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# Production of Milk Clotting Enzyme in Submerged Fermentation with *Streptococcus Lactis* by Using Whey Medium

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**Abstract:** In this study, utilization of whey for the production of milk clotting enzyme by Streptococcus lactis was carried out under submerged fermentation. Effects of different medium components for the production of milk clotting enzyme were determined under stationary and shaking conditions. Highest milk clotting activity was observed in the whey medium containing casein under shaking conditions. Central composite design experiments were carried out to examine the mutual interaction between the variables and to determine the optimal values that brings out high yield milk clotting enzyme. Maximum milk clotting activity of 0.4975 units/mg was obtained at optimum process conditions namely initial substrate concentration 29 g/l, initial pH 6.2, temperature 39.9ºC and biomass concentration 1.6 g/l.

**Keywords:** Milk clotting enzyme, Whey, Response Surface Methodology, Milk clotting activity, Proteolytic activity.

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## I. INTRODUCTION

Milk clotting enzyme is known as Rennet which is composed of rennin and pepsin. Rennin with high milk clotting activity and low proteolytic activity is the potent enzyme source which is commercially acceptable for cheese making in the food processing Industries. The calf rennet used for cheese making is the oldest method and it is extracted from the fourth stomach of young calves. Due to the legal problems against the animal sacrifice for the research purpose leads to the new search for alternate rennet production from plant and microbial sources.The present study deals with the Production of Milk clotting enzyme by *Streptococcus lactis* using whey water as a medium for the submerged fermentation. The *Streptococcus lactis* is the bacterial culture which is widely used in dairy industries.

Whey contains highly nutritious constituents and the most valuable components are the whey proteins which are designated superior to most of the other proteins such as egg, beef, casein and soya proteins in nutritive value [1]. Gupta *et al.* [2] reported that whey proteins are generally regarded as safe for food applications [3,4] because of their good nutritional and functional properties. Whey proteins are next to egg protein in terms of nutritive value. Whey Lactose is the milk sugar which is the primary milk constituent in whey which contributes around 80-90% of whey solids. The major mineral components in liquid whey are the mineral cations like sodium, potassium, calcium and magnesium as well as anions like chlorine, citrate and phosphate. [5] Whey Proteins exhibit excellent functional properties such as solubility, foaming, emulsifying, gelling and water binding etc. apart from their nutritional and therapeutic value [6,7].

Because of the presence of large part of organic constituents, the biological oxygen demand (BOD) of whey is very high (40,000-50,000 mg/kg)leads to a major burden of disposal as waste through the regular channel. In recent times, the environmental regulations have become severe across the world necessitating the treatment of whey prior to disposal through sewage system. To eradicate the problems involved with the whey disposal, efforts are being made towards product diversification using whey components without much change in the existing infrastructure. This will be quite feasible to reduce the pollution.

In the present study, different medium namely basal medium, casein and sucrose along with basal medium in whey medium and plain whey were studied under stationary and shaking conditions for the production of milk clotting enzyme by *Streptococcus lactis.* 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 whey as substrate by Response surface methodology.

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 to explain the individual and cumulative effect of the test variable response surfaces and to find out the interaction between the test variables [8,9].

## II. MATERIALS AND METHODS

**2.1 Microorganism**.

The bacterial culture *Streptococcus lactis(NCIM 2114)* was obtained from NCL Pune, India.. This culture was maintained by sub culturing periodically at 30°C for 24 hours and stored at 4°C.

**2.2 Growth and Production medium**

The microorganism was grown aerobically in MRS media containing following composition in 1000 ml distilled water: protease peptone, 10g; yeast extract, 5g; Beef extract, 10g; dextrose, 20g; tween 80, 1.0g; ammonium citrate, 2.0g;sodium acetate, 5.0g; Magnesium sulphate, 0.1g; Manganese sulphate, 0.05g; Dipotassium phosphate, 2.0g. The pH of the medium was adjusted to 6.5 using dilute hydrochloric acid, incubated at 30°C for 24 hours and stored at 4°C.

