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# Application of response surface methodology (RSM) for protease production from *enterococcus hirae* and using algae as substrate

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## ABSTRACT

Protease is an enzyme that degrades protein, which has varied commercial applications in various fields. Protease enzyme is produced by using algae as substrate by various potential bacterial strains isolated from dairy effluent. *Chlorella sp.* is an SCP and has high protein content therefore its potentiality to used as substrate were explored. *Chlorella sp.* was treated with different concentrations of humic acid 0.1%-1.0%, the protein content in algae was estimated and it is observed the protein content has been increased to its peak at 0.5% humic acid treated algae. Protein content isolated from 0.1% - 0.5% were used as substrate for the production of protease. The maximum protein content found in 0.5% treated algae (880 µg/ml). *Enterococcus hirae* was found to be more potent than *Pseudomonas sp*. in the production of protease at all concentration, especially at 0.5% humic acid treated algae (172µg/ml). *Acinetobacter pitti* was found to be least producing than the other species. Media components were optimized to enhance the Protease production by *Enterococcus hirae* with Response surface methodology; three major factors such as Algal protein, Yeast extract and beef extracts were optimized. The optimized media, which resulted in the maximum amount of protease (290 µg/ml), consisted of Algal protein (70ml/L), Glucose (10g/L) and beef extracts (10g/L).



## KEYWORDS

Protease;
Humic acid;
Chlorella sp.;
Enterococcus hirae.;
Acinetobacter Pittii.;
Pseudomonas aeruginosa.

## INTRODUCTION

*Chlorella* is a genus of single-cell green microalgae, belonging to the phylum Chlorophyta. It is spherical in shape, about 2 to 10 µm in diameter, and is without flagella, it has its implementation as a health food use and food supplement as well as in the pharmaceutical and cosmetics industry[1]. Microalgae were the rich source of proteins and other nutrients, similar to higher plants. It is a potential food source because of its high protein content, when dried it is 45 % protein,20% carbohydrate,20% fat,5% fiber and 10 % minerals and vitamins[2]. Humic acid comprises of a mixture of weak aliphatic

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(carbon rings) and aromatic (carbon rings) organic acids, which were insoluble in water under acidic conditions and soluble in water in alkaline conditions. It is a commercial product that is used to increase the soil fertility and plant growth[3]. It is also used to increase the biomass of algae, *Spirulina plantensis*[4].

Protease are the proteolytic (Protein digesting) enzymes. These biocatalysts find wide applications in many industries such as textile, laundry, health care etc[5]. Microbial alkaline protease production dominates the world wide enzyme market, accounting for two-third shwere of detergent industry. Microorganisms were found to be best is protease production because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation[6]. In this study, Humic acid was used to enhance the protein content of chlorella and thereby it can be used as a substrate for protease production by isolated microorganism.

Statistical approaches offer ideal ways for process optimization studies in biotechnology. Response surface methodology (RSM) is now routinely used for optimization in several biotechnological and industrial processes[7]. There are three major media components which influences the production of protease are Carbon source, Nitrogen source and protein source and so they were optimized to enhance production. Statistical approaches were carried out to Organism with better protease production was optimized using response surface methodology.

## MATERIALS AND METHODS

### Microorganism

The microorganism used in this study was isolated from effluent samples collected from dairy industry Chennai and were screened for their alkaline protease production using skim milk agar plate[8]. Three isolates found to be positive and it was identified as *Pseudomonas aeruginosa, Acinetobacter pittii.* and *Enterococcus hirae* according to the morphological and biochemical tests, further confirmed through partial sequence of 16s RNA homology. Cultures were stored in Nutrient agar slant.

These sequence data have been submitted to the Gen Bank Database under accession no. KC991293, KC991294 & KC991295.

### Algal strain

The C*hlorella sp.* Culture was obtained from CAS botany, Madras University, Chennai Tamil Nadu and the strain were maintained in BG11 medium

### Enrichment of algal strains

*Chlorella sp.* were cultured in 100ml of BG11 media in Erlenmeyer flask with varying concentrations of 0.1%,0.2%,0.3%,0.4%,0.5%,0.6%,0.7%, 0.8%,0.9% and 1.0% humic acid and they were inoculated with 1ml of *Chlorella sp.* Inoculated flasks were cultured at pH 7.2 and at temperature 37± 2°C under illuminated light for 15 days.

