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Critical process parameters optimization for hyperthermostable β amylase production by *Bacillus subtilis* DJ5 using response surface methodology

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ABSTRACT. The combined effects of significant physical and chemical factors affect hyperthermostable β amylase production under submerged fermentation by Bacillus subtilis DJ5. The above was studied using the experimental design and response surface methodology. A 23 full-factorial central composite design was chosen to analyze interactions among three factors i.e. substrate concentration, medium pH and incubation temperature. The experimental data were fitted into a polynomial model for the yield of enzyme and an optimum level was arrived at with optimized conditions. Solving the coded values using Excel equation function indicated that maximum enzyme production is possible at a substrate concentration of 7.07 mg mL^{-1}, pH 6.622 and temperature of 35.435°C. Such prediction was validated with practical experiments in which, at the prescribed condition maximum yield of 15.62 U mg^{-1}, nearly 1.5 fold higher than non-optimized condition was observed.

Keywords: central composite design, response surface plot, contour plot, submerged fermentation, thermozymes.

A otimização dos parâmetros do processo crítico para a produção hipertermoestável de β amilase por *Bacillus subtilis* DJ5 utilizando a metodologia de superfície de resposta

RESUMO. Os efeitos combinados de fatores físicos e químicos significativos influenciam a produção hipertermoestável de amilase β sob fermentação submersa por Bacillus subtilis DJ5. O esquema experimental e metodologia de superfície de resposta foram usados, com 23 planejamento fatorial composto, para analisar as interações entre os três fatores, ou seja, concentração de substrato, pH médio e temperatura de incubação. Os dados experimentais foram ajustados a um modelo polinomial para a produção de enzima e chegou-se a um nível ótimo através de condições otimizadas. A solução dos valores codificados por equação de Excel indicou que a produção máxima da enzima é possível a uma concentração de substrato de 7,07 mg mL^{-1}, pH 6,622 e temperatura de 35,435°C. Tal previsão foi validada com experimentos práticos, onde na condição prescrita de rendimento máximo de 15,62 U mg^{-1}, foram observados cerca de 1,5 vezes maior do que o estado não otimizado..

Palavras-chave: projeto composto central, planejamento de superfície de resposta, planejamento contorno, fermentação submersa, termoenzimas.

Introduction

Biotechnological industry has always given thrust on the application of thermostable enzymes (thermozymes) for bioprocess technologies (HARRIS, et al., 2010; BRUINS et al., 2001). Among the currently available commercial enzymes, thermostable amylases have occupied most market share due to their wide application in different industries. Crave towards thermostable amylases are well understood with the prediction that expects that amylases business will reach $1.7 billion in 2016, with a future growth rate of 8.2% (DEWAN, 2012). To meet such growing demand, applied research has always focused its attention towards findings of thermostable amylases because of their ability to (i) withstand harsh and robust conditions of industrial processing; (ii) show significant catalytic efficiency at high temperatures; (iii) show stability at high temperatures especially the temperature of starch gelatinization (100-110°C) and liquefaction (80-90°C) (HAKI; RAKSHIT, 2003).

In our previous investigation, we reported production of hyperthermostable β amylase enzyme from mesophilic bacterium *Bacillus subtilis* DJ5 (PODDAR et al., 2011). Crude enzyme showed thermal stability at 121°C for 15 minutes To the best of our knowledge, there is no report of such

thermostability of amylases even from hyperthemophiles (BERTOLDO; ANTRANIKIAN, 2001; PARK et al., 2010). This has given the enzyme a dual economic advantage from the industrial point of view i.e. enzyme thermostability and ease of maintenance of mesophilic organism compared to thermophiles (TURNER et al., 2007).

In order to achieve commercial benefit, it is totally necessary to economize production (greater volumetric productivity within a very short time) in the easiest available way. Enzyme overproduction may be achieved by both genetic manipulations and media engineering. Genetic manipulation methodology is complex and rather expensive. Moreover stability of recombinants is an issue of doubt that sometime less popularizes use of overproducers in industry. As an alternative way, manipulation of physico-chemical conditions that positively influence the production machinery has been considered a better strategy (DEY et al., 2001). Such optimizations not only allow overproduction but also lead to optimized selection and use of nutrients by minimizing their losses.

