OPTIMIZATION OF EXTRACTION CONDITIONS FOR LIQUID-LIQUID EXTRACTION OF PERSIPEPTIDES FROM *STREPTOMYCES ZAGROSENSIS* FERMENTATION BROTH

Hamed Kazemi Shariat Panahi[a], Fatemeh Mohammadipanah[a]* and Mona Dehhaghi[a]

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Generally, during drug discovery programs, after identification of new antibiotic metabolite, its high quantity production is obtained by various approaches, including production or extraction improvement or even strain genetic manipulation. To provide enough amounts of two novel non-toxic anti-MRSA pentapeptides named persipeptides (A and B) required for drug discovery programs, seven different fermentation broths examined. CM1 medium considerably enhanced the biosynthesis of persipeptides up to 219.63 ± 2.48, compared with ISP2 medium (36.31 ± 1.37), showing a six-fold increase. Additionally, at the extraction level, results of experimental design indicated that liquid-liquid extraction (LLE) of persipeptides by 34 % BuOH at 228 rpm (Stirrer speed), temperature 28 ˚C, and pH 9-9.5 for 78 min (stirring time) was equal to 264 ± 9.85 µg mL^{-1}, which was the most favorable combination for their extraction. Compared with un-optimized extraction process (219.63 ± 2.48 µg mL^{-1}), the optimized conditions improved the yield of the extraction by 20.20 %, while saving both time and solvent usage up to 67 % (162 min) and 16 %, respectively. The total sum of persipeptides enhancements resulted from the replacement of fermentation broth and subsequent optimization of their extraction by LLE reached almost seven-time, compared to conventional method (36.31 ± 1.37 µg mL^{-1}). Therefore, relatively large amounts of persipeptides can be economically produced and extracted for various future experiments.

* Corresponding Authors
Fax: +982166415081
E-Mail: fmohammadipanah@ut.ac.ir
[a] Department of Microbial Biotechnology, School of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, 14155-6455, Tehran, Iran

Introduction

The treatment of drug-resistant bacteria, including MRSA, in terms of their resistance to the current antibiotics is among major challenges worldwide.[1] New cyclo-pentapeptides, named as persipeptides, consist of two types of amino acids repetition of valine and phenylalanin, two of which are *N*-methylated. So far; two isomers, A and B, has been produced[2] by *Streptomyces zagrosensis* UTMC 1154 with bioactivity against methicillin resistance *Staphylococcus aureus* (MRSA) DSM 23622 (UTMC 1401).[3] Existence of *N*-methylated residues in peptides usually led to higher interesting therapeutic profiles[4] and consequently improved pharmacokinetic properties such as enzymatic stability,[5] receptor selectivity,[6] enhanced potency,[7] membrane permeability,[8] and bioavailability.[9]

The development and the validation of analytical methods play vital roles in discoveries, developments and manufactures of pharmaceuticals. High-performance liquid chromatography (HPLC) is extensively applied as a versatile analytical technology for quantitative analysis of target biomolecules and other compounds in biological matrixes as well as fermentation culture media of microorganisms producing them.[10] HPLC-based methods has previously been developed and validated for persipeptides determination from fermentation broth of *S. zagrosensis* UTMC 1154. The reported assay method requires sample pretreatment using LLE with the aid of *n*-butanol (*n*-BuOH).[3] This pretreatment is required for separating and concentrating of the target analyte and removing interferences, or even increasing the life of HPLC column by the elimination of damaging compounds.[11] However, the LLE method used for sample pretreatment or extraction of persipeptides has not been optimized yet; therefore, has some drawbacks, including lower efficiency and time consumption. These drawbacks directly affect the extraction yield and expenses, including materials usage and equipment depreciation. Improvement of a system performance or a process in order to maximize the exploitation from it is referred as optimization. Process optimization will obtain conditions that produce the best possible response when applied to a production/extraction procedure.[12] Traditional optimization of analytical chemistry are accomplished by one-variable-at-a-time, in which at any given time, influence of only one factor has been examined while all other factors have been kept at a constant level.[13] An alternative to this is response surface methodology (RSM), which decreases the number of experiments while increases the effectiveness for responses that are confounded by many factors and their interactions.[13] Additionally, analysis of variance (ANOVA) provides the statistical results and diagnostic checking tests, which enable researchers to evaluate the adequacy of the models.[14] Although, RSM has been successfully applied in the optimization of culture media to enhance the production of *Streptomyces* secondary metabolites, including streptolydigin,[15] virginiamycin,[16] daptomycin,[17] clavulanic acid,[18] streptomycin,[19] and neomycin;[20] RSM in the optimization of solvent extraction of antibiotics produced by *Streptomyces* has been rarely exploited and the only example is the optimization of extractive fermentation of clavulanic acid.[21]

