Production and statistical optimization of culture condition for Nattokinase from *Bacillus subtilis* MTCC 2616

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Production and statistical optimization of culture condition for Nattokinase from *Bacillus subtilis* MTCC 2616

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Prof. Kasturi Dutta

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May 24, 2017

Supervisors' Certificate

This is to certify that the work presented in the thesis entitled *Production and statistical optimization of culture condition for Nattokinase from Bacillus subtilis MTCC 2616*, submitted by *Sharayu Moharkar,* Roll Number *215BM2456,* is a record of original research carried out by her under my supervision and guidance in partial fulfillment of the requirements of the degree of *Master of Technology* in Biotechnology at *Department of Biotechnology and Medical Engineering of National Institute of Technology Rourkela*. Neither this thesis nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

Prof. Kasturi Dutta
Assistant Professor

Declaration of Originality

I, *Sharayu Moharkar*, Roll Number *215BM2456* hereby declare that this thesis entitled *Production and statistical optimization of culture condition for Nattokinase from Bacillus subtilis MTCC 2616,* presents my original work carried out as a M.Tech student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the thesis. Works of other authors cited in this dissertation have been duly acknowledged under the section "References". I have also submitted my original research records to the scrutiny committee for evaluation of my thesis.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present thesis.

May 24, 2017
NIT Rourkela *Sharayu Moharkar*

Dedication

To my beloved family and friends…

Acknowledgement

I would like to take this opportunity to express my sincere thanks to all those who have supported me in this endeavour.

I would like to begin by expressing my gratitude, indebtedness and respect to my guide **Prof. Kasturi Dutta**, without whose constant support, encouragement and guidance during the difficult times, this project would not have been possible. I would also like to thank Head of Department of Biotechnology and Medical Engineering, NIT Rourkela, **Prof. Mukesh Kumar Gupta** and all the **teachers of NIT Rourkela** for providing me with this opportunity and necessary facilities for the completion of my research work.

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May 24, 2017 *Sharayu Moharkar*

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Abstract

Nattokinase which is a thrombolytic enzyme has a wide range of application in medicine, health care and pharmaceutical industry. It provides many health benefits like chronic inflammation, muscle spasms, poor healing, cure of hemorrhoids, helps to improve blood circulation, blood vicocity etc. In this study, a bacterium named *Bacillus subtilis* MTCC 2616 was employed for optimization of fermentation media and physical parameter in order to be applied for maximum Nattokinase enzyme production. Different carbon sources like lactose and glycerol was used in production media. Maximum nattokinase activity was obtained in lactose conataing media. Six factors including Lactose, Tryptone,Yeast extract, K_2HPO_4,$MgSO_4$ and $CaCl_2$ were screened using placket burman design. In range studied, Lactose, Tryptone, Yeast extract, K_2HPO_4 and $CaCl_2$ had significant effect on Nattokinase activity. Significant factors were optimized using Response surface methodology in central composite design. The optimized media containing (g/L): Lactose (7.50),Tryptone (12.00),Yeast extract (12.00),K_2HPO_4 (4.50) and $CaCl_2$ (0.20) which showed maximum Nattokinae activity and Specific activity 576.88 U/ml and 25.66 U/mg respectively. Central composite design was used to build statistical model to study the effect of two variables pH and temperature on Nattokinase production. The optimal conditions for pH and temperature were found to be 7.4 and 34.85°C respectively. Maximum nattokinase activity and specific activity was found to be 593.08 U/ml and 25.96 U/mg respectively. Caesinolytic activity of Nattokinase in optimal conditions was increased from 321 U/ml to 593.08 U/ml in optimized condition. The fibrinolytic activity using synthetic substrate was enhanced from 1089.50 U/ml to 1565.16 U/ml in optimal conditions. Structural and binding analysis of Nattokinase with substarte caesin was studied using Docking which showed that Nattokinase is the enzyme which binds to caesin more favorably to the tyrosine residue.

Keywords: Nattokinase, screening, optimization, docking

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1. Cardiovascular diseases

Cardiovascular diseases are mainly associated with dysfunctional of blood vessels which are prone to develop thrombi (Blood clot) [1]. Cardiovascular disease compromises a broad spectrum of conditions. It includes congenital heart disease, atherosclerotic cardiovascular disease and rheumatic heart disease. The atherosclerotic cardiovascular disease includes periphery artery disease, cerebral artery disease and coronary artery disease. Atherosclerotic cardiovascular disease is the most common type of cardiovascular disease. Cardiovascular disease contributes to approximately one third of world mortality. It is leading cause of premature death [2].

1.2. Thrombolytic and Fibrinolytic agents

1.2.1. Thrombolytic agents

Thrombolytic agents dissolves the thrombus or blood clot formed due to thrombosis. Thrombosis is a multistage process in which platelets remain adhere to the vessel wall and secrete a stimulatory mediator. Thrombolytic agents directly acts on blood clot. Thrombolytic agents can be used to treat cardiovascular diseases like heart attack, pulmonary embolism, stroke etc.

1.2.2. Fibrinolytic agents

Fibrinolytic agents are the most effective for the treatment of cardiovascular diseases. They are classified in two classes: plasminogen activator and plasmin like protein. Plasminogen activators convert plasminogen to plasmin which helps to degrade fibrin clot while plasmin like protein enzymes directly degrade fibrin in thrombi more rapidly.

Following are the Plasminogen activators:

- Streptokinase
- Tissue plasminogen activator
- Urokinase

Following are plasmin like protein:

- Nattokinase
- Lumbrokinase
- Fibrolase

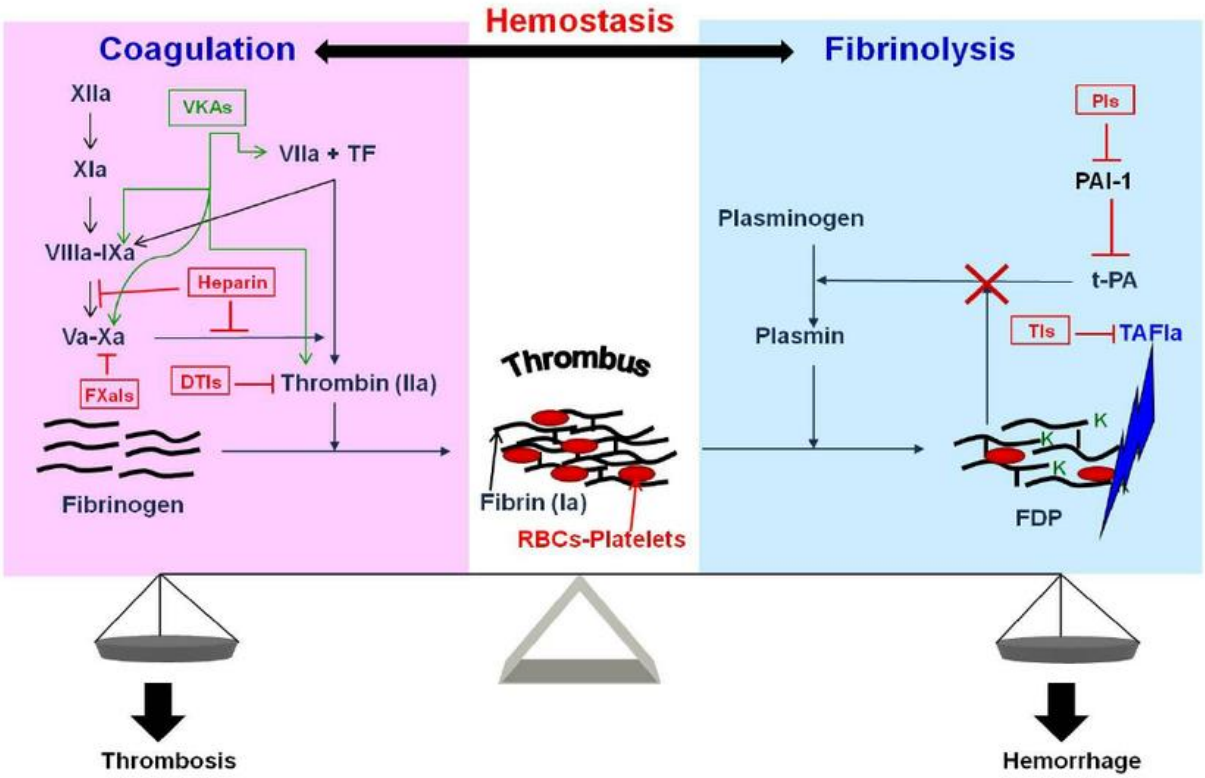

Figure 1.2: Targets of anticoagulant agents [2]

1.3. Problem statement

For treatment of cardiovascular diseases, thrombolytic agents like streptokinase and urokinase have been in utilization since 1960 [2]. However it possess low specificity and it may led to hemorrhagic complications. It also possess short half- life period [3].For industrial production of Nattokinase it is necessary to optimize the culture condition for production of Nattokinase enzyme. Nattokinase production can be optimized using statistical methods. However, there are few studies regarding the optimization of culture condition using statistical methods have been reported [4, 5].

For development of economical medium for microorganisms, carbon, nitrogen and trace element sources are required. For development of bioprocesses and improvement of their performance, optimization of environmental and nutritional conditions play important role. Nutritional and environmental requirement can be optimized using conventional method. There are several limitations of this method as it is time consuming and it often shows slightly variation from true optima. Statistical methods overcome the limitations of conventional methods by shortlisting the significant nutrient factors and reduce the number of experiments for saving resources and time [6].

1.4. Optimization of culture condition using statistical method

The optimal conditions are selected based on the combinations of variables involving optimization of one factor at a time method. This approach often results in time consuming, expensive and tedious method. Moreover, it does not give proper optimized values [7]. It is necessary to develop fermentation process which gives maximum yield of Nattokinase for industrial production of Nattokinase. On comparison to conventional method of optimization, it is advantageous to use statistically planed experiment which gives reduce number of experiments for large number of variables. Among various optimization techniques like nonlinear and quadratic technique, Response Surface Methodology is most employed and well-studied technique optimization of culture condition [8]. The statistical experiment design is a most efficient method to treat optimization of large number of factors and it allows systemic and simultaneous analysis of factors [9].

The present study was to optimize the culture condition using sequential optimization method to maximize the production of Nattokinase enzyme.

1.5. Objective

- Primary optimization for screening of carbon source in fermentation media
- Optimization of fermentation media using statistical optimization
- Optimization of physical parameter using Response surface methodology

Chapter 2

Review of Literature

2.1. About Nattokinase

Nattokinase is a serine alkaline protease with 275 amino acid residue. It is isolated from *Bacillus subtilis* which is a soil bacteria [10]. It possess four times greater activity to dissolve blood clot than plasmin [1]. It enhances the activity of plasmin through endogenous tissue plasminogen activator, which is a pro-urokinase. It mainly helps to increase the concentration of tissue plasminogen activator. It helps to lyse the fibrin strands to slow down clotting time. Nattokinase enhances the process of clot regulation that occur naturally [11].

