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Research article

Optimization of multiple valuable biogenic products using experimental design

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Key words: Biosurfactants; *Pseudomonas aeruginosa (PAO1)*; PlackettBurman; rhamnolipid; protease.

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Abstract

During the last few decades, biosurfactants (e.g. rhamnolipids) have received much more attention as an alternative to the conventional synthetic surfactants. The production cost of biosurfactant is the main hindrance for its commercialization. The production of multiple valuable products in a single process may contribute in reduction of the production cost. The scope of the current work was to use Plackett Burman design for selection of the most significant factors with highest contributionsfor production of rhamno lipids in parallel with protease using *Pseudomonas aeruginosa (PAO1)*. Nineteen factors have been selected for this study. According to the obtained results, six obvious factors; glucose, casein, peptone, yeast extract, pH and NaCl, had to be added to the production medium and three others; urea, $NaNO_3$, and $(NH_4)_2SO_4$, should not. Some of the remaining factors were not significant and the others had contrary effects on rhamnolipids and protease production. The final results showed that, the use of factorial design resulted in 213.27% increase in rhamnolipid productivity and 296.65% increase in protease productivity compared with the original production conditions.

Introduction

The industrial interest in microbial products has been stimulated by their unique properties and the opportunity to develop new materials, which can be used for specific applications in many industries [1]. For example, biosurfactants are natural, safe, biodegradable and had many other advantages over their synthetic counterparts including antimicrobial activity [2].

Biosurfactants are surface-active compounds that can reduce the surface tension, stabilize emulsions, and promote foaming. Despite of being environmentally friendly; nontoxic and biodegradable, biosurfactants are widely diverse, selective and effective under extreme conditions even in small quantities [3]. Thereafter, biosurfactants can be a trusty alternative for the chemically synthesized surfactants [2].

Rhamnolipids are potent natural glycolipid biosurfactants with high potential industrial applications [4]. *Pseudomonas* strains are the best producers of glycolipid containing rhamnose and 3-hydroxy fatty acids. However, the most promising bacterial strain, *P. aeruginosa*, possesses characteristics that allow for multiple potential uses in various industrial and commercial sectors and was investigated and recommended as the best microorganism to produce two classes of rhamnolipids: monorhamnolipids and dirhamnolipids with excellent surface activity [5-7]. In addition to rhamnolipids, *P. aerugenosa* was reported to produce other valuable products including lipase, protease and amylase [8-10]. Proteases comprise a group of industrial enzymes, which alone form about 60% of the total world-wide enzyme yield [11]. Among the different types of proteases, bacterial proteases play a significant role in physiological, commercial and biotechnological processes [12].

The main factor limiting commercialization of biosurfactants is associated with their non-economic large-scale production. To overcome this obstacle and to compete with synthetic surfactants, an inexpensive substrate and effective microorganism have to be intensively developed for biosurfactant production [13, 14]. Several studies have been carried out to define the best nutritional requirements needed to obtain efficient and cost-effective rhamnolipid production by *Pseudomonas aerugenosa* [15]. However, the operational cost is still a hindrance for an economic production of rhamnolipids.

In the current work, we aim to use of Plackett Burman factorial design method for description and statistical modeling of the factors affecting rhamnolipids and protease production by *P. aerugenosa* PAO1. This multi-product bioprocess should contribute in lowering production cost. A process with multiple final products is an "out of the box" solution of the operational high cost problem. Although, easy talk, it is harder to be applied.

Materials and Methods

Organism and culture conditions

Pseudomonas aeruginosa PAO1 with accession number (NR 074828.1) was, kindly, obtained from Prof. Dr. Nagwa Sidkey, Biotechnology Lab, Faculty of Science, Al-Azhar University (Girls Branch) [7].

Pseudomonas aeruginosa PAO1 was pre-cultured and stored on glycerol-nitrate (GN) medium [16] containing (g/l):glycerol, 45; $NaNO_3$, 4.5; K_2HPO_4, 5.2; KH_2PO_4, 4; $MgSO_4.7H_2O$,0.4; $CaCl_2.2H_2O$, 0.1; KCl, 1.0; NaCl, 1.0 and Yeast extract,2.0. The GN medium pH was adjusted to 6.8. Cultures were incubated at 37°C for 72 hours. Rhamnolipid and protease were determined under these conditions to be compared with model results.

