Original Article

Assessment of Optimized Process Parameters for Superior Bioactive Metabolite Production by *Nonomuraea longicatena* VSM-16 using Response Surface Methodology

Ushakiranmayi Managamuri[1], Muvva Vijayalakshmi[1], Venkata Siva Rama Krishna Ganduri[2], Satish Babu Rajulapati[3], Sudhakar Poda[4]*

[1]Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna nagar, Guntur, Andhra Pradesh, INDIA.
[2]Department of Biotechnology, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur, Andhra Pradesh, INDIA.
[3]Department of Biotechnology, National Institute of Technology, Warangal, Telangana, INDIA.
[4]Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, INDIA.

ABSTRACT

Objectives: The present work was carried out to check the capability of novel actinobacterium, *Nonomuraea longicatena* (VSM-16) for bioactive metabolite production and optimization of its process parameters by statistical and mathematical modeling. **Methods:** Response Surface Methodology (RSM) regression evaluation was done to fit the experimental data of each response with the aid of second order polynomial. Unstructured kinetic models had been developed for growth, substrate utilization and bioactive metabolite production (in terms of responses). Model based kinetic parameters were estimated and the profiles of bioactive metabolite production, substrate utilization and growth had been drawn. **Results:** The results have shown accurate interaction among process variables at optimized values of incubation time at 8-9 days, pH at 8-9, temperature at 30-31°C, concentrations of Mannitol at 2-2.2% and Biopeptone at 1.5-1.7% and the data have been effectively fitted into second-order polynomial models. Under these conditions, the responses (zones of inhibition) of various organisms *Staphylococcus aureus, Streptococcus mutans,*

Xanthomonas campestris, Pseudomonas aeruginosa and *Candida albicans* have been also matched with experimental and predicted consequences. **Conclusion:** The zones of inhibition (responses) for the organisms had been also determined to be best fitted with experiment and model values.

Key words: Bioactive metabolites, Mathematical modeling, *Nonomuraea longicatena,* Optimization, Regression Analysis, Response Surface Methodology.

Correspondence

Dr. Sudhakar Poda,

Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, INDIA.

Phone: +91 9000122929

Email: sudhakarpodha@gmail.com

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INTRODUCTION

To combat and reverse the spread of antibiotic resistance pathogens and to cure dreadful diseases like cancer, new drugs are at a great demand. Nature remains as the ingenious source of new compounds.[1] The most promising source for the biologically active natural compounds are the marine micro-organisms which are currently of great interest due to their efficiency to produce array of various metabolites. Biologically active natural compounds produced by actinobacterium are structurally exciting, many of them have been exploited by the pharmaceutical industry as potent drugs and lead molecules that cannot be found in the terrestrial micro-organisms[2] and some of which can be used in the process of drug development.[3]

Marine actinobacterium are found to be a rich source of numerous secondary metabolites with robust biological activities.[4] Actinobacterium are considered to be wealthy abundant source for the production of diverse novel bioactive compounds, of which most of them are reported to be antibacterial, antifungal, anticancer and immunosuppressive compounds.[5] The unconventional compounds simplify the improvement of new and novel drugs with greater therapeutic efficiency and few side effects.[6] Hence there is an urgent need for exploring the secondary metabolites produced by these micro-organisms. Production of the bioactive metabolites is notably stimulated by nutritional and cultural conditions. Several environmental factors which includes temperature, pH and incubation period play a major role in the production of antimicrobial agents.[7] In addition to the physico-chemical factors the

components like carbon, nitrogen, concentration of sodium chloride also play a prime position in production of bioactive metabolites.[8] The optimization of the process parameters is very critical in order to achieve highest level of metabolite production.[9]

RSM is a critical analytical tool (a set of statistical and mathematical techniques) in designing the optimization process characteristic of multiple independent and dependent variables.[10,11] The relative significance of several variables and the interactive effects among the variables can be evaluated concurrently.[12] Conventional method based optimization involves the analysis of one-factor-at-a-time which is time consuming and laborious and the factors interactive effects of the variables are ignored and misleading conclusions may be drawn. It is therefore indispensable to design the procedure to maximize the highest yielding quantities of bioactive metabolites with conserved functional properties. Hence RSM is considered more favorable compared to the classical approach-based optimization and is an ideal strategy for standardizing the process variables.[13]

