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Research Article

In-Situ Recovery of Persipeptides from *Streptomyces zagrosensis* Fermentation Broth by Enhanced Adsorption

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Background: Drug discovery process is growing considerably due to the noteworthy resource of natural products. Persipeptides A and B are cyclopeptide antibiotics, which are produced by *Streptomyces zagrosensis* UTMC 1154. Although extraction of culture broth with the help of solvent has been optimized previously, no effort for *in-situ* extraction of persipeptides has been done yet.
Objective: To produce a high quantity of persipeptides for further drug evaluation, it is crucial to design approaches aimed at improvement of the extraction yield.
Materials and Methods: Amberlite XAD-16N was employed into the fermentation culture medium of *S. zagrosensis* in order to enhance the *in-situ* extraction of persipeptides. Effects of resin content (%), resin addition time (h), and fermentation time (day) were investigated by a two-level full factorial experimental design.
Results: The main factors of resin content (%) and the interaction of resin content (%) with resin addition time (day) were found to be important using ANOVA. The results showed the amount of 0.33 % ($w.v^{-1}$) amberlite XAD-16N added at 27.2 h post-inoculation was the most effective combination to increase the efficiency of *in-situ* adsorption capacity of persipeptides.
Conclusions: The provided method requires 3.3 g resin and 200 mL methanol for the extraction of persipeptides from each liter of fermentation culture of *S. zagrosensis* in less than 15 min. Apart from cost-efficiently and simplicity, this procedure enhanced the recovery of persipeptides by 7 % and 3 times, compared to ISP2 medium without any resin after 4 and 7 days of fermentation, respectively. Therefore, this method can be regarded as a cost-efficient enhancement approach for the production of these newly-discovered metabolites before implementing the genetic manipulation or intensive media optimization, demanding considerable time and effort.
Keywords: Amberlite Resin; Experimental design; Full factorial optimization; *In situ* metabolic extraction; Natural product; Persipeptides

1. Background

Microorganisms are the potent producers of various natural products such as pigments (1), antibiotics (2), and neuroprotectants (3, 4). Among them, persipeptides A and B, two new peptide antibiotics with cyclic structures, are produced by *Streptomyces zagrosensis* UTMC 1154 and comprise N-methylvaline, N-methyl phenylalanine, phenylalanine, and valine with two repetitions (5).

The pipeline of drug discovery is significantly supplied by a rich source of novel natural compounds or their semisynthetic derivatives, which counts 27% of all novel chemical structures approved by the Food and Drug Administration (FDA) calculated from 1981 to 2006. This statistic covers 68% and 34 % of new antibacterial and anticancer agents, respectively (6). Natural products have resulted in the production of prominent therapeutics such as statins in lipid disorders and cyclosporine-class or rapamycin-class in immunosuppression diseases. Nowadays, many pharmaceutical companies have altered their policies from acute to chronic diseases for a higher profit of business perspective (7). Challenges in achieving adequate amounts of natural product to support drug development in its early stages are among the main perceived technical confrontations in natural product

based drug discovery program (8, 9). However, the increased occurrence of drug resistance strains in recent years underlines the continuous requirement for new antibiotics development. To date, only a single class of totally synthetic antibiotics, fluoroquinolones, has reached the clinical application comparing to natural products (10). This highlights the significance of natural products.

A natural product starting material equal to several tens of grams is required to support the early stage of medicinal chemistry experiments. This amount will increase to several times higher as it enters into preclinical investigation studies. The frequent structure complexities of natural products hinder the application of multi-gram-scale chemical synthesis. Moreover, wild-type bacteria producing a specific natural product initially only provide little amounts when cultured at un-optimized conditions (10). The improvement encompasses the employment of various process optimizations, chemical composition and physical parameter, in addition to fermentation operation mode (11-14). In spite of more attempts and advances in extraction and separation of natural products from various bio-resources, it is still challenging and there is necessity to introduce new extraction and isolation approaches for this purpose. Although by developing the identification methods of natural products, the amounts needed from purified form have significantly decreased for stereochemistry or 2NMR analysis, but for *in vitro* and *in vivo* biological activity assessment of novel discovered bio-compounds, adequate amount of them for completion of identification assays is very urgent and notable. Therefore, design and investigation of modern approaches for natural product extraction should be attempted much more than ago (15). A relatively prompt solution is the elevation of the secondary metabolites titers in fermentation broth and therefore, providing the required amounts of natural products for early drug development stage by *in-situ* adsorption of product. Furthermore, this technique has the advantage of feasibility for scale-up as well as commercialization potential. The principle mechanisms involved include; decrease or elevation of feedback inhibition, increased product stability or decreased autotoxicity through adsorption of secondary metabolites as quick as it is synthesized during fermentation (10).

