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Research Article

Optimization of microbial milk clotting enzyme production by *Aspergillus candidus* MTCC1989 using statistical method

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Abstract

Microbial milk clotting enzymes have been highly valued as calf rennet substitutes in the cheese industry. The present study was aimed to optimize the fermentation media components for the production of milk clotting enzyme by *Aspergillus candidus* MTCC 1989. A 5-level central composite design of response surface methodology was employed to optimize the fermentation media components namely yeast extract, skimmed milk powder, potassium di-hydrogen phosphate and initial pH. The various effects of media components on milk clotting enzyme production were fit into second order polynomial model and statistically analysed using student's t-test and Fisher's F-test for analysis of variance. The high value coefficient of determination (R^2=0.89) justified an excellent correlation between media components and milk clotting enzyme production, and the model fitted well with high statistical reliability and significance. The optimal concentration were yeast extract 1.5% (w/v), skimmed milk powder 0.87% (w/v), potassium di-hydrogen phosphate 0.11% (w/v) and an initial pH 6.1. The 774.19 MCU of milk clotting enzyme was obtained by *A. candidus* at the optimal conditions.

Keywords: Milk clotting enzyme; *Aspergillus candidus*; Statistical methods; Optimization.

Introduction

Rennet is an extract from the fourth stomach of young ruminants, such as cows, goats, and sheep. Rennet is an extract from the fourth stomach of young ruminants, such as cows, goats, and sheep. This extract contains a number of enzymes which are designed to help these animals digest their mother's milk. Rennet is usually made from the fourth stomach of young calves. The calves are milk fed and are usually less than 10 days old [1,2]. Owing to the limited availability of proper stomachs for rennet production, cheese makers have looked for other ways to coagulate the milk since at least Roman times. An increase in demand for cheese production worldwide per annum over the past 20 years – coupled with reduced supply of calf rennet, has led to a search for rennet substitutes, such as microbial rennet [3].

There are many sources of enzymes, ranging from plants, fungi and other microbial sources, that will substitute for animal rennet [4-7]. Cheeses produced from any of these varieties of rennet are suitable for lacto-vegetarians to consume. Many plants have coagulating properties. Greeks used an extract of fig juice to coagulate milk [8]. Other examples include fresen seeds, nettles, thistles, mallow and Ground Ivy. Enzymes from thistle or cynara are used in some traditional cheese production in the Mediterranean [3,9].

The cheese industry is seeking novel sources of enzymes for cheese production. microbial rennet have several advantages over animal rennet. They are easy to generate and purify and do not rely on the availability of animal material. The production of microbial clotting enzymes can be improved [10]. Moulds such as *Rhizomucor miehei, Mucor bacilliformis* and *Mucor miehei* used for commercial production of microbial rennet [11-14]. The flavour and taste of cheeses produced with microbial rennets tend towards some bitterness, especially after longer maturation periods [15]. Microbial rennet is suitable for vegetarians. Microbial milk clotting enzymes have long been highly valued as calf rennet substitutes in the cheese industry. At present, microbial rennet is used for one-third of all the cheese produced worldwide. A majority of global reports has predicted a boom in the cheese market with consumption projected to grow more than 20% between 2008 and 2015 [3,16]. Approximately

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33 % of global demand of cheese produced using microbial rennet [17,18]. The present study was aimed to produce microbial rennet by *Aspergillus candidus* MTCC 1989 and optimize the important media components media components namely yeast extract, skimmed milk powder, potassium di-hydrogen phosphate and initial pH using five-level Central Composite Design (CCD) of response Surface Methodology (RSM).

Material and methods

Microorganism

Aspergillus candidus MTCC 1989 was obtained from CSIR-Microbial Type Culture Collection and Gene Bank, IMTECH, Chandigarh, India.

Slant preparation

A. candidus MTCC 1989 strain was cultivated in yeast extract agar slants at 25°C for 7 days, refrigerated and sub-cultured periodically.

Preparation of inoculum culture

A. candidus MTCC 1989 strain was cultivated in skimmed milk powder-yeast extract agar slants at 25°C for 5 days.

Substrate preparation

Soya bean meal was purchased from local market Chennai, India was dried in oven at 98°C and finely powdered. The powder was sieved using the sieve shaker for 20 min. The sieved material in 120 mesh size was then used in the preparation of culture media.