Currently the classical approach of media optimization has lost its significance, mainly due to its failure of finding interactions between independent variables i.e. physical (temperature, pH etc.) and chemical (media components) factors (KENNEDY; KROUSE, 1999). Bio-statistical analysis has successfully filled such lacunas with very few experimental trials with the help of response surface methodology (MYERS et al., 2009). With increasing availability of computer-based bio-statistical designing and analytical softwares (Design Expert, STASTICA, SPSS, MatLab etc) (MARQUES DE SA, 2003; STROBEL; SULLIVAN, 1999) such process is now a 'few key press away' more scientific and easy-to-perform method.

In this study, interactive effects of three major factors (substrate concentration, media pH and incubation temperature) influencing production of hyperthermostable β amylase from *Bacillus subtilis* DJ5 under submerged fermentation were evaluated and optimized using full factorial central composite design and response surface methodology.

Material and methods

Microorganisms

Bacillus subtilis DJ5 (GenBank Accession Number GU357825) was used in this study (PODDAR et al., 2011). The organism was maintained on Starch Peptone agar medium with the following composition (g L^{-1}): Peptone, 0.9; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4,7H_2O$, 0.1; NaH_2PO_4, $2H_2O$, 0.5; soluble starch (Sigma, USA), 5; agar-agar, 15; pH 7.

Inoculum preparation and enzyme extraction

A 24-h-old culture of *Bacillus subtilis* DJ5 was transferred to a 250 mL Erlenmayer flask containing 100 mL of starch peptone broth media (composition mentioned previously). The initial pH of the medium was adjusted to 7.0 and inoculum load was 6%. The flasks were then placed in an orbital shaker at 37°C, at 160 rpm, for 6.5h. This setup was considered as non-optimized condition where substrate concentration (Starch, Sigma, USA), media pH and incubation temperature were 5%, 7 and 37°C respectively. Enzyme was collected after centrifugation of fermented broth at 10,000 rpm at 4°C in a cooling centrifuge (Remi C-24BL).

Enzyme assay

β amylolytic activity was measured by the Bernfeld (1955) method. Assay mixture contains 0.5 mL of 0.1 M phosphate buffer (pH 6.9), 1 mL soluble starch (0.5% w/v, Sigma Chemicals, USA) and 0.1 mL of crude enzyme. Control was prepared with the same conditions without adding the substrate. The reaction mixture was incubated at 100°C for 15 minutes Enzyme-substrate reaction was then stopped by addition of 1 mL 2 M NaOH. Both the assay mixture and control were then allowed to boil in a boiling water bath for 10 minutes after the addition of 0.5 mL of 3, 5-dinitrosalisylic acid reagent (Merck, Germany). After cooling the assay mixture at room temperature (25°C), absorbance were measured spectrophotometrically (Elico, India) at 540 nm. Amount of maltose released was measured from standard curve of maltose. One unit (U) of β amylolytic activity was defined as the amount of enzyme releasing 1 μmol of maltose equivalent per minute per mL from soluble starch (Sigma) under the standard assay conditions. The specific activity of the enzyme (U mg^{-1} of protein) was also determined by measuring protein content of enzyme using bovine serum albumin (BSA) as the standard, according to Lowry et al. (1951). All the experiments were performed in triplicate. The relative β amylase activity was defined as percentage of maximum specific activity measured in assay.

Optimization of process parameters

Various process parameters affecting enzyme production during submerged fermentation (SmF) were optimized by response surface technique. Tested process parameters were initial starch concentration, pH and incubation temperature.

A 2^3 factorial central composite experimental design with 14 non-central points and six replicates at the central point resulted in 20 experiments generated by Design Expert (Version 8.0.6.1 Stat-Ease Inc., Minneapolis, MN) statistical software. Each set of experiment was performed in triplicate and their values were averaged. The experimental design is shown in Table 1 and the coded variables are reflected in Table 2. A regression model containing 3 linear (β_1, β_2, β_3), 3 quadratic (β_{11}, β_{22}, β_{33}), 3 interactions (β_{12}, β_{23}, β_{13}) and β_0 intercept term was used. The overall second order polynomial mathematical relationship of response Y (U mg^{-1}) and three variables i.e. initial moisture content (A, %), medium pH (B) and incubation temperature (C, °C) could be approximated by following quadratic equation 1:

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+ +\beta_{33}C^2+\beta_{12}AB+\beta_{23}BC+\beta_{13}AC \quad 1$$

Experimental data were analyzed to plot response surface. ANOVA was used to estimate statistical parameters.