Solid adsorbents are one of the promising agents for *in-situ* separation techniques, which have been applied to elevate the production yield and rate of recovery for secondary metabolites (16-19). A number of studies have outlined this method as a successful method for improvement in production of desired metabolites, including BMS-182123, cercosporamide, dynemicins, esperamicin A_1, kirromycin, nisin, pristinamycin, teicoplanin, and xinghaiamine A (18, 20-27). Amberlite XAD-16N resin was successfully applied in enhanced production of epothilone by *Sorangium cellulosum* and *Myxococcus xanthus*, migrastatin and isomigrastatin by *Streptomyces platensis*, rubradirin by *Streptomyces achromagenes* v. *rubradiris*, and tirandamycin by *Streptomyces* sp. 307-9 (16, 28-30). Moreover, the *ex-situ* extraction of dorrigocins by *Streptomyces platensis* subsp. *Rosaceus* has been reported (31). Another possible advantage of *in-situ* extraction using amberlite over classical liquid-liquid extraction would be the lower requirement for extraction solvent, which is usually toxic and/or expensive.

2. Objectives

Regarding the demand of enough quantity of the pure persipeptides for biological activity analysis, the *in-situ* extraction of them was subjected to the optimization. In this study, the possibility of amberlite XAD-16N resins for the enhancement of persipeptides production during the fermentation process of *S. zagrosensis* UTMC 1154 was investigated.

3. Materials and Methods

3.1. Chemicals

Acetonitrile (HPLC grade), methanol (extra pure) and *n*-butanol (extra pure) were purchased from Merck (Darmstadt, Germany). The HPLC grade deionized water was produced using Barnstead/thermolyne, USA (Model: d8992-33 Nano pure infinity). Amberlite XAD-16N resin, particle size of 20-60 mesh, pore size of 200 Aº and 800 $m^2.g^{-1}$, was purchased from Sigma.

3.2. Bacterial Strain and Culture Media

The persipeptides-producing actinobacterium, *S. zagrosensis* UTMC 1154, was obtained from the University of Tehran Microorganism Collection. ISP2 medium, consisted of ($g.L^{-1}$): glucose, 4; yeast extract, 4; malt extract, 10; and pH adjusted to 7.4, was used as seeding and fermentation media after sterilization at 121 ºC for 20 min. Additionally, CM1 medium, consisted of ($g.L^{-1}$): starch, 20; soybean, 30; $MgSO_4.7H_2O$, 1; $CaCO_3$, 10; and pH adjusted to 7.0 was also investigated as possible fermentation medium (32) for *in-situ* extraction of persipeptides.

3.3. Culture Conditions

One milliliter of spore suspension of *S. zagrosensis* UTMC 1154 (containing ~1×10^7 $CFU.ml^{-1}$) was

inoculated into each 100-mL flasks containing 9 mL seeding medium and were shaken at 28 ºC with 220 rpm for 36 h. The seeding material (10 % $v.v^{-1}$) was transferred to 250-mL flasks containing 50 mL fermentation medium. The inoculated flasks were incubated at 28 ºC with 220 rpm for 7 to 11 days and no control on pH, *i.e.,* pH was free to change, reaching about 9.1±1 at maximum. All inoculations, including adsorbents, were transferred aseptically.

3.4. Preparation of Adsorbents

The amberlite XAD-16N resins were washed with methanol twice and were allowed to soak in acetone for 24 h. Subsequently, acetone was removed via washing the swollen resins with adequate distilled water. The prepared resins were sterilized separately at 121 °C for 30 min and added to the fermentation broth.

3.5. Analytical Assay of Persipeptides

At the end of fermentation, the broth containing resins was passed through a stainless steel sieve with 0.3 mm mesh size and washed with distilled water in order to remove resins with no culture medium contamination. The resins, then, were extracted using 10 mL methanol, vortexed for 5 min, and centrifuged at 10956 × g for 10 min. Extracted persipeptides from resin were quantified by HPLC. For comparison purpose, liquid-liquid extraction (LLE) method previously described (33) has been conducted for culture media lacking resin. Quantification of persipeptides carried out by a Cecil HPLC instrument, equipped with a Lichrosorb C18 column (250 mm × 4.6 mm I.D., particle size 5 µm, ACE-121-2546) and protected by a Hichrom C18 guard column, and thermostated at 27 ºC. The acetonitrile and water under a gradient program were employed as the mobile phase at 0.8 $mL.min^{-1}$ and the eluent was monitored at 210 nm (33). The concentration of persipeptides was calculated using a calibration curve according to Mohammadipanah *et al*. (33).

