بهینه‌سازی آماری تولید گلوکز اکسیداز نوترکیب توسط مخمر *یاررووییا لیپولیتیکا* با استفاده از روش سطح پاسخ

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چکیده

مقدمه: گلوکز اکسیداز به‌عنوان یک آنزیم تجاری در صنایع پزشکی، دارویی و غذایی مورد توجه است. بهینه‌سازی محیط و شرایط تخمیر برای افزایش تولید گلوکز اکسیداز هترولوگ مهم است. هدف از مطالعهٔ حاضر، بهینه‌سازی تولید گلوکز اکسیداز در یک سویهٔ نوترکیب مخمر *یاررووییا لیپولیتیکا* ازطریق روش‌های آماری بود.

مواد و روش‌ها: روش سطح پاسخ به‌عنوان یک روش آماری به‌منظور طراحی آزمایش برای بهینه‌سازی تولید گلوکز اکسیداز نوترکیب توسط مخمر *یاررووییا لیپولیتیکا* استفاده شد. سپس از شرایط بهینه‌شده برای تولید گلوکز اکسیداز در کشت بیوراکتور استفاده شد.

نتایج: تولید گلوکز اکسیداز پس از بهینه‌سازی محیط در کشت ارلن تا ۵۷۰ واحد در لیتر افزایش یافت که ۲ برابر بیشتر از محیط غیربهینه‌شده است. در کشت بیوراکتور با استفاده از محیط بهینه‌شدهٔ حاوی گلوکز و ساکارز به‌عنوان منابع کربن، تولید گلوکز اکسیداز پس از دو و شش روز به‌ترتیب به ۷۲۰ و ۷۳۰ واحد در لیتر رسید.

بحث و نتیجه‌گیری: نتایج این مطالعه، اطلاعات ارزشمندی دربارهٔ بهینه‌سازی آماری توسعهٔ فرایند زیستی برای تولید پروتئین هترولوگ در مخمر *یاررووییا لیپولیتیکا* ارائه می‌دهد.

واژه‌های کلیدی: *یاررووییا لیپولیتیکا*، گلوکز اکسیداز، بهینه‌سازی، روش سطح پاسخ، بیوراکتور

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Statistical optimization of recombinant glucose oxidase production by yeast *Yarrowia lipolytica* using response surface methodology

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Abstract

Introduction: Glucose oxidase (GOX) is considered a commercial enzyme in the medicine, pharmaceutical, and food industries. Optimization of the fermentation medium and conditions is important to increase heterologous GOX production. The current study aimed to optimize GOX production in a recombinant strain of *Yarrowia lipolytica* via statistical techniques.

Materials and Methods: Response surface methodology (RSM) as a statistical technique for the design of the experiment was used for the optimization of recombinant GOX production by *Y. lipolytica*. Then, the optimized conditions were used for the production of GOX in bioreactor cultivation.

Results: GOX production was increased up to 570 U/L after optimization of the medium in flask culture which is 2 times more than the non-optimized medium. In the bioreactor culture using optimized medium containing glucose and sucrose as carbon sources, GOX production was reached 720 and 730 U/L after two and six days, respectively.

Discussion and Conclusion: Results of the study provided valuable information about the statistical optimization of bioprocess development for the production of heterologous protein in *Y. lipolytica*.

Key words: *Yarrowia lipolytica*, Glucose oxidase, Optimization, Response surface methodology, Bioreactor

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Introduction

Glucose oxidase (EC 1.1.3.4) catalyzes the oxidation of D-glucose to D-glucono-δ-lactone and hydrogen peroxide (H_2O_2). Glucose oxidase (GOX) as an important commercial enzyme has many applications in the pharmaceutical, medicine, and food industries as well as biosensor design (1). GOX is produced by a few fungi, especially *Aspergillus niger* and *Penicillium amagasakiense* (2). Low production efficiency and high-cost purification because of co-production of amylase, catalase, and cellulase are two problems for industrial production of the enzyme by *A. niger* as a main microbial producer (1). Many efforts have been made to heterologous production of the enzyme in microbial hosts to overcome these obstacles (3). GOX was expressed in different yeasts including *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, and *Kluyveromyces marxianus*. Over-glycosylation and no cost-effective production medium are the main problems in the expression and production of the enzyme in the mentioned yeasts (4-7).

