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OPTIMIZATION OF GROWTH MEDIUM FOR PROTEASE PRODUCTION BY *HALOFERAX LUCENTENSIS* VKMM 007 BY RESPONSE SURFACE METHODOLOGY

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ABSTRACT

The production of halophilic thermostable protease by *Haloferax lucentensis* VKMM 007 was optimized using a statistical approach. In accordance with factorial design, soluble starch, gelatin, KCl and $MgSO_4$ were selected among 27 variables tested. Next, a second-order quadratic model was estimated and optimal medium concentrations were determined based on quadratic regression equation generated by model. These were 5.14 g L^{-1} of KCl, 6.57 g $L^{-1}$of $MgSO_4$, 9.05 g $L^{-1}$of gelatin and 5.27 g $L^{-1}$of soluble starch in high salts media supplemented with 0.5% (w/v) of beef extract and peptone, respectively. In these optimal conditions, the obtained protease concentration of 6.80 U mL^{-1} was in agreement with the predicted protease concentration and was further improved to 7.02 U mL^{-1} by increasing the concentration of NaCl in the medium to 25% (w/v). An overall 4.0-fold increase in protease production was achieved in the optimized medium compared to activity obtained in initial medium.

Key words: cultivation media; *Haloferax lucentensis*; Response surface methodology; optimization; halophilic protease.

INTRODUCTION

Proteases from halophilic extremophiles retain activity at conditions of high salt, offer the possibility of cost-reduction by allowing for production under non-sterile conditions (7) and are more likely to aid in industrial processes where high salt concentrations inhibit mesophilic enzymes (11). However, low growth rates of halophilic extremophiles, in particular halophilic archaea, often act to hamper all further biotechnological advances. In a previous study, we studied protease production in a halophilic euryarchaeon *Haloferax lucentensis* VKMM 007 belonging to *Halobacteriaceae* family. The purified protease was stable in a wide range of temperatures (20°C-70°C), NaCl concentrations (0.85 M-5.13 M) and pH (5.0–9.0). Additionally, it remained stable or only marginally inhibited in the presence of various polar and non-polar solvents, surfactants and reducing agents (6). However, the observed protease production (1.78 U mL^{-1}) was low.

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As each organism or strain has its own set of conditions required for maximal enzyme production, extracellular protease production is under influence of physical growth factors or growth media composition. In particular, variations in the C/N ratio, the presence of some easily metabolizable sugars and the presence of metal ions can influence the amount of protease produced (5, 13). Growth medium parameters with significant impact on growth rate and enzyme production can be selected using statistical evaluation of experimental design. Furthermore, calculations of the optimal level of each parameter for a given target can be performed in order to improve product yield, reduce development time and overall process costs. To this aim, response surface methodology (9) is widely used (e.g. 10, 12). Accordingly, the present study presents a sequential optimization strategy to improve the production of halophilic, thermotolerant and organic-solvent tolerant protease produced by *Haloferax lucentensis* VKMM 007. As a first step, we have used a one-variable-at-a-time approach to address the most important among the variables studied. In a second step, we have determined the optimum levels of significant variables identified through response-surface methodology. The last step involved experimental verification of the theoretical solution to validate the quadratic model.

MATERIALS AND METHODS

The experiments conducted in this study were carried out in triplicates. All media components were purchased from Himedia Laboratories, Mumbai, India.

For the optimization of medium components and their concentration, the cells of *Haloferax lucentensis* VKMM 007 (GenBank accession number DQ915814) were propagated in initial medium pH 7.5 containing (g L^{-1}): beef extract 5.0, peptone 5.0, NaCl 18.0, $MgCl_2$ 5.14, Na_2SO_4 4.06, $NaHCO_3$ 0.2, H_3BO_3 0.03, KBr 0.1, KCl 0.69, $CaCl_2$ 1.14, $SrCl_2$ 0.026, NaF 0.003, $NaSiO_3$ 0.002, $FeSO_4$ 0.001. The microorganisms were grown in 125 ml Erlenmeyer flasks containing 25 ml growth medium, incubated at 40°C for 48 hours under shaking conditions of 150 rpm in a Thermostatic orbital shaker (Sub zero Inc, Chennai, India). The flasks were inoculated with 1% (v/v) of 48-hours-old culture of *Haloferax lucentensis* VKMM 007 grown in initial growth medium. Upon cultivation, the cells were harvested by centrifugation (10000 × g, 15 min) and the cell-free supernatant was used for the enzyme determination. The quantitative estimation of protease activity was carried out according to McDonald and Chen (8). One unit (U) of protease activity was expressed as the amount of enzyme that liberated 1 µM tyrosine min^{-1} under assay conditions.