In the present study, six culture media consisting of novel complex carbon sources and insoluble nitrogen sources were examined and compared with ISP2 medium. After determination of the most productive medium, the

optimization of parameters in the process of LLE, including volume percentage of extraction solvent, stirring rate, sample pH, extraction temperature, and process time for LLE of persipeptides from fermentation culture medium samples of *S. zagrosensis* UTMC 1154[3] were done in two steps. In the first step, primary evaluation of mentioned factors using half-fraction of factorial design was performed and striking factors were screened. In the second step, selected factors were investigated by RSM using central composite rotatable design (CCRD) in order to maximize the extraction efficiency of persipeptides.

Experimentals

HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). All other organic solvent used for LLE of persipeptides, including *n*-buthanol (*n*-BuOH), 1-propanol, 2-propanol, cyclohexane, dichlromethane, methanol, and chloroform were extra-pure grade and obtained from Merck (Darmstadt, Germany). HPLC grade water was produced by Barnstead/Thermolyne, USA (Model: d8992-33 Nanopure infinity).

Strain and culture conditions

The commonly used ISP2 medium (consisted of (g L^{-1}): glucose (4); yeast extract (4); and malt (10), pH 7.2) was used as growth and maintenance (supplemented with 2 g L^{-1}) and seeding media. Persipeptides were produced by the inoculation of spore suspension (1 mL of 1×10^7 CFU mL^{-1}) of *S. zagrosensis* UTMC 1154 in 100-mL Erlenmeyer flasks containing 9 mL of ISP2 liquid medium, followed by 36 h of shaking (220 rpm) at 28 ºC to develop seeding culture. This pre-culture was used for the subsequent inoculation of various fermentation media (50 mL) in 250-mL Erlenmeyer flasks with inoculant size of 10 % (5 mL). The inoculated production media were incubated at 28 ºC on shaker incubator with 220 rpm for seven days.[3] In order to examine persipeptides production, seven different media, named as candidate media (CM) 1-6 were investigated (Table 1). The optimization was done using the most productive medium.

Experimental designs and statistical analyses

In the previously reported method, the effect of critical parameters, including pH of the fermentation broth, extraction temperature, percentage of organic solvent, stirring rate, and extraction time on extraction process has not been determined.[3] Therefore, an experimental design using half fraction of factorial design was employed to screen significant variables with the minimum required number of experiments. After the determination of variables with significant effect on extraction process, a three factors CCRD was employed to determine optimal conditions for critical factors. Design-Expert Version® 7.0.0 was used to fit the quadratic response surface model to the experimental information as well as to generate response surfaces, analysis of data, and contour plots diagrams, while keeping a variable constant in the second-order polynomial model. The statistical significance of an effect was evaluated by *p*-values <0.05.

The response was persipeptides peak area and actual values of independent variables (X_i) were coded to x_i according to Eqn. (1).

Table 1. Compositions of seven different production media used for enhancing persipeptides production.

Medium	C-source, g L^{-1}	N-source, g L^{-1}	Salt g L^{-1}	pH
CM1	Starch (20)	Soybean (30)	MgSO4.7H2O (1) and $CaCO_3$ (10)	7.0
CM2	Acorn (20)	Yeast Extract (4)	-	7.0
CM3	Rape seed (10)	Malt (10)	-	7.0
CM4	Cotton seed (10)	Malt (10)	-	7.0
CM5	Sesame (20)	Malt (10)	-	7.0
CM6	Glycerol (15)	Soybean (10)	$CaCO_3$ (1), NaCl (5), and $COCl_2.7H_2O$ (1)	7.0
ISP2	Glucose (4)	Yeast extract (4)	-	7.4

$$x_{\mathrm{i}} = \frac{X_{\mathrm{i}} - \bar{X}_{\mathrm{i}}}{(X_{\mathrm{iHi}} - X_{\mathrm{iLow}})/2}, i = 1,2,3 \ldots k \quad (1)$$

where,

x_i= coded value of independent variable;