3.6. Persipeptides Production Curves

Persipeptides-producing capability of *S. zagrosensis* was investigated in an eight-day period in two different media, including CM1 and ISP2, containing no resins. During this period, concentration of persipeptides was determined every 24 h with three replicates using LLE method (32), and the trend of production was monitored.

3.7. Modeling by Full Factorial Design

A number of factors, including resin content, resin addition time, and fermentation age were selected as independent variables, and their influences on the dependent variable of persipeptides HPLC peak area were investigated using a two-level 2^3 full factorial design. The transformation of actual values of experimental factors (uncoded units) into coded units, -1 (low level), 0 (central point), and +1 (high level) were performed and range of variables was decided based on our knowledge of persipeptides fermentation process (**Table 1**).

Table 1. Experimental codification ranges and levels of variables used in the factorial design

Independent variable	Coded symbol	Range and level		
		-1	0	+1
Resin content (% w/v)	A	0.33	0.67	1
Fermentation time (day)	B	7	9	11
Resin addition time (day)	C	1.13	2.05	2.97

Each experiment was performed with two replicates, and the full factorial design consisted of 16 experimental runs. Additionally, in order to validate the assumption that the factor effect relationships are linear, five center points were included; therefore, the total number of experimental runs was increased to 21 trials (**Table 2**). Design-Expert Version® 10.0.7 was employed to create and analyze the effects, coefficients, standard deviation of coefficients, and other statistical parameters of the fitted model as well as the statistical plots. The statistical significance of an effect was determined by *p*-values <0.05.

4. Results

4.1. Selection of Resin

In the selection process of appropriate adsorbents, various parameters can be considered. The product binding capacities of the adsorbents and their resistance to destructive physical forces into culture medium are among the properties of importance. In this viewpoint, it would be assumed that the pore size and three-dimensional geometry of the resins, the viscosity of fermentation medium and the existence of insoluble components or cell clumps are influential and effective

Table 2. Experimental design matrix and results of in-situ extraction of persipeptides(μg.mL^{-1})

	Factor A	Factor B	Factor C	Response
Run	**Resin content**	**Fermentation time**	**Resin addition time**	**Persipeptides concentration**
	% (w/v)	**Day**	**h**	**μg.mL^{-1}**
1	1.00	11	71.28	59
2	0.33	11	71.28	68.8
3	0.66	9	49.2	60.84
4	0.66	9	49.2	66.26
5	0.33	7	27.12	87.75
6	0.33	11	71.28	76.74
7	0.33	11	27.12	91.76
8	0.33	7	71.28	68.4
9	1.00	7	71.28	66.15
10	0.66	9	49.2	66.21
11	0.66	9	49.2	55.136
12	1.00	7	71.28	59.16
13	1.00	11	71.28	59
14	0.33	7	71.28	83.49
15	1.00	11	27.12	41.74
16	0.66	9	49.2	75.9
17	1.00	11	27.12	42.95
18	1.00	7	27.12	58.18
19	0.33	7	27.12	88.91
20	0.33	11	27.12	98.94
21	1.00	7	27.12	57.97

on the appropriate metabolites capture of the adsorbents (29). Correspondingly, the chemical and structural properties of adsorbent, as well as interaction with biomolecule, are other main factors in determination of the binding capacity of the adsorbents (34). According to persipeptides structure, the existence of phenyl groups and π–π bonding, the resins with adsorption mechanism based on the hydrophobic interactions are more suitable. Therefore, XAD-16N resins was selected owing to their high affinity for hydrophobic and aromatic compounds, as well as their capacity to bind with persipeptides through establishing hydrophobic interaction and π–π bonding (10). This resin was more conveniently recyclable and recovered more concentration of persipeptides than other previously investigated resins (35).