In a factorial experiment, 27 different media components were screened to select for nutrients that significantly influenced the growth of model organism. These were considered as explanatory variables at concentration levels of 0.5% (w/v), 1.0% (w/v) and 1.5% (w/v), respectively. A response variable, protease activity (U mL^{-1}) of *Haloferax lucentensis* VKMM 007 cell culture supernatant, was measured after 48 hours of incubation.

To determine the response pattern and synergy of variables the full 2^k composite design was performed giving 2^k+2k+n_0 combinations where k is the number of independent variables and n_0 is the number of replications of the experiments at centre point. This provided 30 experimental runs performed with four factors at five coded levels (–2 –1, 0, +1 and +2) in duplicate, with central points in triplicate to determine the experimental error (1). The coded and actual values of the variables are presented in Table 1. The responses of the input variables were evaluated as a function of protease production, measured as protease activity obtained after 48 hours of cultivation and coded by Y_p (U mL^{-1}). The relationship of variables was determined by fitting a second order polynomial equation to data obtained from the 30 runs. Design-based experimental data were matched according to the following second-order polynomial equation Eq. (1):

$$Y_p = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i,j=1}^{4} \beta_{ij} x_i \; x_j \qquad (1)$$

Where Y_p is the predicted response (protease activity of *Haloferax lucentensis* obtained in growth medium and measured as the amount of units per ml of culture broth), β_0 is a constant; β_i, linear terms coefficients; β_{ii}, quadratic terms coefficients and β_{ij}, interaction coefficients. The relation between the coded forms of the input variable and the actual values of chosen variables is described as Eq. (2).

$$x_i = \frac{X_i - X_0}{\delta X} \qquad i = 1,2..\, k \qquad (2)$$

Where x_i is the coded value and X_i the actual value of an independent variable, X_0 is the value of X_i at the center point and δX is the step change of the variable. The above calculations were performed using Design Expert 7.0, (Stat-Ease, Minneapolis, USA).

Table 1. Coded and uncoded values of experimental variables used in the central composite experiment design

Independent variables	Coded levels				
	-2	**-1**	**0**	**+1**	**+2**
X_1, KCl (g L^{-1})	0.0	2.5	5.0	7.5	10.0
X_2, $MgSO_4$ (g L^{-1})	0.0	3.0	6.0	9.0	12.0
X_3, gelatin (g L^{-1})	0.0	5.0	10.0	15.0	20.0
X_4, soluble starch (g L^{-1})	0.0	2.5	5.0	7.5	10.0

RESULTS AND DISCUSSION

Amongst 27 media components tested, KCl (X_1), $MgSO_4$ (X_2), gelatin (X_3) and soluble starch (X_4) showed significant effect on protease production (Figure 1) and were chosen as four variables (denominated as X_1, X_2, X_3 and X_4) for response surface methodology based growth medium optimization. The experimental design and predicted responses for each combination of the variables are given in Table 2. The maximal protease production in cell culture of model organism was noted in experimental runs in which the concentrations of all four tested variables were at zero concentration level. In these experiments, the protease concentration levels ranged from 6.2 U mL^{-1} to 6.8 U mL^{-1}.

Based on above responses, Sequential model sum of squares (type I), Lack of Fit tests and model summary statistics a quadratic model was suggested. The ANOVA of the quadratic regression model demonstrated that the computed *F*-value was several times greater of tabulated *F*-value of 2.34 indicating that the model was significant at a high confidence level. The significance of the model was also indicated by low probability *P*-value ($P<0.0001$) and the value of the adjusted determination coefficient (Adj $R^2=0.887$) (3). The 'Lack of Fit *F*-value' of 2.34 was not significant at all observed limits of variables for protease production, indicating that the model thus found fit may significantly describe the variation of the responses. The value of the determination coefficient ($R^2=0.9417$) demonstrated that only 5.83% of the total variations were not explained by the model. A lower value of coefficient of variation (CV=6.91%) showed that the experiments conducted were precise and reliable (1).