$X_{iHi/Low}$ = real values of the independent variable;

X_i = real values of the independent variable at the center point of the domain; and

x_1 (coded value of percentage of organic solvent),

x_2 (coded value of stirring rate), and

x_3 (coded value of extraction time) were given in Eqn. (2), (3) and (4).

$$x_1 = \frac{X_1 - 50}{25} \quad (2)$$

$$x_2 = \frac{X_2 - 225}{75} \quad (3)$$

$$x_3 = \frac{X_3 - 49}{29} \quad (4)$$

Response surface analysis of a five coded level CCRD for three factors, 20 runs (Table 2), was done using the generalized second-order polynomial model of Eqn. (5), and economically optimum conditions for LLE of persipeptides was determined by same equation.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{ij=1(i\neq j)}^{k} \beta_{ij} X_i X_j \quad (5)$$

where,

Y = the experimental response;

β_0, β_i, β_{ii}, and β_{ij} = the constant (intercept) coefficient, the linear coefficient, the quadratic coefficient, and the coefficient of interaction effect, respectively; and

X_i and X_j = independent variables

The quality of the fitted model was evaluated through analysis of variance (ANOVA). Additionally, the statistical analysis of the result, the evaluation of model and factors involved, and the determination of the influence of individual factors and their interaction with other factors on persipeptides extraction from the fermentation culture matrix by LLE were performed by determination of the coefficients from eqn. (5).

Table 2. Experimental sheet for five coded level CCRD of significant factors selected by screening design.

Run	Factor 1 x_1: BuOH %	Factor 2 x_2: stirring rate	Factor 3 x_3: stirring time
1	50	225	49
2	50	225	0.23
3	92	225	49
4	8	225	49
5	50	225	49
6	50	352	49
7	75	300	20
8	50	99	49
9	50	225	49
10	50	225	98
11	50	225	49
12	25	150	78
13	25	300	20
14	75	300	78
15	25	300	78
16	75	150	78
17	25	150	20
18	50	225	49
19	75	150	20
20	50	225	49

Sample preparation

The fermentation medium was harvested and divided for further experiments. Samples were prepared based on experimental conditions defined by experimental designs, then butanol containing fermentation broths were centrifuged at 2937 ×g for 10 min. One-hundred-fifty-µL of each supernatants was separated and the solvent was removed using N_2 gas. The obtained precipitate was dissolved in 150 µL acetonitrile-water (1:1 v/v) and analyzed by HPLC method.

HPLC instrument and analysis

A Cecil instrument with in-line-degasser (CE 4040) consisted of manual Rheodyne (Rohnert Park, CA, USA) injector with 20 µL loop was employed. The sample was retained on an ACE (Aberdeen, UK) LiChrosob C18 column (250 × 4.6 mm ID, particle size 5 µm, ACE-121-2546) protected by Hichrom (Reading, UK) C18 column (NC100-5C18) and thermostated at 27 ˚C. The analytes were eluted by water (A) and acetonitrile (B) as mobile phase using a gradient elution, in which B was 50 % at start point, increased to 64 % in 5.5 min, then to 95 % within 1.5 min, followed by 5 min isocratic at 95 % (purge time), finally decreased to 50 % within 3 min and kept at this B % for as long as 10 min to equilibrate the column to prepare the system for next injection. Measurements were held at 210 nm and data was collected and processed by chromatography system manager and power stream software version 3.1, respectively. [3]