Production culture for media optimization

The production media of 100 ml was prepared in 250 ml flasks using varying concentration of media components such as yeast extract (X_1), skimmed milk powder (X_2), potassium dihydrogen phosphate (X_3) and initial pH (X_4) as given in Table 1. All other media components are maintained constant as soya bean meal flour 1% (w/v), glucose 0.5%, magnesium sulphate 0.05% and mineral salt solution 150 µl. Microbial culture sample of 5 ml was collected after five days of incubation in a temperature controlled incubator shaker at 30°C and 150 rpm and filtered using Whatman No. 1 filter paper. The clear filtrate obtained was used as crude enzyme to determine milk clotting activity.

Media optimization using Response Surface Methodology

RSM is an efficient statistical technique for optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments. CCD is one of the response surface methodologies usually utilized to obtain data that fits a full second order polynomial model. The graphical representation of the model equation represent the individual and interactive effects of test variables on the response.The variables are coded to lie "±1" for factorial points, "0" for the centre points and "±2" for axial points. The CCD in actual unit of the 4 variables was (Table 1) developed using MINITAB 15.0 version software. The experimental result was used to find the optimum level of these variables. The various effects of these 4 variables on milk clotting enzyme production fit to the second order polynomial model according to equation 1.

$$Y=b_0+\sum b_i X_i+\sum b_{ii} X_i^2+\sum b_{ij} X_i X_j \quad \ldots\ldots (1)$$

Where Y is the response variable to be modeled, X_i and X_j are independent variable in coded units and b_i , b_{ii} , b_{ij} are the measures of the X_i , X_j , X_i^2 and X_i X_j of linear, quadratic and interaction effects respectively [19,20]. The second polynomial equation was maximized by a constraint search procedure using the MINITAB 15.0 version to obtain the optimal levels of the independent variables and the predicted maximum milk clotting enzyme production.

Estimation of milk clotting activity

The $CaCl_2$-SMP solution was pre-incubated for 10 min at 35°C. 5 ml of $CaCl_2$-SMP solution was added to 0.5 ml of enzyme sample and the time for first appearance of milk clots of was noted. The enzyme activity was reported in terms of milk clotting unit (MKU) [21].

Results and discussions

The yeast extract, skimmed milk powder, potassium dihydrogen phosphate and pH were selected for optimization using CCD. Experimental design was developed using five-level CCD for four variables and is given in table 1. The coefficients, t and p values for linear, quadratic and combined effects are given in the table 2, at 95% significance level. The p-values are used as a tool to check the significance of each of the coefficients, which in turn may indicate the pattern of the interactions between

the variable. The smaller the p-value more significant is the corresponding coefficient. It was observed that the coefficient for overall effect of the variables was highly significant ($p<0.001$). The individual effects of yeast extract ($p=0.003$), skimmed milk powder ($p<0.001$) and pH ($p=0.042$) and the quadratic effects of yeast extract ($p<0.001$), potassium dihydrogen phosphate ($p=0.037$) and pH ($p<0.001$) were also found to be highly significant. It was found from the student's t-test that there was no interaction effect between the variables.

The high R^2 (0.89) implies high degree of correlation between the observed and predicted values. The adjusted determination coefficient (Adj R^2) corrects the R^2 value for the sample size and the number of terms in the model. There are many terms in the model and the sample size is not very large, therefore the adjusted R^2 noticeably smaller than the determination coefficient R^2. The adjusted R^2 in this study was 0.79, which is close to the R^2 value. Hence the model was well fitted to represent the effect of these four variables on milk clotting enzyme production using CCD statistical optimization.