For this type of fermentation, the standard differential mass balance equations for three components had been well established by applying Monod equation linking substrate concentration to the specific growth rate. Further the evaluation of assumed unstructured mathematical models with experimental facts for comparison in order to find the best model that could describe the whole microbial system, which are more

effective in elucidating the fermentation profiles of microbial process for bioproducts[14-17]

Thus, the present study is targeted to evaluate the optimized conditions such as temperature, pH, concentrations of mannitol and biopeptone for the bioactive metabolite production by VSM 16 and to further optimize the conditions for maximizing the yield of the bioactive metabolite using RSM. The purpose of research is also to assess the kinetic parameters of bioactive metabolite production from *Nonomuraea longicatena* strain VSM 16 (KU507597).

MATERIALS AND METHODS

Isolation

The marine sediment samples were collected at different depths from the Bay of Bengal of coastal Andhra Pradesh, India and air dried. The air-dried sediment sample was pre-treated with calcium carbonate (10:1 w/w) and incubated at 37°C for 4 days. The pre-treated sediment sample was suspended in sterile distilled water (1g in 100 ml). Serial dilutions were prepared and 100 μl of 10^{-4} dilution was spread onto the surface of Hemic acid vitamin agar containing 0.1% Hemic acid, 0.05% disodium hydrogen phosphate, 0.17% potassium chloride, 0.005% $MgSO_4$. $7H_2O$, 0.001% $FeSO_4$. $7H_2O$, 0.002% $CaCO_3$, 0.05% B Vitamins, 0.005% cycloheximide and 2% agar (pH 8) supplemented with nalidixic acid (50 μgml⁻¹) and secnidazole (50 μgml⁻¹). After incubation for two weeks at 30°C, distinct strain was selected and maintained by sub culturing on yeast extract malt extract dextrose (YMD) agar medium at 4°C for further study.

Identification

The promising actinomycetes strain VSM-16 was identified as *Nonomuraea longicatena* VSM 16 with the aid of cultural, morphological, physiological and biochemical studies along with 16 S rDNA analysis. The rDNA sequence was deposited in the NCBI GenBank with an accession number KU507597. The strain was maintained on YMD agar medium at 4°C. The purpose of the present work was to optimize the culture conditions for enhancing the production of bioactive metabolites by the strain using RSM.

Statistical optimization design

RSM is used for modeling and analysis of the problems in which the production of the response is influenced by several variables. RSM is implemented to optimize the bioactive metabolite production by VSM-16 with a purpose for finding the favorable conditions. Central Composite Design (CCD) of the RSM was employed to optimize the process parameters, as per Table 1 for the bioactive metabolite production by VSM-16 and its antimicrobial activity against 5 responses *Staphylococcus aureus*, *Streptococcus mutans*, *Xanthomonas campestris*, *Pseudomonas aeruginosa* and *Candida albicans* (in terms of Zone of inhibition) (Table S1). Range and central point value of all the 5 variables are shown in the Table 1.

The following equation represents the coded process variables

$$x = (X_i - X_0)/\Delta x \quad (1)$$

Where x is dimensionless coded value, X_i is actual value of variables, X_0 is the actual value of the variables at the centre point and ΔX is Step change value. The information obtained from experiments was fitted with the second order polynomial equation that is as follows

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \left(\sum_{i=1}^{n} \beta_{ii} X_i \right)^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \quad (2)$$

Where Y is predicted response, β_0 is intercept coefficient, β_i is the linear coefficient, βij is the interaction coefficients, β_{ii} is the quadratic coefficients, X_i and X_j are coded values of the five additive variables under

Table 1: Experimental range of factors studied using CCD in terms of coded and actual factors.