### Protein estimation

5ml of algal culture was taken and the pellets were homogenized with 5ml of sodium phosphate buffer and centrifuged at 5000rpm for 10minutes. The supernatant was isolated and the protein was estimated through Bradford protein assay method. The absorbance was read at 595nm and the amount of protein was calculated using standard graph prewired with BSA.

### Protein isolation

100ml of humic acid treated *Chlorella sp*. was taken from each flask and centrifuged at 5000rpm for 10 minutes, the pellets were homogenized with $H_2O$ in sonicator and the samples were centrifuged at 5000rpm for 10 minutes, the protein was isolated.

### Media Preparation and cultivation

7ml of isolated protein sample from algae treated with different concentration of Humic acid is added to media of composition with Beef extract (1 g), Yeast extract (1 g) & NaCl (0.5 g) and made to 100ml. They were inoculated with 1ml of three isolated Bacterial cultures and incubated at 37°C and 1ml of samples were taken at time interval of 24, 48, 72 and 96 hours, Protease assay was performed and compeered with casein as substrate under similar composition and conditions.

### Protease assay

Protease assay was carried out for the potential protease producing bacterial strains to determine the protease activity using Universal protease assay. 1 ml of bacterial sample was taken. Solution with 0.2 M glycine, and 0.2 M sodium hydroxide was prewired and 5 ml of this casein solution with glycine - NaOH buffer solution was added and incubated for 10 minutes at 60°C. 110 mM trichloroacetic acid (4 ml) was added. 5 ml of 500 mM sodium carbonate solution was added and the test tubes were vortexes in vortex shaker and incubated for 30 minutes at 37°C in incubator. The samples were observed under UV spectrophotometer at 660 nm. The enzymatic activity of the bacterial strains were determined with the moles of tyrosine released, One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per mL per min under standard assay conditions. Similarly, the protease activity for the 3 bacterial strains was determined after 24hrs, 48 hours, 72 hours and 96 hours of incubation and centrifuged[7]

### Experimental design and optimization

In order to characterize how significant factors affect the responses, we attempted to improve the composition of the media by comparing different levels of several factors that are found to influence the protease production by the bacterium which produces maximum protease from the different percentage of H.A treated algae. Based on the results obtained from preliminary experiments, glucose, algal protein and beef extract were found to be major variables in protease production. We used CCD to find the optimal concentrations of these three factors. Each variable was studied at two different levels (-1, +1) and centre point (0) which is the midpoint of each factor range. The minimum and maximum range of variables investigated and the full experimental are listed in TABLE 1. All experiments were carried out in triplicates.

A multiple regression analysis of the data was carried out with the statistical package (Stat – Ease Inc., Minneapolis, MN, USA) and the second order polynomial equation thet defines predicted response ($R_1$) interims of independent variables ($X_1$, $X_2$ & $X_3$were obtained).

$$R1 = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

Where $b_0$ is intercept term, $b_1$, $b_2$, $b_3$ linear coefficients, $b_{11}$, $b_{22}$, $b_{33}$ squared coefficients and $b_{12}b_{23}b_{13}$ are interaction coefficient. Combinations of factors (such as $X_1X_2$) represent an interaction between the individual factors in that term. That response is a function of the levels of factors.

The response surface graphs indicate the effect of variables individually and in combination and determine their optimum levels for maximal protease production. To validate these predictions, flask cultivation using the completely optimized medium composition was carried out thrice.

## RESULTS AND DISCUSSION

### Effect of humic acid on algal protein enrichment

At the end of the enrichment process, Protein content was estimated and it has been Humic acid had a positive influence at is lower concentration and descending at the higher concentration. It is evidently found that the total protein content has been increased than that of untreated algae. Highest increase in protein is found in 0.5% humic acid treated algae (880 µg/ml) see Figure 1.