Factorial design and experiment

Plackett Burman factorial design was used for modeling of rhamnolipids and protease production by *Pseudomonas aeruginosa* PAO1. In this design, nineteen factors (glucose, sucrose, soybean, soluble starch, casein, glycerol, sunflower oil, sugarcane molasses, peptone, malt extract, yeast extract, NH_4Cl, urea, $NaNO_3$, $(NH_4)_2SO_4$, $CaCl_2$, KCl, NaCl and pH) with two levels, and three center points were used. To all runs, 5.2 g/l, K_2HPO_4; 4 g/l, KH_2PO_4 and 0.4 g/l $MgSO_4.7H_2O$were added and performed at 37°C and 150 rpm in rotary shaking incubator (New Brunswick Innova 43 Incubator Shaker, Eppendorf Co., USA). Data represented in table (1) shows the factors used in the design with their ranges. Experimental design was performed using Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, ver 7.0.0). Analysis of variance (ANOVA) was used to estimate the statistical parameters of the design.

Table 1. Summary of Plackett Burman design used for rhamnolipid and protease production

Factor	Name	Units	Low Actual	High Actual	Mean
A	Glucose	g/l	0	10	5
B	Sucrose	g/l	0	10	5
C	Soybean	g/l	0	10	5
D	Soluble starch	g/l	0	10	5
E	Casein	g/l	0	10	5
F	Glycerol	g/l	0	10	5
G	Sunflower oil	g/l	0	10	5
H	Molasses	g/l	0	10	5
J	Peptone	g/l	0	2	1
K	Malt extract	g/l	0	2	1
L	Yeast extract	g/l	0	2	1
M	NH_4Cl	g/l	0	1	0.5
N	Urea	g/l	0	1	0.5
O	$NaNO_3$	g/l	0	1	0.5
P	$(NH_4)_2SO_4$	g/l	0	1	0.5
Q	$CaCl_2$	g/l	0	0.1	0.05
R	pH	Unit	6	8	7
S	KCl	g/l	0	1	0.5
T	NaCl	g/l	0	1	0.5

Quantification of rhamnolipids

The concentration of rhamnolipid in the sample was estimated using Orcinol (0.19% (w/v) in 53% Sulphuric acid (v/v)) method [3]. 500 µl of the clear supernatant was extracted twice with 1ml of diethyl ether. The extract was allowed to dry and then dissolved in 0.5ml of deionised water. To 100µl of each sample, 900 µl of Orcinol solution was added. The sample was heated at 80°C in a water bath for 30 minutes and cooled for 15 minutes at room temperature and the absorbance measured at 420 nm using Spectro UV-Vis Double Beam UVD 3500, Labomed, Inc USA. The rhamnolipid concentration was quantified from the standard L-rhamnose calibration curve between 0 and 50 µg/ml and the result was expressed as rhamnose equivalents (µg/ml) by multiplying rhamnose values by a coefficient of 3.4, which obtained from the correlation of pure rhamnolipids/rhamnose [3].

Protease Assay

To 0.5 ml of the enzyme solution in test tube, 0.5ml of 1% casein solution (in 0.05M phosphate buffer pH 7and boiled for 15minutes) was added. The mixture was allowed to stand for one hour in water bath (at 37°C) with occasional shaking. Only 3.0 ml of 10% trichloroacetic acid (TCA) was added to terminate the reaction. The tubes were allowed to stand for one hour in the cold chamber at 2°C.The tubes were then centrifuged at 3000 rpm and the absorbance of the protein content of the supernatant was quantified using Bradford method [17] and read against standard solution of Bovine serum albumin (BSA).

One protease unit is defined as the amount of enzyme required to hydrolyse one micro-gram of casein (BSA equivalent) under assay conditions.