Results and discussion

The core aim of current study was the enhancement of persipeptides retrieval. At the production step, the most effective medium basis for persipeptides biosynthesis was chosen amongst seven candidate media listed in Table 1. After cultivation, persipeptides were separately isolated from them using un-optimized LLE method previously provided.[3] Results, in Table 3, indicate the CM1 medium as the most productive fermentation culture medium for persipeptides biosynthesis, which enhances its production by more than six times, reaching to 219.63 ± 2.48, compared with ISP2 medium (36.31 ± 1.37). All other examined novel carbon sources; including acorn, sesame, cotton seed, and rape seed failed to increase the persipeptides production, compared with ISP2 and the production was in range of 33 to 39 µg mL^{-1}. The second most promising medium after CM1 was CM6, which composed of glycerol and soybean as carbon and nitrogen sources, respectively. CM6 considerably enhanced persipeptides production (159.69 ± 27.36), and four times increased in their production was resulted, compared to that of ISP2 medium (36.31 ± 1.37). Therefore, CM1 medium was selected as the final production medium and used for further optimization of LLE.

In this study, the LLE followed by HPLC-UV was applied for the extraction and the quantification of persipeptides in fermentation broth samples, respectively. Effects of multiple factors, including volume ratio of extraction solvent, stirring rate, sample pH, extraction temperature and process time were investigated. Optimized conditions were obtained by

screening design, and subsequently, CCRD was applied for the evaluation of significant factors along with their interactions thereof.

Table 3. Production of persipeptides in different fermentation media tested.

Name	1st trial	2nd trial	3rd trial	Average
CM1	220.36	216.86	221.66	219.63 ± 2.48
CM2	38.39	35.258	37.93	37.93 ± 1.69
CM3	33.65	33.57	33.97	33.73 ± 0.21
CM4	42.42	33.62	39.14	38.39 ± 4.45
CM5	36.84	37.14	37.31	37.10 ± 0.24
CM6	132.27	159.81	186.99	159.69 ± 27.36
ISP2	34.93	36.33	37.68	36.31 ± 1.37

Before starting optimization procedures, broad range of solvents, including ethyl acetate, 1-propanol, 2-propanol, cyclohexane, dichloromethane, methanol, *n*-BuOH, and chloroform were examined for obtaining maximum extraction (Data not shown). Among them, *n*-butanol was selected as the extracting solvent, as it has a high boiling point, which prevent solvent loss during extraction, and low melting point (less than -89 ˚C); is immiscible with aqueous solution and its density is lower than water; and is compatible with the RP-HPLC used in the quantification of persipeptides.

Screening design

Half fraction of factorial (resolution V) design is useful for preliminary purposes or in initial optimization steps owing to its great power in estimation of effects as well as considerable reduction in the number of experimental runs to be performed. Applying this design allows the estimation of all the main effects and two-factor interactions (2 Fl) with the assumption that no three-factor or/and higher interactions occur/s. Therefore, half fraction factorial design was used for the screening step. Major factors, which are assumed to influence the LLE of persipeptides, include volume percentage of extraction solvent, stirring rate, sample pH, extraction temperature and process time. Levels of factors for the screening design were selected according to our knowledge, and are presented in Table 4. The overall design matrix, consisted of 20 runs of which four of them were center runs, was randomly performed in order to minimize unexplained variability effects in obtained responses due to systematic errors. Half-normal probability plot was used to choose significant effects, which were further analyzed by ANOVA and obtained results were evaluated to determine main effects (Table 5).

The standard effect was estimated for calculating a *t*-statistic for each effect. Normalized results of the performed experimental design were investigated at a 5 % of significance and analyzed by standardized Pareto chart (Figure 1). On this plot (Figure 1), effects that are now above the second vertical line, Bonferroni limit, and those between second and first vertical lines, *t*-value limit, are almost certainly and possibly significant parameters, respectively.

Table 4. Levels of factors for the screening design.

Independent Factors	CS	Levels and Ranges		
		-1 (L)	0 (M)	+1 (H)
Broth pH	A	7	9	11
Temperature (ºC)	B	20	40	60
BuOH % (v/v)	C	50	63	75
Stirring rate (rpm)	D	100	200	300
Process time (min)	E	20	70	120

CS, L, M, and H stand for Coded Symbol, Low, Middle, and High, respectively.

