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Production of Raw Starch-Saccharifying Thermostable and Neutral Glucoamylase by the Thermophilic Mold *Thermomucor indicae-seudaticae* in Submerged Fermentation

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Abstract Among physical and nutritional parameters optimized by "one variable at a time" approach, four cultural variables (sucrose, $MgSO_4 \cdot 7H_2O$, inoculum size, and incubation period) significantly affected glucoamylase production. These variables were, therefore, selected for optimization using response surface methodology. The *p*-values of the coefficients for linear effect of sucrose and inoculum size were less than 0.0001, suggesting them to be the key experimental variables in glucoamylase production. The enzyme production (34 U/ml) attained under optimized conditions (sucrose, 2%; $MgSO_4 \cdot 7H_2O$, 0.13%; yeast extract, 0.1%; inoculum size, 5×10^6 spores per 50 ml production medium; incubation time, 48 h; temperature, 40°C; and pH 7.0) was comparable with the value predicted by polynomial model (34.2 U/ml). An over all 3.1-fold higher enzyme titers were attained due to response surface optimization. The experimental model was validated by carrying out glucoamylase production in shake flasks of increasing capacity (0.25–2.0 l) and 22-l laboratory bioreactors (stirred tank and airlift), where the enzyme production was sustainable. Furthermore, the fermentation time was reduced from 48 h in shake flasks to 32 h in bioreactors.

Keywords Glucoamylase · *Thermomucor indicae-seudaticae* · Thermophilic mold · Response surface methodology · Shake flasks · Stirred tank fermenter · Airlift fermenter · Bioreactor

Introduction

Starch is the major carbohydrate reserve polymer in maize, wheat, oat, rice, potato, and cassava and a potential substrate for the production of gaseous or liquid fuels, proteins, and chemicals [1], along with its major application in the production of sugars [2]. Starch-hydrolyzing enzymes have been widely used in industrial applications and account for about

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30% of the worldwide industrial enzyme production [3, 4]. Glucoamylase (α-1,4-D-glucan glucohydrolase, EC 3.2.1.3) is a typical fungal enzyme required in high tonnage [5], holding second position (14%) after proteases in terms of worldwide distribution and sale of industrial enzymes [6]. The enzyme is specifically used for saccharifying starch to produce glucose and other sugar syrups required in food and beverage industries [2]. Application of glucoamylase in the starch industry can be benefited from enhanced thermostability and activity in the neutral pH range [5, 7], and therefore, an emphasis has been laid on screening thermophilic fungal glucoamylases. Most of them, however, exhibit optimal activity in the acidic pH range [8, 9].

The efficiency of a fermentation process can be improved considerably by optimizing the cultivation conditions, which can be achieved by employing various strategies. Conventional "one variable at a time" approach has been traditionally used; however, it is time consuming and does not depict the combined effect of all factors involved [10]. Furthermore, for understanding the interactions among variables, a considerable number of experiments are required. The process of optimization can be simplified and the number of experiments reduced using statistical methods. Rotatable central composite design (RCCD) of response surface methodology (RSM) not only helps in determining the optimum level of each variable, but the interactions among variables and their effects on product yield can also be understood [11–13]. Design Expert version 6.0 provides rotatable 3-D plots of response surfaces, which help in establishing the relationship between one or more measured responses and vital input factors [11]. With the development of computing software and their wide applications, RSM has emerged as a valuable technique in many areas of biotechnology for optimizing the process parameters [14, 15].

Glucoamylase of *Thermomucor indicae-seudaticae* is one of the few thermostable glucoamylases which show optimum activity in the neutral pH range, and therefore, it holds a significant potential in simplifying the process of starch saccharification [16]. The present investigation was thus planned to find out the optimum combination of fermentation variables for maximizing glucoamylase production by *Thermomucor indicae-seudaticae.* Physical and nutritional parameters were optimized by "one variable at a time" approach, and the most important factors (sucrose, $MgSO_4.7H_2O$, inoculum size, and incubation period) were selected for response surface optimization. The model was validated by cultivating the mold in shake flasks (0.25–2.0 l) and 22-l stirred tank and airlift bioreactors.