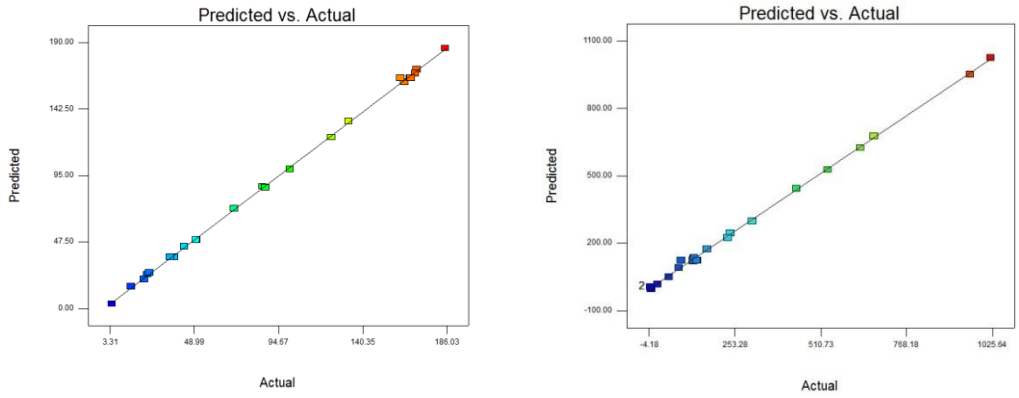
Results and Discussion

Results

Data illustrated graphically in figure (1) and represented in table (2) shows the relation among the actual and predicted rhamnolipids and protease results according to Plackett Burman factorial design. The results show good matching of predicted comparable to the actual results.

Analysis of variance of Plackett Burman design for rhamnolipid production (Table 3) showed that the model F-value of 355.99 implies the model is significant and glucose, sucrose, soybean, casein, sunflower oil, peptone, malt extract, yeast extract, NH_4Cl, urea, $NaNO_3$, $(NH_4)_2SO_4$, $CaCl_2$, pH, KCl and NaCl are all significant model factors. The design lack of fit F-value of 1.12 implies the lack of fit is not significant relative to the pure error. The model R^2 is 0.9993 and the predicted R^2 of 0.9723 is in reasonable agreement with the adjusted R^2 of 0.9965. Adequate precision ratio of 61.044 indicates an adequate signal and the model can be used to navigate the design space.

The most significant contribution of the factors were attributed to sucrose (28.87%), sunflower oil (9.17%), $NaNO_3$ (6.3%), pH (6.65%), NaCl (6.69%), $(NH_4)_2SO_4$ (5.51%), $CaCl_2$ (3.54%), casein (3.18%), Soybean (3.86%), yeast extract (1.01%) and the other factors where less than 1%.



(a) (b)

Figure 1. Relation between rhamnolipids (a) and protease (b) actual and predicted results according Plackett Burman factorial design

Table 2. Actual, predicted and residuals of rhamnolipids and protease results according to PlackettBurman design.

Run Order	Rhamnolipids (µg/ml)			Protease activity (U/ml)		
	Actual value	Predicted value	Residual	Actual value	Predicted value	Residual
1	38.181	36.989	1.19	169.674	174.146	-4.47
2	35.947	36.883	-0.94	231.042	224.820	6.22
3	185.096	186.032	-0.94	670.000	676.222	-6.22
4	132.755	133.947	-1.19	437.310	443.532	-6.22
5	85.947	87.138	-1.19	305.481	299.258	6.22
6	160.840	164.528	-3.69	140.978	123.836	17.14
7	49.883	48.947	0.94	959.229	953.007	6.22
8	123.287	122.351	0.94	239.566	245.788	-6.22
9	87.543	86.351	1.19	131.034	137.256	-6.22
10	43.500	44.436	-0.94	127.056	122.584	4.47
11	24.564	25.755	-1.19	531.636	527.164	4.47
12	23.500	24.436	-0.94	0.000	4.472	-4.47
13	100.840	99.649	1.19	2.046	-4.176	6.22
14	4.245	3.309	0.94	21.081	16.609	4.47
15	166.266	164.528	1.74	91.542	123.836	-32.29
16	14.670	15.861	-1.19	129.045	133.517	-4.47
17	21.691	20.755	0.94	629.940	625.468	4.47
18	169.777	170.968	-1.19	1021.167	1025.639	-4.47
19	168.926	167.989	0.94	0.000	4.472	-4.47
20	166.479	164.528	1.95	138.989	123.836	15.15
21	162.968	161.777	1.19	127.340	121.118	6.22
22	70.628	71.564	-0.94	54.891	50.419	4.47
23	50.096	48.904	1.19	85.007	91.229	-6.22