### Protease production

In the present study, we observed that Enterococcus hirae to produce protease, which is found better than other two species. On the various concentration of humic acid treated algal protein, maximum protease production on 0.1% C1 by *Entero-*

**TABLE 1 : Experimental range of the three variables studied using CCD in terms of actual and coded factors**

| Variables | Coded levels | | | | |
|---|---|---|---|---|---|
| | -1.24 | Low(-1) | Mid(0) | High (1) | +1.24 |
| Algae | 10.08 | 60 | 70 | 80 | 13.44 |
| Glucose | 0.84 | 5 | 10 | 15 | 2.52 |
| Beef Extract | 0.84 | 5 | 10 | 15 | 2.52 |

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**TABLE 2 : Experimental design used in RSM studies by using three independent variables with six center points**

| | | | Factor 1 | Factor 2 | Factor 3 |
|---|---|---|---|---|---|
| Std | Run | Block | A: Algae ml L-1 | B: Glucose g L-1 | C: Beef extract g L-1 |
| 17 | 1 | Block 1 | 70 | 10 | 10 |
| 11 | 2 | Block 1 | 70 | 1.59 | 10 |
| 5 | 3 | Block 1 | 60 | 5 | 15 |
| 19 | 4 | Block 1 | 70 | 10 | 10 |
| 14 | 5 | Block 1 | 70 | 10 | 18.41 |
| 7 | 6 | Block 1 | 60 | 15 | 15 |
| 13 | 7 | Block 1 | 70 | 10 | 1.59 |
| 9 | 8 | Block 1 | 53.18 | 10 | 10 |
| 2 | 9 | Block 1 | 80 | 5 | 5 |
| 18 | 10 | Block 1 | 70 | 10 | 10 |
| 4 | 11 | Block 1 | 80 | 15 | 5 |
| 8 | 12 | Block 1 | 80 | 15 | 15 |
| 10 | 13 | Block 1 | 86.82 | 10 | 10 |
| 6 | 14 | Block 1 | 80 | 5 | 15 |
| 12 | 15 | Block 1 | 70 | 18.41 | 10 |
| 3 | 16 | Block 1 | 60 | 15 | 5 |
| 1 | 17 | Block 1 | 60 | 5 | 5 |
| 20 | 18 | Block 1 | 70 | 10 | 10 |
| 15 | 19 | Block 1 | 70 | 15 | 5 |
| 16 | 20 | Block 1 | 70 | 10 | 10 |

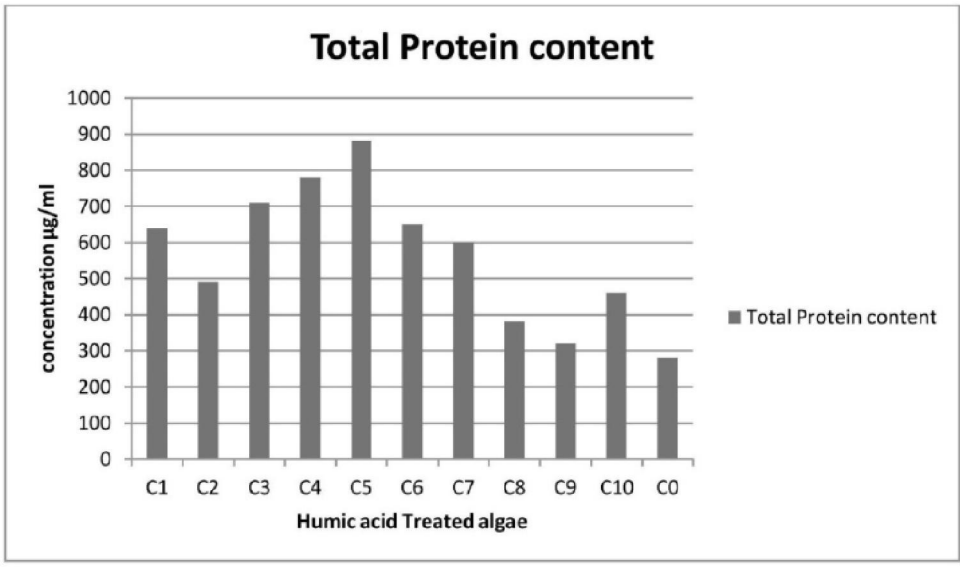


Fig 1: Total Protein content of Algae treated with 0.1%-1.0% Humic acid

*coccus hirae* at 72hrs (126µg/ml), 0.2% C2 by *Enterococcus hirae at* 72hrs (104µg/ml), 0.3% C3 by *Pseudomonas aeruginosa at 48hrs* (142 µg/ml), 0.4% C4 by *Enterococcus hirae at* 72hrs (151 µg/ml), 0.5% C5 by *Enterococcus hirae at* 72hrs (175 µg/ml) and on untreated algae at 48hrs by *Acinitobacter pittii (102 µg/ml)* which is the least on comparison. *Enterococcus hirae* is found to produce maximum amount of protease at 0.5% at 72hrs (175µg/ml). Statistical approach was carried out in