The model F-value of 10.25 implies the significance of the model. There is only a 0.05 % chance that the model F-value of this large could have been occurred due to noise. The measured curvature F-value of 37.33 as the difference between the average of the center points and that of the factorial points implies that the curvature in the design space is significant, and there is a 0.01 % probability that it could occur due to noise. Therefore, there was a need for higher resolution design (RSM) to optimize the significant factors. Furthermore, the lack of fit F-value of 0.35 implies that it is not significant relative to the pure error, and there is an 89.72 % chance that it could occur due to noise, therefore, the model can fit. The model had the predicted R-square of 0.74, which is in reasonable agreement with the adjusted R-square of 0.78. Moreover, model adequate precision measuring the signal to noise ratio had the value of 10.217 being considerably greater than 4, and therefore, the model can be used to navigate the designed space.

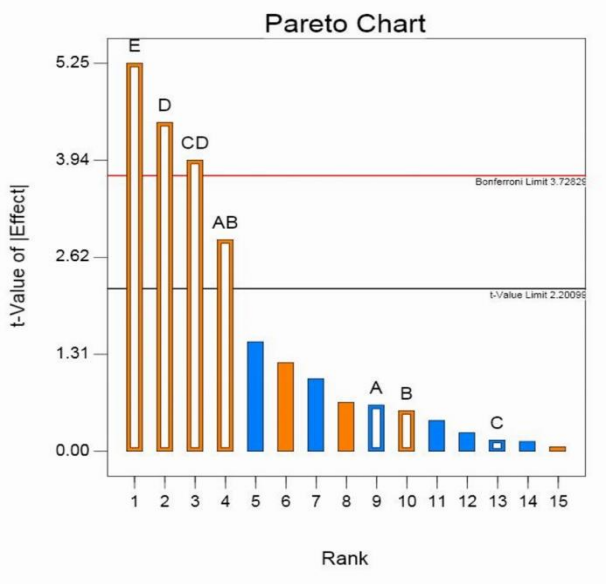

Figure 1. Pareto chart with selected main effects obtained from the half fraction of factorial design for LLE of persipeptides; A: sample pH; B: extraction temperature; C: percentage of BuOH; D: stirring rate; E: extraction time; AB and CD are 2FI between sample pH and extraction temperature, and percentage of BuOH and stirring time, respectively; 1: extraction time; 2: stirring rate; 3: 2FI between percentage of BuOH and extraction time; 4: 2FI between sample pH and extraction temperature; 5: 2FI between sample pH and extraction time; 7: 2FI between extraction temperature and stirring rate; 8: sample pH; 9: 2FI between sample pH and percentage of BuOH; 10: 2FI between sample pH and stirring rate; 11: 2FI between percentage of BuOH and extraction time; 12: 2FI between extraction temperature and percentage of BuOH; 13: 2FI between extraction temperature and extraction time; 14: extraction temperature; and 15: percentage of BuOH.

Table 5. Estimated ANOVA for half fraction of factorial design relationship between response variable (extraction yield) and independent variables (A, B, C, D, and E) in determination of significant factors to be optimized by RSM.

Source	SST	df	MS	F-Value	p-value Prob > F
Model	2438730	7	348390	10.24817	0.0005
A-pH	13219.25	1	13219.25	0.388855	0.5456
B-Temperature	10287.03	1	10287.03	0.302601	0.5933
C- BuOH %	771.4506	1	771.4506	0.022693	0.8830
D-Stirring rate	672359	1	672359	19.77798	0.0010
E-Stirring time	936685.2	1	936685.2	27.55335	0.0003
AB	278440.9	1	278440.9	8.190562	0.0155
CD	526967.1	1	526967.1	15.50116	0.0023
Curvature	1268896	1	1268896	37.32559	<0.0001
Residual	373948.7	11	33995.33		
Lack of Fit	179699.1	8	22462.39	0.34691	0.8972
Pure error	194249.6	3	64749.85		
Cor total	4081575	19			

SST, MS, and df stand for Sum of Squires, Mean Squire, and Degree of Freedom, respectively.

According to Figure 1 and Table 5, duration of stirring time was the most significant factor with a negative effect on the extraction efficiency of persipeptides. The second most important negative-effect variable was the rate of stirring. Interestingly, 2 FI between percentage of BuOH and stirring rate was significant with a negative effect on the extraction process. In contrast, both pH and temperature were non-significant factors with positive and negative effect, respectively. In low temperatures, extraction is slow process with lower yield, whereas at higher temperature, solvent is more soluble in aqueous phase, and therefore its separation from aqueous solution is more complicated. Additionally, persipeptides degradation is apparent at high temperatures;[3] therefore, temperature for their extraction was set at 28 ˚C. Moreover, the pH of the extraction process was kept the same as the pH of fermentation broth of *S. zagrosensis* (9 and 9.5).

