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Production of Milk Clotting Enzyme from Distiller's Sludge by *Penicillium camemberti* using Response Surface Methodology

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Abstract: In this study, the production of milk clotting enzyme was carried out by the Distillers yeast sludge using the fungal culture *Penicillium camemberti*. Effects of different substrates on the production of milk clotting enzyme were studied under stationary and shaking conditions. Highest milk clotting activity was observed in the medium containing casein under static conditions. Central composite design experiments werecarried out to optimize the parameters that brings outhigh yield milk clotting enzyme. Maximum milk clotting activity of 0.785units/mg was obtained at optimum process conditions namely initial substrate concentration 15 g/l, initial pH 5.9, temperature 39ºC and biomass concentration 15g/l. The Logistic model for cell growth and Leudeking-Piret model for product formation kinetics were evaluated for the prediction of experimental data .

Keywords: Milk clotting enzyme, Distiller's sludge, Response surface methodology, Cell growth and Product formation kinetics.

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I. INTRODUCTION

Enzyme utilization in the food, beverage, detergent, pharmaceutical, chemical, leather, paper, pulp, feed, and silk industries is remarkable. Proteases are an important group of enzymes which constitutes a large product segment in the global industrial enzymes market[1]. The use of enzymes in different industries increased due to industrialization and the different types of protease and amylase are randomly used in industries including food, textile, and paper[2].Proteases become important industrial enzymes because of their characteristics of biotechnological interest .

The rapid developments of food technology leads to increase the applications of enzyme technology create the availability of food grade enzymes. Enzymes for food applications are obtained from animal, plant and microbial sources. The majority of microbial industrial enzymes are extracellular hydrolases. Milk clotting enzymes produced by microorganisms have been developed industrially to replace calf rennet to improve the ratio of milk-clotting activity to proteolytic activity .Milk clotting enzyme is an important Protease enzyme plays vital role in the cheese manufacturing Industry. Traditional cheese production by using Bovine rennet is the oldest method.The growing demand for natural milk coagulants led to the necessity for rennet substitutes, promoting a search for new sources of milk clotting proteases [3]

In this paper, the fungal culture *Penicilliumcamembert*iis used for the milk clotting enzyme production which is used for the cheese making. Fungal enzymes contribute the good flavour and texture formation of the food products due to the enzymatic activities of protease and lipases[4] .The distillers yeast sludge is used as a substrate for the submerged production. The majority of the distillers uses molasses (from sugarcane) as a feed stock.[5] and disposes the yeast sludge as the waste which is mainly composed of the rich minerals proteins and rich carbon source, there is scope for using it advantageously as a good source for the industrial production of the valuable bioproducts[6].

Response surface methodology (RSM) is an empirical statistical method utilized for multiple regression analysis of quantitative data obtained from statistically designed experiments by solving the multivariate equations simultaneously. The response surfaces are the graphical representation of the quadratic equations could be applied to explain the individual and cumulative effect of the test variable response surfaces and to find out the interaction between the test variables.[7,8]

1.1 Kinetics and modelling

The performances of microbial systems in the fermentation process are stated by various kinetic models contain kinetics of growth, substrate uptake and product formation.[9]

1.1.1Logistic Growth Model

Logistic equation is a substrate independent model. The Logistic curve is sigmoidal and leads to a stationary population of size $x_s = 1/\beta$. Rate of growth of cell is proportional to the cell mass concentration present at that time. The rate will stop when the cell mass concentration reaches stationary phase. When the cell mass concentration is near the stationary phase rate will slow down.

$$x = \frac{x_o e^{kt}}{1 - \beta x_o \left(1 - e^{kt}\right)} \quad \ldots (1)$$

Where x_o is the initial biomass concentration (g/l) and t is time (h). Monod and the other models predict that the growth will stop only when the limiting substrate concentration is exhausted. In reality due to the accumulation of toxic metabolites or due to inhibition, the growth may stop even when substrate is present. These conditions are taken care of by the Logistic model. The advantage of this model that it provides the exponential phase and endogenous metabolic phase accurately.

1.1.2 Leudeking-Piret Kinetic Model

This model was originally developed for the formation of lactic acid by *Lactobacillus delbrucckii*. In fermentations, especially those involving secondary metabolites, significant product formation does not occur during the log phase where product formation is proportional to the growth rate of cells. The product formation occurs late in the log phase or in the stationary phase. One such behaviour is the Leudeking-piret kinetic model. This model combines both growth-associated and non-growth-associated models and proved extremely useful and versatile in fitting product formation data from much different fermentation.

$$p(t) - p_o - \beta_{LP}\left(\frac{x_s}{k}\right)\left[1 - \frac{x_o}{x_s}\left(1 - e^{kt}\right)\right] = \alpha_{LP}\left[x(t) - x_o\right] \quad \ldots (2)$$

where P(t) = product concentration at any time t (g/l), P_o = initial product concentration (g/l), X_s = the biomass concentration in stationery phase (g/l), X_o = initial biomass concentration (g/l), X(t) = biomass concentration at any time (g/l), and β α, & k = constants.

