Optimized Bioconversion of Soybean Meal Waste to Valued Biosurfactant by Pseudomonas Aeruginosa (PTCC 1074)

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ABSTRACT: *Recently, microbial surface-active molecules called biosurfactants, have gained significant attention due to their structural diversity, biodegradability, low toxicity, and several environmental and industrial applications. However, despite their advantages, they are not widely used because of high production costs, which can be overcome by bioconversion of agro-industrial wastes as low-cost substrates. The current study aimed to overcome the challenges of biosurfactant production by bioconversion of soybean meal, as a low-cost renewable substrate, and to optimize the significant parameters. Rhamnolipid biosurfactant was produced by Pseudomonas aeruginosa (PTCC 1074) using soybean meal under solid-state fermentation and Response Surface Methodology (RSM) by Central Composite Design (CCD) was employed to optimize the significant parameters. The experimental value of biosurfactant production and Emulsification Index were 17.05 (g/kg dry substrate) and 54 % respectively under the optimal conditions (temperature 33 ºC, Initial substrate moisture 80%, and carbon-to-nitrogen ratio (C/N ratio) 54). Regression analysis with RSM resulted in quadratic models and the coefficient of determination (R^2), adjusted R^2, and predicted R^2 were respectively calculated as 0.9767, 0.9557, and 0.9088, indicating that the model fitted the experimental data well. An increase in temperature from 25 to 34°C led to a rise in rhamnolipid production, which implies the significant influence of temperature. The results demonstrated that the production of biosurfactants increased with increasing the initial moisture content at high temperatures and also at low C/N ratios. The current study confirmed the considerable potential of soybean meal for biosurfactant production and also enhanced the production yield by optimizing the significant process parameters.*

KEYWORDS: *Bioconversion; Biosurfactants; Optimization; Pseudomonas aeruginosa.*

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INTRODUCTION

Biosurfactants are surface-active molecules with hydrophobic and hydrophilic moieties which can reduce the liquid surface tension, interfacial tension of one or two liquids, and a solid [1, 2]. Low toxicity, biodegradability, large diversity, effectiveness in a wide range of pH, temperature and salt concentration [3, 4], and the capability to be produced from renewable feedstock are the notable advantages of biosurfactants only to make them appropriate alternatives to synthetic surfactants [5]. The variety of chemical structures and the properties of biosurfactants have provided the choice of a wide range of surfactants for different applications [6]. One of the important applications of biosurfactants is in the crude oil industry. Since bio-surfactants can reduce the surface and interfacial tension of hydrocarbon mixtures, they are considered as potential candidates for enhanced oil recovery, pumping, and transportation of the oil as well as improvement in the oil refinery [7]**.** From an environmental point of view, the use of biosurfactant-producing bacteria is of special importance in increasing biodegradability and eliminating the hydrocarbon pollutants, dyes, and heavy metals in the environment [3, 8]. Moreover, biosurfactants have been identified for commercial importance in the pharmaceutical fields, food processing and cosmetic industry [9-11]. However, despite all these properties, biosurfactants production faces some limitations due to the production costs. To overcome this problem, bioconversion of low-cost agricultural wastes via Solid State Fermentation (SSF) has received considerable attention during the recent years [12, 13]. SSF is defined as the growth of micro-organisms on a solid support in the absence (or near absence) of free water; however, substrate must have adequate moisture for microbial growth and metabolic activity [14]. Although SSF provides numerous opportunities in the processing of agro-industrial wastes to produce value added bioproducts such as secondary metabolites, biopesticides and enzymes, limited studies have been reported on the production of biosurfactant by SSF using waste compounds or by-products from agro-industrial processes (Table 1) [15-22]. The use of agro-industrial residues has economic values to these wastes, while eliminating their disposal problem [20]. Another approach to reduce the cost of biosurfactant production is the optimization of effective parameters for cultivation via the Response Surface Methodology (RSM) based on mathematical and statistical techniques to determine the effective factors and their interactions [23]. RSM determines the optimum levels of operational conditions or determines an area that satisfies the operating specifications as its substantial purpose [24]. In this regard, two main strategies including SSF using soybean meal as a sustainable low-cost substrate and optimization of the process parameters were performed to enhance biosurfactant production. Central Composite Design (CCD) based Response Surface Methodology (RSM) was applied to analyze the effects of the parameters.