Factors	Symbols	Actual levels of coded factors		
		−1 (Low)	0 (Middle)	+1 (High)
Time of incubation (days)	A	9	10	11
pH	B	7	8	9
Temperature (°C)	C	30	35	40
Concentration of Mannitol (%w/v)	D	1	1.5	2
Concentration of Biopeptone (%w/v)	E	0.5	1	1.5

study. 3D plots were generated by varying two variables with the experimental range with the other variable constant at the central point. Fractional factorial design with center points that are augmented with a group of "star points" allows estimation of curvature.[18]

Statistical analysis

Second order polynomial model was applied to predict the response variables and the data analysis was carried out by means of Analysis of Variance (ANOVA) and the adequacy of the model was predicted through regression analysis (*R2*). The values were considered to be significant while the *p*-value <0.05. The quality of the fit of the polynomial model was expressed by value of correlation coefficient (*R2*). The main indicators for determining the significance and the adequacy of the model applied include F-value (Fisher variation ratio), probability value (Prob> F) and Adequate Precision. Additionally, the numerical optimization was carried out to determine the optimum values of the independent variable.

Kinetic Model development

Primary mathematical and unstructured kinetic models quantitatively elucidate the substrate utilization and growth-related production formation kinetics through Monod model and logistic equations, in a batch system.[19-21] Models of Logistic (L) and Modified Luedeking-Piret (MLP) and Logistic Incorporated Modified Leudeking–Piret (LIMLP) were used to duplicate the cell proliferation, Substrate utilization and anti-microbial metabolite productions of the strain, as per Mangamuri *et al.* 2016, 2017.[22,23] The data obtained from the models were used to calculate all the kinetic parameters involved in fermentation.

RESULTS

Statistical optimization design

The relationship between the process variables and the response functions in terms of antimicrobial activity of bioactive metabolites measured as zone of inhibition against test pathogens *S. aureus*, *S. mutans*, *X. campestris*, *P. aeruginosa* and *C. albicans*. The process variables were identified by five factor inscribed central composite design. The experimental values of the bioactive metabolite production by the strain and its antimicrobial effects against the 5 test pathogens (responses) is measured as zones of inhibition (mm). The experimental values were subjected to multiple regression analysis using RSM to fit the second-order polynomial equations (Equation 2). Experimental values of the 5 responses obtained were nearer to the anticipated values that indicate the model is satisfactory according to the experimental design (Supplementary Table 1). The effect of the variables on the five responses was executed through a randomized experimental run. The impact of the bioactive metabolite produced by the strain was measured as zone of inhibition against the

five responses ranged from 14 mm to 17 mm for *S. aureus*, 14 mm to 18 mm for *S. mutans*, 14 mm to 18 mm for *X. campestris*, 13 mm to 16 mm for *P. aeruginosa* and 13 mm to 16 mm for *C. albicans*.

To analyse the significance of all the factors under study, ANOVA was performed and the results are presented in the Table 2. The table summarizes the regression coefficient (R^2); Sum of Squares (SS), Standard Errors (SE), F-value and *P*-Value. *P*-value is the probability value which determines the effect in the model that was significant statistically.[24] The smaller the value of *p* the corresponding coefficient is considered to be more significant.[25] To have higher confidence levels of 95% to 99% the value of p should be equal to 0.05 and 0.01 for the effect to be statistically significant.[26] The Fisher's statistical test was employed to determine the significance of each factor where the significance degree was ranked based on the F-ratio value.[27]

Optimum operating conditions and corresponding results

Following the same procedure reported by[25] the optimum values of incubation time, pH, temperature, concentration of mannitol and biopeptone were obtained. As referred to desirability function a multi-response method for optimizing the operational conditions are employed. Finally, the optimum values of incubation time, pH, temperature have been found to be 8 days, 30°C, pH 8.0 and concentration of mannitol at 2% and biopeptone at 1.5 %, respectively.

Checking the model adequacy

Statistics used to check the adequacy of the models at a confidence level of 99% are summarized in Table 2. The coefficient of determination (R^2) indicate the proportion of the total variability of the model explained and suggested that for a good fit model. $R2$ should be close to the value 1 and should be at least 0.8.[28] Hence the *R* value reflected to be a very good fit between the observed and the predicted response values. Potential problem of the fit model is that it always increases with added factors even if the factors are not significant.[29] Hence adjusted $R2$ is used to assess the model adequacy since it is adjusted for the number of terms in the model.[26] Adjusted $R2$ is adjusted for the size of the model in way it decreases the insignificant factors added to the model.[29] The $R2$ value higher than 0.9 indicates that, the regression model fit the data very well.[30] The Coefficient of Variation (CV) is the standard deviation expressed as a percentage of the mean and should be less than 10%.[26] The ANOVA variance statistics are given in the Table 2. The suggested sequential model sum of squares and lack of fit tests (showing degrees of freedom; Mean square, F-value, *p*-value), model summary statistics for the best outcome quadratic model, for all the five responses, is given in (Supplementary Table 2). The model was found to be statistically significant since the Prob>*F* value of the model for all the five responses was found to be <0.00001 and *F* values of the model for five responses are given in Table S2 and imply that the model is significant.

