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Research article

TAGUCHI DESIGN OF EXPERIMENTS FOR THE OPTIMIZATION OF LIPASE PRODUCTION BY *EMERICELLA NIDULANS* DAOM 222012 ISOLATED FROM PALM OIL MILL EFFLUENT (POME) DUMP SITES

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ABSTRACT: Lipases with their multifarious applications in a wide variety of fields are gaining the attention of industrial biotechnologists thus necessitating the need for screening new isolates with potential industrial applications. In the present study Taguchi DOE was employed to optimize the process parameters for lipase production by *Emericella nidulans* DAOM 222012, screened and isolated from Palm Oil Mill Effluent (POME) dump sites. This method allows the simultaneous study of several cultural factors while considering the mutual interaction among them and enhances the production within few experimental runs thereby greatly reducing the cost and time for process optimization. Four factors Viz., pH, carbon, nitrogen and surfactants each at 5 levels were considered and an orthogonal layout of L_{25} (5^4) was performed. The results indicated that Maltose (1%), Yeast extract (1%), Tween 80 (0.5%) and pH 6 are the significant factors for lipase production by *Emericella nidulans* DAOM 222012 using submerged fermentation studies.

Key Words: *Emericella nidulans* DAOM 222012, Lipase, POME, Process optimization, Taguchi experimental design.

INTRODUCTION

Lipases are ubiquitous and are widely distributed in nature from prokaryotes to eukaryotes (Saxena *et al.,* 2003; Salihu *et al.,* 2012). They are serine hydrolases (triacyl glycerol acyl hydrolases, EC 3.1.1.3) and catalyze the reversible hydrolysis of fats and oils at oil and water interface (Reetz, 2002). Among different sources, microbial lipases are more advantageous because of their rapid growth on inexpensive media, high yield, greater stability, uninterrupted supply due to absence of seasonal fluctuations and stability in organic solvents etc (Shu *et al*., 2010). The ability of these enzymes to catalyze the reactions in both aqueous and non aqueous media facilitate these wonderful biocatalysts to use for various industrial applications (Hasan *et al*., 2006) like detergent industries, cosmetic industries, food industries as butter substitutes, for the modification of fats, synthesis of cocoa, as flavour enhancers (Mahapatra *et al*., 2009a; Kumari *et al*., 2009b; Dheeman *et al*., 2011), as biofuels (Kumari *et al*., 2009a; Lee *et al*., 2011), in pharmaceutical industries for the resolution of chiral drugs (Barbosa *et al*., 2011) etc. Owing to their immense demand to use in a wide variety of fields, lipases are attracting industrial biotechnologists there by necessitating the need for isolation of novel lipases from new sources.

Optimization of fermentation medium is critical to enhance production yields and this is usually done by employing submerged fermentation studies (Li and Zong, 2010). Optimization of bio process variables involves optimizing chemical parameters like carbon, nitrogen, surfactants and as well physical parameters like pH, temperature, agitation etc. Carbon sources, nitrogen sources and surfactants were reported to be main factors for enhancing lipase production by Guerzoni *et al*., (2001). Apart from these cultural factors, there were also reports that the presence of various lipidic substrates such as oils, presence of certain fattyacids, Tweens etc also enhances lipase production (Gupta *et al*., 2004).

Optimization of process parameters can be done by using traditional OFAT (One Factor at a Time) method and as well with various statistical experimental design methods. Statistical methods have become best alternatives to conventional methods in enhancing production yields in a few trials (Gupta *et al*., 2004b). The traditional OFAT approach is laborious and time consuming and as well overlooks the interaction among independent factors (Vishwanatha *et al*., 2010).

By using statistical Design of Experiments (DOE), optimization of bioprocess variables can be done in a systematic way with efficient plan. Statistical DOE allows the simultaneous study of many control factors while considering mutual interactions among these cultural factors which was lacking in the traditional OFAT methods (Rao *et al*., 2001; Abdel-Fattah *et al*., 2005). Of all the various statistical methods, Taguchi DOE, offers additional advantages like many factors can be considered simultaneously and much of the information can be obtained with a few experimental trials (Houng *et al.,* 2006). In addition, we can also identify the crucial factors that mainly effect production yields with only few runs (Dasu *et al*., 2003). The success of Taguchi DOE mainly involves exploitation of Orthogonal Arrays (OA) to minimize experimental errors and for enhancing production yields (Krishna Prasad *et al*., 2005). Sabbaghian and roostaazad (2005) employed Taguchi method for optimizing the lipase production by *Pseudomonas aeroginosa* B-3556.