The significance of each coefficient in the model was established by estimating *P*-values (Table 3.). The quadratic effects (X_1^2, X_3^2) of KCl and gelatin had significant effect on protease production by model organism. These values were followed in *P*-values by interactive effect of $MgSO_4$ and gelatin (X_2 X_3), linear and quadratic effects of $MgSO_4$ (X_2, X_2^2) and interactive effect of KCl and $MgSO_4$ (X_1 X_2). The remaining probability values had less significant effect on the model.

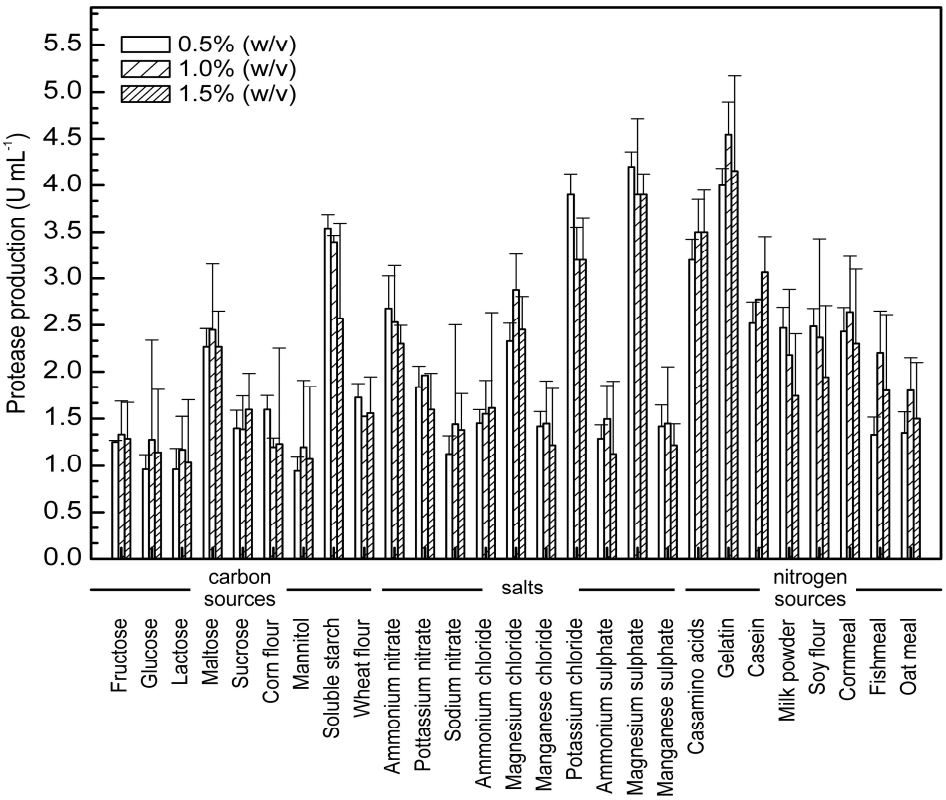

Figure 1. Effects of different nutrient sources and their concentration in culture media on average maximal protease production in cell cultures of *Haloferax lucent*ensis VKMM 007. Each bar represents the mean ± SD of three replicates.

Table 2. Central composite design and experimental values obtained

STD order	Run order	X_1 KCl	X_2 $MgSO_4$	X_3 gelatin	X_4 starch	Protease production (U mL^{-1}) Actual	Predicted
1	24	-1	-1	-1	-1	3.5	3.529
2	6	+1	-1	-1	-1	4.0	4.038
3	4	-1	1	-1	-1	6.2	5.987
4	21	+1	1	-1	-1	5.0	5.295
5	17	-1	-1	1	-1	4.3	4.220
6	25	+1	-1	1	-1	4.8	4.829
7	19	-1	1	1	-1	4.3	4.779
8	23	+1	1	1	-1	4.3	4.187
9	15	-1	-1	-1	1	3.8	4.037
10	14	+1	-1	-1	1	5.6	4.895
11	18	-1	1	-1	1	6.0	5.745
12	26	+1	1	-1	1	5.2	5.404
13	3	-1	-1	1	1	5.1	4.579
14	5	+1	-1	1	1	5.2	5.537
15	30	-1	1	1	1	4.3	4.387
16	2	+1	1	1	1	4.4	4.145
17	8	-2	-2	0	0	3.2	3.266
18	9	+2	-2	0	0	3.5	3.533
19	27	0	-2	0	0	4.6	4.866
20	13	0	2	0	0	6.1	5.933
21	1	0	0	-2	0	4.5	4.633
22	12	0	0	2	0	4.1	4.066
23	10	0	0	0	-2	5.8	5.516
24	11	0	0	0	2	5.6	5.983
25*	**7**	**0**	**0**	**0**	**0**	**6.8**	**6.350**
26	20	0	0	0	0	6.2	6.350
27	16	0	0	0	0	6.5	6.350
28	29	0	0	0	0	6.2	6.350
29	28	0	0	0	0	6.0	6.350
30	22	0	0	0	0	6.4	6.350