The production was carried out by using known volume of 1 day inoculum in the above medium using whey at 30'C both static and shaker at 120 rpm for 3 days. Fermentation medium of above composition namely plain whey medium, Sucrose and Casein along with the basal medium in whey and plain basal medium were used for this study. Samples were taken from the solution at regular time intervals for the analysis of milk clotting activity, proteolytic activity, biomass concentration and protein content. All experiments were carried out in duplicates.

**2.3 Experimental Design and Statistical Analysis**

The factors affecting the production of milk clotting enzyme from whey by *Streptococcus lactis* 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 ($\alpha$ =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 submerged batch reactor.

The experiments with various initial substrate concentrations (whey medium) namely 10,,20,30.40 and 50(%v/v), 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 0.5, 1.0,1.5, 2.0,and2.5 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. (1):

$$x_i = \frac{X_i - X_o}{\Delta x} \quad \ldots (1)$$

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. (2):

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

Where Y is the predicted response, $\beta_o$ is the offset term, $\beta_i$ is the coefficient of linear effect, $\beta_{ii}$ is the coefficient of squared effect and $\beta_{ij}$ is the coefficient of interaction effect. This regression model can be used to estimate the elliptical contours of a constant surface. 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 ANOVA, to determine the significance of each term in the equation and to estimate the goodness of fit in each variable. Response surfaces were drawn to determine the individual and interactive effects of test variables on milk clotting activity.

**2.4 Preparation of whey**

The milk whey was provided by Ponlait Dairy products Ltd., Pondicherry, India. The whey was filtered by Whatmann No. 1 filter paper to remove the suspended particles. The clarified whey was used as a substrate for milk clotting enzyme production.

**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$^{o}$C for 10 min at 10000 rpm in the cooling centrifuge. Then the supernatant was used for the further analysis.

**2.6 Analysis of crude enzyme**

**2.6.1 Estimation of Milk clotting activity**:

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

$$MCU / mg = \frac{M}{T(\text{minutes}) x W(g)} \qquad (3)$$

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 by using casein as a substrate. The reaction mixture containing 5 ml of 0.65% pre incubated casein solution (37 $^{o}$C/10min) and 1ml of enzyme was incubated for 10 min at 37$^{o}$C. And 5 ml of TCA was added to stop the reaction and incubated at 37$^{o}$C for 30 min. The tyrosine standard was set up (0.2mg/ml) in the range of 0.1-0.5ml, made up to 2ml with distilled water. Then the test solutions are centrifuged at4$^{o}$C at 10000rpm for 10 min and the 2ml of aliquots are used for PA. To all the tubes (including standard), 5 ml of sodium carbonate, 1ml of Folin's phenol is added and incubated at 37$^{o}$C for 30 min . Then the optical density was measured at 660 nm by using uv-Biospectrophotometer [12,13].

$$Units / ml \quad enzyme = \frac{(\mu \text{ mole} \quad \text{tyrosine} \quad \text{equivalents} \quad \text{released}) X (11)}{(1) X (10) X (2)} \qquad (4)$$

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] by using BSA as a standard. 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

**3.1 Effect of different medium components on the production of milk clotting enzyme**

The effect of different medium components on the Production of milk clotting enzyme was carried out by supplemented with scurose and casein along with the basal medium of whey, plain basal mediun and plain whey medium under agitated and stationary condition .Fig 1 shows the milk clotting activity and proteolytic activity levels obtained in a whey. The plain whey medium, plain basal medium, casein and sucrose along with the basal medium were denoted as S1,S2,S3 and S4 respectively. Maximum milk clotting enzyme concentration 0.498 units/mg was obtained for *S.lactis* under shaking condition. Fig 1. clearly indicates the addition of

casein(S3) gives the higher milk clotting activity than the other components of medium(S1,S2,S4) Dutt et al [15] reported that the increased MCA was found in the casein containing medium.Fig2. Shows the biomass concentration under static and shaking conditions. Maximum biomass concentration of 15.2 g/l was obtained in the presence of casein, followed by 11.5 g/l in sucrose, 11.0g/l in basal medium ,while plain whey had the least yield of 8.9 g/l under shaking conditions.