4.2. Experimental Design and Value of Parameters

Forgoing literature, resin concentration for *in-situ* extraction purpose is in a range from 0.8 to 20 % (10). According to our previous study (information not provided), it was determined that the addition of a high amount of XAD-16N into culture medium has a negative effect on both the *S. zagrosensis* growth as well as persipeptides production. Also, it can be deduced that the resin content (**Fig. 1a**) and the additional time (**Fig. 1b**) had a negative effect on each other (**Fig. 1c**). This phenomenon is possibly due to the adsorption of medium nutrients as postponed addition of higher amounts of resin relieved this repression. In contrast, addition of a very low amount of resin resulted in insufficient adsorption of persipeptides in the fermentation medium, as a considerable fraction of these peptides was still present in the culture medium. Therefore, in this study, the value range of 0.33 to 1.0 % (w.v^{-1}) was selected for the resin content.

A noticeable surge in persipeptides production was recorded in both mentioned media between days one and two with the highest production in days four (86.66 ± 11.51 μg.mL^{-1}) and six (205.06 ± 1 μg.mL^{-1}) for ISP2 and CM1 media, respectively (**Fig. 2a**). Both of these media contained no resin and persipetides were extracted using conventional LLE method developed previously (32). Studying the biomass production in ISP2 medium

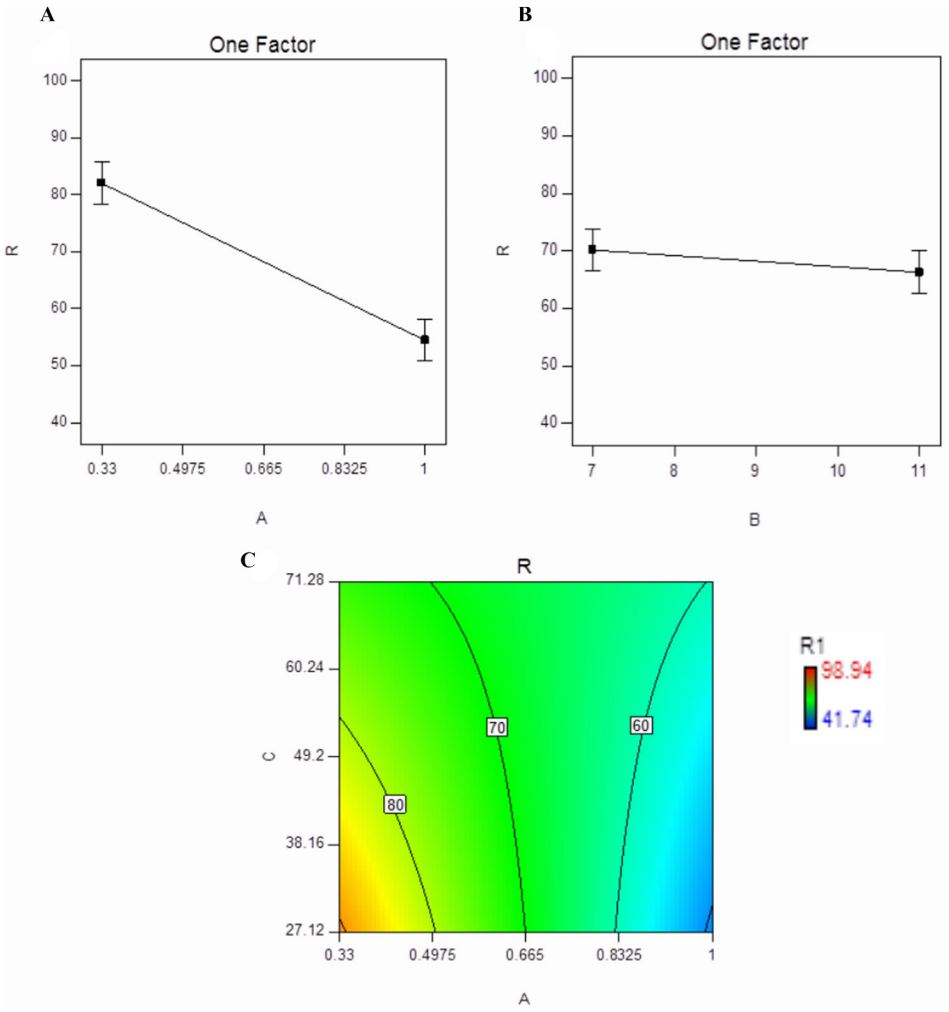

Figure 1. Main effects of the principal parameters (a, resin content when B and C are kept constant at 9 and 49.2, respectively; b, resin addition time when A and B are kept constant at 0.67 and 49.2, respectively; c, interaction effects between A and C when B is 9) on *in-situ* extraction of persipeptides. A: resin content (% w.v^{-1}), B: fermentation time, and C: resin addition time (h).

