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Media Optimization for Serratiopeptidase by Statistical Approach followed by Isolation and Product Purification

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Abstract: *Serratiopeptidase is a potent anti-inflammatory , proteolytic enzyme isolated from Serratia marcescens. The main goal of this study was to optimize the medium to increase the productivity of serratiopeptidase, which was carried out by using Plackett-Burman design for screening of nine medium components out of which the concentration of three significant factors (soya oil, soya flour and casein) were narrowed down for CCD and RSM analysis to understand the mutual interactions between selected factors.Maximum productivity of i.e.15,000 SPU/ml/ml was achieved by applying statistical approach.*

Keywords: Serratiopeptidase, Plackett-Burman, CCD.

[1] Introduction

Serratiopeptidase (Serratia E-15 protease, also known as serralysin, serratiapeptase, serratiopeptidase, serratio peptidase, or serrapeptidase) is a proteolytic enzyme (protease) produced by enterobacterium *Serratia* sp. E-15(1)present in the gut wall of the silk worm [1]. Initially it was discovered in 1819 by Bartolomeo Bizio in Padua, Italy. Bizio named the genus Serratia in honor of and Italian physicist named Serratia, and chose marcescens for the species name after the Latin word for decay [2]. *Serratia marcescens* was first thought to be harmless (non-pathogenic). Due to its ability to produce red pigmentation, it was first used in 1906 as a marker in order to trace bacterial activity or transmission. It was discovered by human researchers in the 1960s and is produced synthetically by bacteria through fermentation process.

Serrapeptase (commonly misspelled as serapeptase) is an enzyme that is produced in the intestine of silk worms to break down cocoon walls. This enzyme is proving to be a superior alternative to NSAIDs (Non Steroidal Anti-Inflammatory Drugs) traditionally used to treat rheumatoid arthritis and osteoarthritis[3]. Serrapeptase has been used to treat chronic sinusitis, carpal tunnel syndrome, sprains and torn ligaments, fibrocystic breast disease, ovarian cysts, ear, nose and throat infections, fibromyalgia, varicose veins, emphysema, asthma, bronchitis, migraines (vascular), Inflammatory Bowel Diseases (IBD) including Crohn's, colitis and cystitis, enlarged prostate, pain, and postoperative inflammation. Some researchers believe Serrapeptase can play an important role in arterial plaque (hardening of the arteries) prevention and removal [4].

Production of an anti-inflammatory enzyme serratiopeptidase by fermentation with *Serratia marcescens* was studied to ascertain optimal nutritional conditions for large scale production. Different physicochemical parameters were studied and optimized. The optimized lab medium comprised of g/L : Beef extract-5.0, Peptone-8.0 , Sodium chloride (NaCl) -2.0, Dextrose-5.0 and Casein-7.5.The pH of the media was adjusted to 7.0 ± 0.05. Inoculated flasks were incubated in shaking incubator at 240 rpm and 30^{O}C for 72hrs.

The modified fermentation medium produced 15,000 SPU/ml/ml of serratiopeptidase compared to 5000 SPU/ml/ml in basal medium and the molecular weight of the purified serratiopeptidase was found to be 52 kD.It is an extra cellular product ,separation of cell mass from fermenter broth is required to get maximum product recovery.different filtration techniques were studied with various polar solvent in order to obtain maximum product.