Optimization of significant factors

In the next step, a CCRD design was applied to optimize the values of three factors (percentage of BuOH, stirring rate, and extraction time), selected from the prior screening design. The process variable of LLE of persipeptides examined using CCRD presented in Table 6. The number of experiments is determined by the expression: (2^n + $2n$ + C), where n represents the number of factors (6) and C denoted the number of center points (3). The design of CCRD consisted of a factorial design (2^n) augmented with ($2n$) run per axial points, which is the number of times each axial run will be performed and located at +1.682α and -1.682α from the center of the experiment domain to satisfy the rotatability condition of the CCRD, and central points (C). Center runs with six repetitions were employed to estimate pure error for the lack of fit test as well as provides rather uniform precision designs. This means that the error inside a sphere that has a radius equal to +/-1 level is nearly uniform. Thus, predictions in this region are equally good.

Table 6. The significant variables and the level of the central composite response design used in the optimization of the persipeptides extraction.

Factor	Level			Star points*	
	-1 (L)	0 (C)	+1 (H)	-α	+α
x_1: BuOH %	25	50	75	8	92
x_2: Stirring rate	150	225	300	99	351
x_3: Stirring time	20	49	78	0.3	98

*The value is (α = 1.682). L, C, and H stand for Low, Central, and High, respectively.

The data obtained were analyzed by ANOVA (Table 7) and then backward elimination regression, with alpha to exit equal to 0.100, was employed to improve the ANOVA results, in which the quadratic response was reduced by the elimination of x_1x_2 (2FI) and x_3^2 (Table 8).

Fitting the model

A regression evaluation (Table 8) was performed for fitting mathematical models to the experimental data aiming at an optimal area, and a quadratic model was suggested according to the results. The predicted model of the regression equation for the peak area of persipeptides was expressed as eqn. (6) in terms of coded factors.

$$Y_1 = 1604.08 - 18.95x_1 + 231.51x_2 + 267.34x_3 - 225.41x_1x_3 - 220.52x_2x_3 - 183.06x_1^2 - 138.61x_2^2 \quad (6)$$

The F-test and p-value (Table 8) were used to determine the significance of each coefficient. If the p-value becomes smaller and the absolute F-value becomes higher, the corresponding variable would be of more significant.[22]

Table 7. Estimated ANOVA of relationship between response variables (extraction yield) and independent variables (x_1, x_2 and x_3) for response surface quadratic model.

Source	SST	df	MS	*F*-Value	*p*-value Prob >*F*
Model	3301366	9	366818.5	3.606939	0.029
x_1	4902.709	1	4902.709	0.048209	0.8306
x_2	731993.3	1	731993.3	7.197717	0.023
x_3	976072.3	1	976072.3	9.597755	0.0113
x_1x_2	2266.328	1	2266.328	0.022285	0.8843
x_1x_3	406463.8	1	406463.8	3.996774	0.0735
x_2x_3	389028.2	1	389028.2	3.825328	0.079
x_1^2	443707	1	443707	4.362988	0.0633
x_2^2	247389.7	1	247389.7	2.432592	0.1499
x_3^2	84216.33	1	84216.33	0.828102	0.3842
Residual	1016980	10	101698		
Lack of fit	789641.9	5	157928.4	3.473428	0.099
Pure error	227337.9	5	45467.59		
Cor. total	4318346	19			

SST, MS, and df stand for Sum of Squires, Mean Squire, and Degree of Freedom, respectively.

Table 8. ANOVA of relationship between response variables (extraction yield) and independent variables (x_1, x_2 and x_3) in response surface reduced quadratic model.