Materials and Methods

Source and Maintenance of the Mold

T. indicae-seudaticae, procured from Prof. A. Subrahmanyam (Department of Botany, Kakatiya University, Warangal, India), [17] was routinely grown on Emerson YpSs agar [18] at 40°C and preserved at 4°C. The spore suspension in glycerol was also preserved at −20°C.

Inoculum Preparation and Cultivation Conditions

Spore suspension from a 4-day-old culture was prepared in normal saline containing 0.1% (*v*/*v*) Tween 40 and used for inoculation. Spores were counted under compound microscope using a hemocytometer. Glucoamylase production was carried out in 250-ml Erlenmeyer flasks containing 50 ml sucrose–yeast extract broth [sucrose, 2%; K_2HPO_4, 0.05%; $MgSO_4{\cdot}7H_2O$, 0.1%; yeast extract, 0.1%; microelement solution, 0.1% ($ZnSO_4$, 1%; $MnSO_4$, 0.5%; $CoSO_4$,

Table 1 Range of values for the rotatable central composite design of RSM.

Independent variables	Levels				
	$-\alpha$	−1	0	+1	$+\alpha$
Sucrose (%)	0.50	1.25	2.00	2.75	3.50
$MgSO_4.7H_2O$ (%)	0.05	0.13	0.20	0.27	0.35
Inoculum size ($\times 10^6$)	1.00	3.00	5.00	7.00	9.00
Incubation period (h)	24.00	36.00	48.00	60.00	72.00

0.1%; H_3BO_3, 0.1%) pH 7.0], inoculated with 3×10^6 spores and incubated at 40°C and 250 rev/min in an incubator shaker (these variables were found to be optimum by "one variable at a time" approach). After 48 h, the fermented broth was harvested by filtering through Whatman no. 1 filter paper and the cell-free culture filtrate was used in glucoamylase assays. The biomass was dried at 80°C to constant weight. The fresh mycelial mats were homogenized in phosphate buffer (pH 7.0, 0.1 M) using mortar and pestle, filtered, and centrifuged at 10,000×*g* for 20 min. The supernatant was used for determining intracellular titer of glucoamylase.

Analytical Methods

Glucoamylase was assayed by incubating the reaction mixture containing 0.5 ml starch (0.5% prepared in sodium phosphate buffer, pH 7.0) and 0.5 ml of appropriately diluted glucoamylase for 30 min at 60°C. The reducing sugars liberated were determined using dinitrosalicylic acid reagent [19] with reference to the standard curve of glucose.

One unit (U) of glucoamylase is defined as the amount that liberates 1 μmol of reducing sugar as glucose/ml/min under the assay conditions. Soluble protein content in the cell-free culture filtrate was determined according to Lowry et al. [20] using bovine serum albumin as standard. Fungal biomass was determined gravimetrically by drying at 80°C to a constant weight.

Optimization of Fermentation Variables

Most significant of the various physical and nutritional parameters affecting glucoamylase production in submerged fermentation, optimized previously by "one variable at a time" approach, were selected for statistical optimization using response surface methodology.

Response Surface Optimization

Optimum levels and the interactions among variables (A: sucrose, B: $MgSO_4{\cdot}7H_2O$, C: inoculum size, and D: incubation time), which exerted significant influence on glucoamylase production, were determined by using RCCD of RSM. Each variable in the design was studied at five different levels, with all variables taken at a central coded value of zero (Table 1). The Greek letter alpha (α) was used to represent the distance from the center of the design space to axial point [11, 12]. All the possible combinations of selected factors were included in the full factorial design of RCCD. For four variables, a 2^4 factorial design with eight axial points and six replicates at the center point with a total of 30 experiments was formulated (Table 2).

Table 2 Experimental design and results of RCCD of response surface methodology.