Rhamnolipids final equation in terms of actual values

Rhamnolipids (µg/ml) = -97.22340 + 0.41064*A + 6.98298*B -2.55319*C +2.31915*E + 3.93617*G + 0.32766*H + 5.72340*J + 5.36170*K + 6.52128*L - 10.70213*M - 8.76596*N - 32.61702*O - 30.51064*P + 244.46809*Q + 16.75532*R - 5.6383*S + 33.61702*T

Where, A: Glucose, B:Sucrose, C: Soybean, E: Casein, G: Sunflower oil, H: Molasses, J: Peptone, K: Malt extract, L: Yeast extract, M: NH_4Cl, N: Urea, O: $NaNO_3$, P: $(NH_4)_2SO_4$, Q: $CaCl_2$, R: pH, S: KCl and T: NaCl.

The model final equation indicates that soybean, NH_4Cl, urea, $NaNO_3$, $(NH_4)_2SO_4$ and KCl have negative effects on rhamnolipids production. According to Plackett Burman design, glucose, sucrose, casein, sunflower oil, molasses, peptone, yeast extract, $CaCl_2$ and NaCl where the most significant and have positive contribution in rhamnolipid production. Figure (2) represents 3D model graphs of some factors affecting rhamnolipids production by *Pseudomonas aeruginosa* PAO1 according to Plackett Burman design. The figures show the factor-factor interactions and how it affects rhamnolipids production.

Analysis of variance of Plackett Burman factorial design for protease production (Table 4) showed that, the model F-value of 204.03 implies the model is significant and glucose, sucrose, soybean, soluble starch, casein, glycerol, molasses, peptone, yeast extract, NH_4Cl, urea, $NaNO_3$, $(NH_4)_2SO_4$,

$CaCl_2$, pH, KCl and NaCl are all significant model factors. The design lack of fit F-value of 0.37 implies that the lack of fit is not significant relative to the pure error. The curvature F-value of 139.69 implies there is significant curvature in the design space. The model R^2 is 1.0 and the predicted R^2 of 0.968 is in reasonable agreement with the adjusted R^2 of 0.9940. The model adequate precision ratio of 48.832 indicates an adequate signal and the model can be used to navigate the design space.

The most significant contribution of the factors were attributed to soluble starch (18.42%), casein (15.81%), pH (14.54%), glucose (11.69%), $(NH_4)_2 SO_4$(8.61%), KCl (9.12%), glycerol (3.36%), NaCl (3.19%), sucrose (2.47%), $NaNO_3$(2.02%), $CaCl_2$(2.21%), urea (1.41%) and the other factors where less than 1%.

Table 3. Analysis of Variance (ANOVA) for rhamnolipid production.

Source	Sum of squares	Df	Mean square	F-Value	*p*-value Prob>F*
Model	65638.22	17	3861.072	355.985	< 0.0001
A-Glucose	84.31	1	84.31191	7.773	0.0494
B-Sucrose	24381	1	24381	2247.892	< 0.0001
C-Soybean	3259.40	1	3259.393	300.511	< 0.0001
E-Casein	2689.23	1	2689.226	247.943	< 0.0001
G-Sun flower oil	7746.72	1	7746.718	714.236	< 0.0001
H-Molasses	53.68	1	53.6804	4.9493	0.0901
J-Peptone	655.15	1	655.1471	60.404	0.0015
K-Malt extract	574.96	1	574.957	53.010	0.0019
L-Yeast extract	850.54	1	850.541	78.419	0.0009
M-NH_4Cl	572.68	1	572.6777	52.800	0.0019
N-Urea	384.21	1	384.21	35.424	0.0040
O-$NaNO_3$	5319.35	1	5319.35	490.436	< 0.0001
P-$(NH_4)_2SO_4$	4654.49	1	4654.495	429.138	< 0.0001
Q-$CaCl_2$	2988.23	1	2988.232	275.511	< 0.0001
R-pH	5614.81	1	5614.814	517.678	< 0.0001
S-KCl	158.95	1	158.952	14.655	0.0186
T-NaCl	5650.52	1	5650.521	520.970	< 0.0001
Curvature	18770.85	1	18770.85	1730.645	< 0.0001
Residual	43.385	4	10.846		
Lack of Fit	22.96	2	11.480	1.124197	0.4708
Pure Error	20.42	2	10.212		
Cor Total	84452.45	22			

* Values of "Prob > F" less than 0.05 indicate model terms are significant.