In the present study, Taguchi DOE was used to identify the contribution of the tested cultural factors in lipase production by *Emericella nidulans* DAOM 222012, a strain that was screened and isolated from POME dumpsites of Pedavegi palm oil mill, West Godavari Dist., A.P. India.

MATERIALS AND METHODS

Microorganism, culture condition and production medium

The fungal culture used in the present study, *Emericella nidulans* was screened and isolated from Palm Oil Mill Effluent (POME) dump sites, Pedavegi, West Godavari District. Culture was maintained on the 4% Potato dextrose agar (PDA) slants throughout the study period. Lipase production medium containing (g/l): KNO_3, 2.5; KH_2PO_4, 1.0; $MgSO_4$, 0.5; Olive oil, 15 ml was prepared for the production of lipase from *Emericella nidulans* DAOM 222012. The pH of the medium was adjusted to 5.6 using citrate phosphate buffer. The fermentation was carried out at 30 ℃ for a period of 4 days using shake flasks at 150 rpm.

Production of lipase

45 ml of Lipase production medium was inoculated with 3% inoculum and the flasks were incubated at 30°C for 96 hours. The crude enzyme was then obtained by filtering the culture broth following centrifugation at 10000×g for 10 minutes. The crude enzyme extract was assayed for lipase activity.

Extracellular lipase assay

Yield of p-nitro phenol was used to measure lipase activity with p-nitrophenylpalmitate (pNPP) (Sigma, USA) as the substrate (Maia *et al*., 2001). The assay mixture consisted of 100 µl of sample and 900 µl of substrate solution containing 10 mg of pNPP dissolved in 1 ml of propan-2-ol diluted in 9 ml of 50 mM Tris–HCl pH 7.0 containing 40 mg of Triton X-100 and 10 mg of gum arabic. The assay mixture was incubated at 30°C for 30 min and the p-nitrophenol released was measured at 410 nm.

One unit of activity was defined as the amount of enzyme that liberated 1 µ mol of p-nitrophenol per min under the assay conditions.

Optimization of process parameters by Taguchi DOE

Taguchi DOE methodology

For Taguchi DOE, 4 factors viz., pH, Carbon sources, nitrogen sources and surfactants each at five levels were considered (Table 1). The levels of the factors studied included 1% each of carbon sources such as Fructose, Galactose, Maltose, Glucose and Sucrose; 1% each of nitrogen sources such as Yeast Extract, Peptone, Malt extract, Ammonium Sulfate and Ammonium Chloride; 5,6,7,8 and 9 as initial pH's and 0.5% each of Gum Arabic, SDS, Triton x 100, Tween 20 and Tween 80 as surfactants.

An Orthogonal Array (OA) layout of L_{25} (5^4) with 24 degrees of freedom was performed to examine the influence of these 4 factors in enhancing lipase yield. The L represents Latin square and the subscript (25) represents the number of experimental runs. Table 2 shows the L_{25} (5^4) orthogonal layout. Table 3 represents the matrix layout of L_{25} (5^4) Taguchi orthogonal array design with the levels of the factors that are involved in each of the 25 experimental runs. Each of the twenty-five simultaneous SmF (Submerged fermentation) experiments/ runs was carried out as per the defined values of 4 different parameters in five levels. All the SmF experiments were carried out in 45 mL of fermentation media (LPM 3) containing 1.5% (v/v) olive oil in 250 mL flasks at 150 rpm for 96 hrs at 30°C.

Analysis of the Taguchi Orthogonal Array Experiments (Runs)

Analysis of the Taguchi experimental runs was done by calculating ***S/N*** ratio (**Signal**-to-Noise ratio). For each experiment or run**,** S/N ratio was calculated which served as objective function for optimization and it is the logarithmic function of desired output. The larger the ***S/N*** ratio the better will be the outcome. *S*/*N* ratio was calculated using the formula

S/N = 10 * Log (1/n sum ($1/y^2$)). Where "*y*" is the signal (Lipase activity value) and "*n*" is the number of repetitions in each experiment.