Response surface analysis of bioactive metabolite production

Optimization of the selected five variables viz. incubation time, pH, temperature, concentration of mannitol and biopeptone, a 10-factorial design with central point was constructed. Highest activity (zone of inhibition represented in mm) of the bioactive metabolite produced by the strain against the five responses was obtained when the incubation time of 8 days, pH of 7, temperature of 30°C, concentrations of mannitol at 2% and bio peptone at 1.5%. The maximum zone of inhibitions against all the five responses was found to be 17 mm (for *S. aureus*), 18 mm (for *S. mutans*), 18 mm (for *X. campestris*), 16 mm (for *P. aeruginosa*) and 16 mm (for *C. albicans*).

DISCUSSION

The interactive effects of the process variables on the five responses are illustrated in 3D (three-dimensional surface plots). Analysis of the 3D plots represents that all the three parameters showed the tremendous impact at the beginning and reached maximum and further increase of the range of values and concentration showed a negative effect on the production of bioactive metabolite by VSM-16 and its effect against the responses. Figure 1-3 showed the zones of inhibition (mm) against the five responses. The increase in the production of bioactive metabolite by VSM-16 metabolite could be achieved with incubation time of 8 days, pH at 8, temperature range between 30°C, concentration of mannitol between 2 and concentration of biopeptone IS 1.5% Beyond these ranges, the zone of inhibition produced by the bioactive metabolite decreased.

RSM is a method that is effective statistically and determines the coefficients of the mathematical model and optimum conditions using minimum set of experiments.[21,31] The values of the responses obtained from the confirmation experiments and predicted values obtained through optimization approach are presented in the Supplementary Table 1. The fit summary produced by using the Design-Expert Version 8 recommends the quadratic and linear models to produce the bioactive metabolite by *N. longicatena* VSM 16. ANOVA variance was executed to study the relationships and the results of the second order response surface fitting were given in the Table 2 and Supplementary Table 2. The *P*- value of the models for the bioactive metabolite production is presented in Supplementary Table 2. The model was considered statistically significant if the p-value was less than 0.05.[32] The lower value of *P* narrates the mutual interactions between the variables and indicates the importance of these variables in the model.[33] ANOVA analysis revealed