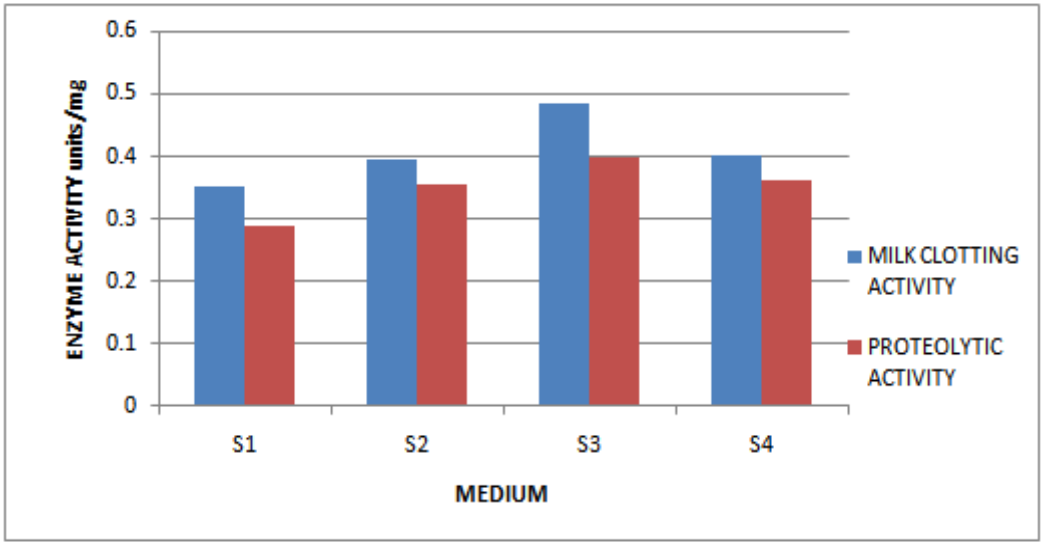


**Fig 1**.Effects different medium components on milk clotting enzyme production by *Streotococcus lactis*

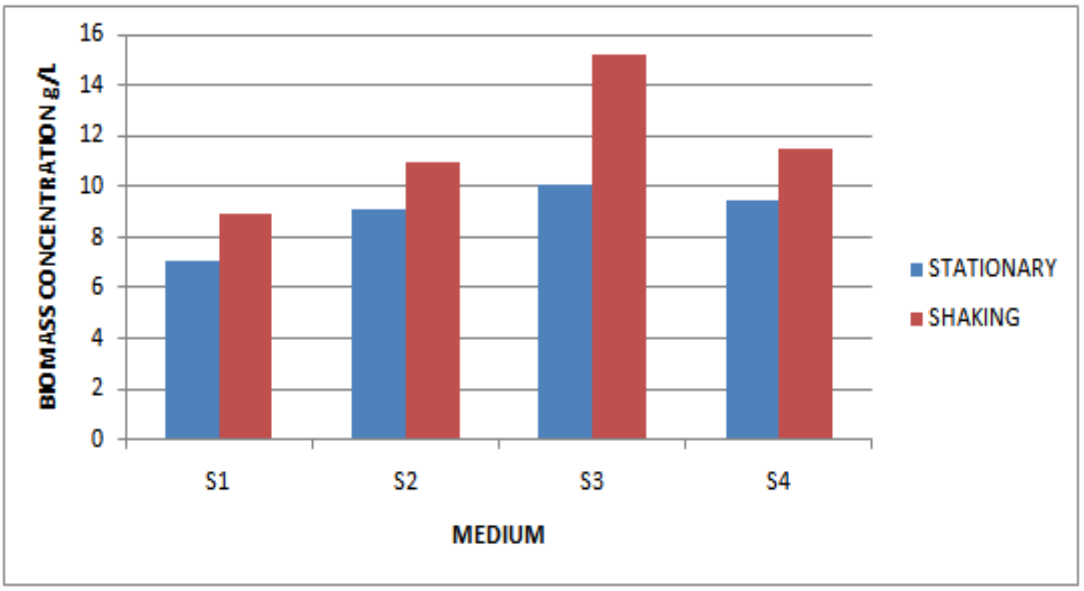


**Fig 2.**Effects different medium components on biomass concentration by *Streptococcus lacris*

**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:

$Y=0.495-0.000A+0.008B-0.003C+0.006D-0.026A^2-0.020B^2-0.020C^2-0.030D^2-0.014AB+0.016AC-0.005AD+0.001BC+0.021BD+0.014CD$ ... (5)

where Y is the milk clotting activity (units/mg), A is the initial substrate concentration(whey medium) (%v/v), B is the initial pH, C is the temperature ($^oC$) and D is the biomass concentration (g/l).