by *S. zagrosensis* showed that the highest biomass weight occurred within three days, after which finally decreased due to cell lysis and spore formation (**Fig. 2b**). It is worth noting that the onset of cell lysis in *S. zagrosensis*, submerge cultured in ISP2, coincided with reaching the persipeptides concentration to the highest titer (**Fig. 2**). This may strengthen the assumption that production of antibiotics in *Streptomyces* is for the protection of generated nutrient for spore development at the cost of cell lysis against competitors; therefore, antibiotics prevent the invasion of competitors to colonies producing them (36). Therefore, the addition of adsorbents in preliminary hours of post inoculation (27.12 to 71.28 h) leads to an increase in yield of recovery, which was due to the exponential production of persipeptides occurred between day one and two. The fermentation beyond 11 days was not reasonable due to nutrient run out as well as high genetic instability of the producing strain, whereas fermentation less than 7 days was not economical since the persipeptides were still synthesized by the strain and therefore, the range of 7 to 11 days were selected.

4.3. Analysis of Variance (ANOVA)

An analysis of variance (ANOVA) was conducted to determine the significant factors affecting the *in-situ* extraction of persipeptides from its production matrix. In **Table 3**, the sum of squares, which was used in the estimation of factors' effects and *p*-value, is presented. It is concluded that for *in-situ* extraction of persipeptides, the effect of resin content and the interaction of resin addition point and resin content present the higher statistical significance.

It can be deduced from the effect of the main factors shown in **Figure 1** that the effect of resin content was characterized by a more considerable degree of departure and in addition had a negative effect on the response. It should be noted that by increasing the resin

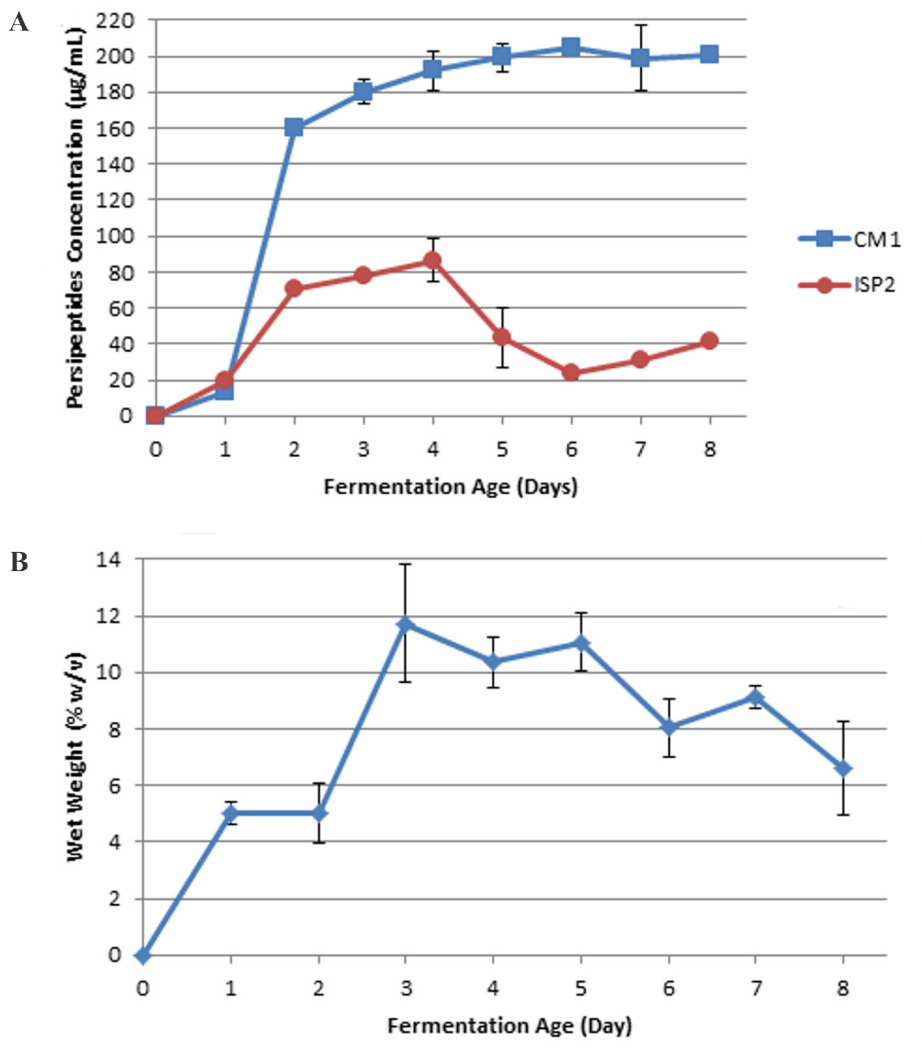

Figure 2. Persipeptides production curves in ISP2 and CM1 **(A)** and biomass production curve in ISP2 **(B)** during eight days of fermentation (Bar standard errors included).