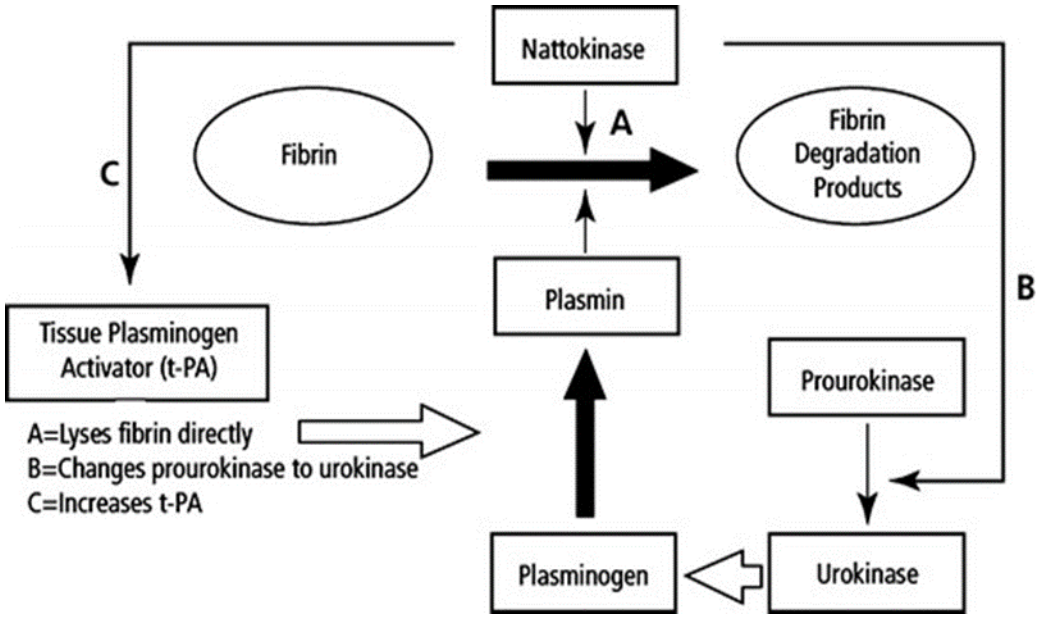

Figure 2.1- Effect of Nattokinase on fibrin [12]

Cardiovascular health benefits of nattokinase

- It possess strong fibrinolytic activity which dissolves blood clot without inhibiting wound healing
- It reduces the increased blood pressure
- It inhibits red blood cell and platelet aggregation
- It decrease the blood viscosity
- It reduces clinical measure of inflammation (CRP)

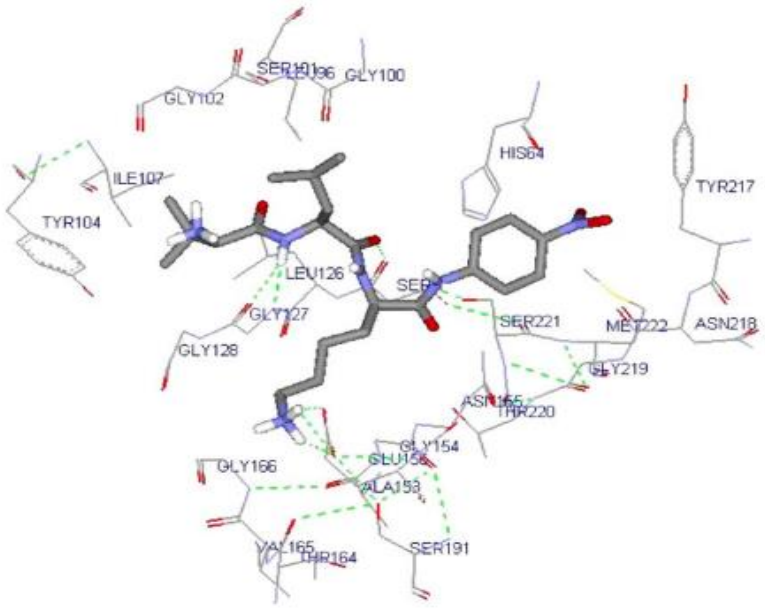

Figure 2.2: Chemical structure of Nattokinase [13]

2.1. Production of Nattokinase

2.1.1. Micro-organisms used for production of thrombolytic enzymes

Fibrinolytic enzymes like Nattokinase, urokinase and streptokinase were isolated from *Bacillus subtilis, Pseudomonas species* and *β-hemolytic Streptococci* respectively and confirmed by various morphological and biochemical tests. Nattokinase and urokinase are serine protease enzyme while

streptokinase is a non-protease enzyme. This fibrinolytic enzymes has been widely used as a thrombolytic agents and can be used as for intravenous instillation [2].

Bacillus natto with NRRL 3666 was used for fermentative production of fibrinolytic enzyme, Nattokinase. Enzyme production was then optimized using Response surface methodology in shake flask culture [6].

For cost effective production of nattokinase, *Bacillus subtilis* was isolated from different soil samples and then identified by using various colony morphology, gram staining and biochemical tests. Different nitrogen sources like sheep fibrin and cow fibrin were used for obtaining optimized production of nattokinase. Maximum production of nattokinase was achieved using glucose and sheep fibrin as a carbon and nitrogen source [11].

For higher production of fibrinolytic enzyme, strain *Bacillus lichiniformis* B4 was selected. The optimal fibrinolytic activity was found to be 50 unit/ml on optimal carbon and nitrogen source, mannitol and soya peptone respectively [14].

Bacillus sp. producing extracellular proteases are of immense interest in a biotechnological perspective. It is important in scientific fields like protein engineering and also in applied fields like pharmaceutical and food industries. The genus *Bacillus* contains many species important for industrial production. Optimization of proteases enzyme is highly important because of its wide application in many industries. Bacterial extracellular proteases are more significant among all fungal, animal, viral and plant proteases [15].

2.2.2. Media components

Efficient production of Nattokinase is dependent on the type of substrate being used in media for enzyme production. Substrate factors are optimized to achieve high production of Nattokinase [16]. Nitrogen and carbon regulation is the critical factors involved in enzyme production as it affects the activity of enzymes. It has been observed that *Bacillus subtilis* use nitrogen source in reduced form for the synthesis of enzymes. After the screening of inorganic nitrogen sources, peptone and yeast extract had shown maximum enzyme activity. Organic nitrogen showed inducing effect for enzyme production [17]. Optimization of yeast extract among inorganic nitrogen sources had shown maximum activity for enzyme production. The variation in fibrinolytic enzyme activity had shown its significance in a media component. Carbon source acts as a source of energy as well as it acts as a important constituent in a cellular membrane [18, 19]. Presence of

magnesium and calcium ions in growth medium plays major role in cell viability. Magnesium ions has important role in cell wall strength and peptidoglycan synthesis while calcium take part in anchoring of surface protein to the cell wall [20].

Six factors of fermentation medium including glucose, soyabean peptone, K_2HPO_4, Nacl, $MgSO_4.7H_2O$ and $CaCl_2$ were used to screen some factors in the media using placket burman design. From the studied range, soybean peptone and $CaCl_2$ was found to be most significant [16].

Production media containing glucose, soy peptone, yeast extract, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ was optimized using Response surface methodology. After optimization, production of nattokinase was enhanced from 188 ± 2.4 to 1,190.68 ± 11 U/mL in shake flask within 40 hr of incubation [6, 21].

Bacillus sphaericus (MTCC 3672) was used to produce extracellular fibrinolytic enzyme which dissolves blood clot. Statistical screening of six independent nutritional variables such as, glucose, yeast extract, NaCl, $MgCl_2$, $MnCl_2$, $CaCl_2$ was studied using Plackett-Burman design. Amongst six variables, yeast extract was found to be significant factor affecting yield of a fibrinolytic enzyme. The fibrinolytic activity achieved showed 2.85 fold increase in the activity after optimization of media using Response surface methodology i.e. from $3.5*10^4$ U/I to $9.98*10^4$ U/I after 24 hour of incubation [16].

Factors including glucose, soyabean peptone, K_2HPO_4, $MgSO_4.7H_2O$, NaCl and $CaCl_2$ were screened using placket burman design. The optimized nattokinase yield was obtained as 31.06 ± 0.297 FU/mL of substrate [19].

Different nitrogen sources like peptone, sheep fibrin, tryptone and cow fibrin were used to obtain maximum Nattokinase activity. It shows maximum caesinolytic activity using tryptone as a nitrogen source. The caesinolytic activity using tryptone as a nitrogen source was found to be 636.73 U/ml [22].

2.2.3. Physical parameter

Most of the *Bacillus sp*. show higher growth rate in slightly basic pH range. Enzyme production will be low at higher temperature due to the change in physiological activities. Bacillus species shows prominent growth in temperature range range between 30-37°C. The optimum caesinolytic activity of Nattokinase was found to be 576.3U at pH 9 and temperature 37°C [2].

The effect of temperature and pH on Nattokinase was studied using central composite design in Response surface methodology. The determination coefficient as shown by analysis of variance was 94.72% showing the model is significant. For maximum production of Nattokinase, optimized value of temperature and pH were 37 °C and 6.36 respectively [14].

2.3. Kinetic modelling

For optimization of production of Nattokinase for industrial production, it is necessary to understand the fundamental key parameter. For fermentative production of Nattokinase, there is need to develop a kinetic model. The kinetic modelling for production of Nattokinase includes variation of substrate (S, Maltodextrin, g/L), product (P, Nattokinase activity U/L) and biomass (X, Dry cell weight g/L) [6].

2.3.1. Microbial growth kinetics

Monad model and logistic equation are widely used to describe the microbial cell growth. Logistic equation which is a substrate independent is well studied for characterization of cell growth in microbial fermentation. Characterization of cell growth in several microbial fermentation processes is well studied.

The growth pattern of logistic kinetics is described as follows:

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right) \quad \text{..... (Eq.1)}$$

Where X is the biomass concentration (gL-1), Xm is maximum biomass concentration (gL-1), μm is maximum specific growth rate (h-1) and t is the time (h). The integration of Eq. (1) yields Eq. (2) with the initial conditions of $X = X_0$ at $t = 0$:

$$ln\frac{X}{(X_m - X)} = \mu_m t - ln\left(\frac{X_m}{X_0} - 1\right) \quad \text{..... (Eq. 2)}$$

The value of μ_m and X_0 can be obtained from the slope and y-intercept of the plot. The value of Xm is determined from the experimental data.

2.3.2. Product formation kinetics

Luedeking- Piret model is used for study of production kinetics which explains that the product formation rate is related linearly with biomass concentration (X) and the growth rate (dX/dt).

$$\frac{dP}{dt} = m\frac{dX}{dt} + nX$$

Where *m* and *n* are the kinetic constants for product formation.

2.3.3. Substrate utilization kinetics

Glycerol was used as a limiting substrate for nattokinase production which acts as carbon source for both biomass growth and product synthesis. The substrate utilization kinetics is usually represented by the following equation.

$$-\frac{dS}{dt} = p\frac{dX}{dt} + qX$$

Where p = 1/YX/S and q is maintenance coefficient (ms) [23,24].

2.4. Enzyme purification

For purification, cells were separated from broth culture by centrifugation at 10000rpm for15 min. Nattokinase enzyme can be purified using ion exchange chromatography and ammonium salt precipitation [11].

2.4.1. Ion exchange chromatography

The chromatography column which is packed with 2% DEAE cellulose, washed twice with distilled water and sonicated for 15min. The matrix was then activated by using activation buffer containing 25 mM Hcl and 25 mM Nacl. The enzyme sample was allowed to flow into column containing elution buffer which contain 25 ml Tris Hcl and higher concentration of Nacl for elution of enzyme. The enzyme then collected in same test tube [22].