Sour-ce	SST	df	MS	*F*-Value	*p*-value
Model	3214883	7	459269.1	4.994487	0.0075
x_1	4902.709	1	4902.709	0.053316	0.8213
x_2	731993.3	1	731993.3	7.960324	0.0154
x_3	976072.3	1	976072.3	10.61465	0.0069
x_1x_3	406463.8	1	406463.8	4.420237	0.0573
x_2x_3	389028.2	1	389028.2	4.230627	0.0621
x_1^2	487734.6	1	487734.6	5.304045	0.0400
x_2^2	279641.6	1	279641.6	3.041063	0.1067
Resi-dual	1103462	12	91955.21		
Lack of fit	876124.5	7	125160.6	2.752744	0.1413
Pure error	227337.9	5	45467.59		
Cor. total	4318346	19			

It was observed that most significant variables were the linear terms of extraction time (x_3) and stirring rate (x_2).

The result suggested that the change in extraction time ($p < 0.0069$) and stirring rate ($p < 0.0154$) had considerable effects on the LLE of persipeptides. Indeed, extraction time (x_3) had a pivotal effect in LLE method and was required to be optimized in order to achieve high efficiency in extraction of the persipeptides. The procedure time was the driving force for transportation of the persipeptides from aqueous solution to organic solvent as a result of the increase in compounds interaction with organic solvent until the extraction equilibrium has been reached. However, if the time is set erroneously high, then persipeptides will be degraded[3] and in turn, the extraction efficiency will decrease. Persipeptides degradation may reach up to 21 % when fermentation broth is kept at room temperature for 24 h prior to extraction or its concentration may be decreased by up to 13 % when the broth is preserved at 4 ˚C in BuOH for same period of time.[3] Results showed that the extraction time is in reverse relationship with the percentage of organic solvent used, justifying the existence of significant interaction between two factors, which was found in the screening design. In high percentage of organic solvent, the time of extraction could be diminished to as low as 33 min. Nevertheless, increasing the rate of stirring as high as possible (up to 300 rpm), which was the second most important factor in this process, enhanced the extraction capability. These are in accordance with other studies conducted on the effect of stirring rate, solvent amount, and hold-up on efficiency of a typical extraction process, which have reported that when stirring speed and solvent amount were increased, the efficiency increased. Additionally, it has been proposed that the efficiency of the compounds extraction increased monotonously with speeding up stirring rate.[23] Following the mathematical model fitting, multiple response method, called the desirability function (*D*), was employed to optimize the studied parameters.

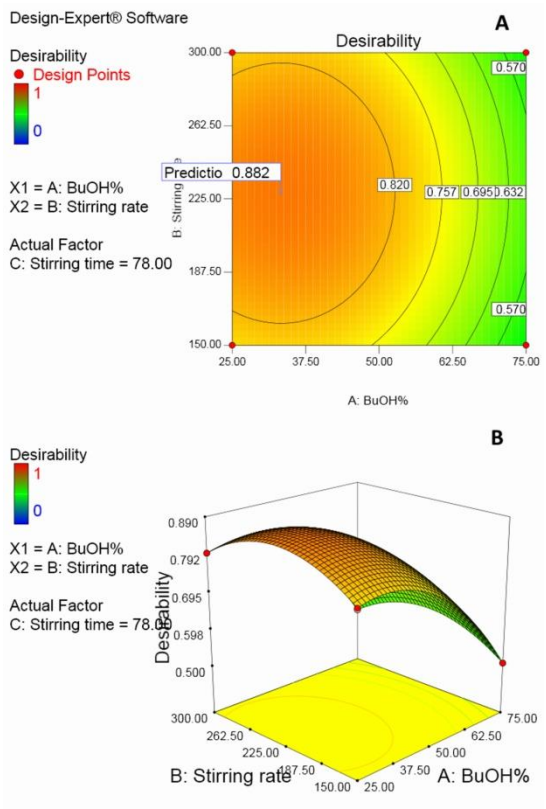

Figure 2. Contour plot (**A**) and 3D surface graph (**B**) of desirability versus ratio of BuOH and stirring rate. Stirring time was 78 min, pH was 9, and temperature was 28 °C were the experiment condition.

This method was applied to meet the requirement for increasing the yield of extraction in as shortest process as possible to both decrease the expenses and prevent persipeptides degradation. The most desired value for the responses is a desirability value of one, whereas a value equal to zero represents an unacceptable value for responses.