In the present study, *S*/*N* ratios of 25 runs were calculated and analyzed to know the main effects of the factors independently. ANOVA (analysis of variance) technique was then employed to determine the contributing factors. The optimal conditions were determined accordingly by combining the levels of factors that had the highest main effect value.

RESULTS OF TAGUCHI DOE

In the present study of optimization of process variables using Taguchi DOE, the effect of 4 factors Viz., pH, Carbon sources, Nitrogen sources and Surfactants was evaluated in 25 experimental runs. From the results it was inferred that the lipase production is ranging from 33.8 U/ml to 498 U/ml (Table 3) corresponding to the combined effect of the 4 factors. The lowest production (33.8 U/ml) was obtained in run 5 with 1% sucrose as carbon source; 1% ammonium chloride as nitrogen source; 0.5% gum arabic as surfactant at pH 5 and the highest production (498 U/ml) was obtained with 1% maltose as carbon source; 1% yeast extract as nitrogen source; 0.5% tween 20 as surfactant at pH 8 in run 18 (Table 3 and Fig. 1). A graph of means of *S/N* ratio Vs Levels (Fig. 2) depicts that the significant contribution is in the order of Carbon sources ? Surfactants ? pH ? Nitrogen sources and among them the best fermentative process parameters were found to be level 2 for pH (pH 6), level 3 for carbon source (1% maltose), level 1 for nitrogen source (1% yeast extract) and level 3 for surfactants (0.5% tween 80).

The Analysis of variance (ANOVA) for the responses in terms of lipase production was carried out as per the contribution of factors by the Taguchi method. ANOVA is used in Taguchi method for the analysis of results obtained by orthogonal array experiments and by this approach one can determine the % contribution of variation by each factor and from this data we can predict the levels of factors that produce the best results. ANOVA was performed for the determination of best parameters on lipase production and the results are shown in Tables 4. From the calculated F ratio in ANOVA table, it can be inferred that the factors considered in the Taguchi DOE are statistically significant at 95% confidence limit. The ANOVA of lipase production has the model F ratio of 25.12 (Prob>F =0.0005) which implies that the model is significant. From the ANOVA table it is clear that carbon sources are the most significant factors for enzyme production and the contribution of nitrogen sources is least. Figure 3 represents the contribution of four factors on lipase production by *Emericella nidulans* DAOM 222012 in submerged fermentation using Taguchi experimental design and the contribution of pH, Carbon sources, Nitrogen sources and Surfactants is 18.3%, 28%, 12.6% and 26.6% respectively. From the figure it is clearly evident that contribution of carbon sources is highest among the 4 factors that were studied and where as the contribution of nitrogen sources towards the lipase production is least.

Validation of Taguchi experimental design was done by comparing the enzyme yields with experimental optimum factors and taguchi optimum factors and from the Table 5 it was clearly evident that the enzyme production for Taguchi optimum factors (512 U/ml) was more than the experimental optimum factors (498 U/ml) and this best validates the Taguchi DOE.

Table 1: Factors and their levels used in the Taguchi's experimental design for extracellular lipase production by *Emericella nidulans* DAOM 222012

Factors	Level 1	Level 2	Level 3	Level 4	Level 5
pH (A)	5	6	7	8	9
Carbon (B)	Fructose	Galactose	Maltose	Glucose	Sucrose
Nitrogen (C)	Yeast Extract	Peptone	Malt extract	Ammonium sulphate	Ammonium chloride
Surfactants (D)	Triton X 100	SDS	Tween 80	Tween 20	Gum Arabic

Four factors (pH, Carbon sources, Nitrogen sources and Surfactants) and five levels (Level 1 to Level 5) selected for optimization of process variables for extracellular lipase production by *Emericella nidulans* DAOM 222012 using Taguchi's Experimental design.