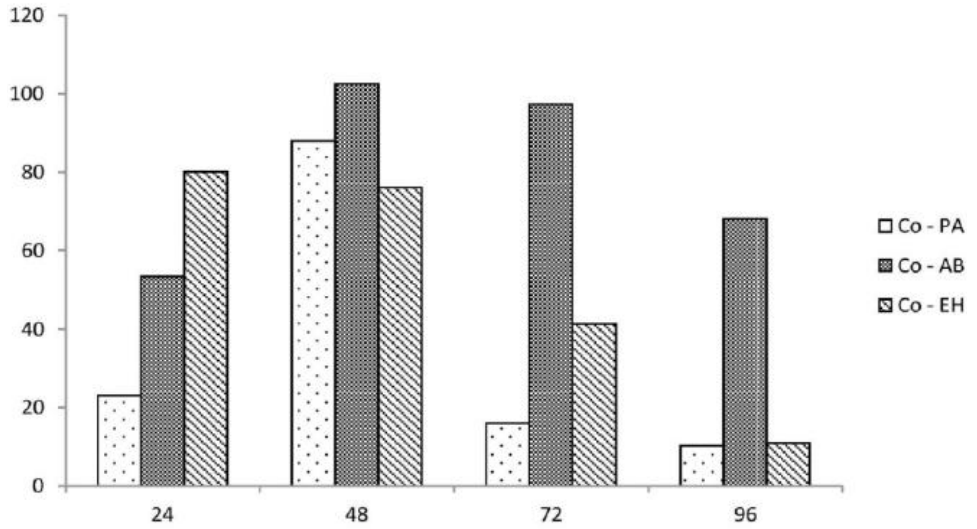


**Figure 2 : Protease production (µg/ml) with the protein isolate from the untreated algae (Control)**

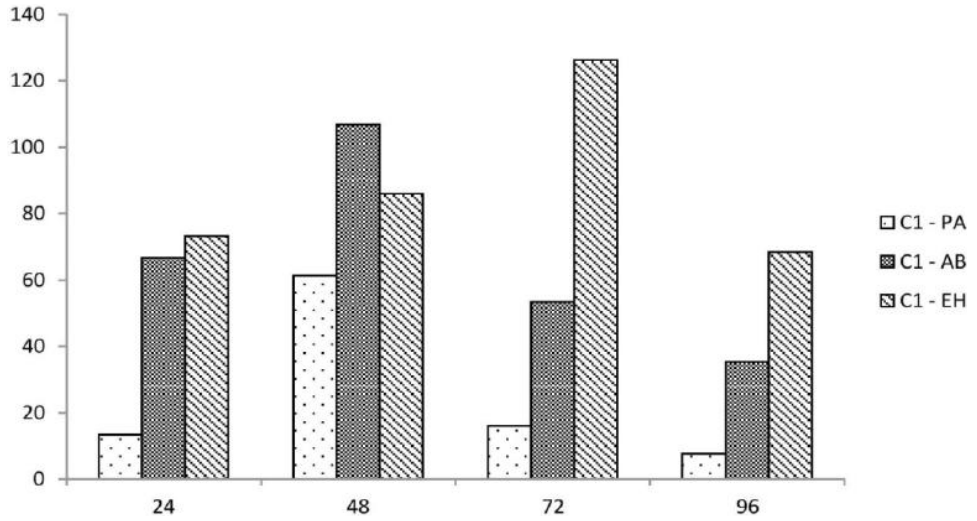


**Figure 3 : Protease production (µg/ml) with the protein isolate from the 0.1% humic