Non-conventional yeast *Yarrowia lipolytica* is a powerful eukaryotic host for heterologous protein production because of its effective secretion system, excellent expression vectors, and suitable transferring strains. It is approved as Generally Recognized As Safe (GRAS) by the food and drug administration (FDA) for human consumption (8,9). For the first time, GOX from *A. niger* was more recently expressed in *Y. lipolytica* (10,11).

Optimization of medium compositions and fermentation conditions are important for the cost-effective production of biotechnological products along with genetic engineering for the creation of new microbial strains and upgrade the desired product level (12) .Optimization of medium compounds has a significant impact on increasing the production of metabolites. Response Surface Methodology (RSM) is a statistical method for the optimization of the bioprocess. RSM could be used to design experiments to reduce the number of experiments and required time for carrying out the experiments as well as understanding the relationship between affecting factors in the bioprocess (13,14).

In the current study, RSM was used for the optimization of recombinant GOX production by *Y. lipolytica* as well as understanding the relationship between main factors affecting that process at the flask level. Then, the optimized medium was used in the bioreactor to scale up the production of GOX using glucose and sucrose as two different carbon sources.

Materials and Methods

Chemicals: 4-Aminoantipyrine 98% (4-AP) and peroxidase from horseradish Type VI (POD) were purchased from Sigma-Aldrich (USA). Phenol, glucose, yeast extract, tryptone, and other chemicals were purchased from Merck (Germany).

Yeast strain and culture conditions: The recombinant *Y. lipolytica* Po1h-GOX was used in this study which was engineered in Darvishi's research group (10). This strain is engineered host strain Po1h (*MatA*, *ura3-302*, *xpr2-322*, *axp1-2*, *Ura*$^-$, ΔAEP, ΔAXP, Suc$^+$ /no Ylt1, no extracellular protease) which can produce recombinant GOX of *A. niger* ATCC 9202 (10).

YPD medium (2% peptone, 2% glucose, 1% yeast extract) was used for the production of recombinant GOX. The yeast strain was grown on YPD agar at 29 °C and stored at 4 °C (15,16).

A colony of *Y. lipolytica* Po1h-GOX was transferred to 20 ml YPD broth as preculture and incubated at 29 °C, 200 rpm for 24 h. The preculture was used to inoculate 50 ml YPD broth as a production medium and incubated at 29 °C, 200 rpm (10).

Design of Experiments: The software Design Expert version 7.0.0 (Stat-Ease, Inc., Minneapolis, USA) was employed for the Central Composite Design (CCD) type of RSM for optimization of four variables, glucose, yeast extract, peptone, and pH of YPD medium (Table 1). The software recommended 30 different compositions of variables to do the experiments (Table 2) (17,18).

Table 1- Real and coded values of four variables of YPD medium for the design of experiment by RSM

Variables	Sign	Low level (−1)	High level (+1)
Glucose (g/L)	A	25	35
Yeast Extract (g/L)	B	10	20
Peptone (g/L)	C	10	20
pH	D	6	8

Bioreactor Cultivation: The *Y. lipolytica* Po1h-GOX was cultivated in a 5-L bioreactor (FS-01-A with double jacketed vessel, Winpact, Taiwan) equipped with two RDT6 Rushton turbines and a built-in digital controller for pH, temperature, agitation, and dissolved oxygen (DO) and with peristaltic pumps for adding acid, base, antifoam and nutrients. The set point for pH and DO concentration was controlled by online monitoring using a pH sensor (Mettler-Toledo InPro3030/325, Urdorf, Switzerland) and a DO sensor (Mettler-Toledo InPro6800/12/320, Urdorf, Switzerland), respectively.

The bioreactor containing 2 L volume of the optimized medium to scale up production of recombinant GOX. The yeast strain was cultured in 200 ml YPD at 29 °C, 200 rpm for 24 h, and inoculated into the bioreactor. All parameters of cultivation such as temperature, agitation, pH, antifoam, and DO were monitored by the online digital controller. Fermentation was done at 29 °C, 500 rpm with an airflow rate of 1 vvm and pH was adjusted 7 by using NaOH or H_3PO_4 (17).