Table 2: L $_{25}(5^4)$ orthogonal array of Taguchi experimental design

Experiments or Runs	A (pH)	B (Carbon sources 1%)	C (Nitrogen sources 1%)	D (Surfactants 0.5%)
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	1	4	4	4
5	1	5	5	5
6	2	1	2	3
7	2	2	3	4
8	2	3	4	5
9	2	4	5	1
10	2	5	1	2
11	3	1	3	5
12	3	2	4	1
13	3	3	5	2
14	3	4	1	3
15	3	5	2	4
16	4	1	4	2
17	4	2	5	3
18	4	3	1	4
19	4	4	2	5
20	4	5	3	1
21	5	1	5	4
22	5	2	1	5
23	5	3	2	1
24	5	4	3	2
25	5	5	4	3

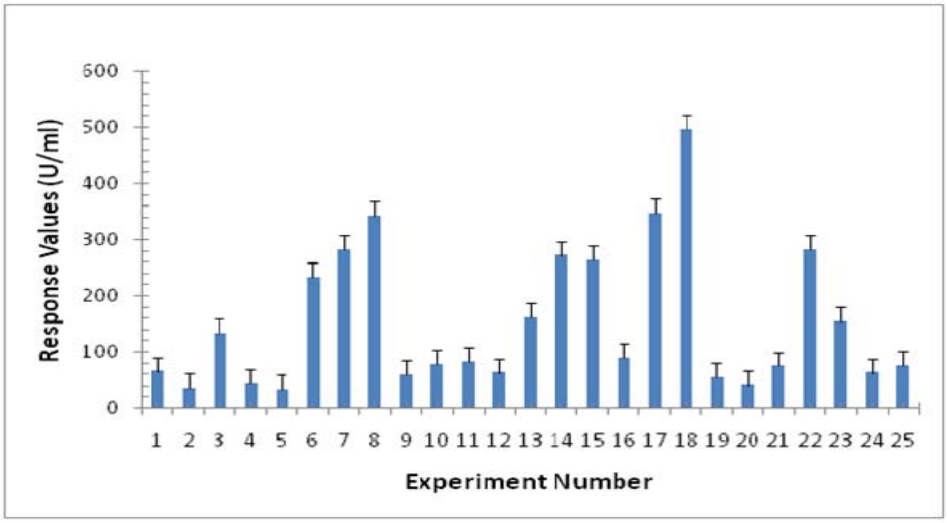

Figure 1: Comparative results of response in terms of Enzyme activity (U/ml) Vs Run/ Experiment No of Taguchi L25 orthogonal array of experiments.

Figure 1 depicts the response values in terms of Enzyme activity in 25 different experimental runs using Taguchi design of experiments

Table 3: Matrix layout of the L $_{25}(5^4)$ Taguchi orthogonal array design with response values

Experiment or Runs	pH	Carbon Sources (1%)	Nitrogen Sources (1%)	Surfactants (0.5%)	Response values (U/ml)	S/N ratio
1	5	Fructose	Yeast extract	Triton X 100	64.58	31.38
2	5	Galactose	Peptone	SDS	36	25.81
3	5	Maltose	Malt extract	Tween 80	134	37.71
4	5	Glucose	Ammonium Sulphate	Tween 20	33.8	25.71
5	5	sucrose	Ammonium Chloride	Gum Arabic	43.8	27.57
6	6	Fructose	Peptone	Tween 80	234	42.57
7	6	Galactose	Malt extract	Tween 20	282	44.23
8	6	Maltose	Ammonium Sulphate	Gum Arabic	344	45.94
9	6	Glucose	Ammonium Chloride	Triton X 100	58.4	30.50
10	6	sucrose	Yeast extract	SDS	77.8	33.04
11	7	Fructose	Malt extract	Gum Arabic	81.61	33.41
12	7	Galactose	Ammonium Sulphate	Triton X 100	62	31.04
13	7	Maltose	Ammonium Chloride	SDS	161.7	39.40
14	7	Glucose	Yeast extract	Tween 80	272	43.90
15	7	sucrose	Peptone	Tween 20	263.86	43.65
16	8	Fructose	Ammonium Sulphate	SDS	89.8	34.28
17	8	Galactose	Ammonium Chloride	Tween 80	349	46.09
18	8	Maltose	Yeast extract	Tween 20	498	49.17
19	8	Glucose	Peptone	Gum Arabic	54.2	29.80
20	8	sucrose	Malt extract	Triton X 100	41.2	27.28
21	9	Fructose	Ammonium Chloride	Tween 20	74.2	32.54
22	9	Galactose	Yeast extract	Gum Arabic	282	44.23
23	9	Maltose	Peptone	Triton X 100	153.66	38.90
24	9	Glucose	Malt extract	SDS	62	30.55
25	9	sucrose	Ammonium Sulphate	Tween 80	84.2	33.61

Matrix layout of the L $_{25}(5^4)$ Taguchi orthogonal array design showing factors, levels and corresponding enzyme activity values in terms of response values and S/N ratio.