Table 1. Central composite design consisting of 20 experiments for the study of three experimental factors in coded units. Actual rates of coded units are given in brackets.

Run	A	B	C
1	0.00 (7)	0.00(7)	2.00 (45)
2	1.00 (9)	-1.00(5)	1.00 (40)
3	-1.00 (5)	1.00(9)	1.00 (40)
4	0.00(7)	0.00(7)	-2.00 (25)
5	0.00(7)	0.00(7)	0.00 (35)
6	0.00(7)	0.00(7)	0.00(35)
7	0.00(7)	-2.00 (3)	0.00(35)
8	1.00(9)	1.00(9)	-1.00 (30)
9	0.00(7)	0.00(7)	0.00(35)
10	-1.00(5)	1.00(9)	-1.00(30)
11	1.00(9)	-1.00(5)	-1.00 (30)
12	0.00(7)	0.00(7)	0.00(35)
13	2.00 (11)	0.00(7)	0.00(35)
14	1.00(9)	1.00(9)	1.00 (40)
15	-1.00(5)	-1.00(5)	-1.00 (30)
16	0.00(7)	2.00 (11)	0.00(35)
17	0.00(7)	0.00(7)	0.00(35)
18	-1.00(5)	-1.00(5)	1.00 (40)
19	-2.00 (3)	0.00(7)	0.00(35)
20	0.00(7)	0.00(7)	0.00(35)

A, substrate concentration ([S]) in mg mL^{-1}; B, pH of media; C, incubation temperature (°C).

Table 2. Experimental range and levels of independent variables.

Experimental variable	Coded symbol	Range and level				
		-2	-1	0	+1	+2
[S] (mg mL^{-1})	A	3	5	7	9	11
pH	B	3	5	7	9	11
Incubation Temperature (°C)	C	25	30	35	40	45

Results and discussion

To detect the effect of 3 major key factors (initial starch concentration, pH, and incubation temperature) responsible for hyperthermostable beta amylase production, each factor was studied at 5 different levels (-2, -1, 0, +1, +2) by central composite design. The experimental and predicted response of β-amylase production after 6.5 hours of cultivation by *Bacillus subtilis* DJ5 under SmF are shown in Table 3. The predicted versus actual value curve (Figure 1) is the graphical representation of Table 3 and indicates little variation between predicted and actual enzymatic activity. On the basis of quadratic polynomial equation of response surface model of Eqn 1, the present model successfully defined optimum fermentation conditions as well as illustrated combined effect of independent variables on enzymatic activity. The coefficients of regression equation were calculated using Design Expert (Version 8.0.6.1 Stat-Ease Inc., Minneapolis, MN) and the following equation were obtained:

$$Y = +9.37 + 0.064A - 0.72B + 0.24C + + 0.20AB + 0.25AC - 0.63BC - - 0.66A^2 - 2.03B^2 - 2.12C^2 \quad 2$$

where Y is the response (β amylase specific activity in U mg^{-1}) and A, B, C are coded values of the test variables, substrate concentration (mg mL^{-1}), medium pH, incubation temperature (°C) respectively.

Table 3. Observed responses and predicted values.

Run	β amylase activity (U mg^{-1})	
	Observed response	Predicted response
1	1.1	1.356
2	6.43	6.261
3	3.3	2.936
4	1.03	0.413
5	9.43	9.370
6	9.43	9.370
7	2.8	2.696
8	3.9	4.244
9	9.43	9.370
10	3.7	4.230
11	3.3	4.025
12	9.43	9.370
13	7.3	6.848
14	3.6	3.965
15	4.8	4.796
16	0.09	-0.167
17	9.43	9.370
18	6	6.018
19	6.5	6.591
20	9.43	9.370