Table 3. Student's t-test values

Model	Sum of Squares	df	Mean Square	F-Value	P-value	
A-Resin content	3879.364	3	1293.121	27.45691	< 0.0001	significant
C-Resin addition time	3042.626	1	3042.626	64.60422	< 0.0001	
AC	47.12822	1	47.12822	1.000676	0.332	
Curvature	789.61	1	789.61	16.76583	0.0008	
Residual	75.08421	1	75.08421	1.594267	0.2248	not significant
Lack of Fit	753.5422	16	47.09639			
Pure Error	320.153	4	80.03824	2.216158	0.1285	not significant
Core Total	433.3893	12	36.11577			

content, delay in resin addition time point will have a positive effect on the response. The contour plot for significant interaction is presented in **Figure 1** and the major impact of resin addition time and resin content on *in-situ* extraction of persipeptides are indicated, which in general, corresponded to early addition of lower concentration of the resins.

4.4. Student's T-test

A Student's *t*-test was employed in order to determine

any significant deviation of calculated effects from zero (37). It was observed that for the level of 5 % risk, the *t*-value was equal to 2.12. There are two different *t* limits plotted on the graph; the lower vertical line is the standard *t* and indicates the minimum statistically significant effect for 5 % risk level, whereas the upper one is the Bonferroni corrected *t* with the value of 3.08 and the effects above this line is almost certainly significant (**Fig. 3a**). Values presented in the vertical axis are Student's *t*-test values (**Fig. 3a**).

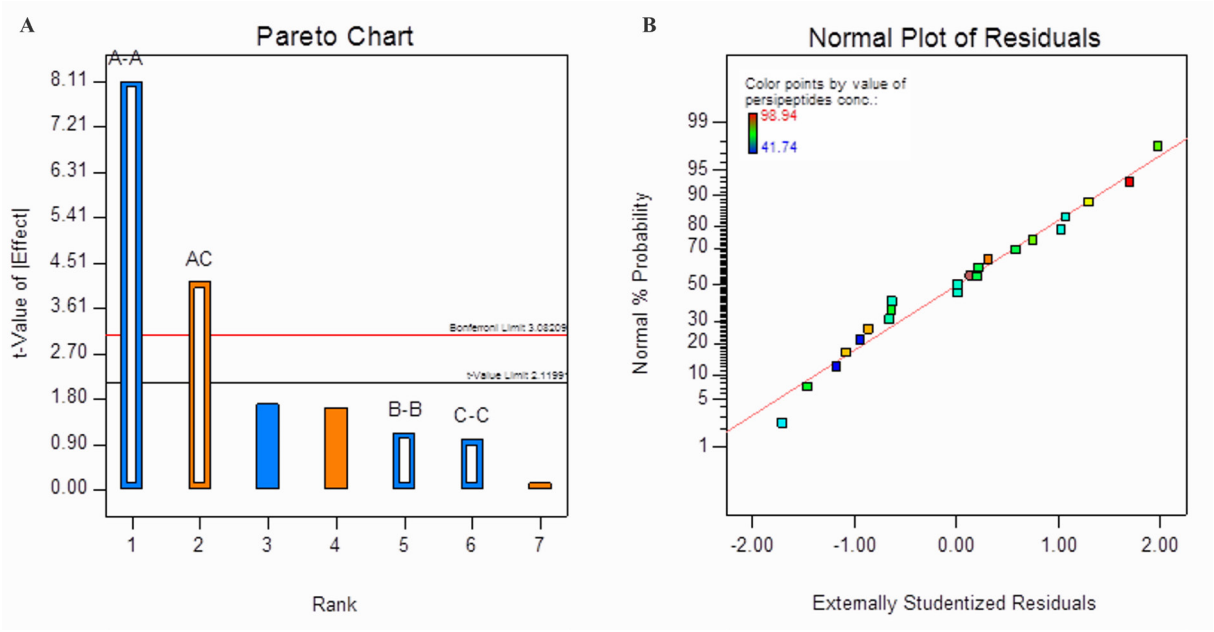

Figure 3. Pareto charts of factors effects **(A)**, and normal probability plot of residual **(B)** for *in-situ* extraction of persipeptides.