2.4.2. Ammonium salt precipitation

In this method, the supernatant obtained after centrifugation was filtered in 0.45µm pore size filter. Ammonium sulfate was slowly poured over the supernatant for 10 min which allows the salt to dissolve slowly. For additional 25 min, supernatant enzyme sample was stirred continually at the room temperature. Precipitates thus obtained were again centrifuged at 10000 rpm for 10 min and

then dissolved in10 mM Tris-Hcl buffer. Now each of $(NH_4)_2SO_4$ fraction was allowed for dialysis overnight. 20% fraction was retained for determination of the precipitation fraction which contain maximum concentration of protease enzyme. Result thus obtained showed that 40%- 70% ammonium sulphate precipitation were used for purifying protease enzyme which showed 20 fold increase in the specific activity compared to unpurified supernatant enzyme sample [23].

2.5. Characterization studies

2.5.1. Effect of temperature

For maximum production of enzyme, temperature is one of the critical factor which has to be maintained in optimum condition [24]. The temperature range showing maximum nattokinase activity was between 37-60°C which showed 31.2, 32.2, 28.5 and 48.6% residual activity at 20,100, 27 and 8°C respectively. So it was concluded that nattokinse enzyme was thermostable protease [2].

2.5.2. Effect of pH

Enzyme production is highly dependent on the pH range because of its influence on the transport of components across cell membrane which significantly affect the product formation [25]. *Bacillus subtilis* was able to produce protease enzyme with a wide range of pH between 5.0 to 12.0. *Bacillus subtilis* can produce enzyme at all range of pH. It could produce 60% enzyme activity even at pH 5.0. It shows higher enzyme activity in slightly alkaline pH [26].

2.5.3. Effect of inhibitor

When enzyme sample was incubated for 10 min at room temperature in phosphate buffer with SDS, ethylenediaminetetraactic acid (EDTA) and phenylmethylsulfonyl fluroids (PMSF), enzyme activity was inhibited. It showed partial inhibition by H_2O_2 and $HgCl_2$ [2].

2.6. Structural analysis by Docking

Docking techniques are used to predict the interaction between protein and a small vitamin like molecule. It used for study of inhibition and enhancement study of significant part of protein

molecule [27]. Optimizing ligand for correct conformation and conformational flexibility of protein and ligand are of paramount important in docking study [28].

Computer based docking are used to speed up the drug discoveries by predicting the effectiveness between protein and ligand binding. Docking study was conducted between B_{12} coenzyme which is present in methionine, diol dehydrate and glutamate with riboflavin molecule by using ArgusLab 4.0.1 to study the possible interaction mode. It showed the maximum binding affinity of riboflavin with glutamate mutase with minimum binding energy which was found to be -7.13 kcal/mol [29]

Chapter 3

Materials and Methods

3.1. Material

Nattokinase producing bacteria bacillus subtilis MTCC 2616 was ordered from IMTECH Chandigarh. Lyophilized strain was revived on nutrient agar medium (g/l): Beef extract (1), Yeast extract (2), Peptone (5), Nacl (5), Agar (1.5). From the nutrient agar plate, agar slant was prepared in the above mentioned media for routine work.

3.2. Enzyme production

Inoculum was prepared in 250-mL Erlenmeyer flask containing 100 mL of liquid media consisted of (g/L): Glucose (10), Tryptone (10), yeast extract (10), $K_2HPO_4 \cdot 3H_2O$ (1), $MgSO_4 \cdot 7H_2O$ (0.5). The inoculum was developed by transferring one loop full of the culture from the agar slant to the above mentioned liquid medium. The inoculated flasks were incubated at 37°C in orbital shaker at 180 rpm for 24 hr and used as the inoculum which was used for all subsequent inoculations
For optimization studies, 1% inoculum culture was added to Erlenmeyer flasks (250-mL) containing 50 mL unoptimized production media composed of (g/L): Lactose (20), Tryptone (10), yeast extract (10), $K_2HPO_4 \cdot 3H_2O$ (1), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCl_2 \cdot 2H_2O$ (0.5). The flasks was then incubated at 37°C and 120 rpm in an orbital shaker for 48 hour incubation period. The pH was adjusted as 7-7.5. The culture thus obtained was centrifuged at 10,000×g and at 4°C for 15 min and the clear supernatant was used for determining enzyme activity and bacterial biomass was used for biomass estimation [6].

3.3. Biomass Estimation

For biomass estimation, cells were recovered from centrifugation in 2 ml micro centrifuge tube. One ml of phosphate buffer saline was added to the micro centrifuge tube. The absorbance was recorded for optical density measurement at 660nm using UV-VIS spectrophotometry.

3.4. In Vitro blood clot degradation analysis

In vitro fibrinolytic activity of Nattokinase is determined by artificial blood clot degradation method. Blood clot was formed by coagulation of freshly collected human blood using 0.2M $CaCl_2$ in a glass tube. 2ml of supernatant enzyme sample was added in test tube used as a test sample containing blood and 3ml of distilled water was added in another test tube which was used as a control. In vitro blood clot degardtion was analysed at room temperature [30].

3.5. Enzyme assay and characterization

3.5.1 Caseinolytic activity

In this method, 1 ml of supernatant enzyme was added in 5 ml of casein solution (0.6%, w/v phosphate buffer pH 7.5). The reaction mixture was incubated for 10 min at 30°C. The reaction between enzyme and substrate was stopped by adding 5 ml of TCA mixture. The reaction mixture was again incubated at 30°C for 30 min. The reaction mixture was centrifuged at 12000 rpm for 15 min and precipitates were removed. 5 ml Folin's ciocalteus reagent was added to the clear solution. The absorbance of supernatant was measured at 620 nm. One unit of enzyme activity was expressed as μmole of tyrosine released per min due to substrate hydrolysis by enzyme [31].

3.5.2. Analytical determination Nattokinase activity using synthetic substrate

Enzyme activity was colorimetrically estimated using synthetic substrate N-succinyl-Ala-Ale-Pro-Phe-p-nitroanilide. 12 μl of enzyme solution was added to 80 μl of phosphate buffer and the reaction mixture was incubated for 10 min at 30°C. After incubation, 16 μl of 3mM synthetic substrate was added to reaction mixture. The reaction was terminated by adding 50 μl of 0.2M glacial acetic acid. The absorbance of released p-nitro aniline was measured using microplate reader at 405nm. One unit of enzyme activity was defined as nmol of p-nitro aniline released per min due to substrate hydrolysis by enzyme [6].

3.6. Optimization of culture condition using statistical methods

Fermentation media and physical parameter were optimized by Response surface methodology using MINITAB version 17 software.

3.6.1. Optimization of fermentation media

Six factors including Lactose (X1), Tryptone (X2),Yeast extract (X3), K_2HPO_4 (X4), $MgSO_4.7H_2O$ (X5) and $CaCl_2$ (X6) were used to screen some effect factors in the fermentation media by placket burman design.

Table 3.1: Values of factors in placket burman design (19)

Name of factors, g/L	**Symbols of factors**	**Values of factors**	
		Low (-1)	**High (+1)**
Lactose	X_1	1.25	10.00
Tryptone	X_2	5.00	15.00
Yeast extract	X_3	5.00	15.00
K_2HPO_4	X_4	1.25	3.00
$MgSO_4.7H_2O$	X_5	0.25	1.50
$Cacl_2$	X_6	0.05	0.40

A 2 level 6 factor placket burman design was used to obtain significant factor. Factors were screened to obtain the optimized values using minimum number of experiments.

Table 3.2: Plackett burman design for fermentation media

StdOrder	RunOrder	PtType	Blocks	Lactose	Tryptone	Yeast Extr	K2HPO4	MgSO4	CaCl2
1	1	1	1	10	5	15	1.5	0.25	0.05
2	2	1	1	10	15	5	3	0.25	0.05
3	3	1	1	1.25	15	15	1.5	1.5	0.05
4	4	1	1	10	5	15	3	0.25	0.4
5	5	1	1	10	15	5	3	1.5	0.05
6	6	1	1	10	15	15	1.5	1.5	0.4
7	7	1	1	1.25	15	15	3	0.25	0.4
8	8	1	1	1.25	5	15	3	1.5	0.05
9	9	1	1	1.25	5	5	3	1.5	0.4
10	10	1	1	10	5	5	1.5	1.5	0.4
11	11	1	1	1.25	15	5	1.5	0.25	0.4
12	12	1	1	1.25	5	5	1.5	0.25	0.05
13	13	0	1	5.625	10	10	2.25	0.875	0.225

The significant factors obtained from placket burman design were further analysed by Response surface methodology to reach the optimized values.

3.6.2. Optimization of physical parameter

The optimization of Temperature and pH for nattokinase activity, the surface response for enzyme production as a function of selected key variable has to be predetermined.

Table 3.3: Values of factors for central composite design

Name of factors	**Symbols of factors**	**Value of factors**	
		Low (-1)	**High (+1)**
Temperature	X_1	25	45
pH	X_2	6.5	8.5

The minimum and maximum ranges of variables are investigated and the full experimental plan with respect to their values in actual and coded form the coded and decoded values was listed.

Table 3.4: Central composite design for physical parameter

StdOrder	RunOrder	PtType	Blocks	pH	Temperature(°C)
5	1	-1	1	6.08579	35
8	2	-1	1	7.5	49.1421
7	3	-1	1	7.5	20.8579
12	4	0	1	7.5	35
9	5	0	1	7.5	35
1	6	1	1	6.5	25
4	7	1	1	8.5	45
11	8	0	1	7.5	35
13	9	0	1	7.5	35
3	10	1	1	6.5	45
10	11	0	1	7.5	35
2	12	1	1	8.5	25
6	13	-1	1	8.91421	35

Response surface methodology was used to obtain the optimum nutrient concentrations for the production of Nattokinase. Responses obtained in the experimental run was analysed using ANOVA table.

The relationship of the independent variables and the response was calculated by the second order polynomial,

$$Y=\beta_0+\sum_{i=1}^{k}\beta_i x_i+\sum_{i=1}^{k}\beta_{ii}x_i^2+\sum\sum\beta_{iij}x_{ji}$$

where, Y is predicted response, β0 is a constant, βi is linear coefficient, βii is squared coefficient, βij is cross-product coefficient, and n is number of factors.

The significance of the model was evaluated by determination of adjusted R^2 coefficient. The "lack of fit test" was compared with the residual to test the significance of model [6].

3.7. Structural analysis by Docking

PDB format files for different protease enzymes found in *Bacillus subtilis* were obtained from protein database. For docking, enzyme and substrate interaction was observed in patch dock. In patch dock, it resulted in several docked files in different sites. Docked files were further analyzed in fire dock which shows the binding energy for each files. Docked files with minimum global energy was selected and analyzed in UCSF chimera. UCSF chimera shows the exact interaction between enzyme and substrate. Binding energy for interaction between different protease enzyme with casein was obtained. Interaction between substrate and each protease enzyme was analyzed by above mentioned method [32, 33].

Chapter 4

Results and discussion

4.1. Primary optimization to screen carbon source in production media

Production media 1: Lactose, Tryptone, yeast extract, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$

Production media 2: Glycerol, Tryptone, yeast extract, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$

Production media 1 and production media 2 was used for screening of carbon source. From figure 4.1, it can be observed that there was increase in biomass with respect to increase in incubation time. It was maximum at 48 hr. After 48 hr, it showed decline in biomass. It can be observed from figure 2 that biomass was increasing with respect to incubation time. From figure 4.3 and figure 4.4, enzyme activity was maximum at 48 hr and 72 hr respectively. So it can be concluded that Nattokinase is a growth associated product. Though enzyme activity was maximum in production media 2, it was taking too long incubation period. So production media containing lactose as a carbon source was used for further optimization study.