In the present study, three different medium namely basal medium, casein and lactose along with basal medium were studied under stationary and shaking conditions for the production of milk clotting enzyme by *Penicillium camemberti.* The aim of this study was to find out the optimum process conditions for the selected operating variables namely initial substrate concentration, initial pH, temperature and biomass concentration for the maximum production of milk clotting enzyme using dried distiller's sludge as substrate.

II. MATERIALS AND METHODS

2.1 Microorganism and its culture conditions

The bacterial culture *Penicillium camemberti(MTCC 418)* was obtained from Chandigarh, India. The microorganism was grown aerobically in enrichment media containing following composition in 1000 ml distilled water: Czapek concentrate, 10 ml; K_2HPO_4, 1.0 g/l; Yeast extract, 5.0 g/l; Sucrose, 30.0 g/l. The compositions of czapek concentrate in 100ml are: Sodium nitrite, 30.0 g; Potassium chloride, 5.0 g; Magnesium sulphate.$7H_2O$, 5.0 g; Ferrous sulphate.$7H_2O$, 0.1 g. The pH of the medium was adjusted to 6.0 using dilute Hydrochloric acid. This strain was incubated at 30^{o}C for 5 days and stored at 4^{o}C.

2.2 Materials

Fermentation experiments were performed using distiller's sludge as substrate, obtained from EID Parry India Ltd, Nellikkuppam, Tamil Nadu, India. The substrate was sun dried, powdered and stored for further use in the experiments.

2.3 Batch Submerged Fermentation Studies

Submerged fermentations were carried out in 250 ml Erlenmeyer flasks with 100 ml of production medium.. Known volume of 5 day old culture of *Penicilliumcamemberti*was transferred to each 100 ml of production medium in sterile conditions.The production was carried by both static and shaking conditions at 120 rpm. Experiments were repeated at least twice. Samples were taken from the solution at regular time intervals for the analysis of milk clotting activity, proteolytic activity, biomass concentration and protein content.

The effect of different medium components on milk clotting enzyme production was investigated using three different fermentation medium components namely plain basal medium (S1), casein (S2) and lactose (S3) along with the basal medium. The culture was incubated at 30^{o}C for 7 days under shaking and stationary conditions. All the experiments were carried out in duplicates.

2.4 Experimental Design and Statistical Analysis

The factors affecting the production of milk clotting enzyme from distiller's sludge by *Penicillium camemberti*was studied using Central Composite Design (CCD) experiments. The initial substrate concentration

(A) g/l, initial pH (B), temperature (C) °C and biomass concentration (D) g/l were chosen as the independent variables as shown in Table 1. Milk clotting activity (Y) was chosen as the dependent output variable. An orthogonal 2^4 full factorial central composite design with eight star points (α =2) and seven replication at the centre point, all in duplicates, resulting in a total of 31 experiments were used to optimize the chosen key variables for the production of Milk clotting enzyme in a batch reactor.

The experiments with various initial substrate concentrations namely 5,10,15,20 and 25g/l, different initial pH values of 5.0, 5.5, 6.0, 6.5 and 7.0, different temperatures of 30, 35, 40, 45 and 50°C and five different biomass concentrations of 3.0, 6.0, 9.0, 12.0 and 15.0 g/l were employed and varied simultaneously to cover the combinations of variables in the design. The range and the levels of the experimental variables investigated in this study were given in Table 1. The chosen independent variables used in this experiment were coded according to Eq. (3):

$$x_i = \frac{X_i - X_o}{\Delta x} \quad \ldots (3)$$

Where x_i is the coded value of the i^{th} variable, $X_{i\,is}$ the uncoded value of the i^{th} test variable and X_o is the uncoded value of the i^{th} test variable at the centre point

The behaviour of the system is explained by the following second- degree polynomial Eq. (4):

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad \ldots (4)$$

Where Y is the predicted response, β_o is the offset term, β_i is the coefficient of linear effect, β_{ii} is the coefficient of squared effect and β_{ij} is the coefficient of interaction effect. This regression model can be used to estimate the elliptical contours of a constant surface.

A statistical design package, Minitab 16 was used for regression analysis of the data obtained and to estimate the coefficients of the second-degree polynomial equation. The equations were validated by the statistical tests called the analysis of variance (ANOVA), to determine the significance of each term in the equation fitted and to estimate the goodness of fit in each case. Response surfaces were drawn to determine the individual and interactive effects of test variables on milk clotting activity.