EXPERIMENTAL SECTION

Microorganism

Pseudomonas aeruginosa PTCC 1074 was obtained from Persian Type Culture Collection (PTCC). The strain was maintained on nutrient agar slants at 4 °C and subculture before use as inoculums for biosurfactant production. A loop of cells was then transferred into 50 mL LB broth in a 250 mL Erlenmeyer flask and incubated at 30 °C until the optical density reached 0.6 to 0.8 at 600 nm, indicating the growth of *Pseudomonas aeruginosa* achieved the mid-exponential phase. This culture was then utilized as inoculum for SSF [25].

Substrate

Soybean meal was obtained from the local market, grinned, passed through the standard sieve (No.10) with mean particle size of 1.4_2 mm, and stored until further use.

Production of biosurfactant by SSF

Production of biosurfactant was conducted by growing cultures in 250 mL Erlenmeyer flasks containing 5 g of soybean meal and a salt solution. Various amounts of water (0 to 45 mL) were added to obtain desired moisture content. The salt solution consisted of (g/L): KH_2PO_4 3, K_2HPO_4 7, $MgSO_4.7H_2O$ 0.2, different amounts of $NaNO_3$, and 2% v/v glycerol. The flasks were then sterilized in an autoclave for 15 min at 121°C and after cooling to room temperature, 1 mL of inoculum of *Pseudomonas aeruginosa* was added to each flask. The inoculated flasks were incubated at various temperatures based on the designed experiment runs for 8 days [26].

Biosurfactant extraction

Acid precipitation followed by liquid–liquid extraction was used to extract biosurfactant. Each SSF flask received

Table 1: Previous reports of biosurfactant production by SSF using agro-industrial wastes.

Substrate	Microorganism	Biosurfactant type	References
Olive leaf residue and olive cake	*Bacillus subtilis* SPB1	Lipopeptides	[15]
Sunflower seed shell, grape wastes or potato peels	*Pleurotus djamor*	Complex structure of biosurfactant	[16]
Rapeseed cake	*Bacillus subtilis* KB1	Surfactin	[17]
Two-phase olive mill waste	*Trametes versicolor*	Biosurfactants	[18]
Winterization oil cake	*Starmerella bombicola* ATCC 22214	Sophorolipids	[19]
mixture of sugarcane bagasse and sunfower seed meal	*Pseudomonas aeruginosa*	Rhamnolipid	[20]
wheat bran and sugar cane molasses	*Aspergillus niger*	Biosurfactants	[21]
Date Molasses	*Bacillus subtilis*	Biosurfactant	[22]

50 mL of distilled water and contents were agitated for 1 h at 200 rpm at 30 °C. The suspension was filtered using cheesecloth and the excess liquid was squeezed out. This procedure was carried out three times and the extract was then centrifuged for 15 min at 12,000x g. The pH of supernatants was adjusted to approximately 2 with 2 N HCl and biosurfactants were extracted with chloroform-methanol (2:1, v/v). The separated solvent layer was removed and the upper aqueous phase was re-extracted as before. The extract was concentrated in a vacuum evaporator [27].

Quantification of rhamnolipids

Rhamnolipid biosurfactants were quantified by measuring the concentration of rhamnose using the orcinol method and also by measuring the emulsification index (% EI_{24}) [28-29]. The rhamnose moiety constitutes only part of the rhamnolipid molecules, therefor rhamnolipid concentration is obtained by multiplying the rhamnose content by a correction factor of 3.2. To measure % EI_{24}, 2 mL of cell-free supernatant was added to 2 mL of kerosene, and the mixture was oscillated by vortex for 2 min. After 24 h, % EI_{24} was calculated using following equation [29]:

$$\%EI_{24} = \frac{\text{Height of emulsified layer}}{\text{Height of total liquid (sum of aqueous, kerosene and emulsified layer)}} \times 100 \quad (1)$$