Experiment number	Sucrose	$MgSO_4$	Inoculum size	Incubation period	Glucoamylase production (U/ml)	
	(%)	(%)	($\times 10^6$)	(h)	Predicted	Experimental
1	1	1	−1	1	28.30	28.12
2	0	0	0	0	35.03	36.33
3	0	0	0	−α	17.01	17.01
4	−1	−1	1	−1	22.36	22.01
5	1	1	1	1	29.99	29.32
6	0	0	0	0	33.23	33.98
7	α	0	0	0	25.87	25.11
8	−α	0	0	0	5.65	6.03
9	0	−α	0	0	31.84	32.21
10	1	−1	−1	1	16.36	18.26
11	−1	1	1	−1	12.00	11.08
12	−1	−1	−1	−1	12.64	14.26
13	0	0	α	0	26.34	26.34
14	0	0	0	0	36.01	34.21
15	−1	−1	1	1	14.40	14.12
16	−1	1	−1	1	27.72	28.32
17	0	0	−α	0	13.78	12.21
18	1	−1	1	1	28.49	28.42
19	1	1	−1	−1	18.92	20.15
20	−1	1	−1	−1	13.79	14.50
21	0	α	0	0	33.48	32.72
22	1	−1	1	−1	31.97	32.32
23	0	0	0	0	35.98	37.23
24	1	1	1	−1	30.96	32.12
25	0	0	0	0	31.23	33.83
26	0	0	0	α	20.87	20.87
27	−1	−1	−1	1	15.07	13.34
28	−1	1	1	1	15.58	15.65
29	1	−1	−1	−1	18.45	17.86
30	0	0	0	0	32.21	30.99

Software and Data Analysis

Design-Expert® 6.0 Stat-Ease Inc., Minneapolis (USA), was used to generate RCCD of experiments. The data obtained from RSM on glucoamylase production was processed by analysis of variance (ANOVA). Glucoamylase production was taken as response (Y), and a multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. The behavior of the system was explained by the following quadratic equation:

$$Y = \beta 0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_1 \beta_4 AD + \beta_2 \beta_3 BC + \beta_2 \beta_4 BD + \beta_3 \beta_4 CD$$

where Y is predicted response, β_0 is intercept, β_1, β_2, β_3, β_4 are linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$, $\beta_{3,3}$, $\beta_{4,4}$ are squared coefficients, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{1,4}$, $\beta_{2,3}$, $\beta_{2,4}$, $\beta_{3,4}$ are interaction coefficients, and A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD, and CD are independent variables.

Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance was explained by the obtained model, which was given by the multiple coefficient of determination R squared (R^2) value, and its significance was checked by F-test. The significance of the regression coefficient was tested by t-test.

Validation of the Experimental Model and Large Scale Glucoamylase Production

Validation of the model and regression equation was performed by taking the optimum values of A (2%), B (0.13%), C (5×10^6 spores per 50 ml medium), and D (48 h). Glucoamylase production was carried out in shake flasks of varied capacity (0.25, 0.50, 1.0, and 2.0 l) containing one-fifth volume production medium. The enzyme production was also carried out in a 22-l stirred tank fermenter (B. Braun Biotech International, Germany) containing 10 l optimized sucrose–yeast extract broth (sucrose, 2%; K_2HPO_4, 0.05%; $MgSO_4{\cdot}7H_2O$, 0.13%; yeast extract, 0.1%; microelement solution, 0.1%; pH 7.0) at 40°C and 250 rev/min. The practicability of cultivating *T. indicae-seudaticae* under airlift conditions and its glucoamylase production performance were also evaluated in a 22-l airlift fermenter (B. Braun Biotech International, Germany) (H:D ratio of 5.4:1) containing 10 l production medium at a constant aeration of 1 vvm.

Results

The data recorded from rotatable central composite design (RCCD) of response surface methodology (RSM) were analyzed by standard analysis of variance (ANOVA), and the mean predicted and observed responses are presented in Table 2. Glucoamylase production varied markedly in the range between 6 and 37 U/ml. The highest enzyme production was attained when all the variables were kept at their optimum ("0") levels, whereas the lowest titers were recorded at the lowest level of sucrose. The center level experiments (where all the variables were used at their optimum levels) showed a little variation in enzyme production (between 31 and 37 U/ml).