4.1.1.Biomass estimation

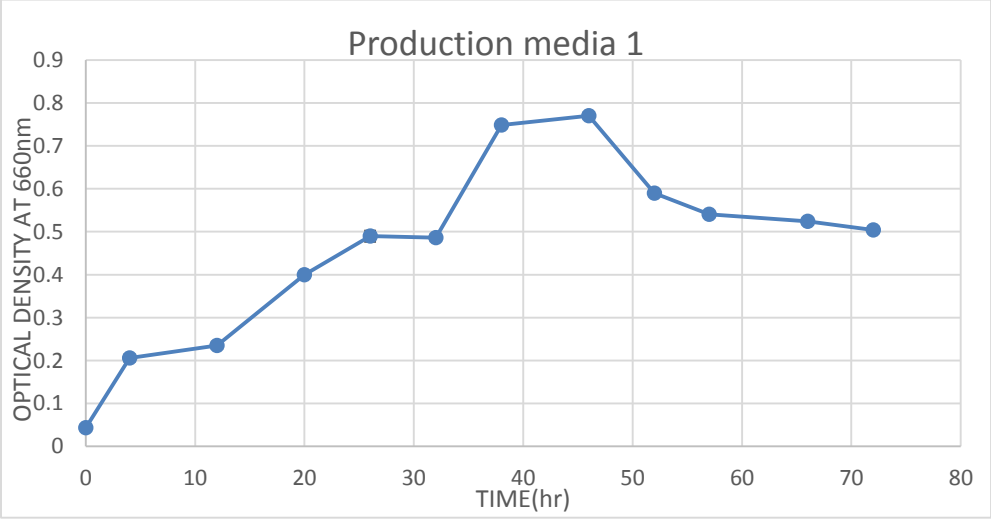

Figure 4.1: Growth curve of bacillus subtilis for lactose as a carbon source for 72 hr incubation period

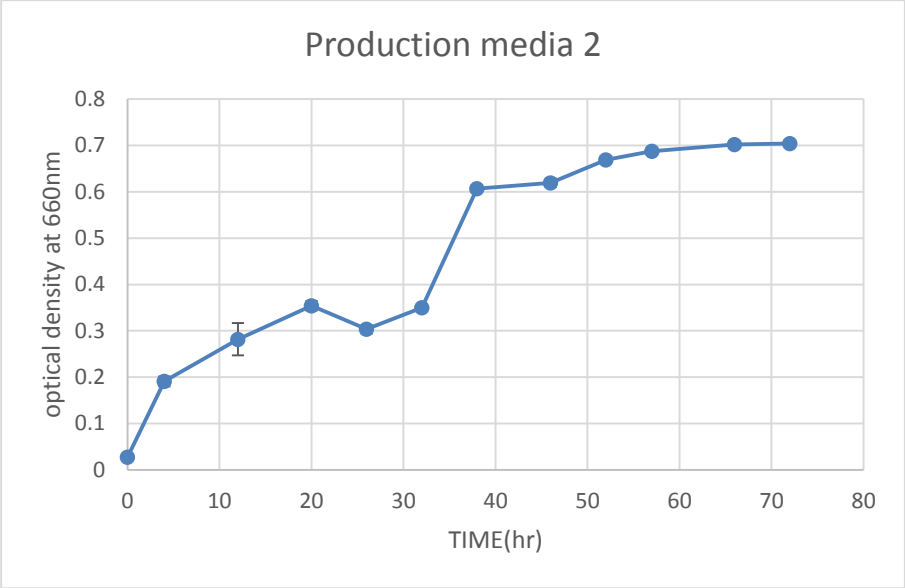

Figure 4.2-Growth curve for bacillus subtilis for glycerol as a carbon source for 72 hr incubation period.

4.2.2 Enzyme activity

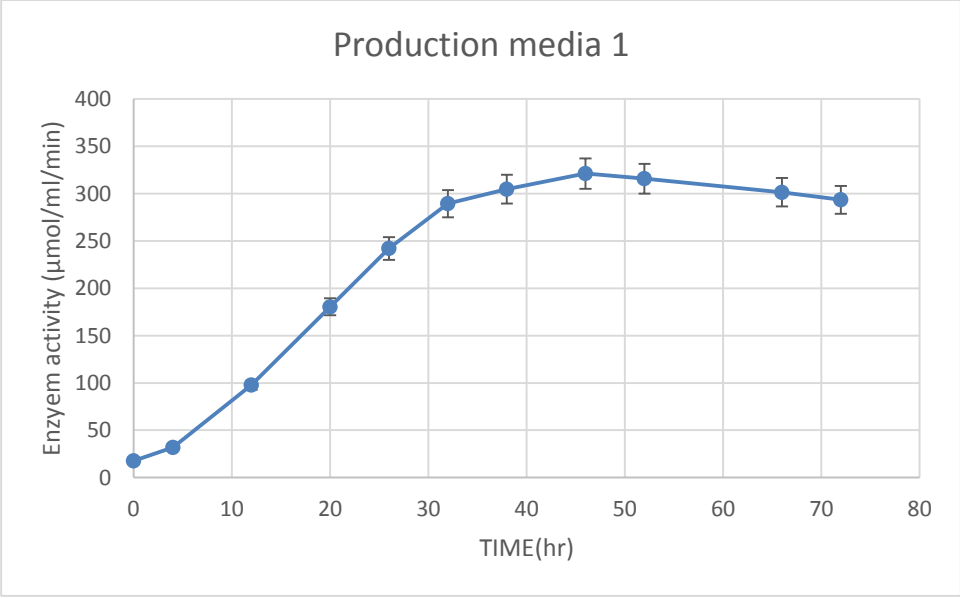

Figure 4.3: Enzyme activity of Bacillus subtilis using lactose as a carbon source

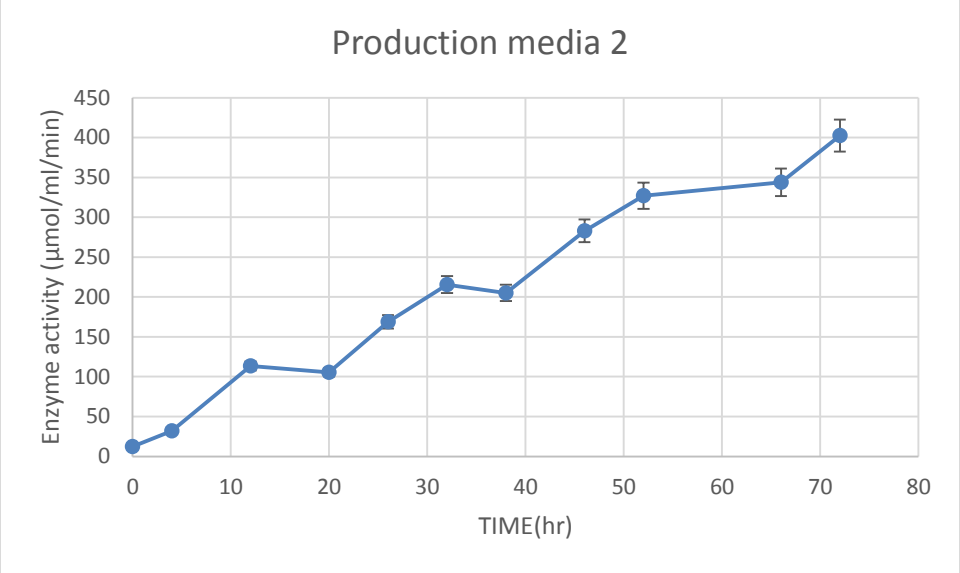

Figure 4.4: Enzyme activity of *Bacillus subtilis* using glycerol as a carbon source

4.2. In-vitro blood clot degradation analysis

Evalution of fibrinolytic activity was carried out using glycerol and lactose as a carbon source in production media as a test samples and distlled water as a control. It was observed that blood clot was degraded in test samples while in control test tube, blood clot was not degraded. It showed that test samples contained fibrinolytic enzyme.

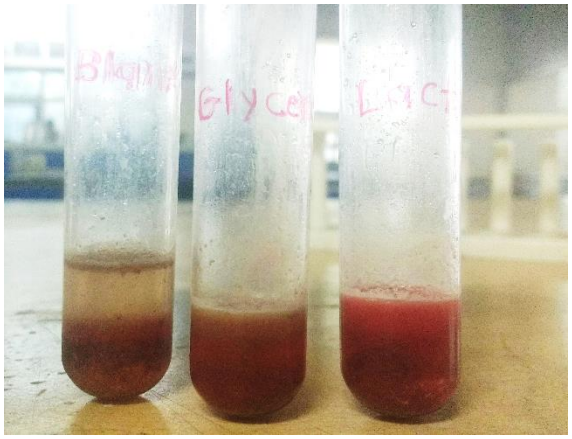

Figure 4.5: Blood clot degradation analysis

4.3. Optimization of fermentation media using statistical method

For optimization of fermentation media, placket burman design was used for screening of factors which affect the enzyme acivity of Nattokinase.

4.3.1. Placket burman design

Six factors including Lactose, Tryptone, Yeast extract, K_2HPO_4, $MgSO_4$ and $CaCl_2$ were screened using plackett burman design. Table 4.1 shows the responses obtained in experimental runs in placket burman design. ANOVA was carried out to determine whether the model is statistically significant or not. From table 4.2 and table 4.3, it showed that regression coefficient are 94.94% and 96.70% respectively. P- value in the ANOVA table is the tool to check wheher the factor is significant or not. A low p-value (< 0.05) suggests that factor has singnificant effect on response. It can be observed that five factors including Lactose,Tryptone,Yeast extract, K_2HPO_4, $MgSO_4$ and $CaCl_2$ are significant.

Table 4.1: The experimental design and response for placket burman design

StdOrder	RunOrder	PtType	Blocks	Lactose	Tryptone	Yeast Extr	K2HPO4	MgSO4	CaCl2	Enzyme activity(U/ml)	Specific Activity (U/mg)
1	1	1	1	10	5	15	1.5	0.25	0.05	506.261542	19.35248187
2	2	1	1	10	15	5	3	0.25	0.05	584.9780702	21.60982897
3	3	1	1	1.25	15	15	1.5	1.5	0.05	559.5856417	18.37398691
4	4	1	1	10	5	15	3	0.25	0.4	517.0533241	18.20716064
5	5	1	1	10	15	5	3	1.5	0.05	576.7255309	18.18108913
6	6	1	1	10	15	15	1.5	1.5	0.4	511.9748384	16.24649115
7	7	1	1	1.25	15	15	3	0.25	0.4	560.8552632	20.38833107
8	8	1	1	1.25	5	15	3	1.5	0.05	566.5685596	15.26877075
9	9	1	1	1.25	5	5	3	1.5	0.4	546.8894275	15.30024105
10	10	1	1	10	5	5	1.5	1.5	0.4	513.2444598	13.48443883
11	11	1	1	1.25	15	5	1.5	0.25	0.4	540.5413204	20.25257851
12	12	1	1	1.25	5	5	1.5	0.25	0.05	555.7767775	20.06414359
13	13	0	1	5.625	10	10	2.25	0.875	0.225	556.4115882	18.80726379

Table 4.2: ANOVA (Analysis of variance) table for Enzyme activity in placket burman design

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	6	7760.07	1293.35	18.76	0.001
Lactose	1	1199.58	1199.58	17.40	0.006
Tryptone	1	1383.88	1383.88	20.07	0.004
Yeast Extract	1	765.70	765.70	11.10	0.016
K2HPO4	1	2287.64	2287.64	33.17	0.001
MgSO4	1	7.56	7.56	0.11	0.752
CaCl2	1	2115.70	2115.70	30.68	0.001
Error	6	413.76	68.96		
Total	12	8173.83			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
8.30418	94.94%	89.88%	77.67%

Table 4.3: ANOVA (Analysis of variance) table for specific activity in placket burman design

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	6	66.5305	11.0884	29.34	0.000
Lactose	1	0.5489	0.5489	1.45	0.273
Tryptone	1	14.9077	14.9077	39.45	0.001
Yeast Extract	1	0.0928	0.0928	0.25	0.638
K2HPO4	1	0.1163	0.1163	0.31	0.599
MgSO4	1	44.1581	44.1581	116.85	0.000
CaCl2	1	6.7067	6.7067	17.75	0.006
Error	6	2.2675	0.3779		
Total	12	68.7980			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.614745	96.70%	93.41%	84.63%

4.3.2.Central composite design

Five factors including Lactose,Tryptone,Yeast extract,$MgSO_4$ and $CaCl_2$ were analysed using Central composite design. Table 4.4 shows the responses obtained in experimental runs in Central composite design.