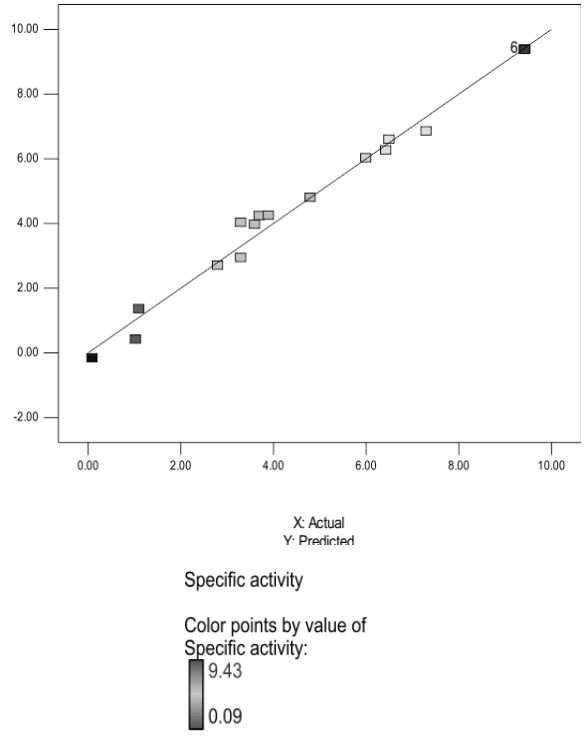

Figure 1. The predicted versus actual response.

The result of the second order response surface model fitting in the form of ANOVA is given in Table 4. The Model F-value of 107.68 implies that the model is significant. There is only a 0.01% chance that such a large "Model F-Value" could occur due to noise. Rates of "Prob > F" less than 0.0500 indicate that model terms are significant. In this case, B, BC, A^2, B^2, C^2 are significant model terms. This model presented a high determination coefficient and explains 98.9% of the variability in the response. Moreover "Pred R-Squared" of 0.9898 is in reasonable agreement with the "Adj R-Squared" of 0.9806, indicating a high significance of the model. "Adeq Precision" of 30.338 indicates an adequate signal for the signal noise ratio. A small coefficient of variation (C.V.) 8.05% clearly indicates a high degree of precision and a good reliability of the experimental values.

The residuals from the least squares also play an important role in judging model accuracy (MYERS et al., 2009). A normal probability plot of the residuals (Figure 2) showed a satisfactory straight line that concludes that the empirical model is adequate to describe the β amylolytic activity by response surface.

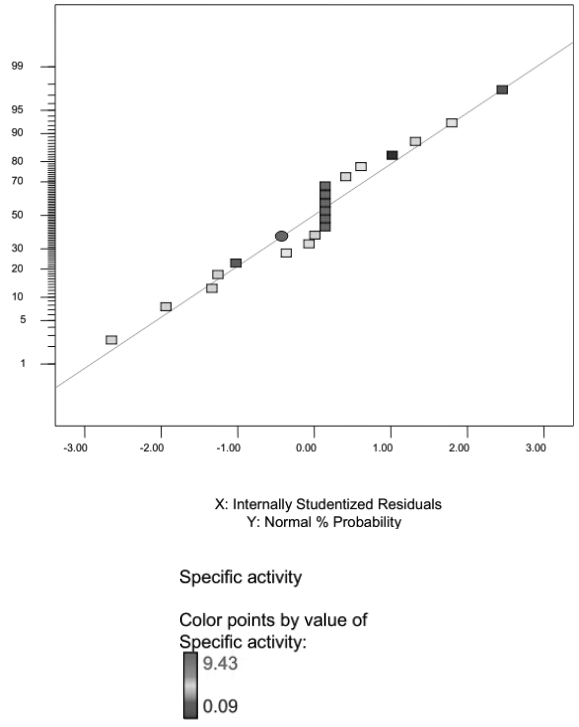

Figure 2. Normal plot of residuals.

Table 4. ANOVA for quadratic model.