Statistical analysis of the experimental data was performed using Fisher's statistical test for ANOVA. The F-value, ratio of the mean square due to regression to the mean square due to error, indicates the influence of each controlled factor on tested model. The p-value corresponding to the F-value indicates the probability that the differences between calculated and tabulated statistics are due only to random experimental error. The statistical analysis of the model showed that sucrose and inoculum size exerted significant effect on glucoamylase secretion. Low probability value of the coefficient of interaction between sucrose and inoculum size also indicated the significance of these variables. High values of probability coefficient were observed for $MgSO_4{\cdot}7H_2O$ and incubation time, while their quadratic effect was significant ($p=0.0001$), and therefore, included in the model. High coefficient values of $\beta_{1,2}$ and $\beta_{1,4}$ indicated that the interactions between sucrose and $MgSO_4.7H_2O$ and sucrose and incubation period are insignificant for glucoamylase secretion. ANOVA for the RSM quadratic model ("Prob>F" value) was less than 0.0001 (99.99% confidence level). The high "R-squared" value of 0.98 suggested that this could explain 98% of the variability in the model responses and is close to the adjusted (0.96) and predicted (0.93) "R squared" values. The "adequate precision" value is at a satisfactory level of 29.52 (Table 3). While calculating the effect of various interactions on glucoamylase production, the nonsignificant interaction coefficients were eliminated, and

Table 3 ANOVA for glucoamylase production in submerged fermentation.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
Model	2,444.94	14	174.64	57.72	0.0001
Error	20.48	5	4.10		
Total	2,490	29			

the deduced model could be expressed in the following final response equation that represented a suitable model for glucoamylase production:

$$\text{Glucoamylase} = 33.95 + 5.05 \times A + 2.84 \times C - 4.55 \times A^2 - 0.32 \times B^2 - 3.62 \times C^2 - 3.72 \times D^2 + 3.45 \times AC - 2.87 \times BC + 2.88 \times BD - 2.60 \times CD$$

where *A*, *B*, *C*, and *D* are sucrose, $MgSO_4.7H_2O$, inoculum size, and incubation time, respectively. Although the elimination of nonsignificant coefficients did not enhance the coefficient of determination of the polynomial model, it is worth mentioning that the lack of fit is insignificant in the deduced model.

Response surface curves were generated by plotting the response (glucoamylase production) on the *z*-axis against any two independent variables, while keeping the other two independent variables at their optimum (0) levels. Six response surface curves were obtained by considering all the possible combinations of four factors. Three-dimensional response surface curve for the interaction between sucrose and $MgSO_4{\cdot}7H_2O$ suggested a linear increase in glucoamylase production with increase in sucrose concentration. Enzyme production stabilized up to 3% sucrose (Fig. 1) and declined sharply at higher levels. At the optimum levels of incubation period and sucrose, the response between $MgSO_4{\cdot}7H_2O$ and

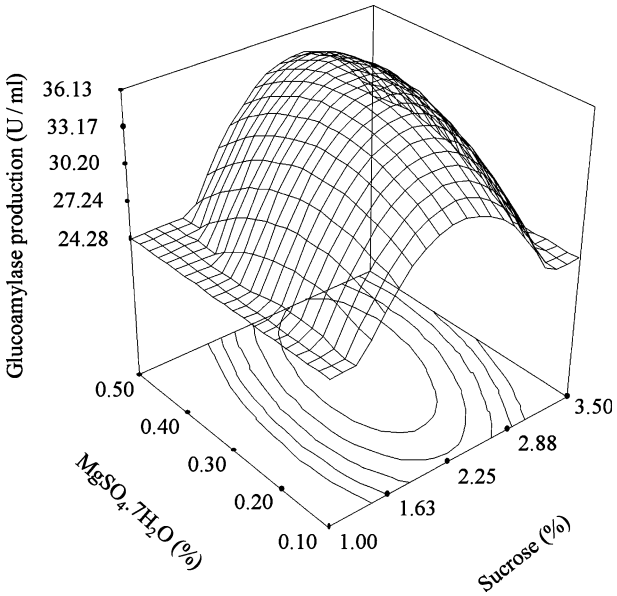

Fig. 1 Response surface graph showing the effect of interaction between sucrose and $MgSO_4{\cdot}7H_2O$ on glucoamylase production