* The values correspond to the maximum Lipase production values that are mean ± SE (n = 3).

Table 4: ANOVA Table

Source	DF	Sum of squares	Mean Square	F-ratio	P Value (%)	Prob>F	
Model	12	2086860.9	173905.1	25.12		0.0005*	Significant
pH (A)	4	69920.8	17480.2	2.52	18.2526	0.1238*	
Carbon (B)	4	107267.1	26816.8	3.87	28.0018	0.049*	
Nitrogen (C)	4	48430.2	12107.6	1.75	12.6426	0.2319	
Surfactants (D)	4	102068.4	25517.1	3.69	26.6447	0.0548*	
Error	8	55386.2	6923.3		14.4584		
Total	24	383072.8	15961.4		100.0000		

*Significant term

ANOVA table showing the statistical significance of the process parameters Viz., pH, Carbon sources, Nitrogen sources and Surfactants in enhancing lipase production by *E. nidulans* DAOM 222012.

Table 5: Validation of Taguchi experimental data values.

Factors	Factors for Experimental optimum	Factors for Taguchi optimum
pH	8	6
Carbon Source (1%)	Maltose (1%)	Maltose (1%)
Nitrogen Source (1%)	Yeast Extract (1%)	Yeast Extract (1%)
Surfactants (0.5%)	Tween 20 (0.5%)	Tween 80 (0.5%)
S/N ratio	53.94	54.19
Enzyme production (U/ml)	498 U/ml	512 U/ml

Table showing the validation of Taguchi model by performing SmF trial employing Taguchi optimized fermentation process parameters and experimental optimum factors in enhancing the lipase production by *E. nidulans* DAOM 222012 in 45 ml of the fermentation medium.

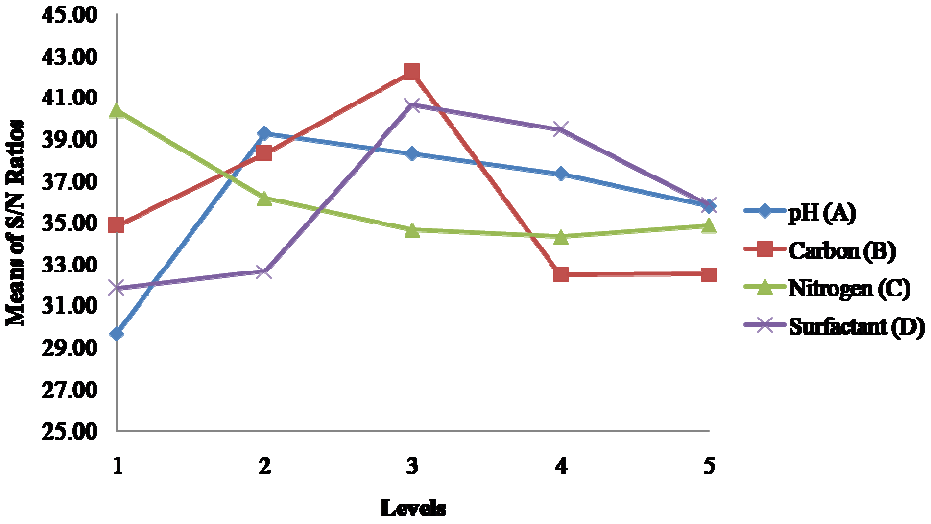

Figure 2: Means of S/N ratios Vs levels for Process parameters on Lipase production

Plot of means of *S/N* ratios Vs levels for different process parameters Viz., pH (A), Carbon sources (B), Nitrogen sources (C) and Surfactants (D) on lipase production by *E. nidulans* DAOM 222012. Larger the means of the *S/N* ratio the better will be the enzyme production at that particular level.