GOX Assay: GOX activity was estimated by measuring the amount of quinoneimine dye via a spectrophotometer (10). The 3 ml reaction mixture was prepared as follows: 1.5 ml sodium citrate buffer 0.2 M (pH 5.5), 0.5 ml glucose solution 10% (W/W), 0.8 ml 4-aminoantipyrine and phenol solution, 0.1 ml horseradish peroxidase enzyme (60 U/ml) and 0.1 ml sample. The reaction mixture was incubated for 10 min at 37 ^{o}C. Finally, the absorbance of the sample was read at 510 nm using a spectrophotometer (UV-1800 Shimadzu, Japan). One unit (U) of GOX was defined as the amount of enzyme, which oxidized 1 µmol of β-D-glucose to D-glucono-δ-lactone and H_2O_2 per min under the assay conditions (11).

Results and Discussion

Maximum production of GOX was 280 U/L in YPD medium by *Y. lipolytica* Po1h-GOX after 7 days in non-optimized culture (Fig. 1).

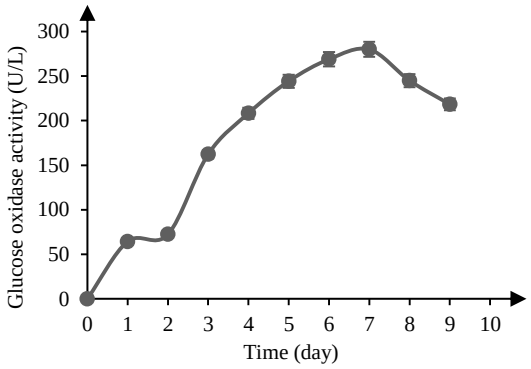

Fig. 1- Glucose oxidase production by *Y. lipolytica* Po1h-*GOX* cultivated in YPD medium for 9 days. All data are the mean values ± SD for three independent replicates.

Glucose, yeast extract, peptone, and pH as four main variables with different levels in YPD medium were selected for optimization by the RSM method. Experiments were done according to the software recommendation (Table 2).

Table 2-The experimental design using RSM for independent variables showing observed and predicted values of GOX production

Run order	Glucose	Yeast Extract	Peptone	pH	Observed	Predicted
1	25	10	20	8	22.89	22.52
2	35	10	10	6	24.08	24.65
3	35	10	10	8	24.49	23.12
4	25	15	15	7	21.33	21.57
5	35	10	20	6	22.76	21.99
6	25	10	20	6	22.93	21.74
7	30	15	15	7	22.67	22.63
8	30	15	20	7	20.00	21.68
9	30	15	15	6	22.38	22.94
10	25	20	20	8	23.07	22.80
11	30	15	15	7	23.22	22.63
12	35	20	20	8	23.23	21.52
13	30	15	15	8	23.35	23.21
14	30	15	10	7	23.69	22.42
15	35	20	20	6	20.57	20.54
16	25	20	10	8	21.26	21.62
17	30	10	15	7	23.92	23.75
18	30	20	15	7	21.98	22.56
19	25	20	20	6	19.75	20.72
20	35	10	20	8	20.00	21.68
21	30	15	15	7	23.66	22.63
22	35	20	10	8	20.25	21.75
23	25	10	10	6	21.68	22.99
24	30	15	15	7	22.36	22.63
25	30	15	15	7	21.98	22.63
26	25	20	10	6	22.14	20.76
27	35	15	15	7	21.59	21.76
28	25	10	10	8	22.23	22.55
29	35	20	10	6	22.02	21.99
30	30	15	15	7	23.13	22.63

After analysis of the results the suggested quadratic model is shown in Equation 1. The coefficients of the polynomial model were calculated using a general form of the second-degree polynomial equation (Table 3 and Equation 2).