Table 3. ANOVA response for linear, quadratic and interactive effect of factors used in the model

Model term	Coefficient estimate	Standard error	*P*-value
Intercept	-6.350	0.160	-
X_1	0.067	0.079	0.4119
X_2	0.027	0.079	0.0042
X_3	-0.140	0.079	0.0931
X_4	0.120	0.079	0.1603
X_1^2	-0.740	0.074	< 0.0001
X_2^2	-0.240	0.074	0.0058
X_3^2	-0.034	0.074	< 0.0001
X_4^2	-0.500	0.074	0.0605
$X_1 X_2$	-0.150	0.097	0.0073
$X_1 X_3$	0.025	0.097	0.7996
$X_1 X_4$	0.088	0.097	0.3800
$X_2 X_3$	-0.480	0.097	0.0002
$X_2 X_4$	-0.190	0.097	0.0716
$X_3 X_4$	-0.037	0.097	0.7037

The quadratic mathematical model, which included all terms regardless of their significance level, can be given as Eq. (3):

$$Y_p=6.35+ 0.067\ x_1 +0.27\ x_2-0.14\ x_3+0.12\ x_4-0.30\ x_1\ x_2+0.025\ x_1\ x_3+0.088\ x_1x_4 -0.48\ x_2x_3 -0.19\ x_2\ x_4-0.037\ x_3\ x_4-0.74\ x_1^2-0.24\ x_2^2-0.50\ x_3^2-0.15x_4^2 \quad (3)$$

Where Y_p is the predicted protease concentration and x_1, x_2, x_3 and x_4, the coded variables of KCl, $MgSO_4$, gelatin and soluble starch, respectively.

This regression equation was solved by the method of Myers and Montgomery (9). Maximum protease production of 6.57 U mL^{-1} was predicted to be obtained in initial medium containing 5.14 g L^{-1} of KCl, 6.57 g L^{-1} of $MgSO_4$, 9.05 g L^{-1} of gelatin and 5.27 g L^{-1} of soluble starch.

The maximal protease concentration obtained experimentally was 6.50 U mL^{-1} and was very close to predicted response obtained at centre points (average of six centre points was 6.53 U mL^{-1}).

In order to further assess the effect of independent variables on the protease production by *Haloferax lucentensis* VKMM 007, two-dimensional contour plots and three-dimensional response surface plots were generated from the regression equation by keeping the two variables at zero and changing the other two variables with different combinations (Figure 2). These interactions indicated that previously predicted medium concentration values were optimal for maximal protease production. All the factor values of four variables were found to be present within the design space. These notion were futher supported by validation experiment conducted using the predicted values for the four variables studied which resulted in the maximal protease production of 6.80 U mL^{-1}. Finally, protease production was observed in optimized medium in NaCl concentration range from 5% to 25% (w/v). In these experiments, maximal protease production was obtained after 48 hours of cell growth and was highest in media supplemented with 20% (w/v) and 25% (w/v) NaCl with respective protease concentrations of 7.00 U mL^{-1} and 7.02 U mL^{-1}.

The time required for cell cultures to reach maximal protease production using optimized medium is significantly shorter than reported for other halophilic protease producers. Protease production in halophilic archaea *Halobacterium salinarum* and *Halobacterium* sp. PB 407 reached maximal levels after 96 hours of incubation (4,2), while cultures of *Halogeometricum* sp. TSS 101 supported maximal protease production after 86 hours of incubation (14). Compared to initial medium optimized medium allowed for a 3.95-fold increase in protease

production. This increase was further improved to 4.08-fold by increasing the concentration of NaCl in the medium to 20% (w/v) and 25% (w/v).

In conclusion, the experimental design presented in this study effectively defined optimal medium composition, which supported enhanced protease production in cultures of *Haloferax lucentensis* VKMM 007. Given the simplicity and low-cost of preparation of optimized medium, we consider the results of this study useful for highly efficient production of this halophilic protease on a bioreactor scale.