From table 4.5 and table 4.6, significance of model was evaluted using regression analysis. Regression coefficient for enzyme activity and specific activity was found to be 92.80% and 94.60% which showed that model was significant. Interaction between lactose and tryptone was significant as P- value was less than 0.05.

Contour plots is graph which can be used to explore the relationship among three variables. Contour plots is used to display the three dimensional relationship in two dimension, with two factors plotted on x-axis and y-axis and response is represented by contour. From contour plots(Figure 4.6 – Figure 4.13), it can be observed that both enzyme activity and specific activity increases with increase in all the factors present in fermentation media.

Table 4.7 shows the optimized predicted and experimental values for factors and also the optimized response for enzyme activity and specific activity. It can be obsserved that there is not significant diffrence between experimental predicted optimum values and response. Both shows maximum response at maximum concentration of factors in fermentation media.

Table 4.4 – Experimental runs and responses of significant factors in CCD

StdOrder	RunOrder	PtType	Blocks	Lactose	Tryptone	Yeast Extract	KH2PO4	CaCl2	Enzyme activity(U/ml)	Specific activity(U/mg)
1	1	1	1	3.75	7.5	7.5	2.75	0.1	526.5011547	17.62584859
2	2	1	1	7.5	7.5	7.5	2.75	0.1	533.169746	18.16292541
3	3	1	1	3.75	12	7.5	2.75	0.1	537.6154734	17.32440715
4	4	1	1	7.5	12	7.5	2.75	0.1	545.8718245	17.88797733
5	5	1	1	3.75	7.5	12	2.75	0.1	540.7909931	17.21203366
6	6	1	1	7.5	7.5	12	2.75	0.1	538.8856813	18.72808982
7	7	1	1	3.75	12	12	2.75	0.1	543.3314088	19.72280289
8	8	1	1	7.5	12	12	2.75	0.1	554.1281755	20.74634474
9	9	1	1	3.75	7.5	7.5	4.5	0.1	534.4399538	15.45488672
10	10	1	1	7.5	7.5	7.5	4.5	0.1	540.7909931	18.38214998
11	11	1	1	3.75	12	7.5	4.5	0.1	543.9665127	17.71319527
12	12	1	1	7.5	12	7.5	4.5	0.1	555.3983834	21.9050253
13	13	1	1	3.75	7.5	12	4.5	0.1	543.9665127	19.60809523
14	14	1	1	7.5	7.5	12	4.5	0.1	550.317552	21.16605969
15	15	1	1	3.75	12	12	4.5	0.1	551.5877598	21.69951847
16	16	1	1	7.5	12	12	4.5	0.1	561.7494226	24.11943504
17	17	1	1	3.75	7.5	7.5	2.75	0.2	534.4399538	20.00922532
18	18	1	1	7.5	7.5	7.5	2.75	0.2	538.8856813	19.2016737
19	19	1	1	3.75	12	7.5	2.75	0.2	547.7771363	18.41766944
20	20	1	1	7.5	12	7.5	2.75	0.2	550.317552	19.25490306
21	21	1	1	3.75	7.5	12	2.75	0.2	545.2367206	20.51254653
22	22	1	1	7.5	7.5	12	2.75	0.2	548.4122402	18.24117966
23	23	1	1	3.75	12	12	2.75	0.2	557.3036952	20.96652251
24	24	1	1	7.5	12	12	2.75	0.2	564.9249423	22.50986274
25	25	1	1	3.75	7.5	7.5	4.5	0.2	540.1558891	17.96655854
26	26	1	1	7.5	7.5	7.5	4.5	0.2	550.317552	20.40651209
27	27	1	1	3.75	12	7.5	4.5	0.2	550.9526559	20.33277659
28	28	1	1	7.5	12	7.5	4.5	0.2	560.4792148	21.99348817
29	29	1	1	3.75	7.5	12	4.5	0.2	556.6685912	19.88102112
30	30	1	1	7.5	7.5	12	4.5	0.2	554.7632794	21.12734848
31	31	1	1	3.75	12	12	4.5	0.2	562.3845266	22.52444486
32	32	1	1	7.5	12	12	4.5	0.2	588.4237875	25.76431838
33	33	0	1	5.625	9.75	9.75	3.625	0.15	545.2367206	16.37823482
34	34	0	1	5.625	9.75	9.75	3.625	0.15	547.1420323	17.23719817
35	35	0	1	5.625	9.75	9.75	3.625	0.15	547.1420323	17.37848668
36	36	0	1	5.625	9.75	9.75	3.625	0.15	547.1420323	17.37848668
37	37	0	1	5.625	9.75	9.75	3.625	0.15	547.1420323	17.37848668
38	38	0	1	5.625	9.75	9.75	3.625	0.15	547.1420323	17.37848668
39	39	0	1	5.625	9.75	9.75	3.625	0.15	547.1420323	17.37848668
40	40	0	1	5.625	9.75	9.75	3.625	0.15	547.1420323	17.37848668
41	41	-1	2	1.18875	9.75	9.75	3.625	0.15	540.7909931	18.92158102
42	42	-1	2	10.06125	9.75	9.75	3.625	0.15	556.6685912	22.76612972
43	43	-1	2	5.625	4.4265	9.75	3.625	0.15	535.7101617	16.06094295
44	44	-1	2	5.625	15.0735	9.75	3.625	0.15	555.3983834	21.20363286
45	45	-1	2	5.625	9.75	4.4265	3.625	0.15	533.8048499	18.84732386
46	46	-1	2	5.625	9.75	15.0735	3.625	0.15	557.3036952	23.42247092
47	47	-1	2	5.625	9.75	9.75	1.55475	0.15	530.6293303	17.42532758
48	48	-1	2	5.625	9.75	9.75	5.69525	0.15	543.3314088	23.59001915
49	49	-1	2	5.625	9.75	9.75	3.625	0.0317	535.0750577	17.9128799
50	50	-1	2	5.625	9.75	9.75	3.625	0.2683	561.7494226	21.44609865
51	51	0	2	5.625	9.75	9.75	3.625	0.15	546.5069284	18.45502699
52	52	0	2	5.625	9.75	9.75	3.625	0.15	547.1420323	17.37848668
53	53	0	2	5.625	9.75	9.75	3.625	0.15	547.1420323	17.37848668
54	54	0	2	5.625	9.75	9.75	3.625	0.15	547.1420323	17.37848668

Table 4.5: ANOVA (Analysis of variance) table for Enzyme activity in CCD

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	20	5264.33	263.22	21.28	0.000
Linear	5	4860.66	972.13	78.60	0.000
Lactose	1	572.70	572.70	46.30	0.000
Tryptone	1	1390.19	1390.19	112.40	0.000
Yeast Extract	1	1197.06	1197.06	96.78	0.000
KH2PO4	1	659.82	659.82	53.35	0.000
CaCl2	1	1040.90	1040.90	84.16	0.000
Square	5	182.90	36.58	2.96	0.026
Lactose*Lactose	1	33.23	33.23	2.69	0.111
Tryptone*Tryptone	1	1.92	1.92	0.15	0.696
Yeast Extract*Yeast Extract	1	1.92	1.92	0.15	0.696
KH2PO4*KH2PO4	1	109.04	109.04	8.82	0.006
CaCl2*CaCl2	1	28.37	28.37	2.29	0.139
2-Way Interaction	10	220.77	22.08	1.78	0.103
Lactose*Tryptone	1	87.88	87.88	7.11	0.012
Lactose*Yeast Extract	1	0.03	0.03	0.00	0.962
Lactose*KH2PO4	1	41.67	41.67	3.37	0.075
Lactose*CaCl2	1	0.38	0.38	0.03	0.862
Tryptone*Yeast Extract	1	3.86	3.86	0.31	0.580
Tryptone*KH2PO4	1	2.30	2.30	0.19	0.669
Tryptone*CaCl2	1	26.10	26.10	2.11	0.156
Yeast Extract*KH2PO4	1	6.96	6.96	0.56	0.458
Yeast Extract*CaCl2	1	44.62	44.62	3.61	0.066
KH2PO4*CaCl2	1	6.96	6.96	0.56	0.458
Error	33	408.17	12.37		
Lack-of-Fit	22	404.67	18.39	57.88	0.000
Pure Error	11	3.50	0.32		
Total	53	5672.49			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.51692	92.80%	88.44%	72.47%

Table 4.6: ANOVA (Analysis of variance) table for specific activity in CCD

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	20	261.939	13.0969	28.92	0.000
Linear	5	160.808	32.1615	71.01	0.000
Lactose	1	23.296	23.2958	51.44	0.000
Tryptone	1	39.610	39.6100	87.46	0.000
Yeast Extract	1	43.435	43.4348	95.90	0.000
KH2PO4	1	33.617	33.6167	74.23	0.000
CaCl2	1	20.850	20.8504	46.04	0.000
Square	5	71.024	14.2049	31.36	0.000
Lactose*Lactose	1	23.213	23.2130	51.25	0.000
Tryptone*Tryptone	1	3.124	3.1238	6.90	0.013
Yeast Extract*Yeast Extract	1	27.243	27.2425	60.15	0.000
KH2PO4*KH2PO4	1	18.960	18.9596	41.86	0.000
CaCl2*CaCl2	1	10.316	10.3163	22.78	0.000
2-Way Interaction	10	30.107	3.0107	6.65	0.000
Lactose*Tryptone	1	2.171	2.1706	4.79	0.036
Lactose*Yeast Extract	1	0.134	0.1345	0.30	0.589
Lactose*KH2PO4	1	8.759	8.7591	19.34	0.000
Lactose*CaCl2	1	1.466	1.4658	3.24	0.081
Tryptone*Yeast Extract	1	6.088	6.0876	13.44	0.001
Tryptone*KH2PO4	1	6.959	6.9589	15.37	0.000
Tryptone*CaCl2	1	0.004	0.0041	0.01	0.925
Yeast Extract*KH2PO4	1	3.768	3.7681	8.32	0.007
Yeast Extract*CaCl2	1	0.662	0.6617	1.46	0.235
KH2PO4*CaCl2	1	0.096	0.0963	0.21	0.648
Error	33	14.946	0.4529		
Lack-of-Fit	22	12.767	0.5803	2.93	0.034
Pure Error	11	2.179	0.1981		
Total	53	276.884			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.672977	94.60%	91.33%	82.28%

.