**Table 2: Analysis of ANOVA variance to test the adequacy of the model.**

Statistics	Response				
	S. aureus	*S. mutans*	*X. Campestris*	*P. aeruginosa*	*C. albicans*
R²	0.9112	0.9581	0.9504	0.9455	0.9247
Adjusted R²	0.9499	0.9292	0.9162	0.9370	0.9652
Predicted R²	0.9447	0.8566	0.8453	0.9642	0.9652
Adequate Precession	13.988	20.004	16.452	9.158	14.288
CV %	2.55	2.73	2.89	2.31	3.03
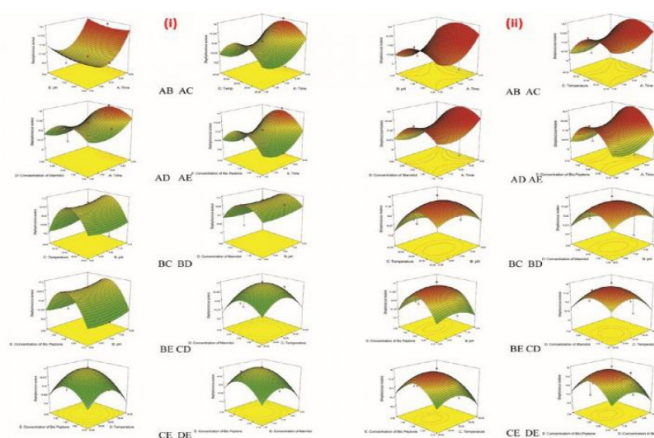
Figure 1: Response surface 3D plots showing the interactive effects of selective variables on Zone of inhibition (mm) of the bioactive compound production by *Nonomuraea longicatena* VSM-16 against (i) *S. aureus*, (ii) *S.mutans* response.
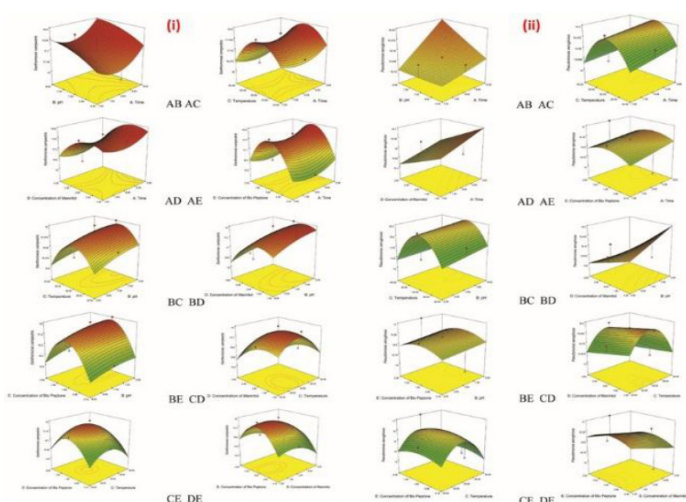
Figure 2: Response surface 3D plots showing the interactive effects of selective variables on Zone of inhibition (mm) of the bioactive compound production by *Nonomuraea longicatena* VSM-16 against (i) *X. campestris*, (ii) *P. aeruginosa* response.
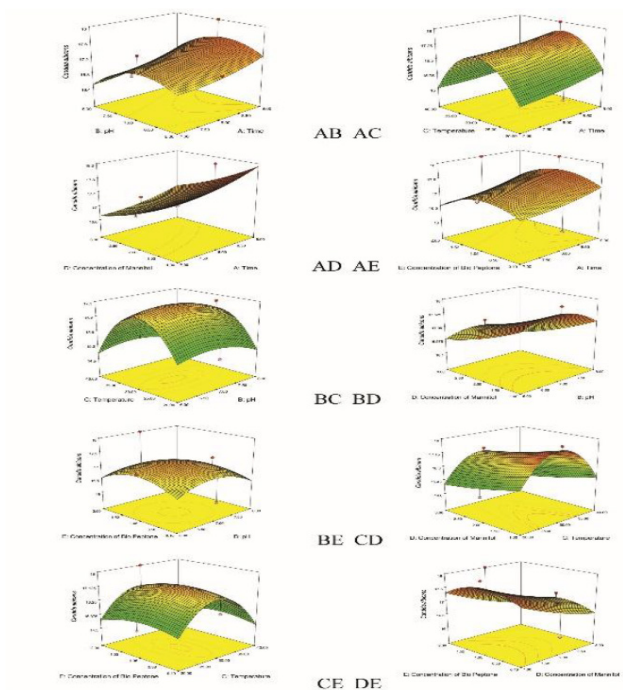
Figure 3: Response surface 3D plots showing the interactive effects of selective variables on Zone of Inhibition (mm) of the bioactive compound production by *Nonomuraea longicatena* VSM-16 against response *C. albicans*.

that the *p*-values of all the six response were < 0.0001 indicating the model was significant.