[2] Materials and method

2.1 Culture Source: Bacterial strain of *Serratia marcescens* .

2.2 Fermentation Medium and Flask Culture Growth media and culture conditions:

Initially the strain was grown in Nutrient broth 35.0 ml of media was dispensed in 250ml of Erlenmeyer flask and sterilized for 20 mins at 121^{o} C. The autoclaved media was inoculated with 0.2 ml preserved cryovial suspension.Inoculated medium was incubated at 30^{o} C for 24±2 hrs in a shaking incubator at 240 rpm. Grown culture (0.5%) was transferred in lab media (35ml in 250ml flasks) ; Lab media comprises of g/L : Beef extract-5.0,Peptone-8.0 , Sodium chloride (NaCl) -2.0, Dextrose-5.0 and Casein-7.5. The pH of media was adjusted to 7.0 ± 0.05 and autoclaved for 30 min. The flasks were incubated at 30^{o} C for 15±2 hrs in a shaking incubator at 240rpm. After checking the physical parameters,i.e pH, PMV and Microscopy .Grown lab (0.5%)was further transferred to 250 ml Erlenmeyer flask containing 35.0 ml of seed media.Seed media comprises of g/L : Soya flour-5.0 , Casein-15.0, Di ammonium hydrogen phosphate [(NH4)2HPO4]-2.5,Soya oil-5.0,Calcium chloride di hydrate (CaCl2.2H2O)-0.1, Sodium chloride (NaCl)-0.2, Potassium chloride (KCl)-0.1 , Magnesium sulphate (MgSO4.7H2O) - 0.2, Zinc sulphate - 7 - hydrate (ZnSO4.7H2O) -0.2 and Dextrose - 20.0.The actual pH of the media was 7.0 - 7.2 which was adjusted to 7.7 ± 0.05 and autoclave for 30 min.The flasks were incubated at 30^{o} C for 24±2 hrs in a shaking incubator at 240rpm. After checking the physical parameters,i.e pH, PMV and Microscopy.Grown seed (1.0purification is maximum with polar solvents as compared to non polar solvents%) was further transferred in production media (50 ml in 500ml wide mouth , base three baffled flasks) ; Production media comprises of g/L : Soya flour-20.0 , Casein-15.0, Diammonium hydrogen phosphate [(NH4)2HPO4]-7.5,Soya oil-15.0,Sodium chloride (NaCl)-0.5, Potassium chloride (KCl)-0.1 , Magnesium sulphate (MgSO4.7H2O) - 0.1, Zinc sulphate - 7 - hydrate (ZnSO4.7H2O) -0.1 and Dextrose - 20.0.The actual pH of the media was 7.1 - 7.4 which was adjusted to 7.7 ± 0.05 and autoclaved for 30 mins.Flasks were incubated in shaking incubator at 30°C and 180 rpm for 72 hrs. The yield was assessed through Enzymatic assay.

2.3 Quantification of serratiopeptidase by enzymatic assay:

Serratiopeptidase yield was estimated by enzymatic assay.The method used for analysis was described below:

2.3.1 Method:

(a) Test solution: Pipette 1.0 ml of stock test solution, allowed it to stand in a water-bath at 37°C for 20minutes. Added 5 ml of substrate solution,mixed immediately and allowed it to stand in water-bath at 37°C for 20minutes. Added 5 ml of protein precipitating solution. Mixed and allowed to stand in water-bath at 37°C for 30 minutes. Measured the absorbance at 660nm.

(b) Blank solution: Pipette 1.0 ml of stock test solution, allowed to stand in a water-bath at 37°for 5 minutes, added 5 ml of protein precipitating solution,mixed immediately and allowed it to stand in water-bath at 37°C for 20 minutes. Added 5 ml of substrate solution. Mixed and allowed to stand in water-bath at 37°C for 30 minutes and filtered. 2 ml of the filtrate was taken ,50 ml of 0.6 percent w/v sodium carbonate solution was added to it. Added 1 ml of diluted Folin's reagent, mixed and allowed to stand at 37 C for 30 minutes. Measured the absorbance at 660nm.

(One serratiopeptidase unit is defined as the amount of enzyme required to liberate 1 µm of free tyrosine per minute under the specified assay conditions.)

Calculated the serratiopeptidase IU/mg by using concentration of tyrosine from graph considering the reaction time and dilution.

2.3.2 Solutions required for the Enzymatic assay:

(a) Sodium borate hydrochloride acid buffer pH 9.0: Dissolved 19.0 g sodium borate in 900 ml of water.The pH was adjusted to 9.0 with 1M hydrochloric acid and was further diluted with water to 1000ml.