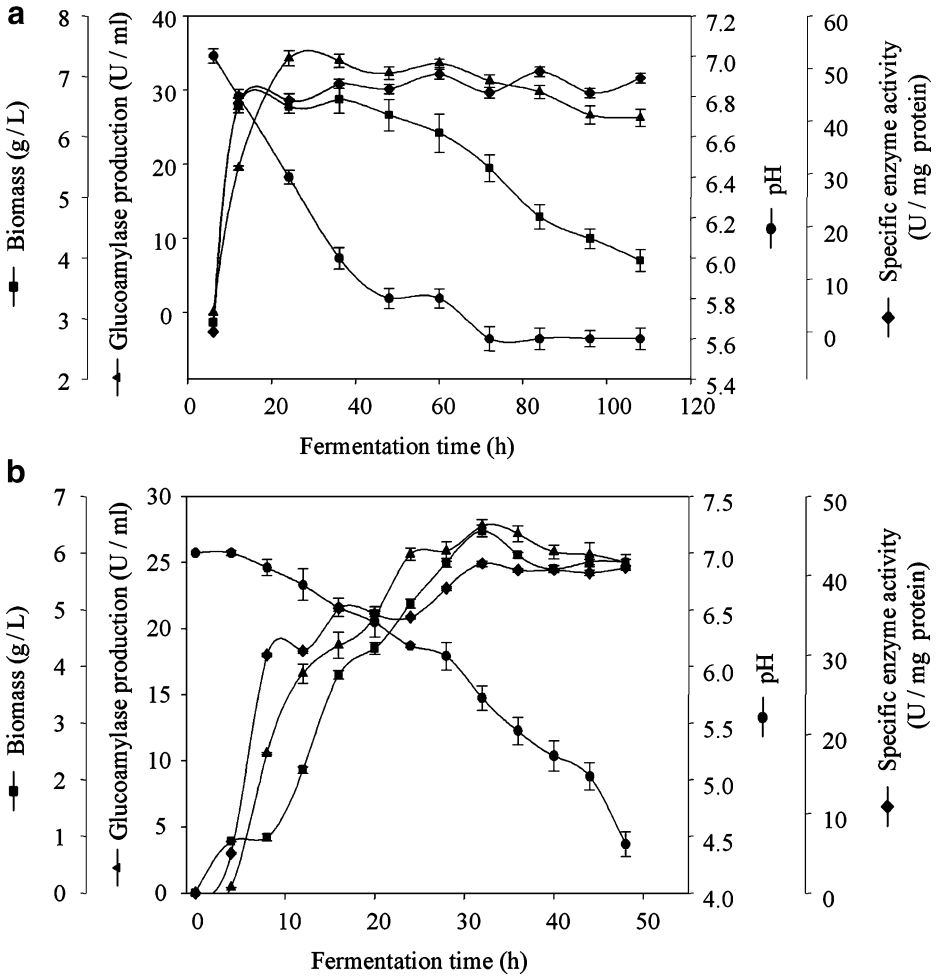

Fig. 2 **a** Glucoamylase production profile of *T. indicae-seudaticae* in stirred tank bioreactor. **b** Glucoamylase production profile of *T. indicae-seudaticae* in airlift bioreactor

inoculum level indicated that a higher $MgSO_4 \cdot 7H_2O$ concentration (around 0.3%) was desirable; however, experimentally, when the concentration was raised beyond a certain level, precipitation of medium components occurred. Three-dimensional graph between inoculum level and incubation time suggested significant model terms. An inoculum size of 5×10^6 spores per 50 ml medium resulted in high enzyme production (34.5 U/ml) after 48 h, which stabilized up to 54 h and declined afterwards.

Validation of the experimental model was attempted by cultivating the mold under optimized conditions in shake flasks of varied volume, where a slight decrease in glucoamylase production was observed with increase in flask/medium volume. Glucoamylase production was sustainable in bioreactors and a significant reduction in fermentation time was observed (Fig. 2a and b). A peak in glucoamylase production was attained after 48 h in Erlenmeyer flasks, while the maximum production in stirred tank and airlift bioreactors was attained in 32 h.