Source	Sum of suqares	df	Mean square	F value	p-value Prob > F	Remarks*
Model	191.52	9	21.28	107.68	< 0.0001	s
A-[S] mg mL^{-1}	0.066	1	0.066	0.34	0.5752	ns
B-pH	8.19	1	8.19	41.46	< 0.0001	s
C-Temp (°C)	0.89	1	0.89	4.49	0.0600	ns
AB	0.31	1	0.31	1.56	0.2402	ns
AC	0.52	1	0.52	2.61	0.1375	ns
BC	3.16	1	3.16	16.00	0.0025	s
A^2	11.04	1	11.04	55.86	< 0.0001	s
B^2	103.24	1	103.24	522.41	< 0.0001	s
C^2	113.15	1	113.15	572.54	< 0.0001	s
Residual	1.98	10	0.20			
Lack of fit	1.98	5	0.40			
Pure error	0.000	5	0.000			
Cor Total	193.49	19				

R^2 = 0.9898, Adj R^2 = 0.9806, Pred R^2 = 0.9188, C.V. = 8.05%, Adeq Precision = 30.338; * ns = not significant, s = significant.

The significance of each coefficient was determined by confidence interval (CI) indicated in Table 5. The smaller the confidence length, the more significant the factor is. Results indicate that interaction between coded factors B and C is more significant than interaction between AC and AB.

Table 5. Confidence interval (CI) of model factors.

Factor	Coefficient estimate	Standard error	95% CI Low	95% CI High
Intercept	9.37	0.18	8.97	9.76
A-[S]	0.064	0.11	-0.18	0.31
B-pH	-0.72	0.11	-0.96	-0.47
C-Temp	0.24	0.11	-0.012	0.48
AB	0.20	0.16	-0.15	0.55
AC	0.25	0.16	-0.096	0.60
BC	-0.63	0.16	-0.98	-0.28
A^2	-0.66	0.089	-0.86	-0.47
B^2	-2.03	0.089	-2.22	-1.83
C^2	-2.12	0.089	-2.32	-1.92

On the other hand, the significance of each coefficient was also determined by the p rates listed in Table 4. The smaller the p value, the higher the significance of corresponding coefficient. From p value, it is clear β amylase production is highly dependent on interaction between pH and incubation temperature (model term BC) ($p < 0.005$). The other two interactions i.e. AC and AB have little influence on enzyme production, as evidenced from higher p values.

The 3D response surface and the 2D contour plots are the graphical representation of the regression equation (Figure 3, 4, and 5). The main goal of these plots is to obtain the optimum values of the variables such that response is maximized. Each contour curve represents an infinite combination number of two test variables with the other maintained at their zero level. The maximum predicted value is indicated by the surface confined within the smallest ellipse in the contour diagram. Elliptical contours are obtained when there is a perfect interaction between the independent variables (DEY et al., 2001) and the maximum predicted value is present within the smallest eclipse in the contour diagram. In current study, each interaction resulted in formation of elliptical contour plots indicating perfect interactions among different variables. Moreover, a smallest circle in each contour diagram represents maximum yield during an interaction. The setup with variables that were chosen thus may be justified as a fact that each interaction was highly perfect, as may also be evidenced from peak point of 3D response plots.

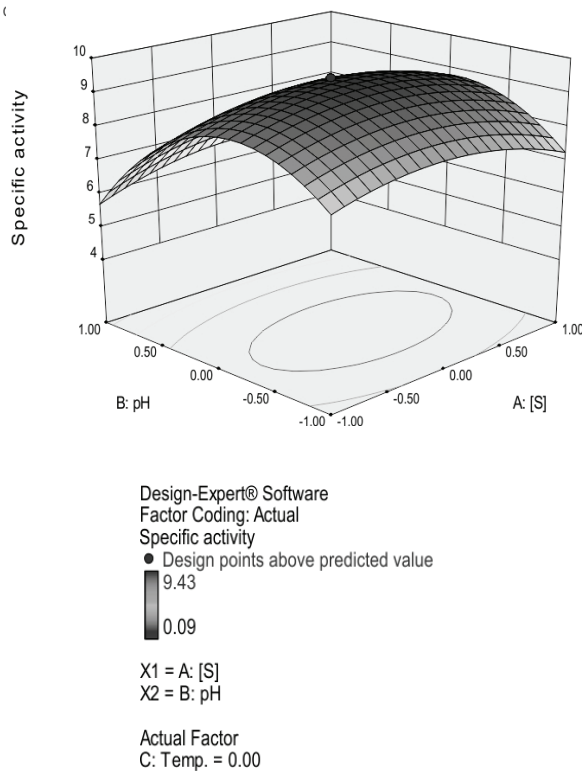

Figure 3. 3D response surface plot showing effects of pH and starch concentration on hyperthermostable β amylase production with other variable (temperature) constant at middle point.