(b) Substrate solution: Dissolved 1.2 g dried casein in 100 ml of sodium borate hydrochloric acid buffer pH 9.0. Kept it in boiling water- bath for 1-2 minutes to get clear solution. Cooled and filtered the solution through cotton and diluted with sodium borate hydrochloric acid buffer pH 9.0 to 200 ml.

(c) Protein precipitating solution: To 18.0 g of trichloroacetic acid, added 30 g of sodium acetate and 20 ml of glacial acetic acid and diluted with water to 100 ml.

(d) Dilute Folin's reagent: 1 ml of Folin's reagent, was added 2 ml of water.

(e) Reference tyrosine solution: Dissolved 10 mg of tyrosine in 1 ml of 1M hydrochloric acid and diluted to 100 ml with sodium borate hydrochloric acid buffer pH 9.0.

(1) Reference tyrosine curve: To 1, 2, 3, 4 and 5 ml of reference tyrosine solution, added 5, 4, 3, 2 and 1 ml of sodium borate hydrochloric acid buffer pH 9.0 respectively. Added 5 ml of protein precipitating reagent in each tube. To 2 ml of these solutions, added 5 ml of sodium carbonate solution. Added 1 ml of diluted folin's reagent mixed and allowed to stand at 37°C for 30minutes, measured the absorbance at 660 nm.Graph was plotted µm of tyrosine per system against the absorbance.

(f) Stock test solution: Weighed about 0.1 g of the substance under examination, dissolved in 100 ml of sodium borate hydrochloric acid buffer pH 9.0 (solution A). Mixed and kept it for 5 minutes.1 ml of solution A was taken and diluted to 200 ml with sodium borate hydrochloric acid buffer pH 9.0 (Solution B).

2.4 Product Extraction and purification :

2.4.1 Effect of solvent on isolation of serratiopeptidase:

Serratiopeptidase is an endopeptidase, having molecular weight of about 60 K Dalton. The recovery process involves microfiltration, ultra-filtration and precipitation by anti solvent. Effect of polarity of solvents is studied on precipitation of serratiopeptidase. In presence of highly polar solvents like methanol, acetonitriles.

Serratiopeptidase breaks into small molecular weight

compound which results in low recovery whereas mid polar solvent like Acetone and Isopropyl has a better stability and gives better recovery.

2.5 Materials and methods for the recovery of serratiopeptidase :

2.5.1 Concentration of serratiopeptidase enzyme

Serratiopeptidase is extra-cellular product. Mycelium is separated by three methods

[3] High speed centrifugation, fermentation broth was centrifuged at 5000 rpm and supernatant was decanted.
[4] Ceramic microfiltration.
[5] Hollow fiber micro filtration system.It gives better filtration rate. The filtrate was concentrated by subjecting it to ultrafiltration system having 5 k dalton cut of membrane.

Concentrated broth was analyzed by Enzymatic assay.

2.5.2 Isolation of serratiopeptidase:

Serratia petidase was isolated by slow addition of clear solution to various water soluble solvents ,the slurry was stirred for 2 hrs, filtered and dried .

Solvent	Methanol	Acetonitrile	Acetone	IPA
Volume ml	500	500	500	500
Units/ml	127821	127821	127821	127821
Solvent used	2 V	2 V	2 V	2 V
Solids isolated	11.3	14.5	14.5	14.5
Activity units/mg	294	2345	3305	3414
Rec on activity	5.21	53.20	74.98	77.46
Serratiopeptidase content by GPC	7.34	68.20	88.54	89.61%

2.6 Experimental Methods and Analysis-

2.6.1 Plackett-Burman Design for Screening Medium Components

A set of nine medium components were screened by Plackett Burman design [5]. Plackett-Burman Design was introduced in this study as a first optimization step to identify the factors that have significant effects on the serratiopeptidase production. The ingredients studied by Placket Burman design were carbon sources (dextrose, casein), nitrogen sources (soya flour, soya oil), minerals
(sodium chloride, potassium chloride,magnesium sulphate, zinc sulphate), and buffering agent (di-ammonium hydrogen phosphate). Based on Plackett-Burman factorial design, each variable was examined in two levels: −1 for low level and +1 for high level.This design was used to screen and evaluate the important factor(s) that influence the response of 09 assigned factors and two dummies to estimate test error in 12 experimental designs.