Table 3- Analysis of variance for response surface model for GOX production

Source	DF	Sum of squares	Mean square	*F* value	*P* value
Model	14	22.49	1.61	0.98	0.0413
Glucose (A)	1	0.17	0.17	0.1	0.754
Yeast extract (B)	1	6.39	6.39	3.89	0.067
Peptone (C)	1	2.44	2.44	1.49	0.241
pH (D)	1	0.33	0.33	0.2	0.658
AB	1	0.19	0.19	0.12	0.738
AC	1	1.98	1.98	1.2	0.289
AD	1	1.21	1.21	0.74	0.404
BC	1	1.47	1.47	0.9	0.359
BD	1	1.68	1.68	1.2	0.327
CD	1	1.49	1.49	0.91	0.356
A^2	1	2.4	2.4	1.46	0.245
B^2	1	0.72	0.72	0.44	0.518
C^2	1	0.87	0.87	0.53	0.479
D^2	1	0.51	0.51	0.31	0.585

Equation (1):

Glucose Oxidase Activity (Y) = 22.6287 + 0.0960518 x A + -0.595693 x B + -0.368487 x C + 0.13639 x D + -0.108757 x AB + -0.351389 x AC + -0.274704 x AD + 0.303083 x BC + 0.323916 x BD + 0.304748 x CD + -0.961777 x A^2 + 0.526233 x B^2 + -0.577939 x C^2 + 0.443474 x D^2

Where A is glucose, B is yeast extract, C is peptone and D is pH.

(a) (2):

$$Y = \beta_0 + \sum \beta_i X_j + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i x_j.$$

i = 1, 2, …, k; j = 1, 2, …, k

Where Y is the predicted response (GOX activity); Xi and Xj are the coded independent factors that affect the response variable Y; β0 is the offset term; βi indicates the linear effect of Xi; βij represents the interaction between Xi and Xj; and βij demonstrates the quadratic effect of Xi (19).

An analysis of variance for the response surface model showed that the model for the production of GOX was significant. In the CCD of RSM, the real values of GOX were determined in the experiments and the predicted values could be predicted by the model equation (Equation 1).

Three-dimensional plots and their relevant contour plots were acquired based on the influence of four variables at three different values each and their interaction on the yield of GOX. The optimum concentration for each variable was recognized based on the hump in the three-dimensional plots (Fig. 2).

After optimization of the medium by RSM, GOX production reached 570 U/L after 7 days, which is 2-times more than the non-optimized medium.

The optimized medium was used in a 5-L bioreactor to scale-up the GOX production. In the bioreactor with glucose used as a carbon source in an optimized medium, 720 U/L of GOX was obtained after 2 days (Fig. 3).

Surprisingly, GOX was produced in the bioreactor after 2 days which is a shorter time than flask cultures. GOX production was reached 730 U/L after 6 days when sucrose was used as the carbon source in an optimized medium (Fig. 4).

Many efforts are being made to industrial production of GOX due to numerous applications in different industries. Researchers tried to solve the problems of GOX production in *A. niger* as an industrial producer using genetic engineering and express it in other microbial hosts (10,11).

It seems that the optimization of the fermentation medium and conditions is important in the next step.

Statistical experiment design methods are a cost-effective way to optimize and increase the products in microbial biotechnology. RSM is one of the statistical experimental design methods which is used to optimize bioprocesses as well as find interactions between different variables affecting that process (20).

Here, the YPD medium was selected for production and optimization of recombinant GOX production by *Y. lipolytica* Po1h-GOX. The software presents a model (Equation 1) and interactions between main variables in the GOX production (Fig. 2) according to the experiment design of RSM.

The model for the RSM method is approved by analysis of variance. Effect of coefficients that show the interaction between variables could be determined via *P*-value. The value of model *F*, model $P > F$ and lack of fit is 0.98, <0.041 and 0.4, respectively which is shown the model is significant.

Bankar et al. used Plackett–Burman design to optimization of GOX production by *A. niger* NCIM545. They studied six nutritional variables including sucrose, sodium nitrate, peptone, calcium carbonate, magnesium sulfate, and potassium dihydrogen phosphate. They obtained 2 U/ml of GOX after optimization of the process (21).