Based on Student's *t*-test, the statistically significant effects were selected. The resultant model can be represented by Eqn. (1) in coded form, in which only the content of the resin and the interaction between this parameter with the resin addition time present influence in the response, therefore the resin addition time also included to hierarchy correct the model:

Concentration of persipeptides =

$$68.25-(13.79 \times A)-(1.94 \times C)+(7.02 \times A \times C) \quad (1)$$

The model F-value of 20.52 implies the significance of the model. There is only 0.01 % chance that the model F-value of this large could occur due to noise. The measured curvature F-value of 1.62 as the difference between the average of the center points and that of the factorial points implies the curvature in the design space is not significant, and there is a 22.48 % chance that it could occur due to noise. Furthermore, the lack of fit F-value of 2.32 implies that it is not significant relative to the pure error, and there is a 11.65 % chance that it could occur due to noise; therefore, the model can fit. The Predicted R-Squared of 0.7269 was in reasonable agreement with the Adjusted R-Squared of 0.7960; *i.e.* the difference is less than 0.2. Moreover, model adequate precision measuring the signal to noise ratio had the value of 13.462 which is considerably greater than 4. Consequently, the model can be used to navigate the designed space.

4.5. Normal Probability Plot of Residuals

The differences between the predicted (model) and the observed (experimental) values as well as an illustration of how closely the set of observed values follows a theoretical distribution were evaluated by the visual examination of the residual value distribution in the normal probability plot of residuals for the efficiency of *in-situ* extraction of persipeptides. As it is presented in **Figure 3b**, *in-situ* extraction of persipeptides exhibited reasonably aligned scattering of experimental points with some expectable moderate scatters suggesting normal distribution.

4.6. Optimizations Using the Desirability Function

The generic aim of any secondary metabolite-manufacturing industry is to yield as the highest product as possible in as shortest process as possible to elevate profit. Therefore, following the mathematical model fitting, multiple response method called the desirability function (*D*) was employed to optimize the studied parameters (32). In this approach, a product with desired characteristics is obtained by combining process parameters, including resin content, addition time of the resin, and fermentation age into a single variable for the prediction of optimal levels of the independent variables. A desirability value of one is the

most desired value for the responses; in contrast, a value equal to zero exhibits an unacceptable value for the responses. The optimization aimed to enhance the *in-situ* extraction of persipeptides adsorption conditions in fermentation flask. To achieve maximum desirability, both resin content and fermentation age were set to minimum levels, whereas resin addition time and the concentration of persipeptides extracted were set to within the range and maximum level, respectively. **Figure 4** illustrates the 3D surface graph of desirability for *in-situ* extraction of persipeptides generated from 59 optimum points through numerical optimization.

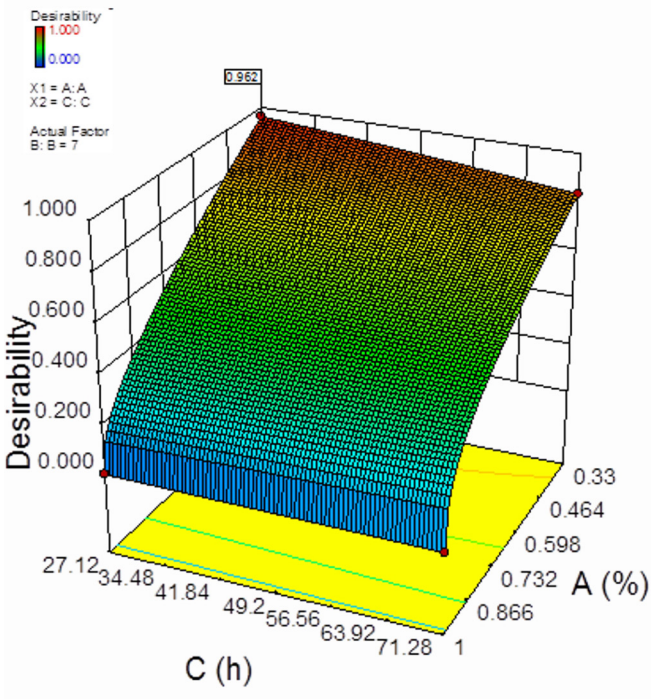

Figure 4. Desirability fitted 3D surface for *in-situ* extraction of persipeptides (B was constant at 27.12 h). **A)** resin content, **B)** fermentation time, and C: resin addition time.