Plackett-Burman experiments were designed by using STAT EASE Version 8.0 USA. All experiments were performed in duplicate and the average of it was taken as the response. The factors included in the screening experiment and their settings are given in Table 1.

Table 1: Two levels of the factors used in Plackett-Burman Design. (High and low concentrations of various medium components used in Plackett Burman)

Code	Factors	Low level (-1)	High level (+1)
A	Casein	20.0	40.0
B	Soya Flour	10.0	20.0
C	Soya Oil	20.0	40.0
D	Dextrose	5.0	20.0
E	NaCl	0.5	2.0
F	(NH4)2HPO4	5.0	15.0
G	MgSO4.7H2O	0.1	0.5
H	KCl	0.1	0.5
I	ZnSO4.7H2O	0.1	0.5

Table 2: Twelve run Plackett-Burman design matrix for 7 variables with coded values along with the yield.

Run	A	B	C	D	E	F	G	Yield
1	+	+	-	+	+	+	-	10,000
2	-	+	+	-	+	+	+	9,500
3	+	-	+	+	-	+	+	8,100
4	-	+	-	+	+	-	+	11,200
5	-	-	+	-	+	+	-	11,800
6	-	-	-	+	-	+	+	13,500
7	+	-	-	-	+	-	+	12,700
8	+	+	-	-	-	+	-	11,700
9	+	+	+	-	-	-	+	12,100
10	-	+	+	+	-	-	-	12,600
11	+	-	+	+	+	-	-	10,800
12	-	-	-	-	-	-	-	10,400

2.6.2 Optimization of the independent variables-

(a) Response surface methodology

A central composite design (CCD) was employed to optimize the three most significant factors (soya flour, soya oil, and casein) screened by Plackett-Burman Design. The CCD is one of the most commonly used response surface designs for fitting second-order models. A central composite design consists of F factorial points, 2k axial points (±α), and nc center points. To optimize a Central Composite Design (CCD), consisting of a set of 17 experiments with replicates at 3 central point was conducted. The following second-order polynomial equation was adopted to study the effects of variables to the response.

Table 3: Coded values for each factor of the central composite design.

Code	Factors	Low level (-1)	High level (+1)
A	Casein	10.0	20.0
B	Soya Flour	5.0	10.0
C	Soya Oil	10.0	20.0

Each parameter was studied at three different levels (−1, 0, +1). The minimum and maximum ranges of parameters were investigated and the full experimental plan with respect to their values. A matrix of seventeen experiments with three factors was generated using the software package STAT EASE Version 8.0 USA. Serratiopeptidase activity was taken as the dependent variable or response (Y). The following second-order polynomial equation was adopted to study the effects of variables to the response.

$$Y= \beta o + \beta 1X1 + \beta 2X2 + \beta 3X3 + \beta 11X12 + \beta 22X22 + \beta 33X32 + \beta 12X1X2 + \beta 23X2X3 + \beta 13X1X3 \quad \text{.....(1)}$$

Where Y is the response (serratiopeptidase yield, SPU/ml/ml), βo is the constant term, β1, β2, β3 the coefficients of linear terms and β11, β22, β33 are the coefficients of quadratic terms and β12, β23, β13 are the coefficients of cross product terms,

X1, X2 and X3 represent the factors casein, Soya flour and Soya oil respectively.