Experimental design for optimization of the biosurfactant production

Rhamnolipid production was optimized by RSM using a CCD design with three independent factors, including initial moisture content, temperature, and Carbon-to-Nitrogen ratio (C/N ratio). C/N ratio was considered as the ratio of the mass of soybean meal to the mass of $NaNO_3$ in the medium. The independent variables were studied at five coded levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) (Table 2) and the experimental plan was carried out by 20 experiments as shown in Table 3. All the experiments were carried out three times and the average concentration of rhamnolipid and % EI_{24} were considered as the responses (Y_1 and Y_2 respectively). Analysis of variance (ANOVA) was used to acquire a practical model that demonstrates the relation between independent variables and response. A second-order polynomial equation for a three-factor system can be presented as follows:

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j \quad (2)$$

Where Y is the predicted response, β_0 is the offset term, β_i, β_{ii}, β_{ij} are respectively the linear coefficients, the quadratic coefficients, and the interaction coefficients and x_i, x_i^2, and $x_i x_j$ are levels of the independent variables [27]. The "Design Expert 7.0" software was applied to the analysis of the experimental data and to obtain the response surface curves for optimization of variables.

RESULTS AND DISCUSSION

Optimization of the process parameters for biosurfactant production

Table 3 shows the responses' actual values gained in the experiments and the model's predicted values. Regression analysis with RSM resulted in the following quadratic models as a function of initial moisture (x_1), temperature (x_2) and C/N ratio (x_3) in terms of actual factors:

Table 2: Experimental range and levels of independent variable.

Independent variables	Symbols	Range and level				
		-α	-1	0	+1	+α
Initial moisture (%)	X_1	49	58	70	82	91
Temperature (°C)	X_2	23	27	32	37	40
C/N ratio	X_3	32.9	50	75	100	117

Table 3: Central composite design (CCD) matrix of independent variables and their corresponding experimental and predicted amounts of biosurfactant production.

Run no.	Independent variables			Responses	
	x_1	x_2	x_3	Rhamnolipid (g/kg of substrate)	Emulsification Index (% EI_{24})
1	70	32	75	13.66	53
2	82	27	50	13.75	41
3	82	27	100	13.33	36
4	49.82	32	75	15.87	53
5	70	32	117	13.45	47
6	70	32	75	14.75	50
7	82	37	100	15.75	52
8	90.18	32	75	20	59
9	70	23.59	75	9.6	21
10	70	32	75	14.91	51
11	70	32	75	14.12	48
12	70	32	75	14.29	53
13	58	27	100	15	37
14	70	32	75	13.5	52
15	70	40.41	75	12.21	25
16	82	37	50	19.67	55
17	58	27	50	12.58	38
18	58	37	50	12	33
19	70	32	32.96	15.33	51
20	58	37	100	12.58	32

$$y_1 = 23.38152 - 1.68482x_1 + 1.92646x_2 + 0.36064x_3 + 0.023625x_1x_2 - 0.003058x_1x_3 - 0.00534x_2x_3 + 0.0090268x_1^2 - 0.04742x_2^2 + 0.000074x_3^2 \quad (3)$$

$$y_2 = -166.2322 - 3.7668x_1 + 20.6635x_2 + 0.0833x_1x_2 + 0.0102x_1^2 - 0.4078x_2^2 \quad (4)$$

F-test (ANOVA) was used to check the statistical significance of driven models, the data of which are listed in Table 4. The model F values of 46.58 and 29.45

Table 4: ANOVA for the response surface quadratic model.

Source of variation	Sum of square		*df*		Mean square		F		P-value		FI
	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	
Model	106.19	2107.04	9	9	11.8	234.12	46.58	29.45	<0.0001	<0.0001	Significant
X_1	21.88	*214.24*	1	1	21.88	214.24	86.36	26.95	<0.0001	0.0002	
X_2	6.93	*52.31*	1	1	6.93	52.31	27.36	6.58	0.0004	0.0285	
X_3	1.48	*20.49*	1	1	1.48	20.49	5.86	2.58	0.0360	0.1395	
X_1X_2	16.07	*200.00*	1	1	16.07	200	63.45	25.15	<0.0001	0.0003	
X_1X3	6.73	*4.50*	1	1	6.73	*4.50*	26.58	0.57	0.0004	0.4692	
X_2X3	3.56	*0.50*	1	1	3.56	*0.50*	14.07	0.063	0.0038	0.8071	
X_1^2	24.35	26.67	1	1	24.35	26.67	96.10	3.35	<0.0001	0.0791	
X_2^2	20.26	*1530.92*	1	1	20.26	*1530.92*	79.98	192.55	<0.0001	<0.0001	
X_3^2	0.031	*17.90*	1	1	0.031	*17.90*	0.12	2.25	0.7335	0.1644	
Residual	2.53	79.51	10	10	0.25	7.95					
Lack of Fit	0.93	*60.67*	5	5	0.19	12.13	0.58	3.22	0.7172	0.1148	Not Significant
Pure Error	1.6	*18.83*	5	5	0.32	3.77					
Cor Total	108.73	2186.55	19	19							