Discussion

T. indicae-seudaticae has been reported to produce a thermostable glucoamylase with optima at 60°C and neutral pH [21, 22]. The enzyme, in combination with α-amylase and amylopullunase of *Geobacillus thermoleovorans*, has shown significant potential in developing simpler starch saccharification process [16]. The optimization of fermentation variables was, therefore, attempted for maximizing glucoamylase titers. The mold secreted around 11 U/ml glucoamylase in unoptimized medium (soluble starch, 0.5%; yeast extract, 0.2%; K_2HPO_4, 0.1%; $MgSO_4$, 0.05%; pH 7.0) after 96 h of incubation at 40°C and 200 rev/min. A 1.7-fold enhancement in glucoamylase titers (19 U/ml) was recorded when fermentation variables were optimized by "one variable at a time" approach. Besides extracellular glucoamylase, the mold also contained around 16.5 U/g dry biomass intracellular glucoamylase. Glucoamylase production was recorded in almost all carbon sources (starch, glucose, maltose, fructose, lactose, mannose, galactose, cellobiose, raffinose, arabinose, inulin, sorbitol, mannitol, inositol, and sucrose) tested. The synthesis of glucoamylase in this mold is constitutive, as reported in *Aspergillus tamarii* [23].

For improving glucoamylase production by *T. indicae-seudaticae* further, the variables which were identified by "one variable at a time" approach to influence glucoamylase production were optimized by response surface methodology. This resulted in around 34 U/ml glucoamylase production (3.1-fold improvement). Glucoamylase production predicted by the statistical design was very close to the experimental enzyme titers, which reflected the accuracy of the model [24]. Response surface optimization has been successfully used earlier for optimizing fermentation variables for the production of α-amylase [15], phytase [25], and chitinase [26].

Among the four variables used in this investigation, the optimum levels for sucrose (2%) and yeast extract (0.1%) were similar to that optimized earlier by "one variable a time" method. The model predicted a slightly higher requirement of $MgSO_4$; however, to overcome precipitation problems, a concentration of 0.13% was used. The enzyme production was significantly affected by inoculum level, which appeared to be the major factor responsible for higher glucoamylase titers. An inoculum size of 5×10^6 spores per 50 ml production medium was optimum, and at higher levels, the enzyme production declined, which could be attributed to the competition among fungal population for nutrients [27].

The experimental model was successfully validated by carrying out glucoamylase production in increasing capacity shake flasks; the slight decrease in production levels could be attributed to improper mixing of nutrients and aeration problems at higher volumes. Enzyme production was sustainable in stirred tank reactor, which further validated the optimization strategy and indicated the successful scale up of glucoamylase production from 250-ml flask (50 ml production medium) to 22-l (10 l production medium) fermenter. It was also possible to cultivate the mold in an airlift bioreactor for the production of glucoamylase. Reduction in fermentation time could mainly be due to the better control of fermentation parameters (uniform distribution of nutrients due to improved agitation/aeration and temperature maintenance) in bioreactors [28] as reported for the production of other enzymes [29, 30].

Glucoamylase production in *T. indicae-seudaticae* was growth-associated, which commenced in log phase and stabilized when the mold entered into stationary phase. Decline in pH of the production medium with the progression of fermentation could be due to the production of acids as by-products or deamination reactions. High α-amylase production by *Aspergillus oryzae* was at controlled pH (6.0) in batch and continuous cultivations [31]. Similarly, *Bacillus thermoleovorans* NP-54 exhibited improved growth rate and a 1.38-fold improvement in α-amylase production when pH was maintained at 7.0 [32].

Optimization of fermentation parameters significantly improved glucoamylase production by *T. indicae-seudaticae*. The results obtained from the conventional "one variable at a time" optimization formed the basis for this investigation to understand the important interactions among critical variables for achieving enhancement in enzyme production.

Conclusions

This investigation demonstrated the applicability of RSM for optimizing cultural variables for maximizing glucoamylase production by *T. indicae-seudaticae*. The enzyme secretion improved 3.1-fold, when the culture variables were optimized by RSM. The statistical model was successfully validated, and enzyme production was scaled up from flask to laboratory fermenter with sustainable enzyme titers.

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