Table 4: Experimental code and levels of factors in CCD

Run	A	B	C	Activity (SPU/ml)
1	-1.00	1.00	1.00	11,200
2	0.00	0.00	0.00	12,500
3	1.00	1.00	-1.00	15,000
4	-1.00	-1.00	-1.00	12,100
5	1.00	-1.00	-1.00	14,600
6	0.00	0.00	0.00	11,800
7	0.00	0.00	1.68	10,600
8	0.00	0.00	-1.68	12,900
9	-1.00	-1.00	1.00	13,500
10	0.00	1.68	0.00	10,900
11	0.00	-1.68	0.00	12,300
12	-1.00	1.00	-1.00	13,800
13	1.68	0.00	0.00	13,600
14	0.00	0.00	0.00	11,900
15	0.00	-1.00	1.00	12,700
16	-1.68	0.00	0.00	11,800
17	1.00	1.00	1.00	10,500

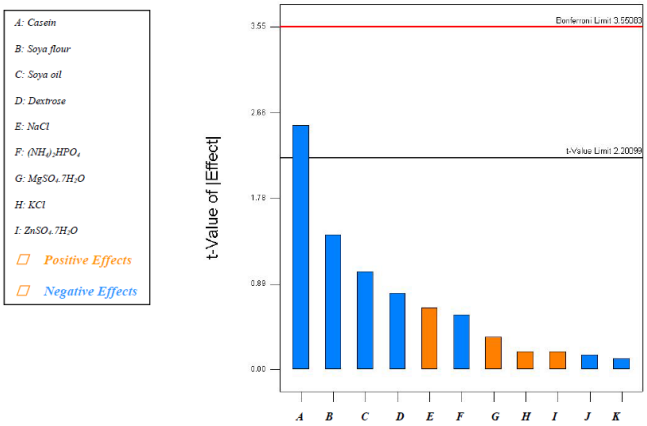
(b) Statistical analysis

All experiments were done in triplicate, and the average concentration. The P-B experimental design and statistical analysis of the data were done with the package STAT EASE Version 8.0 USA., while the CCD and statistical analysis of the data were done with the Design Expert software package (version 8.0, State-Ease Inc., Minneapolis, MN, USA). Statistical analysis of the models was used to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equations was judged statistically by the coefficient of

determination R2, and its statistical significance was determined by the F-test [6].

3. Result and Discussion :

Plackett-Burman Design was used as a screening method to determine which of the 09 components of the fermentation medium significantly effect the product yield..The experimental results with the Plackett-Burman Design are shown in Table 3. These are tested simultaneously by shifting factors from a low value (−1) to a high value (+1). The experimental results were interpreted based on the partition of the overall effect of all the factors to the response into individual factor effect. This partition has been made statistically. When the value of the concentration effect, E(Xi) ,of the tested variable is positive, the conclusion is that the influence of the concerning variable is greater at a high concentration tested, and when negative, this means that the influence of the given variable is greater at a low concentration [7]. Screened variables of PB was further proceeded with CCD to optimize the actual required concentration of selected variables.



3.1 Optimization of the independent variables:

3.1.1 Response surface methodology

To optimize nitrogen source (soya flour, soya oil), and carbon source (casein) which was already optimized with Plackett-Burman were further proceeded with (response surface) a Central Composite Design consisting of a set of 17 experiments with three replicates at central point was conducted. Table 3 shows variables and their levels for central composite design (CCD). The CCD matrix of the independent variables in coded units (experimental design) and experimental values of response is given in Table 4. All the experiments were performed in 500 ml base baffled flask containing 50 ml of media. The quadratic model expressed by equation (2) represents serratiopeptidase yield (Y) as a function of casein (X1), soya flour (X2) and soya oil (X3).