Y_1 : R-square = 0.9846; Adj. R-square = 0.9703; Predicted R-square = 0.9272; Adeq Precision= 29.10
Y_2: R-square = 0.9438; Adj. R-square = 0.9237; Predicted R-square = 0.8228; Adeq Precision= 26.46

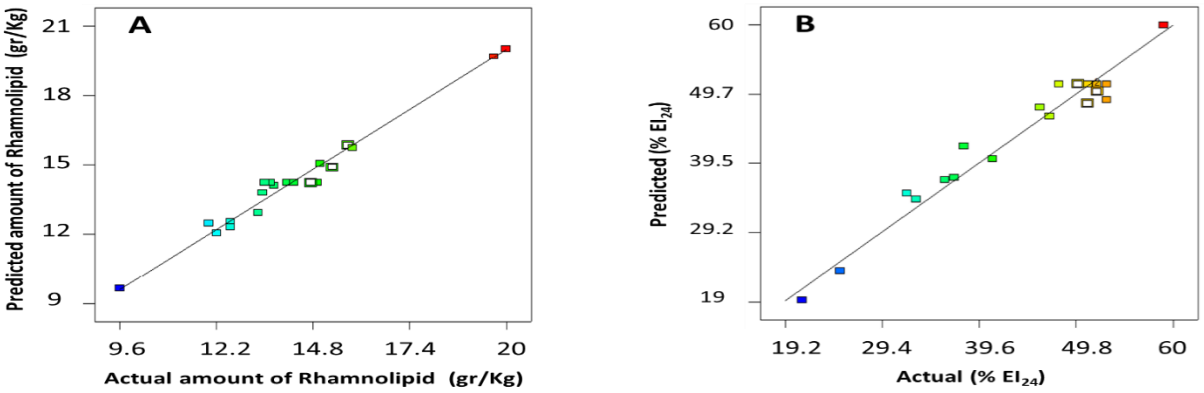


Fig. 1: Relation between actual and predicted values of the amount of rhamnolipids (A) and % EI_{24} (B).

indicated that the models were significant, and there was only a 0.01% probability that this level of fit could occur due to noise. The terms x_1, x_2, x_3, x_1x_2, x_1x_3, x_2x_3, x_1^2, and x_2^2 were statistically significant as their P values are lower than 0.05. The model terms are considered insignificant when their P values are greater than 0.1 [30, 31]. The R^2-value is always between 0 and 1 and the fitness of the models is confirmed with R^2-values >0.8. In the current study, the coefficient of determination (R^2), adjusted R^2, and predicted R^2 were respectively calculated as 0.9767, 0.9557, and 0.9088 for Y_1 and 0.9636, 0.9309and 0.7766 for Y_2. The R^2-values indicate that the models fitted the experimental data well. This fact was also confirmed from the predicted versus actual values plot for the number of rhamnolipids and % EI_{24} in Fig. 1. The normal probability plot of the studentized residuals, illustrated in Fig. 2, is an additional tool to check the appropriateness of the model. The residuals followed the normal distribution

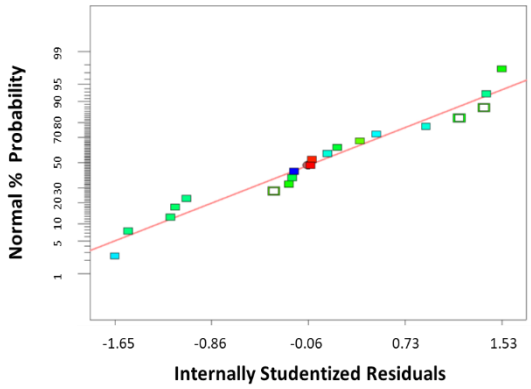


Fig. 2: Normal probability plot of studentized residuals for the quadratic model.

and the assumption of normality was valid. "Adeq Precision" measures the signal (response) to noise (deviation) ratio. A ratio greater than 4 is desirable [28]. The ratio of 29.10 and 21.54 indicate adequate signals and therefore the models are significant for the process.