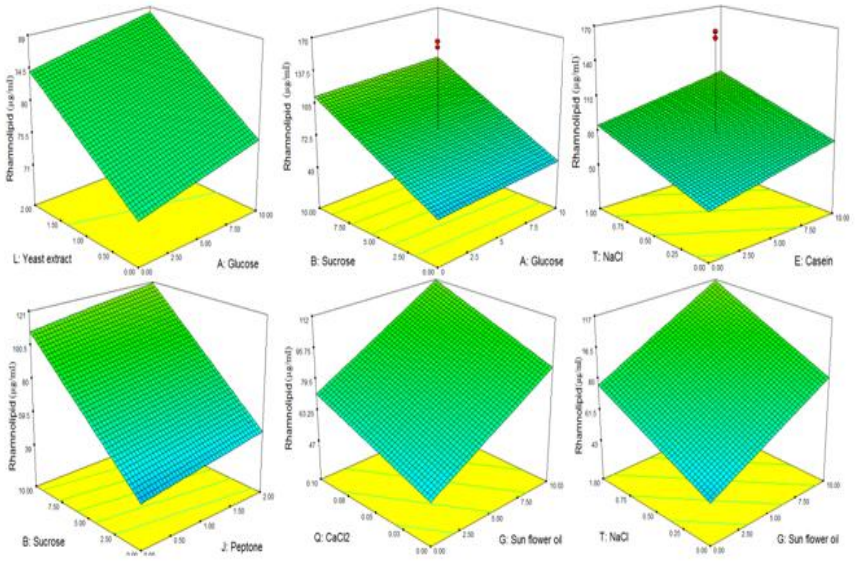


Figure 2. 3D model graphs representing some of the factors affecting rhamnolipids production by *Pseudomonas aeruginosa* PAO1.

Table 4. Results of Plackett Burman design analysis of variance (ANOVA) for protease production.

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob>F
Model	1867326.56	17	109842.7	204.03	< 0.0001
A-Glucose	227416.58	1	227416.6	422.41	< 0.0001
B-Sucrose	48095.04	1	48095.04	89.33	0.0007
C-Soybean	9847.38	1	9847.382	18.29	0.0129
D-Soluble starch	358146.34	1	358146.3	665.23	< 0.0001
E-Casein	307487.87	1	307487.9	571.14	< 0.0001
F-Glycerol	65289.42	1	65289.42	121.27	0.0004
H-Molasses	10254.99	1	10254.99	19.05	0.0120
J-Peptone	7780.15	1	7780.149	14.45	0.0191
L-Yeast extract	15657.22	1	15657.22	29.08	0.0057
M-NH_4Cl	18071.33	1	18071.33	33.57	0.0044
N-Urea	27409.91	1	27409.91	50.91	0.0020
O-$NaNO_3$	39288.72	1	39288.72	72.98	0.0010
P-$(NH_4)_2SO_4$	167411.15	1	167411.2	310.96	< 0.0001
Q-$CaCl_2$	43051.78	1	43051.78	79.97	0.0009
R-pH	282727.54	1	282727.5	525.15	< 0.0001
S-KCl	177434.54	1	177434.5	329.57	< 0.0001
T-NaCl	61956.60	1	61956.6	115.08	0.0004
Curvature	75206.03	1	75206.03	139.69	0.0003
Residual	2153.51	4	538.3763		
Lack of Fit	587.13	2	293.5666	0.37	0.7274
Pure Error	1566.37	2	783.186		
Cor Total	1944686.09	22			

* Values of "Prob > F" less than 0.05 indicate model terms are significant.