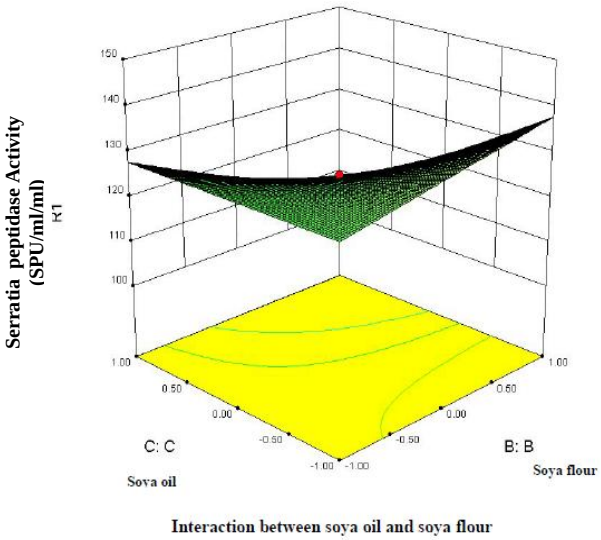
3.2 ANOVA for Response Surface Quadratic Model

Source	Sum of squares	df	Mean square	F value	p value Prob > F
Model	2672.20	9	296.91	5.00	0.0227
A-Casein	200.07	1	200.07	3.37	0.1089
B-Soya flour	165.52	1	165.52	2.79	0.1388
C-Soya oil	1266.18	1	1266.18	21.34	0.0024
AB	18.00	1	18.00	0.30	0.5989
AC	338.00	1	338.00	5.70	0.0484
BC	544.50	1	544.50	9.18	0.0191
A^2	84.41	1	84.41	1.42	0.2718
B^2	*14.98*	1	*14.98*	0.25	0.6308
C^2	14.79	1	14.79	0.25	0.6329
Residual	415.33	7	59.33		
Cor Total	3087.53	16			

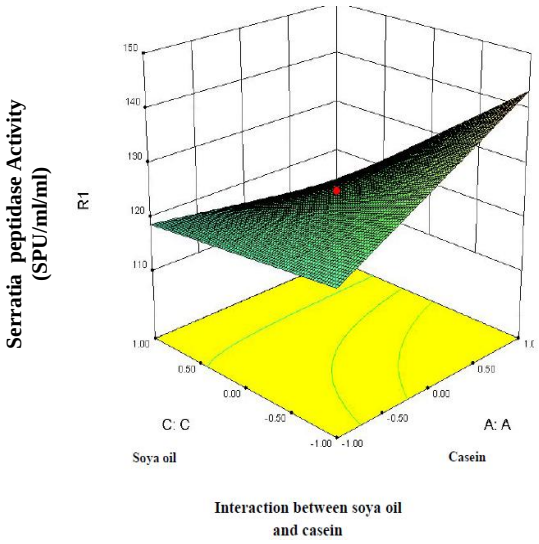
The Model F-value of 5.0 implies the model is significant. There is only a 2.27% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.

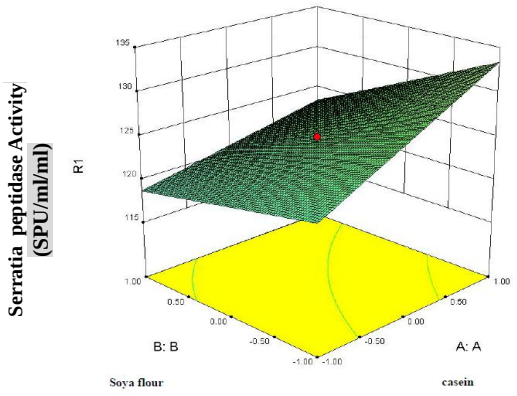
In this case C,AC,BC are significant model terms.Values greater than 0.1000 indicate the model terms are not significant.The "Lack of Fit F-value" of 4.74 implies the Lack of Fit is not significant relative to the pure error.There is a 18.34 % chance that a "Lack of Fit F-value" this large could occur due to noise.The R-Squared value is 0.8655 is and "Adj R-Squared" value is 0.6925. This model can be used to navigate the design space.