Effects of variables on the biosurfactant production

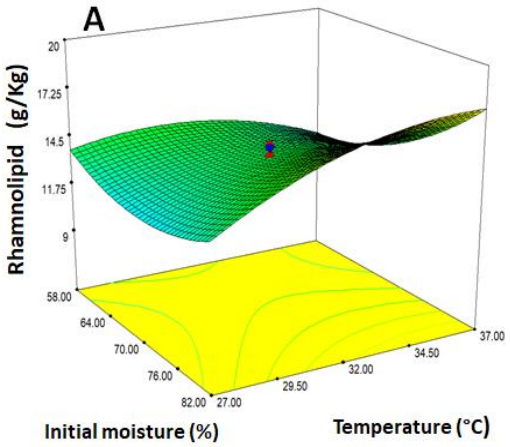
To determine the optimal levels of each variable for maximum production of Rhamnolipid, three-dimensional response surface curves were plotted, each of which was constructed by illustrating the rhamnolipid production (Y_1 and Y_2) on the Z-axis against any two independent variables, while the other one maintained at its respective zero level [32]. Fig. 3A and Fig. 4A show the initial moisture content of the substrate and temperature effects on rhamnolipid production. Fig. 3B and Fig. 4B demonstrate the interaction effects of the initial moisture content and C/N ratio on the rhamnolipid production and the interaction between temperature and C/N ratio is represented in Fig. 3C and Fig. 4C. The results of ANOVA and response surface curves indicated that the initial moisture content of the substrate and temperature significantly affect the production of biosurfactant. The process temperature is one of the considerable and determinant factors, has to be controlled in bioprocesses. This parameter varies from organism to organism and affects the type and amount of produced biosurfactant [33, 34]. As shown in Fig. 3 and Fig. 4, the increase of temperature from 25 to 34°C led to an increase of rhamnolipid production, which implies the significant influence of temperature. Not only does the temperature increment augment the biochemical reaction rate in microorganism cells but also, but it also improves microbial metabolism within limits [35]. The results demonstrated that the production of biosurfactants increased with increasing the initial moisture content at high temperatures and also at low C/N ratios. This dependent variable is almost kept constant at low temperatures. It might have stemmed from the fact that substrate nutrient solubility reduces at low moisture content [36]. Carbon and nitrogen are the most important nutrient requirements in the composition of the medium that regulate and improve the metabolic activities of microorganisms during the fermentation process [37, 38]. The results (Fig. 3B and 3C) demonstrated that at low levels of moisture and temperature, the rhamnolipid production slightly increased with increasing the C/N ratio while at high levels of moisture and temperature, the opposite was achieved.