2.5 Preparation of the Crude enzyme

The fermented medium was filtered to separate the biomass from the culture filtrate using whatman no 40 filter paper. The filtrate was centrifuged at 4°C for 10 min at 10000 rpm in the cooling centrifuge. Then the supernatant was used for the enzyme assays.

2.6 Analysis of crude enzyme

2.6.1 Estimation of Milk clotting activity

Milk clotting activity was determined by the method explained by Arimaet al[10] and Balls et al.[11] using 0.1 (w/v) of rennin std. The substrateconcentration is 10g of skimmed milk powder in 0.01 moles. calcium chloride. The reaction mixture contains 5 ml of skim milk and 1ml of enzyme and kept at 37°C. The curd formation was observed by manually rotating the test tube from time to time and the end point is the semi liquefied film appears on the side of the test tube above the milk. The clotting time was noted.

$$MCU/mg = \frac{M}{T(\text{min}\,utes)xW(g)} \quad \ldots (5)$$

Where M is the milk factor, T is the clotting time of sample (min) and Wis the grams of enzyme added to the substrate in 2.0 ml aliquot (g wt. x 2)

2.6.2 Estimation of Proteolytic activity

Proteolytic activity was determined by the universal protease activity assay using casein as a substrate. The reaction mixture containing 5 ml of 0.65% preincubated casein solution at37°C for 10min and 1ml of enzyme added to standard and crude .The tubes were incubated for 10 min at 37°C. 5 ml of TCA was added to stop the reaction and incubated at 37°C for 30 min. Tyrosine standard was set up (0.2mg/ml) in the range of 0.1-0.5ml and made up to 2ml with distilled water. The test solutions were centrifuged at 4°C at 10000 rpm for 10 min and the 2ml of aliquots were used for finding Proteolytic activity. To all the tubes (including standard), 5 ml of sodium carbonate, 1ml of Folin's phenol was added and incubated at 37°C for 30 min and the optical density was measured at 660 nm using UV-Biospectrophotometer, which directly expresses the Proteolytic activity[12,13]

$$Units/ml \quad enzyme = \frac{(\mu\text{mole} \quad \text{tyrosine} \quad \text{equivalents} \quad \text{released})X(11)}{(1)X(10)X(2)} \quad \ldots (6)$$

Where 11 is the total volume of assay(ml), 10 is the time of assay as per the unit definition (min), 1is the volume of enzyme used(ml) and 2is the volume used in colorimetric determination(ml).

2.6.3 Determination of Protein

Protein was estimated by Lowry method [14]using BSA (200µg per ml concentration) as a standard. 0.2 to 1.0 ml of the working standards and 0.2 ml of the unknown crude sample were taken in a series of test tubes. The volume was made up to 1 ml with distilled water. 5 ml of the alkaline copper reagent was added to all the tubes and incubated for 10 min at room temperature Then 0.5 ml of Folin 's phenol reagent was added to all the tubes and incubated at dark room for 30 min and the optical density was measured for 660 nm.

2.6.4 Estimation of Biomass concentration

Samples from the production medium were filtered through whatmann no .40 filter paper to separate the biomass. The settled biomass was collected and dried and expressing the dry weight as grams per litre of growth medium.

III. RESULTS AND DISCUSSION

3.1Effect of different medium components on milk clotting enzyme production

In order to optimize the medium components and culture conditions capable of inducing high milk clotting and low proteolytic activities, three different media were tested. It was found that static conditions influenced the growth of *Penicillium camemberti* thereby increasing milk clotting enzyme production. Fig 1 shows the maximum biomass concentration of 40.5g/l was obtained in presence of casein, followed by 36g/l culture in plain basal medium, while lactose had the least biomass yield of 35.3g/l culture medium under staticconditions. Fig 2 clearly indicates that the addition of casein provided higher milk clotting activity than lactose & plain basal medium andit was proved that the Distillers yeast sludge with casein is best medium for maximum milk clotting enzyme production under static condition. The combination of casein with the substrates is an enhancer for the enzyme coagulant[15]High milk clotting activity of 0.779units/mg and low proteolytic activity of 0.60units/mg was observed in S2, presence of casein stimulates the milk clotting activity and metabolism of the microorganism during the fermentation process.