acid treated algae**

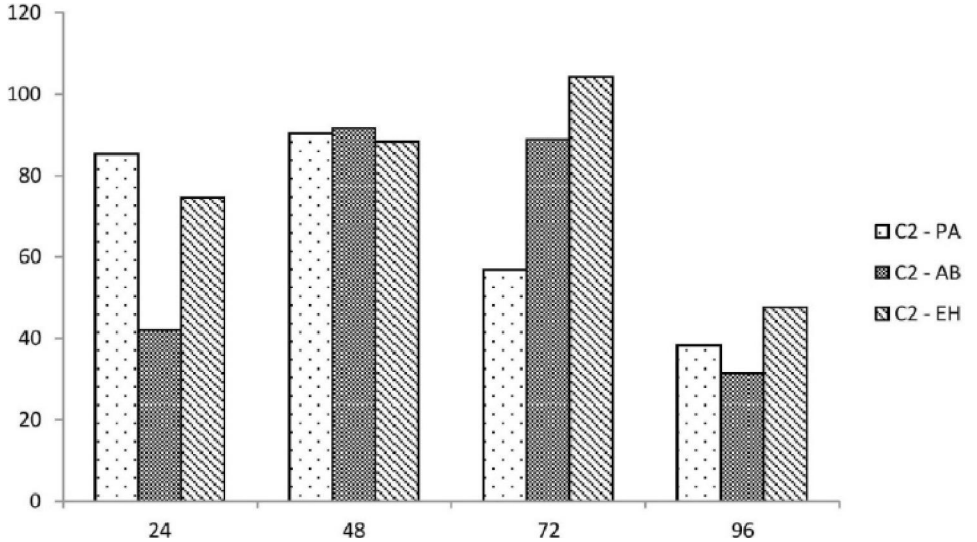


**Figure 4 : Protease production (µg/ml) with the protein isolate from the 0.2% humic acid treated algae**

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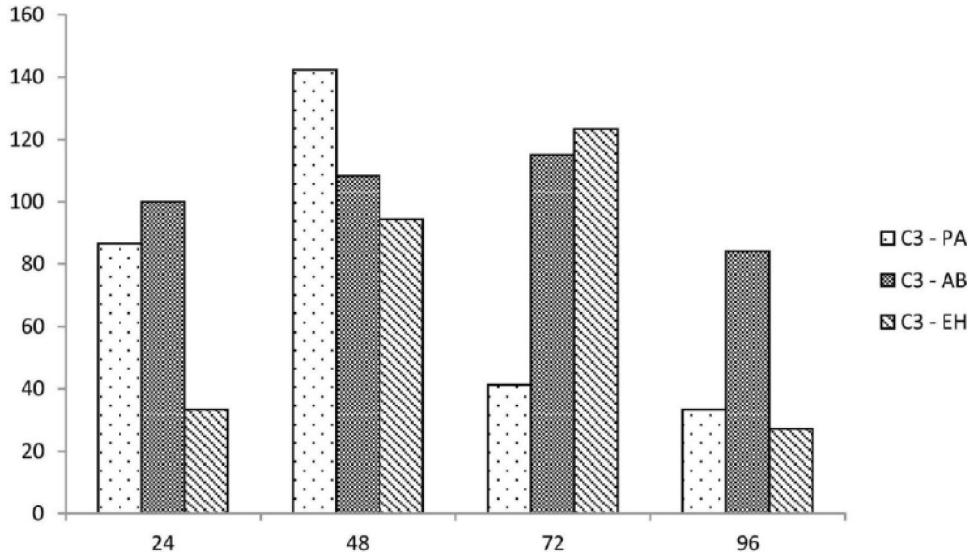


