crude exotoxin extracted from *P. aeruginosa* grown on optimized medium.

Post injection period (day)	Control mice (0.0)	Exotoxin protein concentration (µg ml^{-1})			
		15	30	45	60
1	Active	Active	Active	Active	Active
2	Active	Active	Active	Less active	Not active*
3	Active	Active	Active	No activity*	Death
4	Active	Active	Less active	Death	--
5	Active	Active	No activity*	--	--

*No activity (crusted eyes and ruffled fur).

Conclusions

The results indicated that the quantity of exotoxin in crude culture filtrate is sufficient to elicite severe signs of morbidity shortly after injection. Mice death was observed after 4 and 5 days of post-injection with 45 and 60 µg ml^{-1}, respectively.

References

1Wolf, P., Alt, K., Bühler, P., Katzenwadel, A., Wetterauer, U., Tacke, M. and Elsässer-Bile, U. 2008. Anti-PSMA immunotoxin as novel treatment from prostate cancer high and specific antitumer activity on human prostate xenograft tumors in SCID mice. Prostate **68**:129-138.

2Bitter, W. 2003. Secretions of *Pseudomonas aeruginosa*: Large holes in the outer membrane. Arch. Microbiol. **179**:307-314.

3Chandramohan,V., Sampson, J. H., Pastan, I. and Bigner, D. D. 2012. Toxin-based target therapy for malignant brain tumors. Clin. Dev. Immunol. **2012**:1-15. doi:10.1155/2012/480429.

4Weldon, J. E. and Pastan, I. 2011. A guide to taming a toxin recombinant immunotoxin constructed from Pseudomonas exotoxin A for the treatment of cancer. EEBS J. **278**:4683-4700.

5Gross, T. L., Ihrke, P. J., Walder, E. J. and Affolter, V. K. 2005. Skin

Diseases of the Dog and Cat: Clinical and Histopathologic Diagnosis. 2nd edn. Blackwell Science Ltd., Oxford, UK.
[6]Pavlovskis, O. R. and Gordon, F. B. 1972. *Pseudomonas aeruginosa* exotoxin: Effect on cell cultures. J. Infect. Dis. **125**:631-636.
[7]Welekind, J. E., Christine, B., Trame, M. D., Patrice, K. and David, B. M. 2001. Refined crystallographic structure of *Pseudomonos aeruginosa* extoxin A and its implications for the molecular mechanism of toxicity. J. Mol. Biol. **314**:823-837.
[8]Daniele, B., Young, A., Choi, Y. K., Dana,Y., Sazzad, H. and Michel, S. 2012. Prostate- specific inhibtion of p13 kinase and protein synthesis as a therapy for advanced hormone- refractory prostate cancer. J. Cancer Res. **72**:1-10.
[9]Chaudhary,V. J., Xu, Y. H., Fitz-gerald, D., Adhya, S. and Pastan, I. 1988. Role of domain **22** of *Pseudomonas aeruginosa* exotoxin in the secretion of protein in to the periplasm and medium by *E. coli*. Proc. Natl Acad. Sci. **85**:2939-2943.
[10]Debinski, W., Clay, B. S., Fitzgerald, D. and Pastan, I. 1991. Substitution of foreign protein sequences in to a chimeric toxin composed of transforming growth factor α and Pseudomonas exotoxin. Mol. Cell. Biol. **11**:1751-1753.
[11]Dasu, V. V. and Panda, T. 2000. Optimization of microbiological parameters for enhanced griseofulvin production using response surface methodology. Bioprocess and Biosystems Engineering **22**:45-49.
[12]Sharma, D. C. and Satyanarayana, T. 2006. A marked enhance in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcsr1 in submerged fermentation by using statistial of methods. Bioresource Technology **97**:727-733.
[13]Reddy, J. A., Dorton, R., Dawson, A., Vetzel, M., Parker, N., Nicoson, J. S., Westrick, E., Klein, P. J., Wang, Y., Vlahov, I. R. and Leamon, C. P. 2009. *In vivo* structural activity and optimization studies of folate-tubulysin conjugates. Mol. Pharmaceutics **6**:1518-1525.
[14]Abde-Fattah,Y. R. and Olama, Z. A. 2002. L-asparaginase production by *Pseudomonas aeruginosa* in solid-state culture conditions using factorial designs process. Biochem. **38**:115-122.
[15]Doehlert, D. H. 1970. Uniform shell designs. Applied Statistics **19**:231-239.
[16]Box, G. E. P., Hunder, W. G. and Hunder, S. J. 1978. Statistics for Experiments. JohnWiley and Sons Inc, New York, 664 p.
[17]Lundstedt, T., Seifert, E., Abramo, L., Thelin, B., Nyström, Å., Pettersen, J., Bergman, R. 1998. Experimental design and optimization. Chemometr. Intell. Lab. **42**:3-40.
[18]Zheng, Z. M., Cheng, K. K., Hu, Q. L., Liu, H. J., Guo, N. N. and Liu, D. 2008. Effect of culture conditions on 3-hydroxy propionaldhyde detoxification in 1.3 propandiol fermentation by *Klebsiella pneumoniae*. Biochemicel Engineering J. **39**:305-310.
[19]Prassad, K. K., Mohan, S. V., Rao, R. S., Pati, R. P. and Sarma, P. N. 2005. Laccase production by *Pleurotus ostreatus* 1804: Optimizatien of submerged culture conditions by Taguchi: DO methodology. Biochemical Engineering Journal **24**:17-26.
[20]Callahan, M. A., Slimak, M. W., Gabal, N. W., May, I. P., Fowler, C. F., Freed, J. R., Jennings, P., Durfee, R. L., Whitmore, F. C., Maestri, B., Mabey, W. R., Holt, B. R. and Gould, C. 1979. Water-related Enviromental Fate of 129 Priority Pollutants. Vol. I. Office of Water Planning and Standards, Office of Water and Waste Management, U.S. Environmental Protection Agency, Washington, DC. EPA UO/4-79-029a, pp. 14-1- 14-15.
[21]Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**:265-276.
[22]Roy, R. K. 2010. A Primer on the Taguchi Method. 2nd edn. Society of Manufacturing Engineers, Van Nostrand Reinhold, New York, 200 p.
[23]Ferreira, G. M. and Moreira, A. N. 2004. Application of Plackett-Burman design and response surface methodology to achieve exponential growth for aggregated shipworm bacterium. Biotechnol. Bioeng. **85**:666-675.
[24]Vanot, G. D., Valerie, M., Giuhem, C., Phentan-Luu, R. and Comeau, L. C. 2002. Maximizing production of *Penicillium cyclopium* partial acylglycerol lipase. Appl. Microbiol. Biotechnol. **60**:417-419.
[25]Dutrarl-Maltez, H. F. and Carasek, E. 2006. Development of an on-line preconcentration system for zince determination in biological samples. Talanta **69**:488-493.
[26]Heir, E., Holck, A. L., Omer, M. K., Alvseike, O. and Hoy, M. 2010. Reduction of verotoxigenic *E. coli* by process and recipe optimisation in dry-fermented sausages. Int. J. Food Microbiol. **141**:195-202.
[27]Liu, P. V. 1966. The role of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. 3. Identity of the lethal toxins produced *in vitro* and *in vivo*. J. Infect. Dis. **116**:481-489.