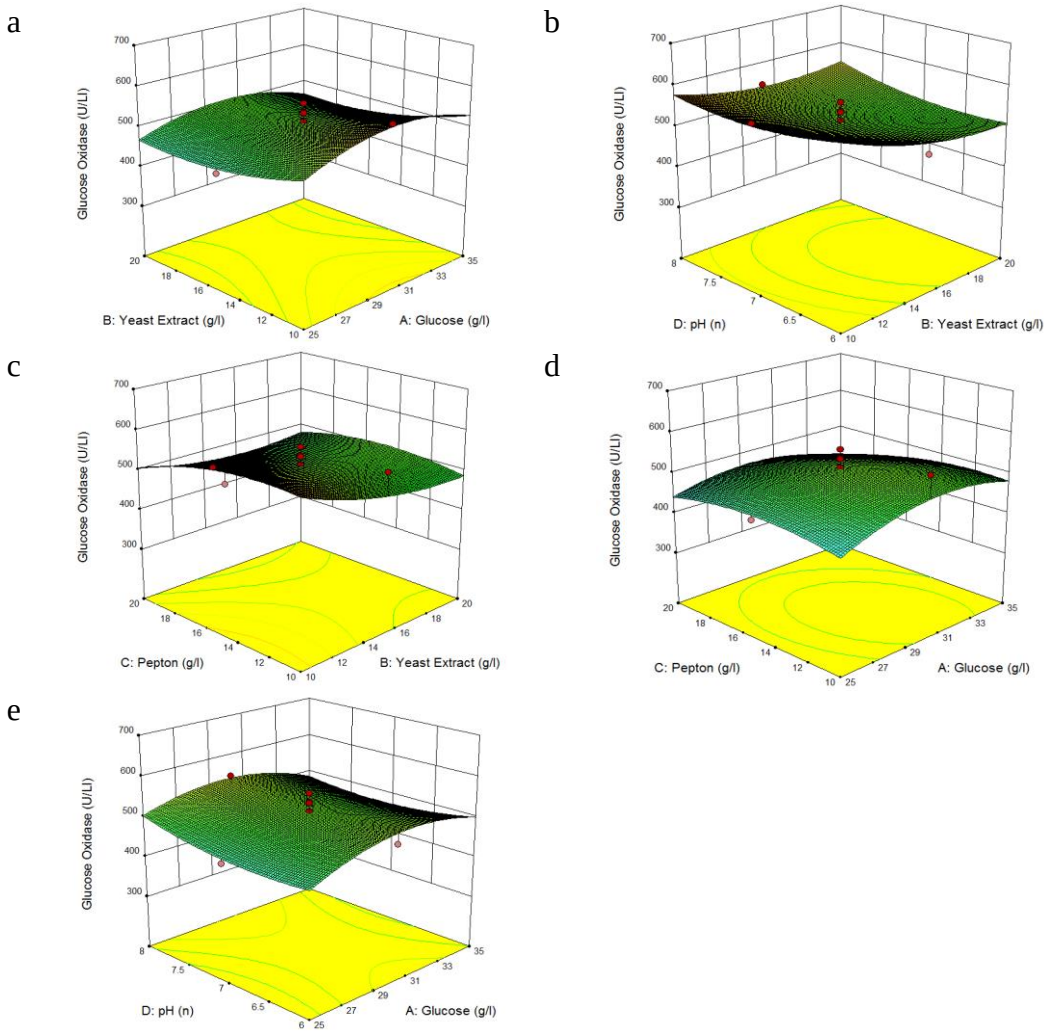

Fig. 2- Response surface plots showing interactions between variables of YPD medium for GOX production by *Y. lipolytica* Po1h-GOX. a) interaction between glucose and yeast extract; b) interaction between pH and yeast extract; c) interaction between peptone and yeast extract; d) interaction between peptone and glucose; e) interaction between pH and glucose.