Regression equation in uncoded units:

Enzyme activity (U/ml) =544.1 - 7.04 A - 2.75 B – 1.49 C + 8.12 D – 210 E + 0.212 A*A + 0.0354 B*B + 0.354 B*B + 0.0354 C*C – 1.766 D*D + 276 E*E + 0.393 A*B + 0.007 A*C + 0.696 A*D + 1.16 A*E + 0.069 B*C + 0.136 B*D + 8.03 B*E + 0.237 C*D + 10.50 C*E + 10.7 D*E

Specific Activity (U/mg) = 64.11 – 2.871 A – 2.487 B – 3.352 C – 9.95 D – 5.2 E + 5.2 A*A + 0.0452 B*B + 0.1335 C*C + 0.737 D*D + 166.4 E*E +0.0617 A*B – 0.0154 A*C + 0.3189 A*D – 2.28 A*E + 0.0862 B*C + 0.2369 C*D – 0.10 B*E +0.1743 C*D – 1.28 C*E – 1.25 D*E

Where Lactose- A, Tryptone- B, Yeast extract- C, KH_2PO_4- D, $CaCl_2$-E

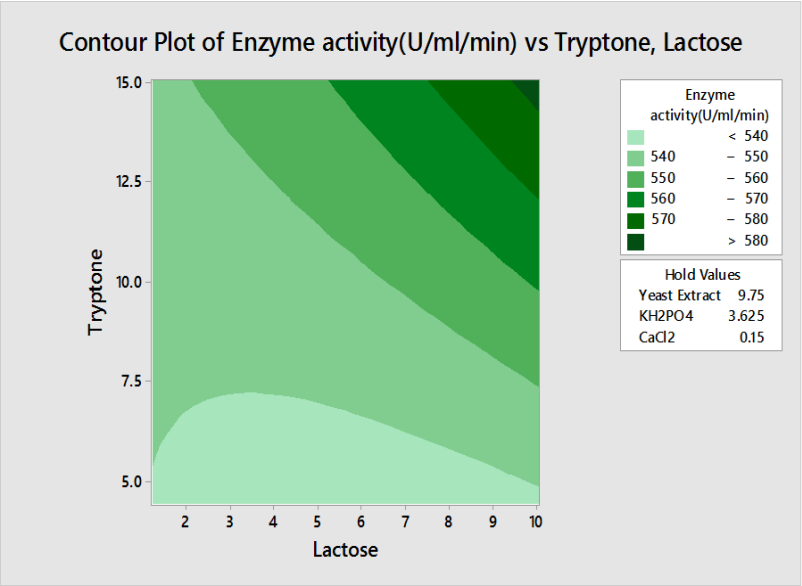

Figure 4.6: Contour plot for Lactose and tryptone interaction for Enzyme activity

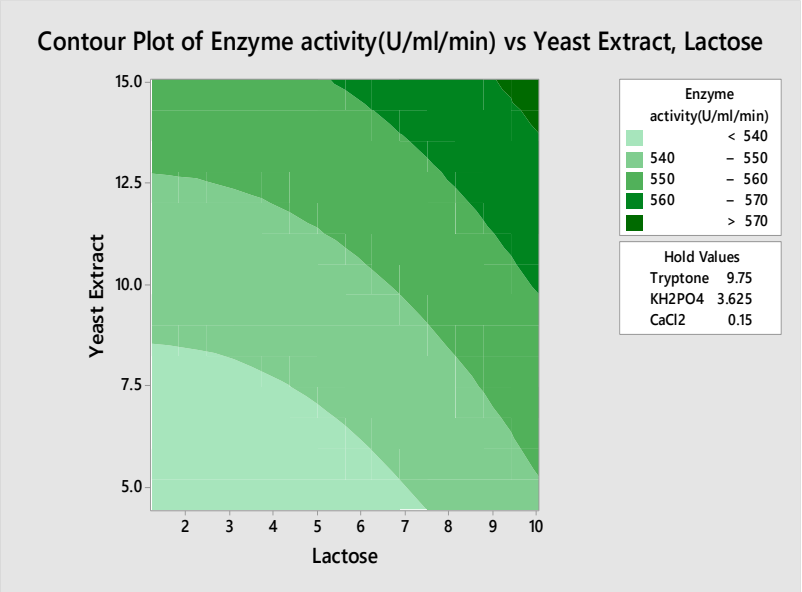

Figure 4.7: Contour plot for Lactose and yeast extract interaction for Enzyme activity

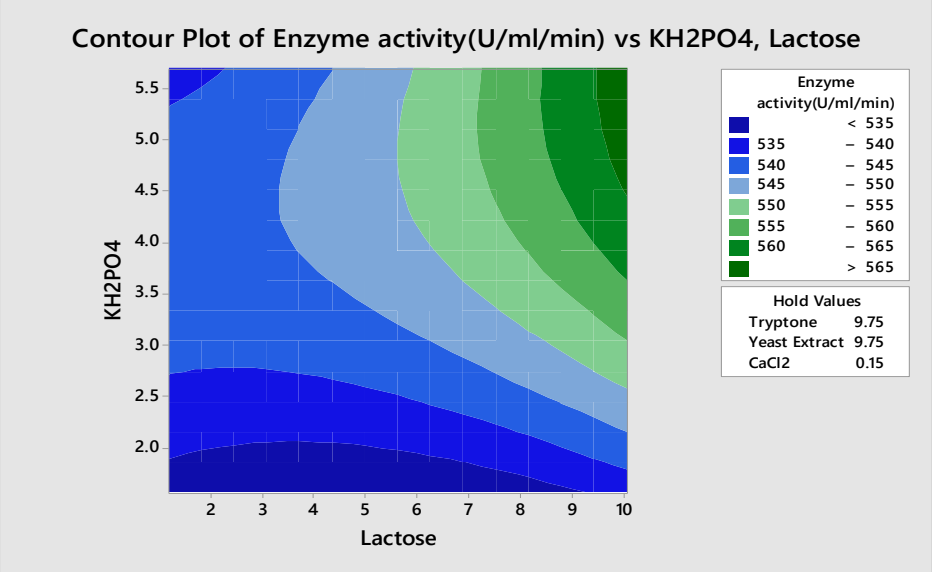

Figure 4.8: Contour plot Lactose and KH_2PO_4 interaction for Enzyme activity

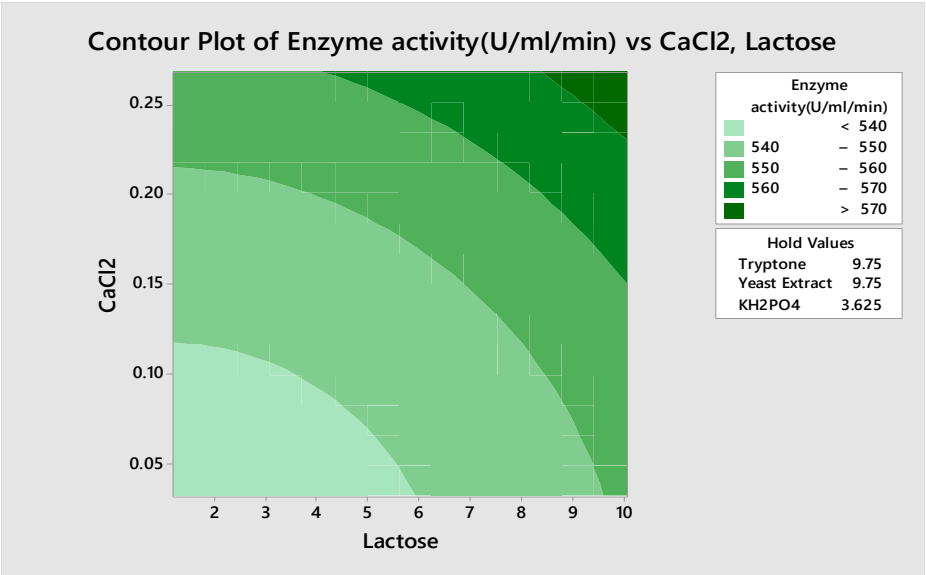

Figure 4.9: Contour plot for Lactose and $CaCl_2$ interaction for Enzyme activity

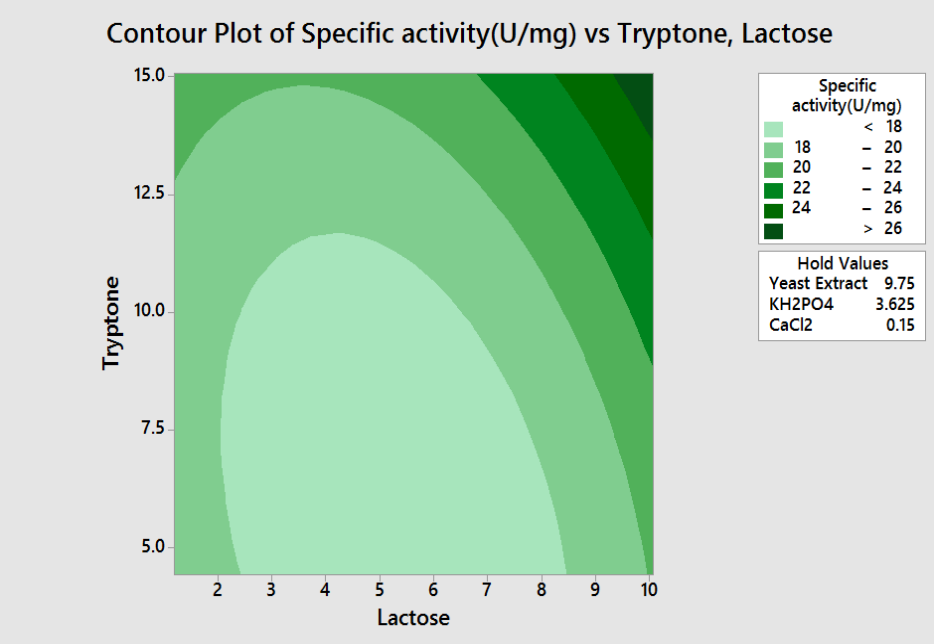

Figure 4.10: Contour plot for Lactose and Tryptone interaction for Specific activity

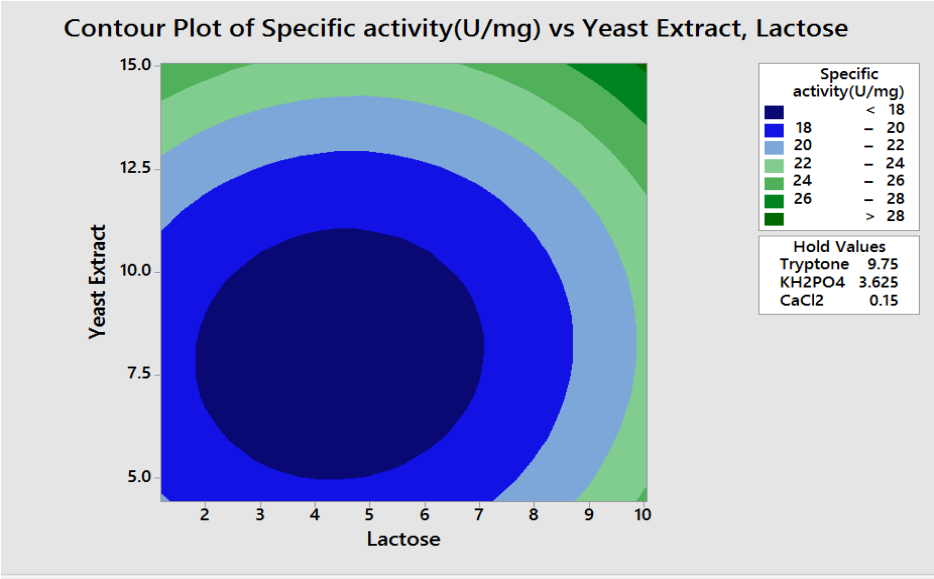

Figure 4.11: Contour plot for Lactose and yeast extract interaction for Specific activity