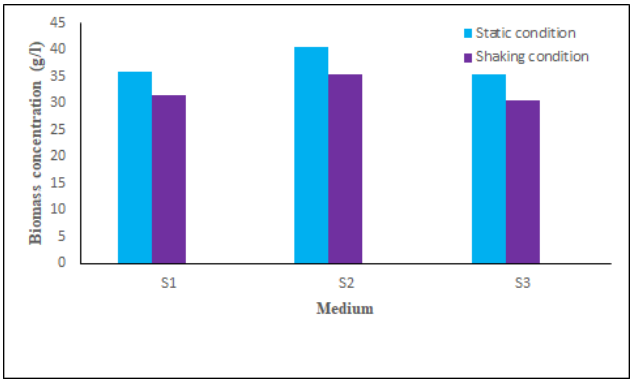


Fig 1 Effect of different medium components on biomass concentration by *Penicillium camemberti*

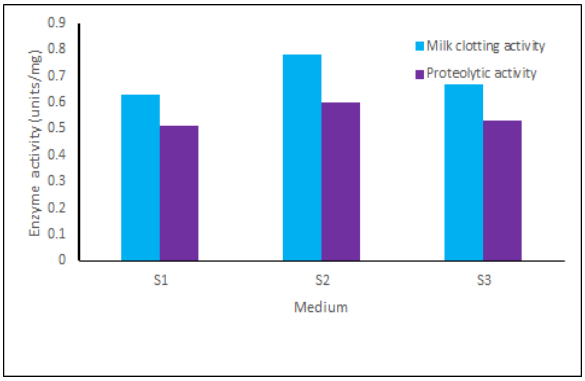


Fig 2 Effects of different medium components on milk clotting enzyme production by *Penicillium camemberti*

3.2 Central composite design and optimization using response surface methodology for the production of milk clotting enzyme

The coded values of the independent variables along with observed responses in each case were given in Table 2. By applying multiple regression analysis, a predictive quadratic model was fitted with experimental results, and the equation for the production of milk clotting enzyme was in the form of the following equation:

$$Y=0.685-0.009A-0.037B-0.014C+0.022D-0.053A^2-0.025B^2-0.106C^2+0.011D^2-0.024AB+0.011AC-0.022AD+0.023BC+0.006BD-0.007CD \quad \ldots (7)$$

where Y is the milk clotting activity (units/mg), A is the initial substrate concentration (g/l), B is the initial pH, C is the temperature (^oC) and D is the biomass concentration (g/l).. The milk clotting observation during enzyme analysis for all 31 experimental run were given in Table 3. It was found that the milk clotting activity mainly depends on clotting time and enzyme concentration. Complete settling with good texture was observed after 5 min when the pH was maintained at 6.0 and all other parameter values were low level. And the complete clotting with fine texture was observed within 2 min .at slightly higher level of temperature and the other

variables were at zero levels. Complete clotting with fine curd within 3 min. was observed at minimum substrate concentration with the p H range between 5.5 to 6.0 at the temperature range of 35-40.

Table 1 Central composite design for the production of milk clotting enzyme by *Penocilliumcamaberti*

Independent Variable	Range and Level				
	-2	**-1**	**0**	**+1**	**+2**
Initial Substrate Concentration (g/l) (A)	10	20	30	40	50
Initial pH (B)	5.0	5.5	6.0	6.5	7.0
Temperature(^{o}C) (C)	30	35	40	45	50
Biomass Concentration (g/l) (D)	3.0	6.0	9.0	12.0	15.0

A = Initial Substrate Concentration (g/l)
B = Initial pH
C = Temperature (^{o}C)
D = Biomass Concentration (g/l)

Table 2 Full factorial central composite design matrix of orthogonal values along with observed responses for the production of milk clotting enzyme

Run No.	Independent Variables Orthogonal Values				Milk Clotting Activity (units/mg)	
	A	**B**	**C**	**D**	**Experimental**	**Predicted**
1	0	0	0	0	0.600	0.569
2	0	0	0	0	0.600	0.590
3	1	1	1	1	0.700	0.684
4	-1	-1	-1	-1	0.600	0.549
5	-2	0	0	0	0.680	0.775
6	-1	1	-1	1	0.480	0.538
7	-1	1	1	1	0.685	0.656
8	0	0	0	0	0.600	0.514
9	1	-1	-1	-1	0.436	0.452
10	1	-1	1	1	0.685	0.685
11	-1	-1	1	-1	0.685	0.685
12	1	-1	1	-1	0.400	0.404
13	0	0	0	0	0.685	0.685
14	0	0	0	0	0.400	0.507
15	1	1	-1	-1	0.480	0.430
16	0	0	0	0	0.685	0.685
17	0	-2	0	0	0.533	0.460
18	1	-1	-1	1	0.600	0.588
19	1	1	-1	1	0.533	0.549
20	-1	1	-1	-1	0.685	0.685
21	-1	-1	1	1	0.400	0.460
22	1	1	1	-1	0.240	0.289
23	0	0	0	0	0.600	0.460
24	-1	1	1	-1	0.428	0.491
25	0	0	-2	0	0.200	0.230
26	0	0	2	0	0.685	0.685
27	0	0	0	-2	0.533	0.451
28	0	0	0	2	0.400	0.451
29	2	0	0	0	0.685	0.629
30	-1	-1	-1	1	0.685	0.685
31	0	2	0	0	0.436	0.522