Protease production final equation in terms of actual values

Protease activity (U/ml) =-696.77 + 21.33*A - 9.81*B + 4.44*C - 26.76*D + 24.80*E + 11.43*F + 4.53*H + 19.72*J + 27.98*L + 60.12*M-74.04*N-88.64*O-182.98*P- 927.92*Q + 118.90*R + 188.38*S + 111.32*T

Where, A: Glucose, B:Sucrose, C: Soybean, D: Soluble starch,E: Casein,F: Glycerol, H: Molasses, J: Peptone, L: Yeast extract, M: NH_4Cl, N: Urea, O: $NaNO_3$, P: $(NH_4)_2SO_4$, Q: $CaCl_2$, R: pH, S: KCl and T: NaCl.

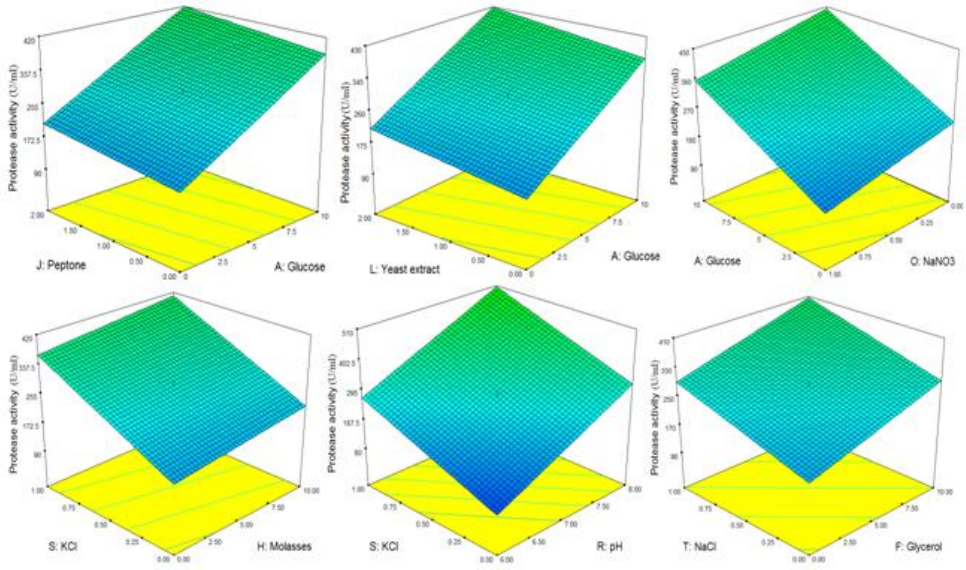


Figure 3. 3D model graphs representing some of the factors affecting protease production by *Pseudomonas aeruginosa* PAO1.

The model final equation showed that glucose, soybean, casein, glycerol, molasses, peptone, yeast extract, NH_4Cl, pH, KCl and NaCl have positive effect on protease production whereas sucrose, soluble starch, urea, $NaNO_3$, $(NH_4)_2SO_4$ and $CaCl_2$ have negative ones. Figure (3) represents 3D model graphs of some factors affecting protease production by *Pseudomonas aeruginosa* PAO1 according to Plackett Burman design. The figures show the factors interaction and its effect on protease production.

Discussion

It was noted from the results of Plackett Burman models that glucose, casein, peptone, yeast extract, pH and NaCl are common significant factors with positive effects on both rhamnolipids and protease production by *P. aeruginosa* PAO1 whereas urea, $NaNO_3$, and $(NH_4)_2SO_4$ are common significant factors with negative effects. Hence, urea, $NaNO_3$, and $(NH_4)_2SO_4$ should be excluded from medium of both processes.

The complexity in the accomplishment of a single process for production of protease and rhamnolipids is the factors of opposite signs in the equations. These factors are sucrose, soybean, NH_4Cl, $CaCl_2$ and KCl. Sucrose had a very good contribution (28.87%) and have been used by many researchers for *P. aerugenosa* production of rhamnolipids [18, 19] and protease [20, 21]. Hence, sucrose should be added to the production medium.

Soybean had a very low contribution (0.51%) in case of protease production and negative effect in case of rhamnolipids and, therefore, omitted from the production medium.

Whooley and coworkers [22] reported inhibition of exoprotease production by *Pseudomonas aeruginosa* using NH_4Cl and Rikalović and coworkers [13] reported inhibition of rhamnolipids production in presence of $NH4^+$. Hence, NH_4Cl should be excluded from the production medium.