Table 1. Five Level CCD in actual unit of the variables for MCA, IU

Std. Exp.	Media Components (%w/v)				MCA, IU	
Order	YE	SMP	KH_2PO_4	pH	Experimental	Predicted
1	0.5	0.5	0.1	5.5	323.075	379.231
2	1.5	0.5	0.1	5.5	500.000	458.219
3	0.5	1.0	0.1	5.5	395.142	341.097
4	1.5	1.0	0.1	5.5	539.939	556.337
5	0.5	0.5	0.2	5.5	386.667	316.548
6	1.5	0.5	0.2	5.5	346.546	394.604
7	0.5	1.0	0.2	5.5	492.611	524.130
8	1.5	1.0	0.2	5.5	777.778	738.438
9	0.5	0.5	0.1	6.5	330.317	346.434
10	1.5	0.5	0.1	6.5	400.111	362.849
11	0.5	1.0	0.1	6.5	367.252	313.451
12	1.5	1.0	0.1	6.5	419.221	466.117
13	0.5	0.5	0.2	6.5	281.443	259.303
14	1.5	0.5	0.2	6.5	243.963	274.785
15	0.5	1.0	0.2	6.5	453.476	472.035
16	1.5	1.0	0.2	6.5	685.668	623.769
17	0.5	0.75	0.15	6.0	665.447	743.199
18	1.5	0.75	0.15	6.0	820.455	858.561
19	1.0	0.5	0.15	6.0	404.075	424.222
20	1.0	1.0	0.15	6.0	483.936	579.647
21	1.0	0.75	0.1	6.0	388.199	439.518
22	1.0	0.75	0.2	6.0	422.464	487.003
23	1.0	0.75	0.15	5.5	333.312	386.463
24	1.0	0.75	0.15	6.5	250.023	312.730
25	1.0	0.75	0.15	6.0	601.504	562.988
26	1.0	0.75	0.15	6.0	615.385	562.988
27	1.0	0.75	0.15	6.0	631.579	562.988
28	1.0	0.75	0.15	6.0	615.385	562.988
29	1.0	0.75	0.15	6.0	585.366	562.988
30	1.0	0.75	0.15	6.0	631.579	562.988
31	1.0	0.75	0.15	6.0	607.692	562.988

Analysis of variance (ANOVA) was used to test the significance and adequacy of the second order polynomial model. The ANOVA result of the model is given in table 3 at 95% confidence level. The regression model is highly significant, it is evident from the calculated F-value (F-model = 8.91) and probability value ($p< 0.001$). It is evident from the ANOVA that the linear ($p<0.001$) and quadratic effect ($p<0.001$) of the variables have greater influence on milk clotting enzyme production and have significant

influence (p = 0.047) due to the interaction effect of the variables. Hence the regression model given in equation 2 is the good prediction of the experimental results and the factor effects are real.

$$Y_{MCA} = 562.988 + 57.681\ X_1 + 77.713\ X_2 + 23.742\ X_3 - 36.866\ X_4 + 237.892\ X_1^2 - 61.053\ X_2^2 - 99.727\ X_3^2 - 213.391 X_4^2 + 34.063\ X_1X_2 - 0.233\ X_1X_3 - 15.643\ X_1X_4 + 61.429\ X_2X_3 + 1.288\ X_2X_4 - 6.112\ X_3X_4 \quad \ldots (2)$$

Table 2. Estimated regression coefficients for optimization of MCA, IU

Term	Coef	SE Coef	T	P	Confidence Level %
Constant	562.988	20.94	26.887	<0.001	
X_1	57.681	16.64	3.467	0.003	99.7
X_2	77.713	16.64	4.671	<0.001	100
X_3	23.742	16.64	1.427	0.173	82.7
X_4	-36.866	16.64	-2.216	0.042	95.8
X_1*X_1	237.892	43.82	5.429	<0.001	100
X_2*X_2	-61.053	43.82	-1.393	0.183	81.7
X_3*X_3	-99.727	43.82	-2.276	0.037	96.3
X_4*X_4	-213.391	43.82	-4.870	<0.001	100
X_1*X_2	34.063	17.65	1.930	0.071	92.9
X_1*X_3	-0.233	17.65	-0.013	0.990	1.0
X_1*X_4	-15.643	17.65	-0.886	0.388	61.2
X_2*X_3	61.429	17.65	3.481	0.003	99.7

Table 3. Analysis of Variance for optimization of MCA, IU

Source	Degree of Freedom	Seq Sum of Squares	Adj Sum of Squares	Adj Mean Square	F	P
Regression	14	621332	621332	44380.9	8.91	<0.001
Linear	4	203204	203204	50801.0	10.20	<0.001
Square	4	334646	334646	83661.6	16.79	<0.001
Interaction	6	83482	83482	13913.6	2.79	0.047
Residual Error	16	79718	79718	4982.4		
Lack-of-Fit	10	78093	78093	7809.3	28.84	<0.001
Pure Error	6	1625	1625	270.8		
Total	30	701050	1625			

The graphical representations of the interaction effect of the variables called the contour plots were developed using MINITAB 15.0 version and interaction between any two variables was studied on milk clotting enzyme production, keeping other two variables constant at their middle values. The shape of the response surface plots, elliptical or circular, indicates the interactions between the variables are significant or not. The circular shape of the contour plots in figures 1, 2 and 3 indicates that there was no significant interaction effects between these set of variables on milk clotting enzyme production.