Table 3 Central composite design matrix of orthogonal values along with respective observed milk clotting observation

Run No.	Independent Variables Orthogonal Values				Milk Clotting Observation
	A	B	C	D	
1	0	0	0	0	Immediate milk clotting along the sides of the test tube and complete ,fine curd after 5 min
2	0	0	0	0	Immediate milk clotting along the sides of the test tube and complete ,fine curd after 5 min
3	1	1	1	1	Immediate floccules appears along the sides of the test tube but no complete milk clotting after 30 min
4	-1	-1	-1	-1	Slight precipitate along the sides of the test tub and complete milk clotting after30 min but no fine curd formation
5	-2	0	0	0	Slight milk clotting on the upper layer of the tube immediately and complete milk clotting with fine curd formation after 20 min
6	-1	1	-1	1	Immediate milk clotting on the top of the tube and complete ,fine curd after 6 min
7	-1	1	1	1	Immediate precipitate along the sides of the test tube but no complete milk clotting
8	0	0	0	0	Immediate milk clotting along the sides of the test tube and complete ,fine curd after 5 min
9	1	-1	-1	-1	Milk clotting is formed after 3 min and complete fine curd formed after 15 min
10	1	-1	1	1	Slight precipitate along the test tube after the addition of the enzyme and fine curd formation after 10 min
11	-1	-1	1	-1	Slight precipitate on the upper layer after the addition of the enzyme but no complete milk clotting after 30 min
12	1	-1	1	-1	Immediate milk clotting on the upper layer and complete clotting with fine curd after 7 min
13	0	0	0	0	Immediate milk clotting along the sides of the test tube and complete ,fine curd after 5 min
14	0	0	0	0	Immediate milk clotting along the sides of the test tube and complete ,fine curd after 5 min
15	1	1	-1	-1	Slight precipitate along the sides of the test tube but no fine curd formation after 30 min
16	0	0	0	0	Immediate milk clotting on the top of the tube and complete milk clotting with fine curd after 6 min
17	0	-2	0	0	Immediate milk clotting. grainy texure and no fine curd after 30 min
18	1	-1	-1	1	Immediate milk clotting on the top of the tube and complete ,fine curd after 7 min
19	1	1	-1	1	Milk clotting solution is formed after 3 min and complete milk clotting with fine curd formation after 5 min
20	-1	1	-1	-1	Slight granules on the sides of the tube after 2 min and complete milk clotting with fine curd formation after 35 min
21	-1	-1	1	1	Immediate milk clotting and complete , fine curd formation after 7 min
22	1	1	1	-1	Slight precipitate along the sides of the test tube and complete milk clotting with fine curd formation after 10 min
23	0	0	0	0	Immediate milk clotting along the sides of the test tube and complete ,fine curd after 6 min
24	-1	1	1	-1	Immediate precipitate along the sides of the test tube and no complete milk clotting after 30 min
25	0	0	-2	0	No proper reaction immediately after the addition of the enzyme and slight granules formed after 10 min
26	0	0	2	0	No reaction occurs immediately after the addition of enzyme but complete milk clotting with fine curd after 2 min
27	0	0	0	-2	Immediate precipitate grainy texure and complete milk clotting after 30 min
28	0	0	0	2	Slight precipitate on the upper layer but no curd formation after 30 min
29	2	0	0	0	Slight precipitate on the upper layer and complete milk clotting ,fine curd formation after 20 min
30	-1	-1	-1	1	Precipitated immediate milk clotting and complete milk clotting with fine curd formation after 5min
31	0	2	0	0	Slight milk clotting on the upper layer after the addition of the enzyme and not settled after 30 min

Table 4 Significance of regression coefficients for the production of milk clotting enzyme using Minitab 16 software

Model Term	Parameter estimate (Coefficients)		
Constant	0.685	23.566	0.000
A	-0.009	-0.616	0.541
B	-0.037	-2.368	0.031
C	-0.014	-0.945	0.359
D	0.022	1.455	0.165
A *A	-0.053	-3.716	0.002
B * B	-0.025	-1.795	0.092
C * C	-0.106	-7.401	0.000
D * D	0.011	0.769	0.453
A * B	-0.024	-1.268	0.223
A * C	0.011	0.611	0.550
A * D	-0.022	-1.157	0.264
B * C	0.023	1.242	0.232
B * D	0.006	0.345	0.735
C * D	-0.007	-0.377	0.711

A, B,C,D = Linear effects
A^2, B^2, C^2, D^2 = Squared effects

AB, AC, AD, BC, BD, CD = Interaction effects
B = Significant
A * A = Significant
B * B = Significant
C * C = Significant

Table 5Analysis of Variance (ANOVA) for the selected quadratic model for the Production of milk clotting enzyme