For *Pseudomonas aerugenosa*, $CaCl_2$ stimulated rhamnolipids production as shown by Sahoo *et al.* [23] and enhanced protease activity by 167% according to Pathak and Sardar [24]. In the current model, $CaCl_2$ has a good contribution (3.54%) in rhamnolipids production. Consequently, $CaCl_2$ should be added to the production medium.

Although, some researchers added KCl to rhamnolipids and protease production medium [16, 24, 25, 26], others [19, 27, 28] abandoned it. In the present work, KCl has adverse effect on rhamnolipids production; therefore, it should be omitted if we aim at rhamnolipid production. KCl, inevitably, should be added in case of protease production due to it high contribution (9.12%) and positive effect.

In the original conditions using glycerol-nitrate medium, rhamnolipid and protease production by *P. aeruginosa* PAO1were 86.79 (µg/ml) and 344.23 (U/ml), respectively. These results are, highly, comparable to the maximum values (185.1 µg/ml and 1021.17 U/ml) obtained from the model which indicates a promising organism and culture conditions. Finally, the use of factorial design resulted in 213.27% increase in rhamnolipid productivity and 296.65% increase in protease productivity compared with the original production conditions. Further studies are going to be performed to improve yields and performance of *P. aeruginosa* PAO1based on the obtained results from Plackett Burman design.

Conclusion

P. aeruginosa PAO1 is a promising bacterial strain for production of multiple products for economic bio-process. Rhamnolipids and protease enzyme are not contrary products and both can be produced in parallel by *P. aeruginosa* PAO1.PlackettBurman factorial design helps in optimization and improvement of products and/or processes.

References

1. Erickson, B., J.E. Nelson and P. Winters: Perspective on opportunities in industrial biotechnology in renewable chemicals. Biotechnol. J. 2012; 7: 176–185.
2. Sidkey, N.M., E.A. Al Hadry: Biosurfactant Production by Bacillus cereus, B7 from Lubricant Oil Waste. International Journal of Science and Research (IJSR) 2014; 3(12):498-509.
3. Rahman K.S., T.J. Rahman, S. McClean, R. Marchant, I.M. Banat: Rhamnolipid biosurfactant production by strains of Pseudomonas aeruginosa using low-cost raw materials. Biotechnol Prog. 2002; 18(6):1277-81.
4. Wittgens A., T. Tiso, T.T. Arndt, P. Wenk, J. Hemmerich, et al.: Growth independent rhamnolipid production from glucose using the non-pathogenic Pseudomonas putida KT2440. Microb Cell Fact. 2011; 10: 80.
5. Siddhartha GVAOC, M. Nitschke, R. Haddad, M.N. Eberlin, J. Contiero: Production of Pseudomonas aeruginosa LBI rhamnolipids following growth on Brazilian native oils. Process Biochem. 2006; 41: 483-488.
6. Irfan-Maqsood M., M. Seddiq-Shams: Rhamnolipids- Well-Characterized Glycolipids with Potential Broad Applicability as Biosurfactants. Ind Biotechnol 2014; 10: 285-291.
7. Sidkey, N.M., H. F. Mohamed and H.I. Elkhouly: Evaluation of Different Screening Methods for Biosurfactant Producers Isolated from Contaminated Egyptian Samples Grown on Industrial Olive Oil Processing Waste. British Microbiology Research Journal 2016; 17(4): 1-19.
8. Raju E.V.N. and G. Divakar: Production of Amylase by using Pseudomonas aeruginosa isolated from garden soil. IJAPBC,2013; Vol. 2(1).
9. Sonja M.J., Z.D. Kneževi-Jugovi, S.Ž. Grbavi, D.I. Bezbradica, N.S. Avramovi, I.M. Karadži: Rhamnolipid and lipase production by Pseudomonas aeruginosasan-ai: The process comparison analysis by statistical approach. Hem. Ind. 2013; 67 (4) 677–685.
10. Karadži I., A. Masui, N. Fujiwara: Purification and characterization of a protease from Pseudomonas aeruginosa grown in cutting oil, J. Biosci. Bioeng. 2004; 98:145–152.
11. Chu W.H.: Optimization of extracellular alkaline protease production from species of Bacillus. Journal of Industrial Microbiology Biotechnology 2007; 340: 241-245.
12. Ibraheem, A.S. and S.O. Malomo: Partial characterization of proteases from Citrus sinensis fruit peel. World Scientific News 2017; 67(2): 250-264.
13. Rikalovi M.G., G.G. Cvijovi, M.M. Vrvi and I. Karadži: Production and characterization of rhamnolipids from Pseudomonas aeruginosasan-ai. J. Serb. Chem. Soc. 2012; 77(1): 27–42.
14. Sinumvayo J.P., N. Ishimwe: Agriculture and food applications of rhamnolipids and its production by Pseudomonas aeruginosa. Journal of Chemical Engineering & Process Technology 2015; 06:223.
15. Kahraman H., S.O. Erenler:Rhamnolipid production by Pseudomonas aeruginosa engineered with the Vitreoscilla hemoglobin gene. PriklBiokhimMikrobiol. 2012; 48(2):212-7.
16. Zhao F., Q. Cui, S. Han, H. Dong, J. Zhang, F. Ma and Y. Zhang: Enhanced rhamnolipidproduction of Pseudomonas aeruginosa SG by increasing copy number of rhlAB genes with modified promoter. RSC Adv 2015; 5: 70546–70552.
17. Bollag, D.M. and S.J. Edelstein: Protein methods. John Wiley & Sons, Inc. New. York, 1991.
18. Babu P.S., A.N. Vaidya, A.S. Bal, R. Kapur, A. Juwarkar, P. Khanna: Kinetics of biosurfactant production by Pseudomonas aeruginosa strain BS2 from industrial waste. Biotechnol. Lett. 1996; 18: 263 – 268.
19. Moussa T.A.A., M.S. Mohamed and N. Samak: Production and characterization of di-rhamnolipid produced By Pseudomonas aeruginosa TMN.Brazilian Journal of Chemical Engineering 2014; 31: (4): 867 – 880.