The irregular shape of the contour plots in figures 4, 5 and 6 indicate significant interaction between pH and yeast extract, KH_2PO_4 and yeast extract, and skimmed milk powder and yeast extract. The regression equation was solved by optimizer in MINITAB 15.0 version software for global optimum value of the variables for production of milk clotting enzyme with maximum MCA.

The confirmation experiment was conducted at the predicted optimum values of the tested variables for validating the model under experimental conditions.

The experimental value of milk clotting enzyme activity obtained for predicted optimum conditions of the variables at standard cultivation conditions used in CCD was 774.19 IU. Milk clotting activity of 778.81 IU was predicted by the MINITAB 15.0 version software. The experimental and predicted values of milk clotting enzyme production showed a good agreement with one another, indicating that the CCD of response surface methodology was effective in media optimization for milk clotting enzyme production by *A.candidus* MTCC 1989.

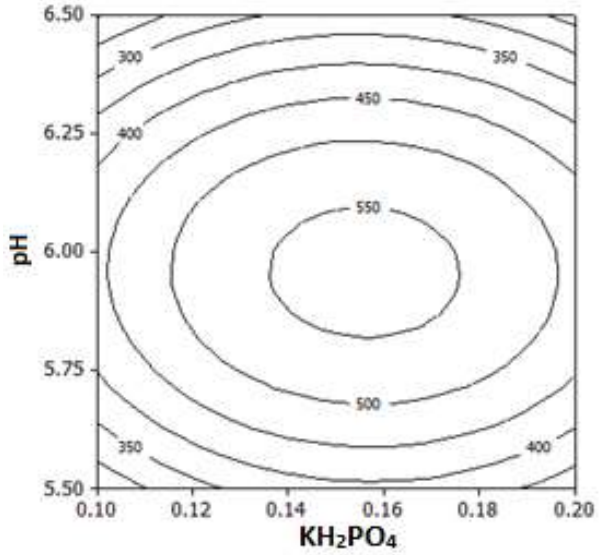

Figure 1. Response contour plot shows the interaction effect of pH and KH_2PO_4

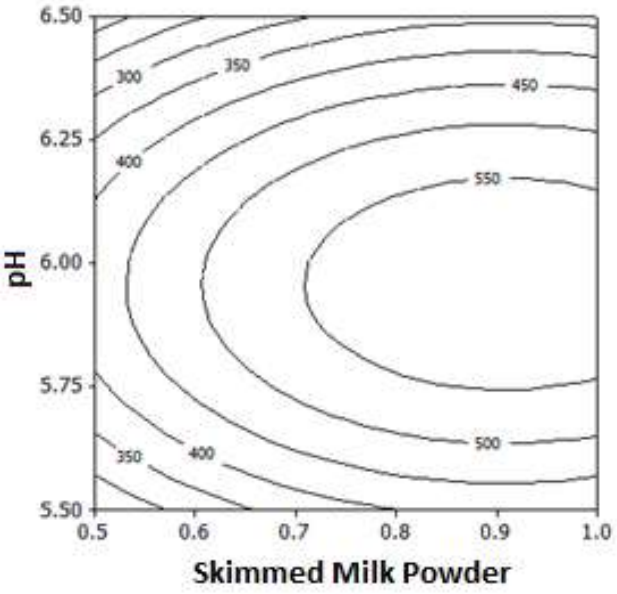

Figure 2. Response contour plot shows the interaction effect of pH and skimmed milk powder

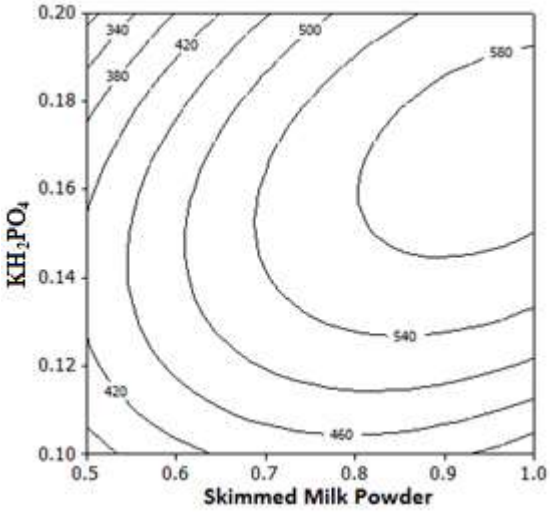

Figure 3. Response contour plot shows the interaction effect of KH_2PO_4 and skimmed milk powder