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

| Independent Variable | Range and Level | | | | |
|---|---|---|---|---|---|
| | -2 | -1 | 0 | +1 | +2 |
| Initial Substrate Concentration (whey medium) (%v/v)) (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) | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 |

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

| Run order | Independent Variable | | | | Milk Clotting Activity (units/mg) | |
|---|---|---|---|---|---|---|
| | Orthogonal Value | | | | Experimental | Predicted |
| | A | B | C | D | | |
| 1 | -1 | 1 | -1 | 1 | 0.450 | 0.458 |
| 2 | 1 | -1 | -1 | 1 | 0.325 | 0.355 |
| 3 | 0 | 0 | 0 | -2 | 0.334 | 0.362 |
| 4 | 1 | -1 | 1 | -1 | 0.422 | 0.421 |
| 5 | 0 | 0 | 0 | 2 | 0.365 | 0.386 |
| 6 | -1 | -1 | -1 | -1 | 0.399 | 0.420 |
| 7 | 0 | -2 | 0 | 0 | 0.401 | 0.395 |
| 8 | -1 | -1 | 1 | 1 | 0.366 | 0.358 |
| 9 | 2 | 0 | 0 | 0 | 0.388 | 0.389 |
| 10 | 0 | 0 | 0 | 0 | 0.499 | 0.495 |
| 11 | 0 | 0 | 0 | 0 | 0.489 | 0.495 |
| 12 | 1 | 1 | -1 | -1 | 0.354 | 0.369 |
| 13 | 0 | 0 | 0 | 0 | 0.490 | 0.495 |
| 14 | 0 | 2 | 0 | 0 | 0.374 | 0.429 |
| 15 | 1 | 1 | -1 | 1 | 0.422 | 0.382 |
| 16 | 0 | 0 | 0 | 0 | 0.495 | 0.495 |
| 17 | -1 | 1 | 1 | -1 | 0.378 | 0.355 |
| 18 | 1 | -1 | -1 | -1 | 0.450 | 0.425 |
| 19 | -1 | 1 | 1 | 1 | 0.480 | 0.449 |
| 20 | 0 | 0 | 0 | 0 | 0.496 | 0.496 |
| 21 | 1 | 1 | 1 | -1 | 0.385 | 0.368 |
| 22 | 0 | 0 | -2 | 0 | 0.399 | 0.418 |
| 23 | 1 | 1 | 1 | 1 | 0.455 | 0.441 |
| 24 | -2 | 0 | 0 | 0 | 0.343 | 0.392 |
| 25 | 0 | 0 | 2 | 0 | 0.375 | 0.405 |
| 26 | 0 | 0 | 0 | 0 | 0.497 | 0.495 |
| 27 | -1 | 1 | -1 | -1 | 0.480 | 0.442 |
| 28 | -1 | -1 | 1 | -1 | 0.364 | 0.348 |
| 29 | -1 | -1 | -1 | 1 | 0.411 | 0.372 |
| 30 | 0 | 0 | 0 | 0 | 0.499 | 0.495 |
| 31 | 1 | -1 | 1 | 1 | 0.407 | 0.409 |

**Table 3** Significance of regression coefficients for the production of milk clotting enzyme using Minitab 16 software

| Model Term | Parameter estimate ( Coefficients) | | |
|---|---|---|---|
| Constant | 0.495 | 37.982 | 0.000 |
| A | -0.000 | -0.107 | 0.0916 |
| B | 0.008 | 1.22 | 0.240 |
| C | -0.003 | -0.485 | 0.634 |
| D | 0.006 | 0.864 | 0.400 |

| | | | |
|---|---|---|---|
| A *A | -0.026 | -4.068 | 0.001 |
| B * B | -0.020 | -3.215 | 0.005 |
| C * C | -0.020 | -3.234 | 0.005 |
| D * D | -0.030 | -0.468 | 0.000 |
| A * B | -0.014 | -1.711 | 0.106 |
| A * C | 0.016 | 1.958 | 0.068 |
| A * D | -0.005 | -0.638 | 0.532 |
| B * C | 0.001 | 0.131 | 0.898 |
| B * D | 0.021 | 2.436 | 0.027 |
| C * D | 0.014 | 1.697 | 0.109 |