20. Ariole, C. N. and E. Ilega Alkaline Protease Production ByPseudomonas aeruginosa isolated from the gut of Pila Ovata. Journal of Global Biosciences 2013; 2(5): 126-131.
21. Abdel-Rhman, S.H.M., A.M. El-Mahdy and E.S.A. Abdelmegeed: Optimization of protease production by P. aeruginosa PAO1 and Physico-chemical characterization of the enzyme. Journal of American Science 2014; 10(7).
22. Whooley M.A., J.A. O'callaghan and A.J. Mcloughlin: Effect of Substrate on the regulation of exoprotease production by Pseudomonas aeruginosa ATCC 10145. Journal of General Microbiology 1983; 129, 981-988.
23. Sahoo S., S. Datta, D. Biswas: Optimization of culture conditions for biosurfactant production from Pseudomonas aeruginosa OCD1. Journal of Advanced Scientific Research 2011; 2(3): 32-36.
24. Pathak A.P. and A.G. Sardar: Isolation and characterization of salt stable protease producing archaea from marine solar saltern of Mulund, Mumbai. Indian Journal of Geo-marine Science 2014; 43(3): 412-417.
25. Zhang G., W. Yue-ting, Q. Xin-ping, M. Qin: Biodegradation of crude oil by Pseudomonas aeruginosa in the presence of rhamnolipids. J Zhejiang Univ SCI 2005; 68(8):725-730.
26. Dutta J.R. and R. Banerjee: Isolation and characterization of a newly isolated Pseudomonas mutant for protease production. Brazilian Archives of Biology And Technology 2006; 49(1): 37-47.
27. Nicodme M., J.P. Grill, G. Humbert and J.L. Gaillard: Extracellular protease activity of different Pseudomonas strains: dependence of proteolytic activity on culture conditions. Journal of Applied Microbiology 2005; 99: 641–648.
28. Onwosi C.O. and F.J.C. Odibo: Effects of carbon and nitrogen sources on rhamnolipidbiosurfactant production by Pseudomonas nitroreducens isolated from soil. World J Microbiol Biotechnol. 2012; 28:937–942.