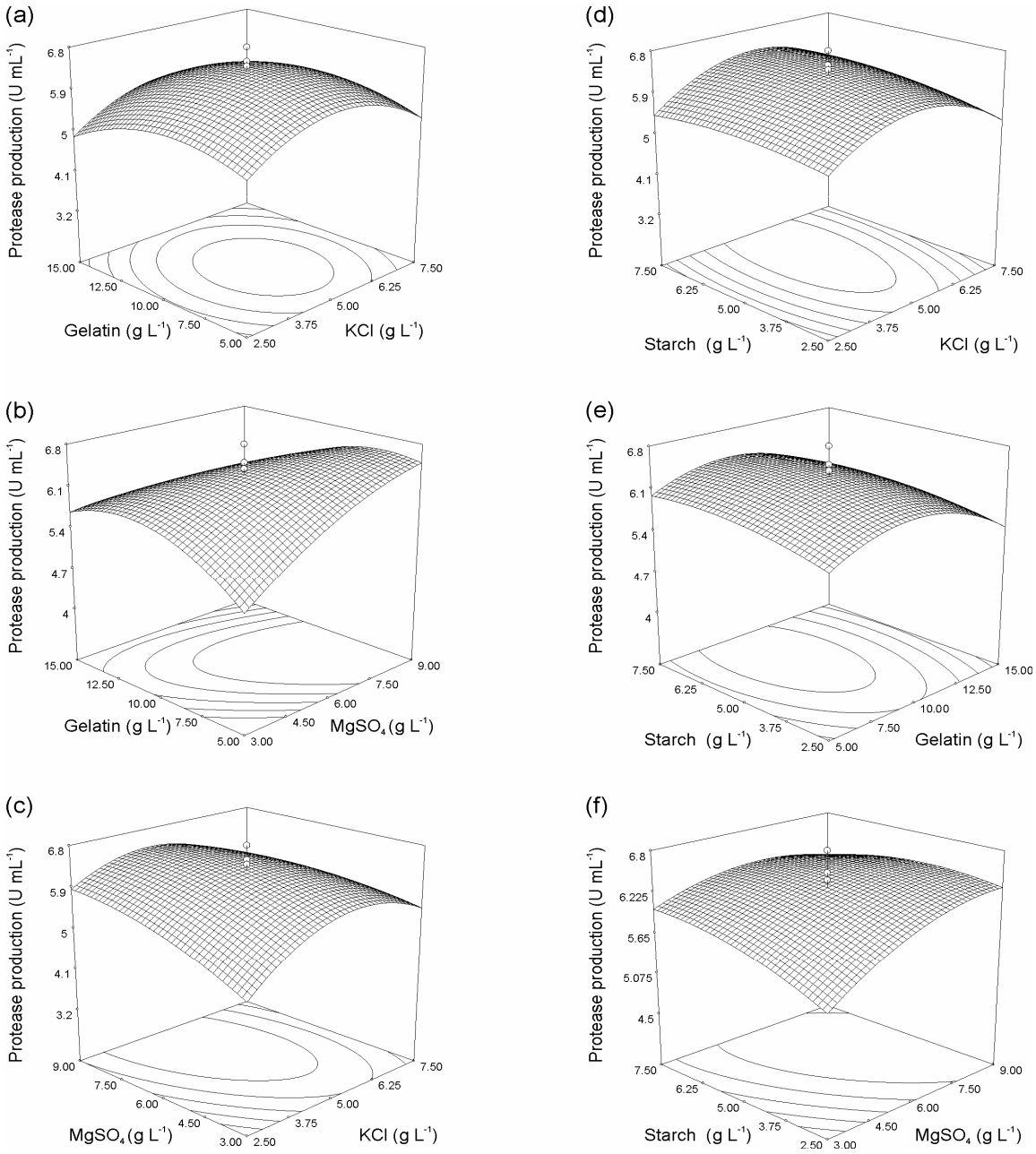

Figure 2. Response-surface and contour plots for the effects on protease production in cell cultures of *Haloferax lucentensis* VKMM 007. From top to bottom left panel: gelatin (X_3) and KCl (X_1); gelatin (X_3) and $MgSO_4$ (X_2); $MgSO_4$ (X_2) and KCl (X_1); right panel: soluble starch (X_4) and KCl (X_1), soluble starch (X_4) and gelatin (X_3), soluble starch (X_4) and $MgSO_4$ (X_2).

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