| | | | |
|---|---|---|---|
| A, B,C,D | = Linear effects | C * C | = Significant |
| $A^2$, $B^2$, $C^2$, $D^2$ | = Squared effects | D * D | = Significant |
| AB, AC, AD, BC, BD, CD | = Interaction effects | A *C | = Significant |
| A *A | = Significant | B * D | = Significant |
| B * B | = Significant | | |

**Table** 4 Analysis 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 | 0.076 | 14 | 0.005 | 4.5 | 0.002 |
| Linear | 0.002 | 4 | 0.000 | 0.62 | 0.655 |
| Square | 0.054 | 4 | 0.013 | 11.38 | 0.000 |
| Interaction | 0.019 | 6 | 0.003 | 2.67 | 0.055 |
| Residual error | 0.019 | 16 | 0.001 | | |
| Total | 0.095 | 30 | | | |

Square = Significant

The student t distribution and corresponding p values, along with the parameter estimate were given in Table 3. The squared effects of all the parameters $A^*A$,$B^*B$,$C^*C$,$D^*D$ were found to be significant and the Interactive effect of the parameters A*C and B*D were also found to be 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 4. ANOVA of the regression model was significant and it was evident from the calculated F value (4.5) 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. Fig 3 to 6 shows the response surface plots against each of the independent variables while keeping the other variables at their '0' levels. The smallest surface curve of the response surface diagram indicated the maximum product yield. The elliptical nature of the contour indicates that this interaction is significant on the response and the optimum range of process variable are found by the response surfaces of the contour plots.

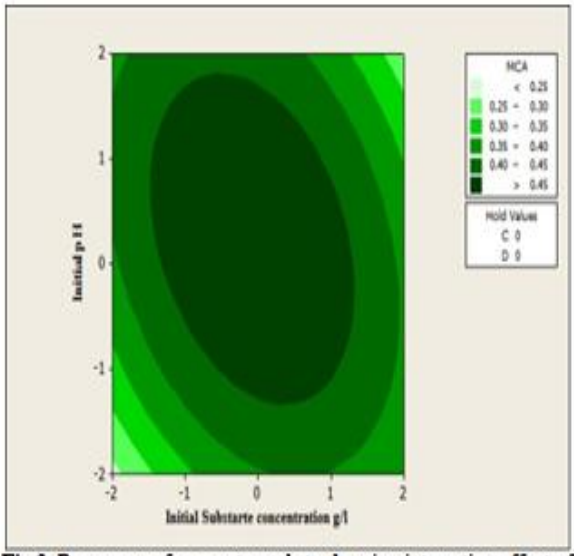


**Fig 3.** Respose surface contour plots showing interactive effect of initial substrate concentration and initial pH on the production of milk clotting enzyme.

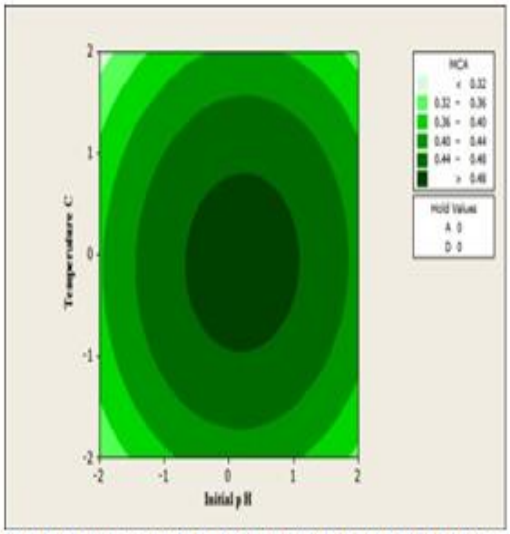


**Fig 4.** Respose surface contour plots showing interactive effect of initial pH and temperature on the production of milk clotting enzyme.