The aim of the optimization was to improve the LLE efficiency of persipeptides. In this approach, a process with desired characteristics is obtained by combining process parameters, which has been evaluated by RSM, into a single variable for the prediction of the optimal levels of the independent variables. To achieve the highest desirability, all factors were set to within range, except the concentration of persipeptides that was set to maximum level. Figures 2A and 2B, respectively, illustrate the contour plot and 3D surface graph of desirability for LLE of persipeptides generated from 15 optimum points through numerical optimization.

Among 30 starting points, the best local maximum for LLE of persipeptides (264 ± 9.85 µg mL^{-1}) was resulting in 34 % BuOH, 228 rpm (stirrer speed), and 78 min (stirring time) with the value of desirability of 0.88. Compared with un-optimized process (persipeptides concentration of 219.63 ± 2.48 µg mL^{-1}), the optimized conditions increased the extraction of persipeptides by 20.20 %, while decreased both time and percentage of BuOH by 67 % (162 min) and 16 %, respectively. Therefore, using optimized LLE method and suggested fermentation broth, a total of seven times increased in persipeptides production was reached. Nevertheless, from large scale prospect, the final reaction conditions *i.e.* broth pH, 9-9.5; temperature, 28 ˚C; stirrer speed, 228 rpm; percentage of BuOH; 34 %; and process time, 78 min have industrial compatibility.

This is because of no pre-pH adjustment requirement for LLE of persipeptides as the ambient pH of *S. zagrosensis* CM1 broth is within the mentioned range. It is worth to note that as pH increased and temperature decreased the corrosion rate of steel from different parts of extraction plants decreased, which is an astonishing feature of this optimized process. Moreover, minimum facilities and energy consumption are required for adjusting temperature at 28 ˚C; therefore, greatly decreases the expense of extraction. The percentage of BuOH was decreased in expense of increasing time from 33 to 78 min to minimize instrument corrosion and solvent usage and its subsequent evaporation expenses. However; despite great effort, the agitation could not be decreased and indeed increased from 150 rpm in un-optimized process to 228 rpm in further optimized method, which is inevitably still not considered as an improving step.

Conclusions

The direct impact of this optimization is on decreasing the cost of extraction as well as the time of process, whereas increasing the yield of extraction. This in turn, facilitates further clinical trial investigations, which require several of grams of purified persipeptides. This was the first attempt reported on retrieval of persipeptides from fermentation broth that has been optimized. It has been previously shown that presence of glucose plus a more slowly utilized carbon source such as malt result in production of higher secondary metabolites, as glucose utilization results in good growth of bacteria and complex carbon source is used for antibiotic synthesis.[24] However, rapid catabolism of glucose has been shown to decrease the rate of antibiotic production.[25, 26] In practice, ISP2 medium, which have been tested on the basis of this assumption failed to increase the production of persipeptides. In contrast, both CM1 and CM6 increased the production of persipeptides. These increases may be due to the fact that application of low solubility carbon sources, such as insoluble starch in CM1 and glycerol in CM6, prevents carbon catabolite regulation.[26] Despite the advantage of utilization of glycerol in fermentation, which contains more energy than starch and glucose on a weight-to-weight basis, it has some disadvantages, including higher oxygen requirement, increased medium viscosity, and more problematic downstream processing.[26] Interestingly, both CM1 and CM6, considerably enhanced persipeptides production by six and four times, respectively, compared to ISP2 medium. CM1 and CM6 contain soybean, which is a rich source of valine and phenylalanine amino acids with percentage of total weight on dry basis of 2.06 ± 0.19 and 2.16 ± 0.21, respectively.[27] These amino acids are present in core structure of persipeptides with repetitions as the only utilized amino acids. CM1 contains three times more soybean than CM6, which may explain its 37 % (approximately 60 µg L^{-1}) more persipeptides production, compared to CM6 by means of providing more valine and phenylalanine. It has been observed that increase in amount of $CaCO_3$ from 2 to 10 g L^{-1} improves the production of persipeptides (Data not shown); however, there is little knowledge on effects of various salts in biosynthesis of persipeptides. Therefore, a systematic study using a number of techniques such as labeled precursors for the study of various media components and its further optimization for exploiting the best result are required.

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