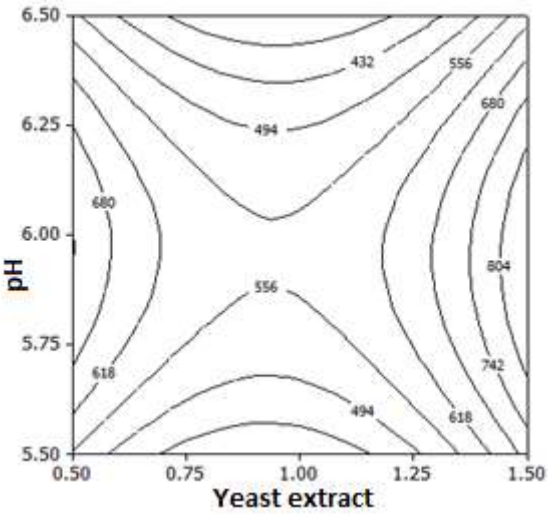

Figure 4 – Response contour plot shows the interaction effect of pH and yeast extract

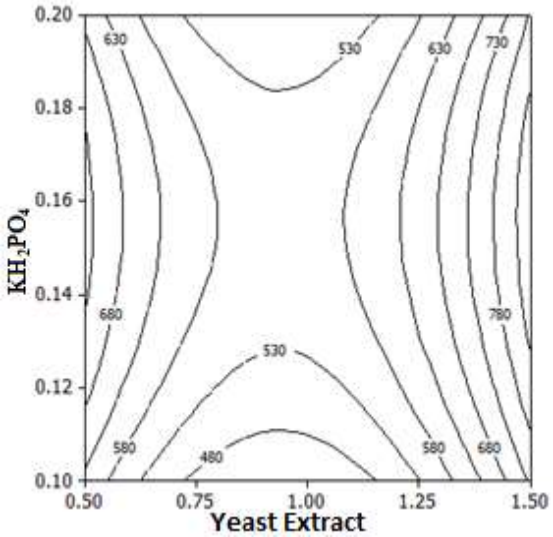

Figure 5. Response contour plot shows the interaction effect of KH_2PO_4 and yeast extract

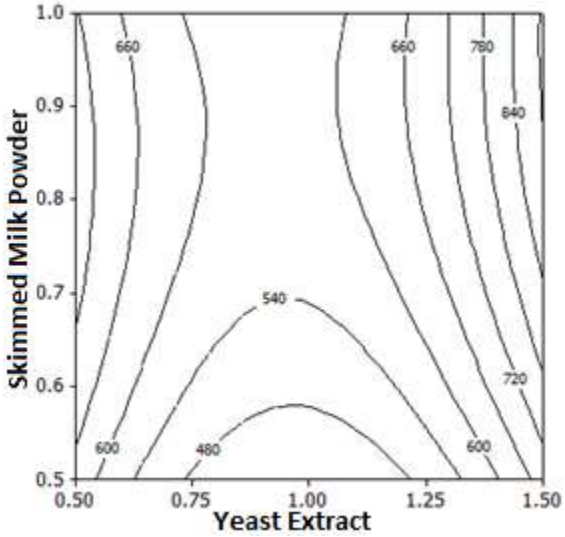

Figure 6. Response contour plot shows the interaction effect of skimmed milk powder and yeast extract

Conclusions

The statistical optimization of media components for the production of milk clotting enzyme by *A. candidus* MTCC 1989 was effectively studied using of design experiments. It was found that the interaction effect of the variables has no significant influence on milk clotting enzyme production. The predicted optimum value of the variables in actual unit is yeast extract - 1.5% (w/v), skimmed milk powder - 0.87% (w/v), potassium dihydrogen phosphate 0.11% (w/v) and an initial pH of 6.1. The experimental value of 774.19 IU and predicted value of 778.810 IU of milk clotting activity of the milk clotting enzyme produced showed good agreement with one another, indicating that the Central Composite Design of response surface methodology was effective in media optimization for milk clotting enzyme production by *A. candidus* MTCC 1989.

Conflicts of Interest

Authors declare no conflict of interest.

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