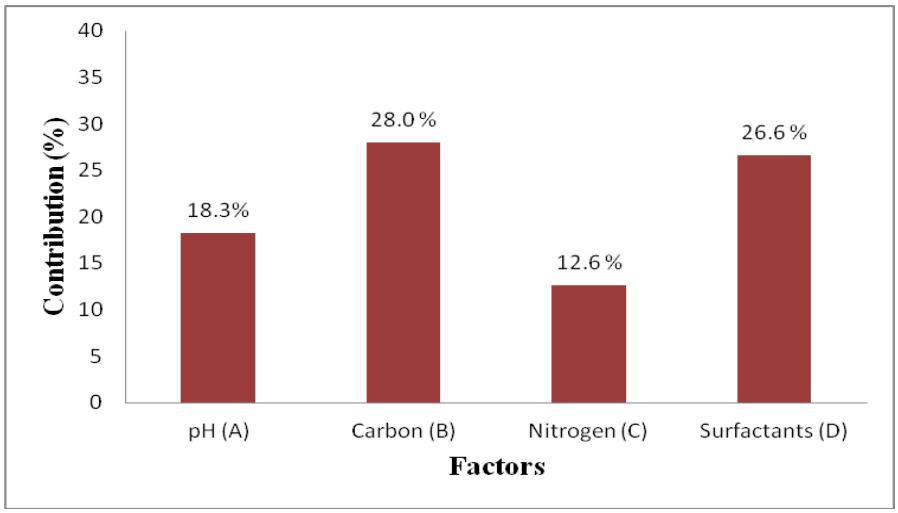

Figure 3: A plot of Factors Vs Contribution % in lipase production

Graph showing the contribution of four factors Viz., pH(A), Carbon sources (B), Nitrogen sources (C) and Surfactants (D) on lipase production by *Emericella nidulans* DAOM 222012 in submerged fermentation using Taguchi experimental design.

DISCUSSION

Optimization of process parameters mainly involves the use of a good reliable statistical method to achieve higher yields (Ghaley *et al.,* 2005). Among various statistical experimental design methods, Taguchi DOE is the most reliable statistical method and offers enhanced yields in few experimental trials considering the interactive effects among the process variables. This drastically reduces both the time required to achieve optimum yields and as well cost of production. Statistical DOE have been employed for increasing production yields in various studies (Kunamneni *et al*., 2005). Taguchi experimental design was successfully employed by Heravi *et al*., (2008) for lipase production by *Bacillus* F3 and observed an increased enzyme production. Teng and Xu (2008) also used Taguchi method for the initial optimization of lipase production using submerged fermentation by *Rhizopus chinensis*. The optimization of cell bound lipase production by *Rhodotorula mucilagenosa* (Nuylert and Hongpattarakere, 2012) was also done by Taguchi experimental design, using orthogonal array (OA) L9 to examine the influence of four factors Viz., carbon sources (A), nitrogen sources (B), initial pH (C) and surfactants (D).

Taguchi method mainly allows the interactions between various factors and evaluates their influence on maximal enzyme production and therefore by this approach we can identify the levels of the factors that produce high yields (Chang *et al*., 2006).

In the present study of optimization of process variables for lipase production by *Emericella nidulans* DAOM 222012 in submerged fermentation, the best parameters comprises level 2 for pH (pH 6), level 3 for carbon sources (1% maltose), level 1 for nitrogen sources (1% yeast extract) and level 3 for surfactants (0.5% tween 80). The lipase production for taguchi optimized parameters was found to be 512 U/ml.

The contribution of 4 factors in lipase production by Taguchi experimental design showed that carbon sources played a leading role than other selected parameters (Carbon sources 28%, Surfactants 26.64%, pH 18.25%, and Nitrogen sources 12.64%).

CONCLUSIONS

Taguchi DOE was applied to test the significance and as well the contribution of the selected cultural parameters on lipase production by *Emericella nidulans* DAOM 222012 employing L_{25} (5^4) orthogonal array and the significant contribution of factors were found to be in the order of Carbon sources Surfactants pH Nitrogen sources and among carbon sources, maltose (1%), pH (6), **tween 80** (0.5%) and **yeast** Extract (1%) were found to be best process parameters. At this optimum condition, the yield of lipase was found to be 512 U/mL.

Abbreviations: POME (Palm Oil Mill Effluent), DOE (Design of experiments)

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