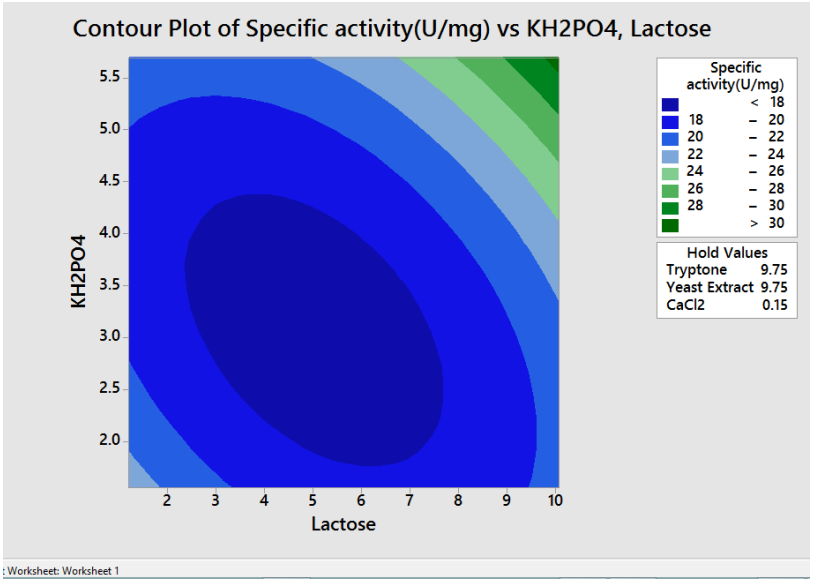

Figure 4.12: Contour plot Lactose and KH_2PO_4 interaction for Specific activity

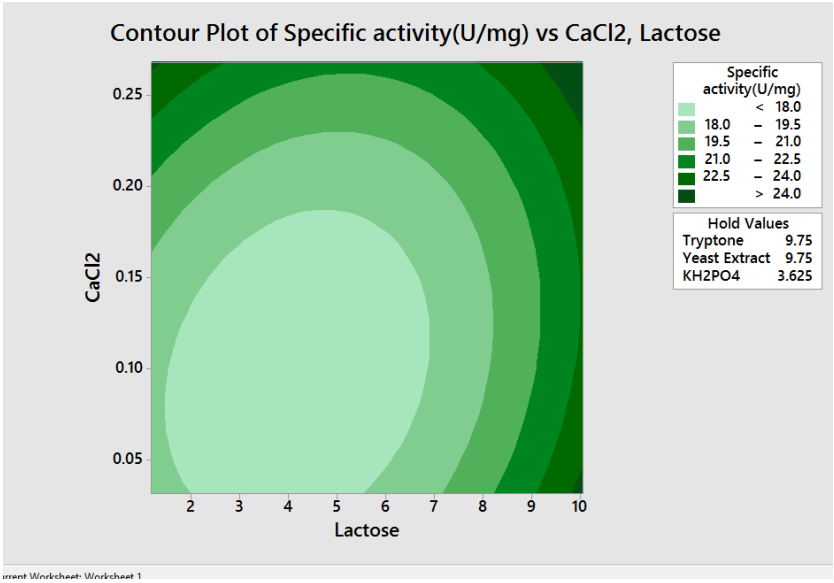

Figure 4.13: Contour plot for Lactose and $CaCl_2$ interaction for specific activity

Table 4.7: Optimized values and response for Factors in fermentation media

Factors	Predicted optimum value	Experimental optimum value
Lactose	7.50	7.50
Tryptone	12.0	12.0
Yeast extract	12.0	12.0
KH_2PO_4	4.50	4.50
$CaCl_2$	0.20	0.20

Response	Predicted optimum response(U/ml)	Experimental optimal response(U/mg)
Enzyme activity	576.88	588.42
Specific activity	25.69	25.76

4.4. Optimization of Physical parameter using Response surface methodology

Optimization of pH and temperature was carried out using optimized values fermentation media component. Table 4.8 shows the responses obtained in experimental runs in Central composite design. Anlysis of variance was evaluted in table 4.9 and table 4.10. From ANOVA table, regression coefficient was found to be 94.63% and 91.73% for enzyme activity and specific activity respectively which shows that model was significant. P-value for pH and temperature was found to be 0.001 and 0.051 which showed that both the factors have significant effect on enzyme activity. From the counter plot(Figure 4.14 and Figure 4.15), it can be concluded that both Enzyme activity and specific activity was maximum in the temperature range 30 - 40°C and in pH range 7 – 8. Beond these range enzyme and specific activity decreases. Figure 4.16 shows the predicted optimized values obtained for pH and temperature which was found to be 7.4 and 34.85°C respectively while the maximum enzyme activity and Specific activity was found to be 593.08 U/ml and 25.95 U/mg respectively.

Table 4.8: Experimental runs and responses for physical parameter in CCD

StdOrder	RunOrder	PtType	Blocks	pH	Temperature	Enzyme activity(U/ml)	Specific activity(U/mg)
5	1	-1	1	6.08579	35	535.7101617	16.09206881
8	2	-1	1	7.5	49.1421	289.2898383	15.19997456
7	3	-1	1	7.5	20.8579	503.9549654	14.30641385
12	4	0	1	7.5	35	590.9642032	25.94885312
9	5	0	1	7.5	35	590.3290993	25.92096612
1	6	1	1	6.5	25	512.8464203	17.5865476
4	7	1	1	8.5	45	410.5946882	10.67821756
11	8	0	1	7.5	35	590.3290993	25.92096612
13	9	0	1	7.5	35	590.3290993	25.92096612
3	10	1	1	6.5	45	458.2274827	18.78975127
10	11	0	1	7.5	35	590.3290993	25.92096612
2	12	1	1	8.5	25	507.130485	15.78418176
6	13	-1	1	8.91421	35	444.2551963	19.23451269

Table 4.9: ANOVA (Analysis of variance) table for Enzyme activity using CCD

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	93361.8	18672.4	24.68	0.000
Linear	2	30020.0	15010.0	19.84	0.001
pH	1	4171.7	4171.7	5.51	0.051
Temperature(°C)	1	25848.2	25848.2	34.16	0.001
Square	2	62902.5	31451.3	41.57	0.000
pH*pH	1	12869.9	12869.9	17.01	0.004
Temperature(°C)*Temperature(°C)	1	55963.4	55963.4	73.96	0.000
2-Way Interaction	1	439.3	439.3	0.58	0.471
pH*Temperature(°C)	1	439.3	439.3	0.58	0.471
Error	7	5296.6	756.7		
Lack-of-Fit	3	5296.2	1765.4	21884.04	0.000
Pure Error	4	0.3	0.1		
Total	12	98658.3			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
27.5073	94.63%	90.80%	61.83%

Table 4.10: ANOVA (Analysis of variance) table for specifec activity using CCD

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	328.731	65.746	15.55	0.001
Linear	2	4.610	2.305	0.55	0.603
pH	1	3.740	3.740	0.88	0.378
Temperature(°C)	1	0.871	0.871	0.21	0.664
Square	2	314.170	157.085	37.14	0.000
pH*pH	1	126.023	126.023	29.80	0.001
Temperature(°C)*Temperature(°C)	1	226.915	226.915	53.66	0.000
2-Way Interaction	1	9.951	9.951	2.35	0.169
pH*Temperature(°C)	1	9.951	9.951	2.35	0.169
Error	7	29.603	4.229		
Lack-of-Fit	3	29.603	9.868	63441.52	0.000
Pure Error	4	0.001	0.000		
Total	12	358.335			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.05646	91.74%	85.84%	41.25%

Regression Equation in Uncoded Units

Enzyme activity (U/ml) = -2833 + 659 A + 65.0 B – 43.0 A*A – 0.897 B*B – 1.05 A*B

Specific activity (U/mg) = -318.6 + 68.7 A + 5.148 B - 4.256 A - 0.05711 B*B - 0.158 A*B