Sources of variation	Sum of squares	Degrees of Freedom	Mean square	F	P
Regression	0.483	14	0.034	5.84	0.001
Linear	0.053	4	0.013	2.25	0.109
Square	0.400	4	0.100	16.93	0.000
Interaction	0.030	6	0.005	0.85	0.548
Residual error	0.094	16	0.005		
Total	0.578	30			

Square = Significant

The student t distribution and corresponding p values, along with the parameter estimate were given in Table 4.The linear effect of pH was found to be significant. The squared effects of theparameters(A^*A,B^*B,C^*C)were also found to be significant and the coefficient of the effect of temperature($p = 0.0001$)was found to be highly significant. The statistical significance of each term in the quadratic model was validated by the statistical tests called the Analysis-of-variance (ANOVA) and the results were given in Table 5. ANOVA of the regression model was highly significant and it was evident from the calculated F value (5.84) and a very low probability. The coefficient for the squared effect was highly significant ($p=0.0001$) when compared with the linear and interactive effects.

Response surface contour plots describe the relationship between the response and experimental levels of each variable and These plots explain the type of interaction between test variables and help to obtain the optimum conditions[16].Fig3 to 5 shows the response surface plots against each of the independent variables while keeping the other variables at their '0' levels. The maximum predicted yield was indicated by the surface confined in the smallest curve of the response surface diagram. It was evident from the circular nature of the contour that the interaction between the individual variables is not significant. The elliptical nature of the contour indicates that this interaction is significant on the response. The response surfaces can also find the optimum range of process variables. Fig 6.clearly show the photographs of milk clotting activity using (a)standard rennin enzyme and(b) fungal rennin.

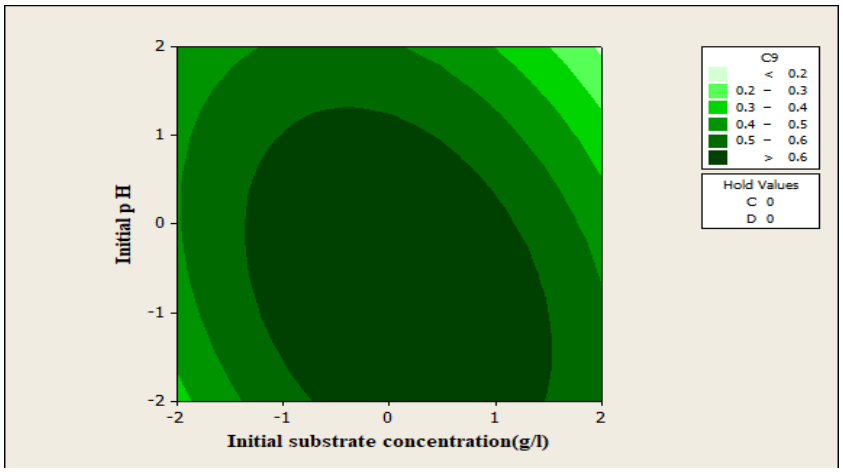


Fig 3 Response surface contour plot showing interactive effect of initial substrate concentration and initial pH on the production of milk clotting enzyme

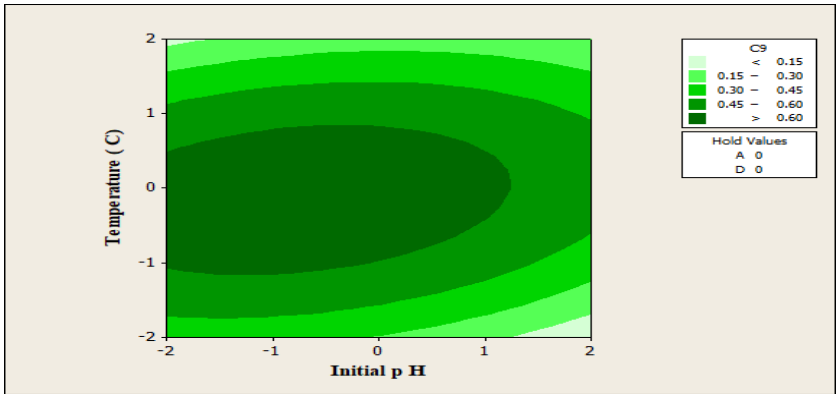


Fig 4 Response surface contour plot showing interactive effect of initial pH and temperature on the production of milk clotting enzyme