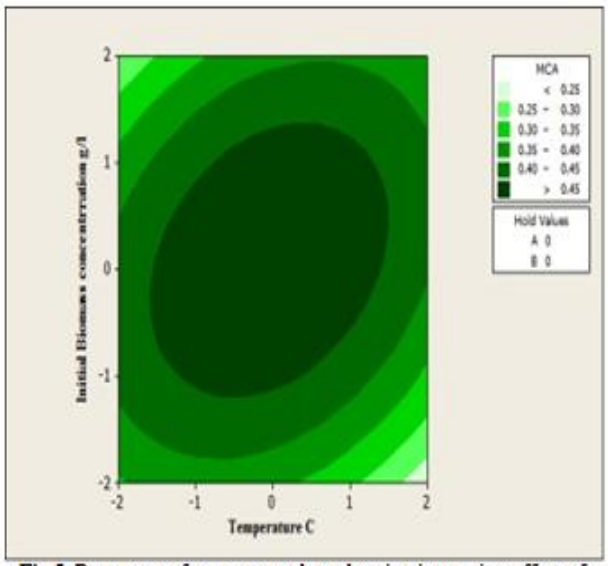


**Fig 5.** Respose surface contour plots showing interactive effect of temperature and initial biomass concentration on the production of milk clotting enzyme.

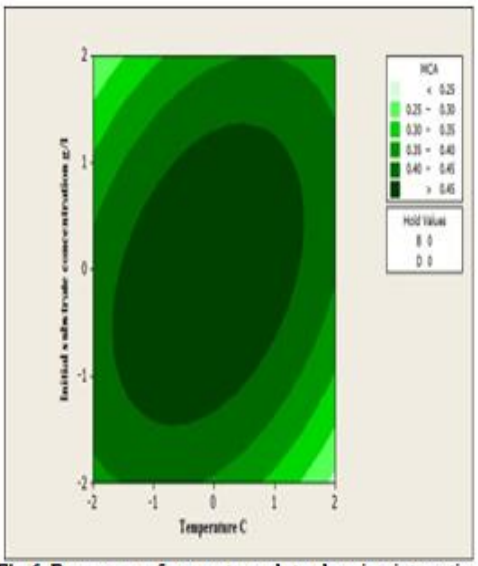


**Fig 6.** Respose surface contour plots showing interactive effect of temperature and initial substrate concentration on the production of milk clotting enzyme.

To validate the optimal parameters, confirmatory experiments were carried out by lab scale production in the Biofermentor. The observed results were compared with the predicted results. The process conditions for the maximum production of milk clotting enzyme by *Streptococcus lactis* under optimized conditions were given in Table 5. Milk Clotting Activity 0.491units/mg(MCA), Proteolytic Activity 0.387units/mg (PA), the ratio MCA/PA1.26 and protein content 0.297mg/ml were found under optimum conditions. These values agree with the values from the response surface analysis (MCA=0.4975units/mg) confirming that the RSM using statistical design is the effective tool to optimize the process parameters and to study the individual, cumulative and interactive effects of the test variables in milk clotting enzyme production. The confirmatory experiments showed the high milk clotting activity and low proteolytic activity which suggests this is a suitable enzyme source for Milk clotting in cheese Industries. Good coagulation was observed after17 min under the optimized conditions.

**Table 5** Optimum values of variables obtained from regression equations for the production of milk clotting enzyme by *Streptococcus lactis*

| Parameter | Optimum value for milk clotting enzyme production |
|---|---|
| Initial Substrate Concentration (whey medium) (%v/v) | 29 |
| Initial pH | 6.2 |
| Temperature($^{o}$C) | 39.9 |
| Biomass Concentration (g/l) | 1.6 |
| Milk Clotting Activity (units/mg) | 0.4975 |

## IV. CONCLUSION

The fermentative production of milk clotting enzyme by *Streptococcus lactis* has been studied using whey as a substrate .The results reported that the whey basal medium containing casein under shaking conditions enhanced the milk clotting activity of 0.491 units/mg with low proteolytic activity 0.387units/mg. Statistical experimental design is a valuable tool for studying the influence of process parameters on milk clotting activity. The results suggested that the whey medium is the high nutrient substrate for the production of milk clotting enzyme by the bacterial culture *Streptococcus lactis* .The whey could be used as a cheap and good source for the production of milk clotting enzyme..

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