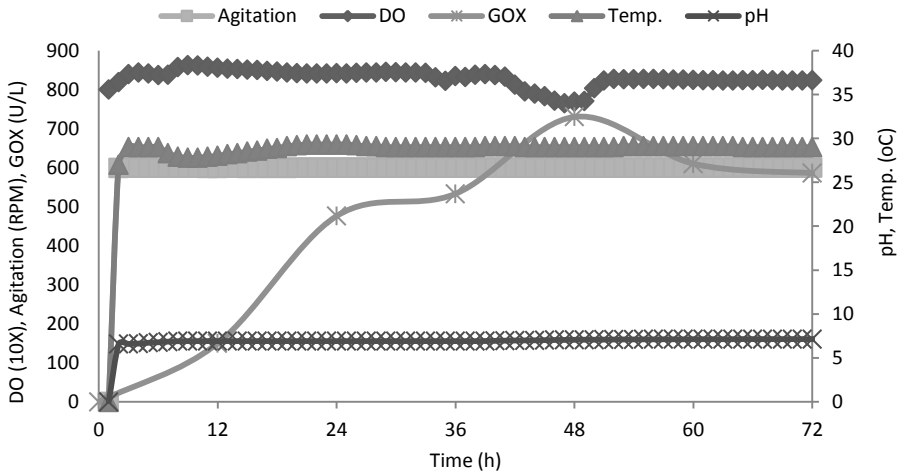

Fig. 3- Time courses of GOX production, pH, DO and temperature in the culture of recombinant *Y. lipolytica* Po1h-GOX in 5-L bioreactor containing the optimized medium and glucose as carbon source.

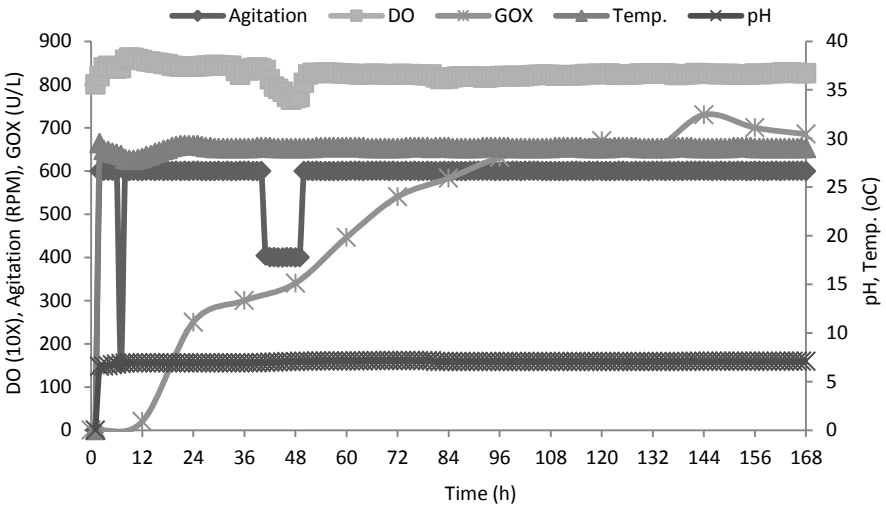

Fig. 4- Time courses of GOX production, pH, DO and temperature in the culture of recombinant *Y. lipolytica* Po1h-GOX in 5-L bioreactor containing the optimized medium and sucrose as carbon source.

Three-dimensional curves showed that increases in glucose and peptone concentrations influence maximum GOX production by the recombinant yeast strain (Fig. 2). The glucose and peptone (AC) had interaction and the highest effect on GOX production.

Liu et al. applied the RSM method for optimization of agitation and aeration in GOX production by *A. niger* in the bioreactor. They found that aeration has a negative effect on GOX production. GOX production was increased to 4.59 U/L in the bioreactor after 12 h (22).

Meng et al. expressed the GOX gene of *A. niger* under *AOX* promoter in *P. pastoris* and the recombinant strain used fed-batch fermentation for increasing GOX production. BMGY medium was optimized and 99 U/L of GOX was obtained in bioreactor after 24 h (5).

GOX production in the bioreactor culture is 1.2- and 2.5-fold more than flask cultures contain optimized and non-optimized media, respectively. The GOX production by *Y. lipolytica* Po1h-GOX in the bioreactor containing optimized medium with glucose as carbon source produces in a shorter time than flask culture. Furthermore, sucrose as a cheap carbon source can be considered for the production of recombinant GOX and other heterologous proteins by *Y. lipolytica*.

Conclusions

genetic engineering and metabolic engineering are important for the production of desired biotechnological products. But the optimization of fermentation medium and conditions can be a significant role in the improvement of their production level. The GOX production was optimized using the statistical experiment design method in recombinant *Y. lipolytica* for the first time. The method is helpful to the optimization of main variables of fermentation medium in a short time and cost-effective way. The results can be used to increase the production of heterologous enzymes and proteins by recombinant *Y. lipolytica* strains.

Acknowledgments

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