**Figure 5 : Protease production (µg/ml) with the protein isolate from the 0.3% humic acid treated algae**

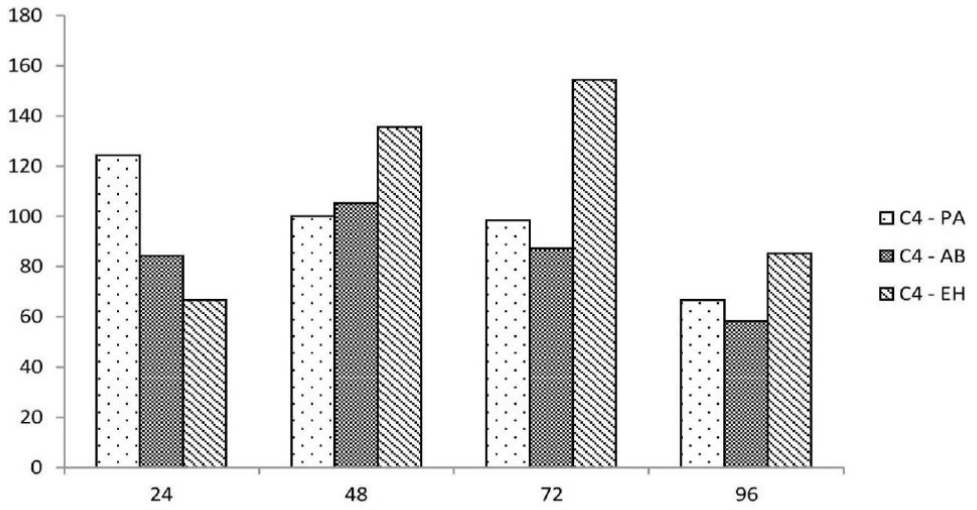


**Figure 6 : Protease production (µg/ml) with the protein isolate from the 0.4% humic acid treated algae**

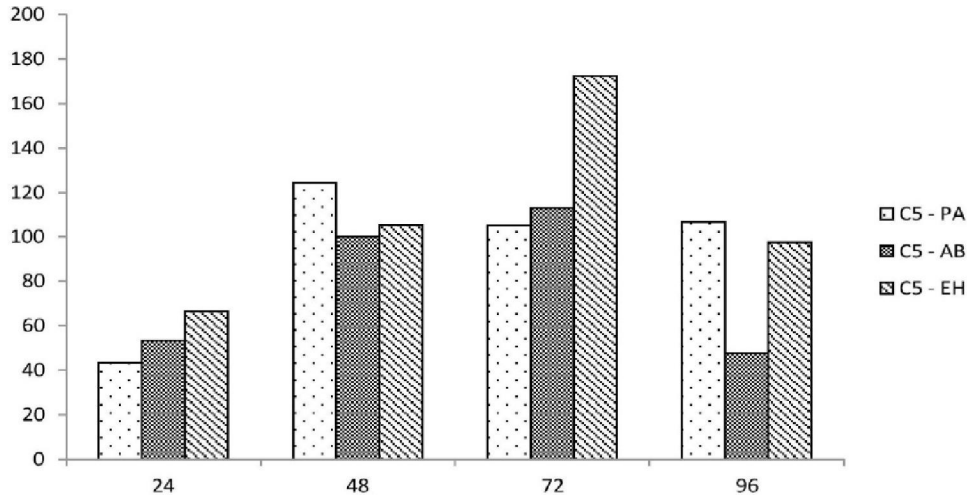


**Figure 7 : Protease production (µg/ml) with the protein isolate from the 0.5% humic acid treated algae**

order to optimize for the maximum producing strain. Figure 2 - 7.

**TABLE 3 : Analysis of variance (ANOVA) for all terms of models**

| ANOVA for Response Surface Quadratic Model<br>Analysis of variance table [Partial sum of squares - Type III] | | | | | |
|---|---|---|---|---|---|
| **Source** | **Sum of Squares** | **df** | **Mean Square** | **F Value** | **p-value Prob > F** |
| Model | 23657.42 | 9 | 2628.6 | 3.97 | 0.0213 |
| A-Algae | 882.34 | 1 | 882.34 | 1.33 | 0.2753 |
| B-Glucose | 3677.33 | 1 | 3677.33 | 5.55 | 0.0402 |
| C-Beef extract | 1029.92 | 1 | 1029.92 | 1.55 | 0.2408 |
| AB | 4.5 | 1 | 4.5 | 6.79E-03 | 0.9359 |
| AC | 3362 | 1 | 3362 | 5.08 | 0.0479 |
| BC | 839.35 | 1 | 839.35 | 1.27 | 0.2866 |
| A^2 | 6425.16 | 1 | 6425.16 | 9.7 | 0.011 |
| B^2 | 4258.36 | 1 | 4258.36 | 6.43 | 0.0296 |
| C^2 | 5933.18 | 1 | 5933.18 | 8.96 | 0.0135 |
| Residual | 6623.53 | 10 | 662.35 | | |
| Lack of Fit | 6074.33 | 6 | 1012.39 | 7.37 | 0.0368 |
| Pure Error | 549.2 | 4 | 137.3 | | |
| Cor Total | 30280.95 | 19 | | | |
| | | | | | |
| Std. Dev. | 25.74 | R-Squared | 0.7813 | | |
| Mean | 233.05 | Adj R-Squared | 0.5844 | | |
| C.V. % | 11.04 | Pred R-Squared | -0.1724 | | |
| PRESS | 35499.94 | Adeq Precision | 5.707 | | |

## Response surface methodology

From the earlier results, the maximum protease production is found in *Enterococcus hirae* is at 0.5% at 72hrs (175µg/ml). The central composite design was used to find the suitable concentrations of the variables on alkaline protease production by *Enterococcus hirae*. The result of Central Composite Design experiments consisted of experimental data for studying the effects of three independent variables such as algal protein, glucose and beef extract on protease production are presented in the TABLE 3.