Where pH- A

Temperature- B

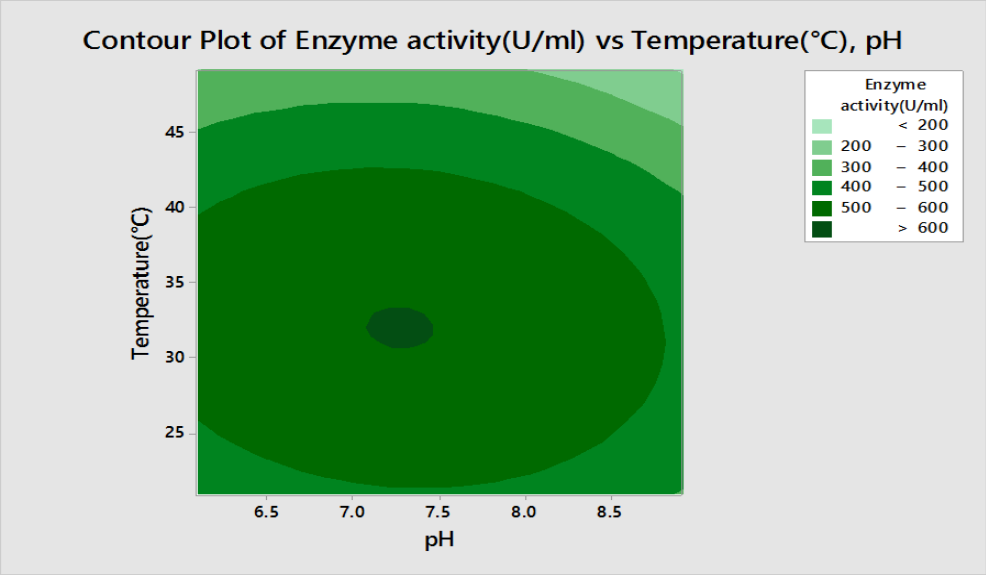

Figure 4.14: Contour plot for pH and temperature interaction for Enzyme activity

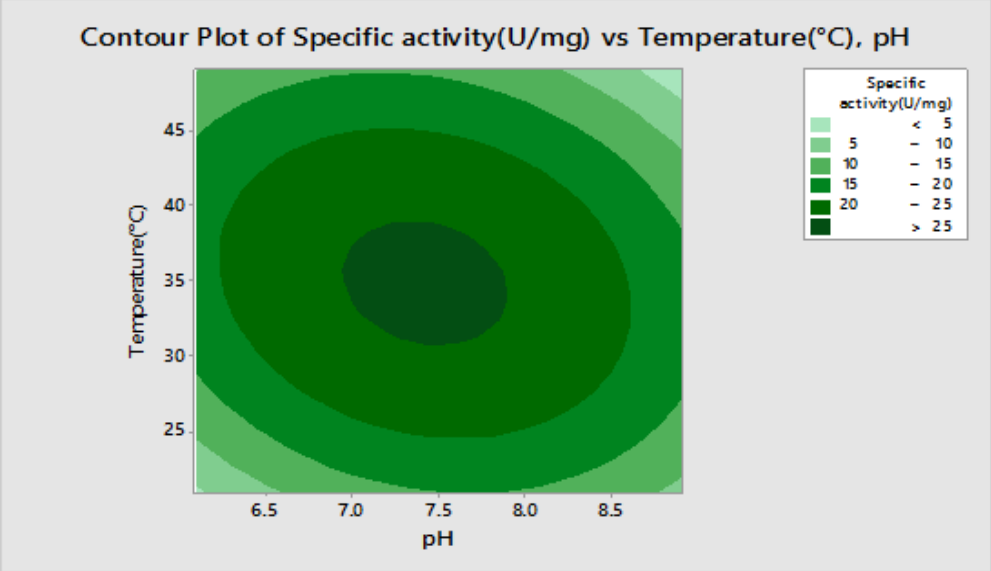

Figure 4.15: Counter plot for pH and temperature for Specific activity

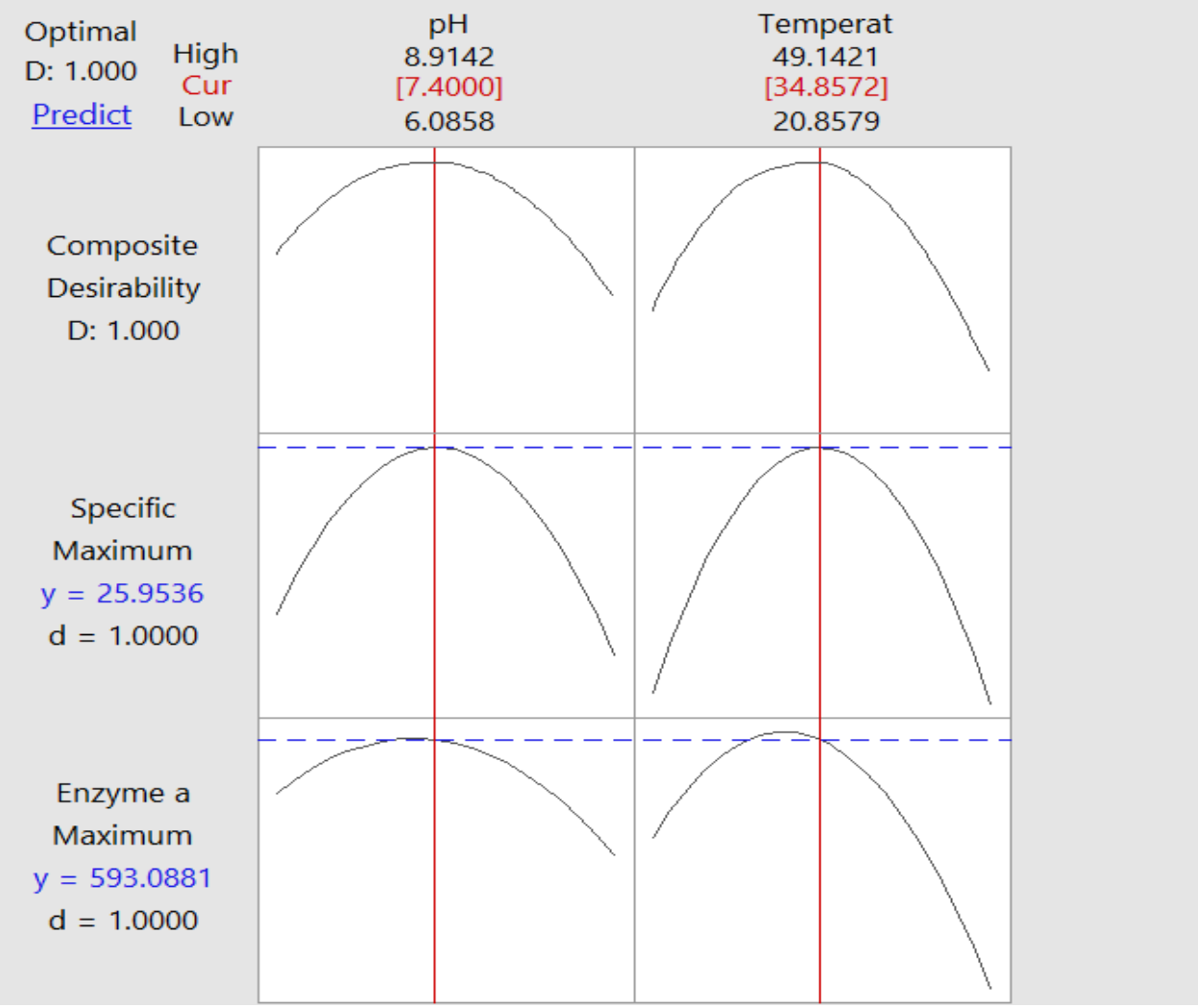

Figure 4.16: Optimized values of Enzyme activity and specific activity

Table 4.11: Optimized values for factors and response for physical parameter

Factors	Predicted optimum value	Experimental optimum value
Ph	7.4	7.5
Temperature	34.84	35

Response	Predicted optimum response(U/ml)	Experimental optimum response(U/mg)
Enzyme activity	593.08	590.96

Specific activity	25.96	25.92

4.5. Detrmination of Nattokinase activity using synthetic substrate N-succinyl-Ala-Ale-Pro-Phe-p-nitroanilide

Nattokinase activity for unoptimized parameter was found to be 1089.5 U/ml. For optimized parameter, Nattokinase activity was found to be 1565.18 U/ml. Nattokinase activity showed enhancement from 1089.5 U/ml to 1565.18 U/ml in optimal culture conditions when N-succinyl-Ala-Ale-Pro-Phe-p-nitroanilid used as a substrate.

Table 4.12: Enhanced enzyme activity in optimized culture condition

Substrate used for determination of Nattokinase activity	Nattokinase activity in unoptimized culture condition	Nattokinase activity in optimized culture condition
Caesin	321 U/ml	593.08 U/ml
N-succinyl-Ala-Ale-Pro-Phe-p-nitroanilide.	1089.50 U/ml	1565.16 U/ml

4.6. Structural and binding analysis using Docking

In this study, we have performed docking analysis to understand the interaction between nattokinase and casein to interpret the data we have collected from the experiments aforementioned. It was done to resolve a question about the specificity of nattokinase towards casein as its substrate and to screen the effect of other proteases which might be present alongside nattokinase in the system. We have collected the binding energy values of nattokinase with the substrate from Firedock. Alongside, we have performed docking study with other proteases as well. The binding energy of the enzymes with the substrate reveal that the affinity of nattokinase

towards casein is significantly less in comparison to other proteases. A negative value of binding energy corresponds to spontaneity of the reaction. We have found that the binding energy of Nattokinase (PDB ID. 4DWW) to casein is -6.17 which is higher than we have found in alkaline serine protease (PDB ID.1WME), serine protease (PDB ID. 1AH2), alkaline protease (PDB ID.1WSD), serine protease (PDB ID. 1GCI) and chymotrypsin (PDB ID.1YPH) we see that the value is positive which means that the reaction is not spontaneous. So, we predict that, even in the presence of other protease enzymes, Nattokinase would bind to casein more favorably. This resolves a question that Nattokinase is the enzyme which bound to casein specifically to the tyrosine residue and cleaved substrate at the site, which is why tyrosine is released in the process.

Table 4.13: Binding energy of Nattokinase with substrate

PDB ID.	Binding energy (Kcal/mol) with substrate caesin	Spontaneity of reaction
4DWW	-6.17	Spontaneous

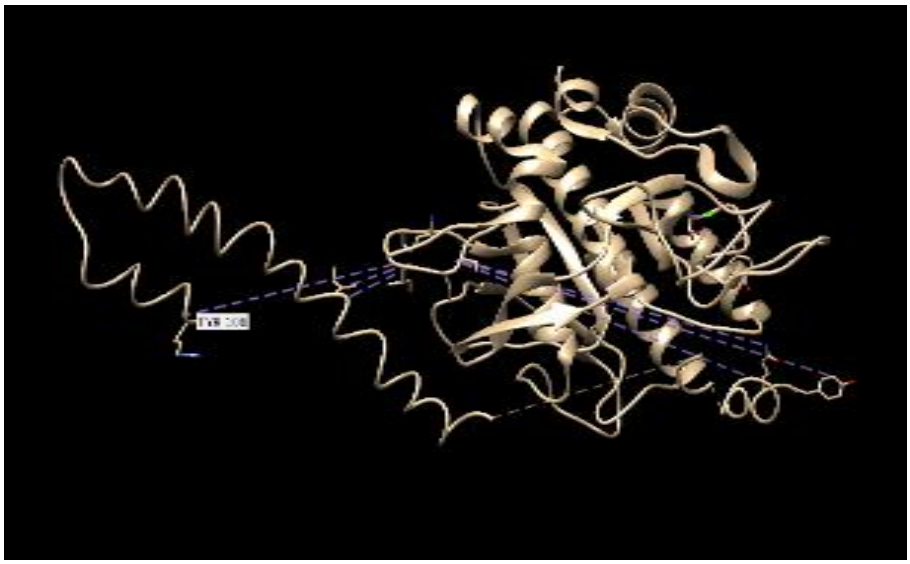

Figure 4.17: 3D representation of Nattokinase with tyrosine residue of the alpha domain of casein

Table 4.14: Binding energy of different protease enzymes found in Bacillus subtilis

PDB ID.	Binding energy (Kcal/mol) with substrate caesin	Spontaneity of reaction
1WME	-1.62	Spontaneous
1WSD	1.42	Non-spontaneous
1AH2	4.17	Non-spontaneous
1GCI	6.32	Non-spontaneous
1YPH	14.26	Non-spontaneous

Chapter 5

Conclusion

In this study, culture condition for Nattokinase production using *Bacillus subtilis* MTCC 2616 was successfully optimized using statistical method. Different carbon sources like lactose and glycerol were used for production of Nattokinase. Maximum production was obtained in lactose containing media. The unoptimized media obtained after screening of carbon source was used for further optimization studies. For optimization of fermentation media, placket burman design was used for screening of factors which affect the enzyme acivity of Nattokinase. The screened factors were further optimized using Central composite design. The optimized media containing (g/L): Lactose (7.50),Tryptone (12.00),Yeast extract (12.00),K_2HPO_4 (4.50) and $CaCl_2$ (0.20) which showed maximum Nattokinae activity and Specific activity 576.88 U/ml and 25.66 U/mg respectively. Physical parameter like pH and temperature was optimized in optimized fermentation media using central composite design. . Maximum nattokinase activity and specific activity was found to be 593.08 U/ml and 25.96 U/mg respectively. Caesinolytic activity of Nattokinase in optimal conditions was increased from 321 U/ml to 593.08 U/ml. The fibrinolytic activity using synthetic substarte was enhanced from 1089.50 U/ml to 1565.16 U/ml in optimal conditions. The enhancement in Nattokinase activity after the optimization proved that Nattokinase production was successfully optimized. In structural and binding analysis of Nattokinase with substrate caesin, we have performed docking of various protease enzymes found in *Bacillus subtilis*. Docking analysis showed that the binding energy of Nattokinase (PDB ID. 4DWW) to casein is -6.17 which is higher than other protease enzyme present in *Bacillus subtilis*. It resolves the question about the specificity of Nattokinase towards its substrate caesin.

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