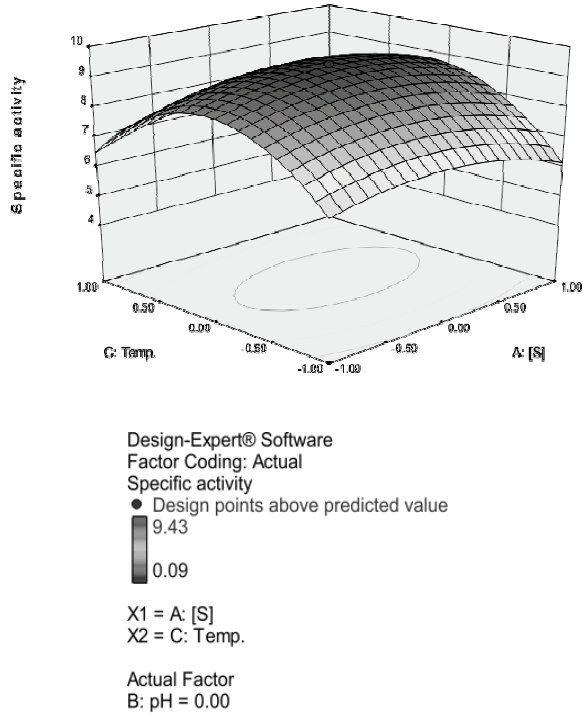

Figure 4. 3D response surface plot showing effects of temperature and starch concentration on hyperthermostable β amylase production with other variable (pH) constant at middle point.

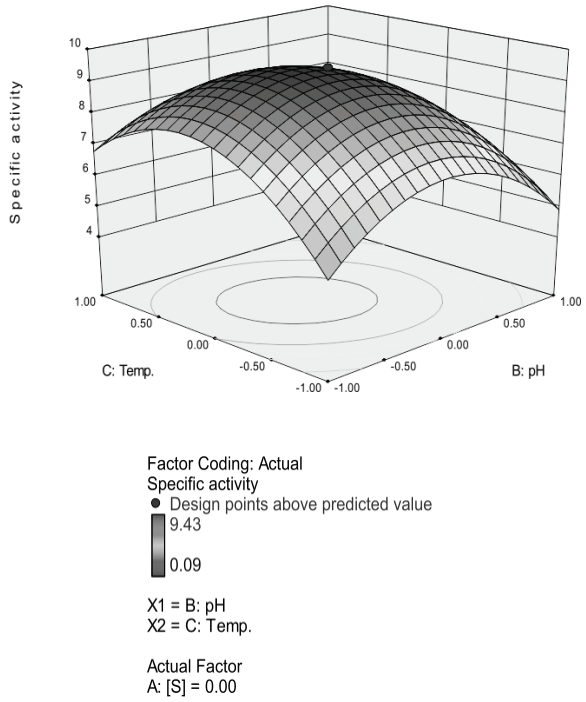

Figure 5. 3D response surface plot showing effects of temperature and pH on hyperthermostable β amylase production with other variable ([S]) constant at middle point.

Validation of model

Regression equation was applied using equation functions of Microsoft Excel 2007 to determine optimum values of test variables (A, B, and C). The optimum rates of test variables in coded units were An = 0.36289, B = - 0.189 and C = 0.08683. At these rates, the actual substrate concentration, pH and incubation temperature were 7.07 mg mL^{-1}, 6.622 and 35.435°C, respectively. Laboratory experiment conducted at such optimal condition, highest enzyme production of 15.46 U mL^{-1} was recorded after 6.5 hours of incubation. Highest production obtained after optimization was nearly 1.5 fold higher than production (10.97 U mL^{-1}) observed under non-optimized condition.

Conclusion

Current report has evaluated efficiency of response surface methodology for optimization of physico-chemical factors affecting hyperthermostable β amylase production under submerged fermentation. The study suggests that the amount of carbon source, pH and temperature affects submerged fermentation significantly. Such optimization method has successfully maximized production up to 1.5 fold and avoided wastage of media constituents. Application of response surface methodology was highly efficient and relatively simple.

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