The profiles of *Nonomuraea longicatena* (VSM 16) (KU507597) growth and limiting substrate utilization results obtained from shake flask experiments and model kinetics were compared in Figure 4 (a) – (f) display the comparison of experimental versus model predicted zones of inhibition of bioactive metabolite on media inoculated with *S. aureus*, *S. mutans*, *X. campestris*, *P. aeruginosa* and *C. albicans* over the time. From all the profiles, it was observed that model predicted and experimental acquired values show very good fit. Biokinetic parameters used in the mathematical model equations were also estimated and are tabulated
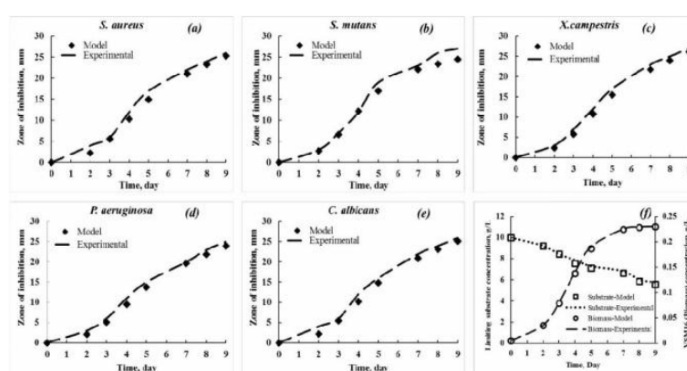
Figure 4: Comparison of experimental and model predicted kinetics *(a)-(e)*: for zone of inhibition (mm); *(f)*: for biomass growth (g/L), substrate utilization (g/L).

Table 3: Estimated kinetic parameters using L, LILP, LIMLP model equations.

Kinetic Parameters	*S. aureus*	*S. mutans*	*X. campestris*	*P. aeruginosa*	*C. albicans*
Logistic (L) Model Parameters					
μmax (d^{-1})			1.05		
$R2$			0.99		
$X0$ (g/L)			0.005		
Xm (g/L)			0.231		
Logistics incorporated Modified Luedeking-Piret (LIMLP) Model parameters					
γ (g.S/g.X)			12.46		
$R2$			0.91		
η (g.S/(g.X.d))			1.125		
Logistics incorporated Luedeking-Piret (LILP) Model parameters					
α (mm/g.X)	65.06	85.08	68.26	58.84	64.25
$R2$	0.91	0.88	0.93	0.93	0.92
β (mm/(g.X.d))	8.65	4.32	8.65	8.65	8.65

Table 4: Comparison of zones of inhibition (mm) from shake-flask experiments and from model.

Maximum Zone of Inhibition (mm)	*S. aureus*	*S. mutans*	*X. campestris*	*P. aeruginosa*	*C. albicans*
Experimental	26	27	27	25	26
Model fitted	25.36	24.5	26.07	23.95	25.17

in Table 3 which shows determination coefficient (*R2*) values obtained by fitting L, LILP and LIMLP models to the experimental data were found to be high, thus revealing good precision of the models. Values of growth and non-growth-related product parameters, α and β, were estimated using LILP model and a higher α values than β confirmed that bioactive metabolite production by the strain is more growth related than non-growth related in shake flask. The simulated parameters, γ and η, of LIMLP model are also in good agreement with the experimental values, implies that this model is more appropriate to symbolize restraining substrate utilization kinetics in bioactive metabolite production by *N. longicatena* VSM 16. Further, zones of inhibition from agar diffusion assessments are much like model anticipated values (Table 4).

CONCLUSION

A novel actinobacterium *Nonomuraea longicatena* VSM-16 was able to produce bioactive metabolites under the influence of the Incubation time, pH, Temperature, Concentrations of mannitol and biopeptone. Application of the RSM based optimization of the significant factors and their interactive effects to produce bioactive metabolites and parameters obtained were in consistent with experimental values of the bioactive metabolite production using VSM-16. The statistical parameters values obtained indicate that the models for responses match the experimental facts accurately. The optimized process parameters were 8 days of incubation time, pH at 8, temperature at 30°C and concentrations of mannitol and biopeptone was found to be 2% and 1.5%, respectively. Under this optimal point the maximum responses represented as zones of inhibition (in mm) from the bioactive metabolite produced using VSM-16. This is the first report on the kinetic modelling for bioactive metabolite production (in terms of zones of inhibition studies) by *N. longicatena* VSM 16. The results obtained are promising and merit for large scale production of the bioactive natural metabolites for novel VSM-16 that can be used to find new compounds for the medical application.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

RSM: Response Surface Methodology; **ANOVA:** Analysis of Variance; **L:** Logistic; **LILP:** Logistic Incorporated Leudeking Piret; **LIMLP:** Logistic Incorporated Modified Leudeking Piret; **YMD:** Yeast Extract Malt Extract Dextrose.

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