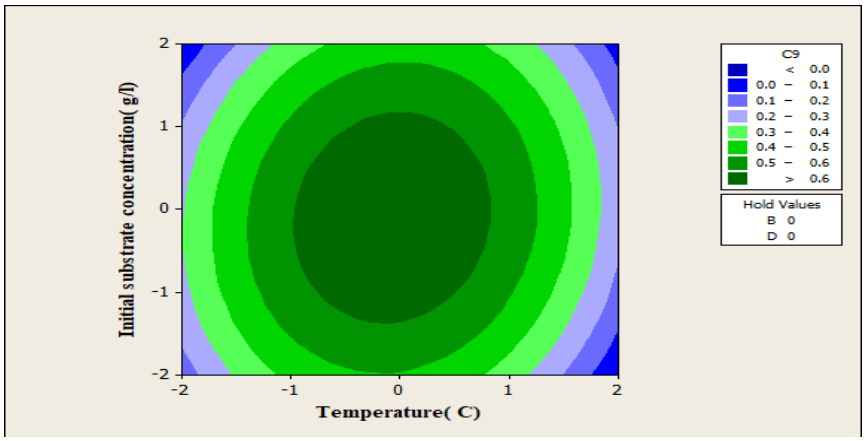
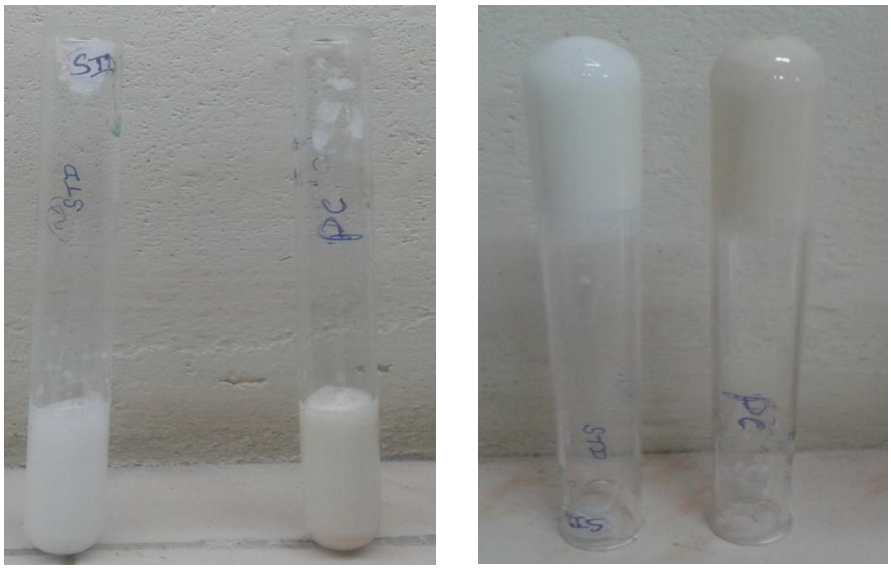


Fig 5 Response surface contour plot showing interactive effect of temperature and initial substrate concentration on the production of milk clotting enzyme



(a) (b) (a) (b)

(a) Standard Rennin (b) Fungal Rennin

Fig 6 Milk clotting observation for the produced enzyme using distiller's sludge and Standard Rennin (Calf Chymosin)

To validate the optimal parameters, confirmatory experiments were carried out by lab scale production. The observed results were compared with the predicted results. The process conditions for the maximum production of milk clotting enzyme by *Penicillium camemberti*under optimized conditions were given in Table 6. Milk Clotting Activity 0.785units/mg(MCA), Proteolytic Activity0.610units/mg (PA), the ratio MCA/PA1.28 and protein content 0.882mg/ml were found under optimum conditions . These values agree with the values from the response surface analysis (MCA=0.7941units/mg) confirming that the RSM using statistical design of experiments can be effectively used to optimize the process parameters and to study the importance of individual, cumulative and interactive effects of the test variables in milk clotting enzyme production.

Table 6 Optimum values of variables obtained from regression equations for the production of milk clotting enzyme by *Penicillium camemberti*

Parameter	Optimum value for milk clotting enzyme production
Initial Substrate Concentration(g/l)	25g/l
Initial pH	5.9
Temperature(^{o}C)	39
Biomass Concentration (g/l)	15g/l
Milk Clotting Activity (units/mg)	0.7941

3.3 Kinetics and Modeling

The kinetics of milk clotting enzyme production by *Penicillium camemberti* were studied under optimum process conditions obtained from RSM and the modeling was attempted using logistic and Leudeking-piret kinetic model. Fig.7 shows that there is a good agreement between the experimental data with the simulation data and the Logistic model appeared to provide adequate representation of growth and fermentation kinetic of *Penicillium camemberti.* The kinetic parameters of logistic equation constants Kc and β were found to be 0.08 $h^{-1}$and 0.0165g/l respectively. The experimental biomass concentration is well fitted with predicted biomass concentration with high regression coefficient 0.9966 and it is most suited for milk clotting enzyme production from distiller's by *Penicillium camemberti.*