Interaction between soya oil and soya flour



Interaction between soya oil and casein



Interaction between soya flour and casein

4. Conclusion:

This work has demonstrated the use of statistical approach to obtain optimum yield of serratiopeptidase production from *Serratia marescenes*. This methodology could therefore be successfully employed for process development where an analysis of effects and interactions of many experimental factors are required. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments required. Response curves are very helpful in visualizing the main effects and interaction of factors. Thus, less time consuming experimental designs could generally suffice for the optimization of many processes. From the above Pareto chart of standardized effects, it can be seen that nitrogen sources like Soya flour and Soya oil ,Carbon source casein have significant effect on serratiopeptidase production.Therefore,to increase the production, it is necessary to optimize the concentrations of Soya flour , Soya oil and Casein with CCD whereas other components of the medium can be kept constant.The optimization of the analyzed responses demonstrated that the maximum Serratia peptidase production (14,900 SPU/ml/ml) were obtained with Casein - 20.0 g/l, Soya flour 20.0 g/l and Soya oil-10.0 g/l .All points were located near the central point of the design. The significant interactions between three variables were also observed from the contour plots. The methodology as a whole proved that the screening of medium components from experiment ,optimization is adequate responsibility of results obtained from final concentration of the medium.

In downstream process of product extraction and purification it was observed that in presence of high polar solvents like methanol and acetonitrile ,Serratiopeptidase breaks into low molecular weight compound which results in low activity as well low recovery.Hence, polar solvents shows better recovery as compared to non polar solvents.

Hence, statistical experimental designs are powerful tools for the rapid search of key factors from and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner [9].

[6] References:

[7] Nakahama K, Yoshimura K, Marumoto R, Kikuchi M, Lee IS, Hase T, Matsubara H (July 1986). "Cloning and sequencing of Serratia protease gene". *Nucleic Acids Res.* **14** (14): 5843–55. doi:10.1093/nar/14.14.5843. PMC 311595. PMID 3016665.

[8] Bry, Lynn. "Re: How and why did Serratia marcescens produce prodigiosin?" 5 June 2005. 2 Dec. 2008. <http://www.madsci.org/posts/archives/2005-06/1117999360.Mi.r.html>

[9] http://www.aviva.ca/article.asp?articleid=9#ixzz3DYBwSEsm

[10] http://www.naturalnews.com/033498_SerratioPeptidase_inflammation.html#ixzz3BP0HFMA2

[11] R. L. Plackett and J. P. Burman, "The design of optimum multifactorial experiments," Biometrika, vol. 33, no. 4, pp. 305–325, 1946. View at Publisher · View at Google Scholar

[12] GANGADHARAN, Dhanya; SIVARAMAKRISHNAN, Swetha; NAMPOOTHIRI, K. Madhavan; SUKUMARAN, Rajeev and PANDEY, Ashok. Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*. *Bioresource Technology*, July 2008, vol. 99, no. 11, p. 4597-4602.

[13] S. S. Deshmukh, M. V. Deshpande, and V. Shankar, "Medium optimization for the production of glucose isomerase from thermophilic Streptomyces thermonitrificans," World Journal of Microbiology and Biotechnology, vol. 10, no. 3, pp. 264–267, 1994. View at Scopus

[14] Cappuccino, James and Natalie Sherman. Microbiology: a Laboratory Manuel. 7th edition. ©2005 Pearson Education, Inc. pp.146,151,165

[15] 9.Statistical Optimization of Medium Components by Response Surface Methodology for Enhanced Production of Bacterial Cellulose by Gluconacetobacter persimmonis **Swati Hegde, Gururaj Bhadri, Kavita Narsapur, Shanta Koppal, Princy Oswal, Naina Turmuri, Veena Jumnal, Basavaraj Hungund*** B.V.B College of Engineering & Technology, Hubli-580031, Karnataka, India (rsm equation).