Among 109 starting points, the best local maximum for *in-situ* extraction of persipeptides (92.73 µg.mL^{-1}) was resulted from resin content of 0.33 % (w.v^{-1}), added after 27.12 h from starting of fermentation with the final fermentation age of 7 days. The value of desirability obtained for this solution was 0.962.

5. Discussion

Factorial Design can be used as a systematic approach for designing and optimizing the desired formulation based on different process variables. The main goal of this approach is exploring the significant parameters to achieve the desired values of results on the basis of principal parameters (38). Unlike one factor experimental designs, all parameters affecting the process, for example, interfering factors are considered by multi factor experimental designs and therefore, the full factorial design was selected which produced high resolution power to study the selected parameters.

Generally, antibiotic biosynthesis often coincides with the cessation of exponential growth and when relatively high population density has been achieved (36). It may thus be concluded that more expenses, both time and cost, are imposed on industrial production of antibiotics. Interestingly, in *S. zagrosensis*, the production of persipeptides occurs right from the beginning of growth with higher titer in CM1 compared to ISP2 medium, which justify their future commercial production (**Fig. 2a**). Additionally, the optimized *in-situ* adsorption of persipeptides containing a minute amount of resin 0.33 % (w.v^{-1}) increased the production of these novel pentapeptides by *S. zagrosensis* up to 3 times (92.73 µg.mL^{-1}) after 7 days fermentation, compared to ISP2 medium with no supplemented resin (31.01 µg.mL^{-1}) (**Fig. 2a**). It is worth to mention that up to 28.1 mg of persipeptide could be adsorbed by each gram of amberlite in this study. Compared to the highest titer of persipeptides in ISP2, which was observed in the fourth day of fermentation (86.62 µg.mL^{-1}), the recent method enhanced the production by 7.05 %. In the previous study, ISP2 medium as the fermentation medium for *S. zagrosensis* was replaced by another medium, named CM1, and the subsequent extraction of secreted persipeptides by an optimized LLE was performed, provided a yield up to 219.63 µg.mL^{-1} (32). This amount is approximately twice the yield obtained in the current study. However, the recent developed and optimized *in-situ* extraction of persipeptides has provided some notable advantages over previous LLE method, which include; shortening the extraction time to almost one-sixth (from 88 to 15 min) of that of optimized LLE method, and reducing the volume of required solvent for the extraction of cultivation medium (32). In previous optimized LLE method, 515 mL butanol was required for the extraction of one liter of the fermentation broth (32), whereas for the same amount of fermentation medium only 200 mL methanol is utilized in the recovery of persipeptides from resins (3.3 g.L^{-1}). Additionally, methanol has a lower boiling point at 64.7 ˚C, compared with that of butanol at 117.7 ˚C; therefore, from the industrial viewpoint, this means the process needs lower energy for vaporing and recycling the extraction solvents.

Despite the observation that CM1 medium considerably enhances the biosynthesis of these novel peptides (32), we were unable to take advantage of CM1 medium due to its medium components, including starch, soybean,

and $CaCO_3$. These compounds interfered with resin separation, extraction, and recycle; consequently, a complicated process would have resulted. Therefore, different resins with abilities for aided-separation from fermentation broth, for example, magnetic resin should be investigated coupled with more sophisticated media such as CM1.

6. Conclusion

A full-factorial experimental design was applied to optimize the factors impacting on persipeptides *in-situ* extraction. Regarding to the new chemical structure of persipeptides and the desperate demand of more amount of them for further evaluation and identification, approaches towards their enhanced production yield such as optimized *in-situ* extraction condition, can contribute to their future clinical evaluations. It has been concluded that the addition of amberlite XAD-16N resins at a concentration of 0.33 % ($w.v^{-1}$) after 27.12 h post-inoculation with harvesting time of 7-day fermentation had maximum desirability values of obtained interest metabolites. Scaling-up of this *in-situ* adsorption process for the pilot or industrial level based on this optimization can significantly be cost-effective, especially from the viewpoint of solvent usage in addition to the increased yield of production. The obtained results showed the *in-situ* adsorption/induction can be an efficient approach for production enhancement of newly discovered metabolites before implementing the intensive media optimization or genetic manipulation, which demand considerable time and effort. Such approaches for increasing the amount and recovery of secondary metabolite production can project the endeavors more toward the *in-situ* extraction before exploring the other time consuming alternative approaches. This approach can accelerate the conventional process for obtaining the sufficient quantity of compounds that are required for preclinical and toxicity studies.

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