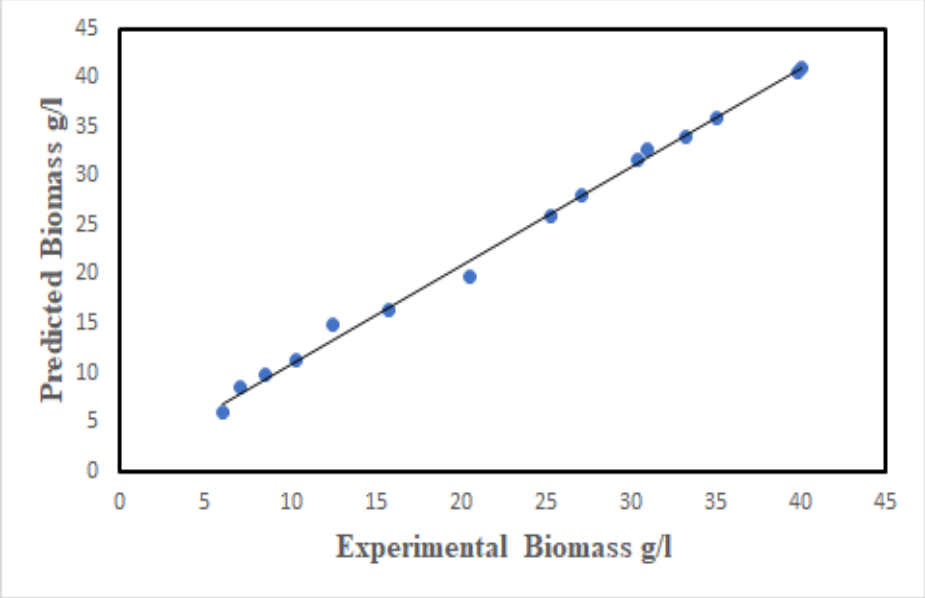


Fig 7 Comparison between experimental results and predicted data in logistic growth model

Fig 8 shows the experimental and predicted product formation rate for milk clotting enzyme production using Leudeking-piret model. The kinetic parameter values of β_{LP} and α_{LP} were found to be 0.0011 and 0.021 respectively. The constants indicate that growth associated product formation depends on biomass growth and

milk clotting enzyme. The experimental data fitted with predicted product formation rate with high regression coefficient of 0.9934

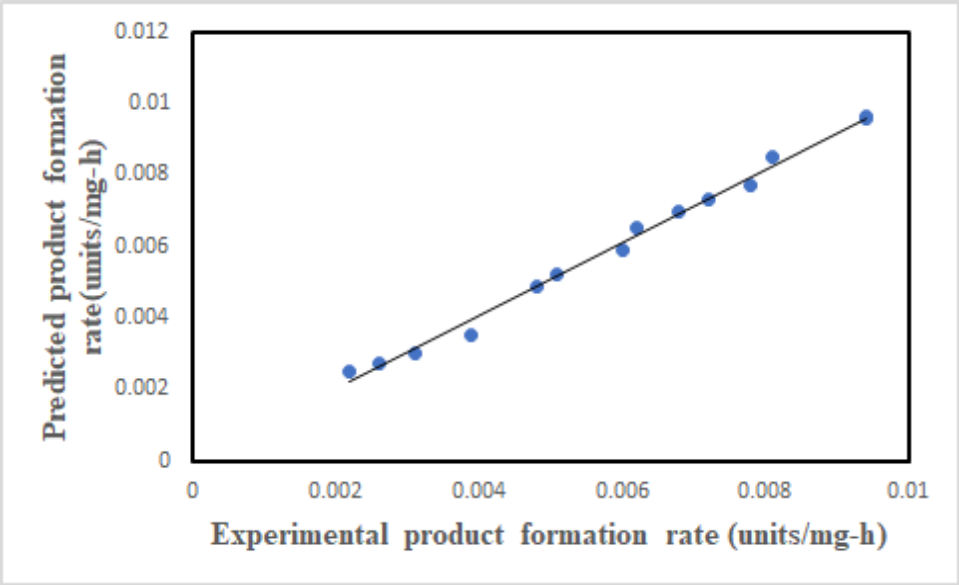


Fig 8 Correlation between experimental product formation rate and predicted product formation rate in Leudeking-piret kinetic model

IV. CONCLUSIONS

The Distillers sludge with casein shows the high milk clotting activity and it is an effective substrate for the production of milk clotting enzyme by *Penicillium camemberti.* The results reported that the distillers sludge medium containing casein under static conditions enhanced the milk clotting activity of 0.785 units/mg with low proteolytic activity 0.61units/mg.Statistical experimental design is an effective tool for studying the influence of process parameters on milk clotting activity. Logistic model and Leudeking-Piret model were found to represent the experimental data of cell growth and product formation kinetics. The results recommended that the distillers sludge is the high nutrient substrate for the production of milk clotting enzyme by the fungal culture*Penicillium camemeberti.*

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The authors have no conflicts of interest to declare.

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