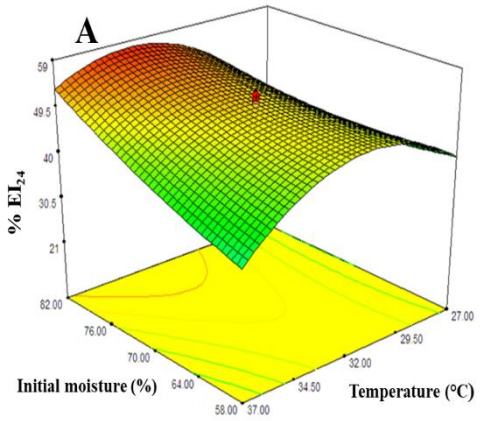
Validation of the model

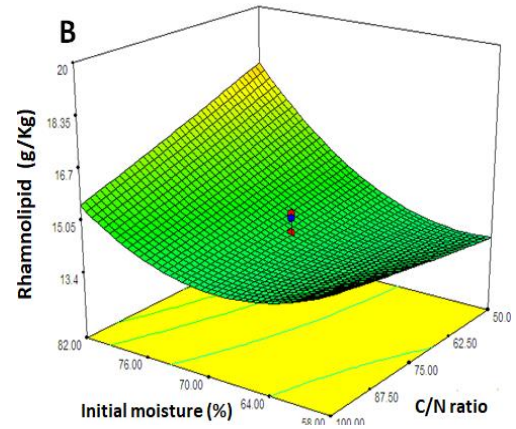
The model predicted that the optimal values of variables were the temperature of 33 °C, Initial substrate moisture of 80%, and C/N ratio of 54, which were obtained by solving the regression equation. To evaluate the accuracy of the model, additional experiments were performed under optimal conditions. The observed value of 17.05 g/kg dry substrate and experimental emulsification index of 54 % were in good agreement with the models' predicted value (17.21 g/kg dry substrate and 56% respectively).

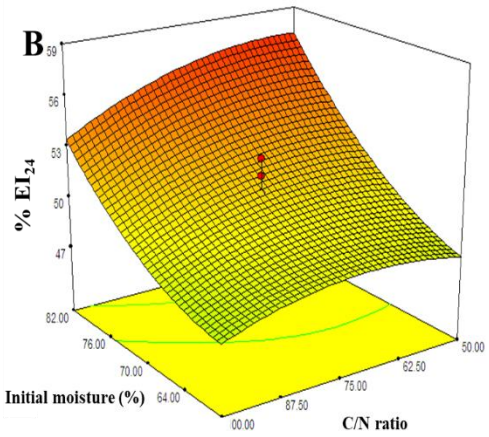
CONCLUSIONS

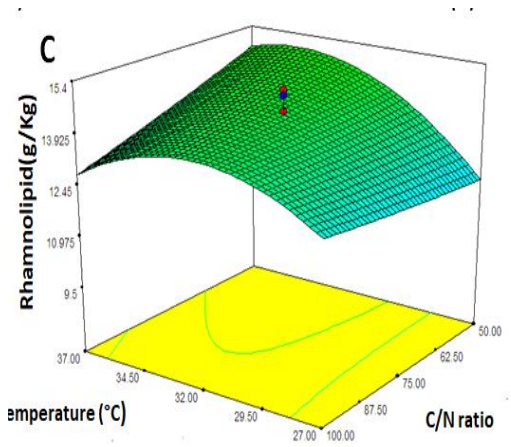
The results showed the potential use of soybean meal as the renewable substrate for rhamnolipid production by *Pseudomonas aeruginosa* (PTCC 1047) under SSF. In order to investigate the effects of initial moisture of the substrate, temperature, and C/N ratio on biosurfactant production, a Central Composite Design (CCD) was employed. In optimum conditions (temperature 33 ºC, Initial substrate moisture content 80%, and C/N ratio 54), the experimental value of biosurfactant production and Emulsification Index were 17.05 g/kg dry substrate and 54 % respectively. Validation experiments confirmed the adequacy and accuracy of the quadratic model, and the results showed that the predicted values had good agreement with the experimental data.











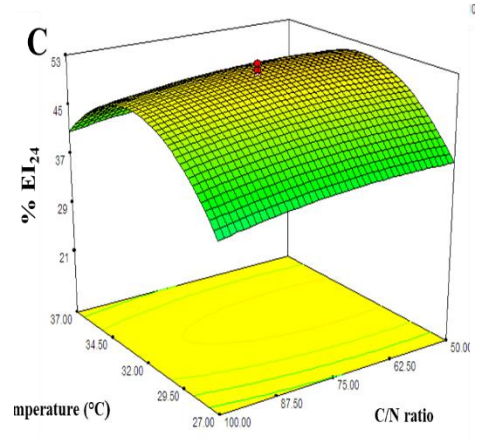


Fig. 3: Rhamnolipid production (g/kg of substrate) as a function of (A) initial moisture content and temperature), (B) initial moisture content and C/N ratio and (C) temperature and C/N ratio.

Fig. 4: % EI_{24} as a function of (A) initial moisture content and temperature), (B) initial moisture content and C/N ratio and (C) temperature and C/N ratio

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