The data were fitted with the second order polynomial function(Eqn (2)). Therefore, the simplified second-order polynomial equation for protease production ($R_1$) in terms of actual factors was expressed as follows:

$$R1 = -765.007 + 26.89636X_1 + 22.26016X_2 - 9.82663X_3 - 0.015X_1X_2 + 0.41X_1X_3 - 0.39053X_2X_3 - 0.21459X_1^2 - 0.70561X_2^2 - 0.83289X_3^2 \quad (2)$$

Where $X_1$, $X_2$, $X_3$ are algal protein, Glucose and beef extract respectively.

The model F – Value is 0.16 ("P- value > F" less than 0.05) implies the model is significant. The regression equation obtained from the analysis of variance (ANOVA) indicated that multiple correlation coefficient of $R^2$ is 0.7813 i.e. the model can explain 78.13% variation in the response. The adjusted $R^2$ value is -0.5844 and the adequate precision value is 5.737. The signal to noise ratio (adequate precision value) value > 4 is a prerequisite for a model to be a good fit. The model showed standard deviation, mean, C.V., Predicted residual sum of squares (PRESS) values of 25.74, 233.05, 11.04 and 35499.94 respectively. ANOVA results confirmed a satisfactory adjustment of the simplified quadratic model to the experimental data. (TABLE 3)

The three dimensional response surface graphs were plotted to show the interaction of the medium composition and the optimum concentrations of determined components on protease production.

Figure 8a shows the response for the interactive factors; Glucose and Algae (Protein), when Beef extract was kept constant at 10g/l. similarly, the other

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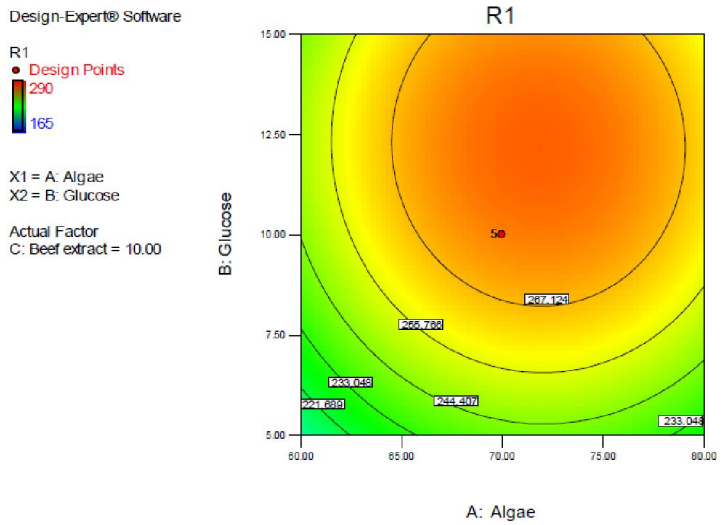


**Figure 8.a : Contour interaction between glucose and algae protein with beef extract as constant**

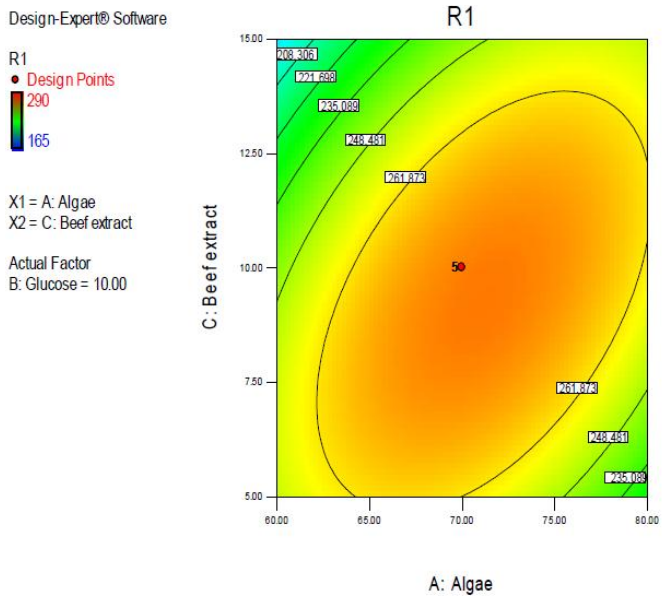


**Figure 8.b : Contour interaction between beef extract and algae protein with glucose as constant**

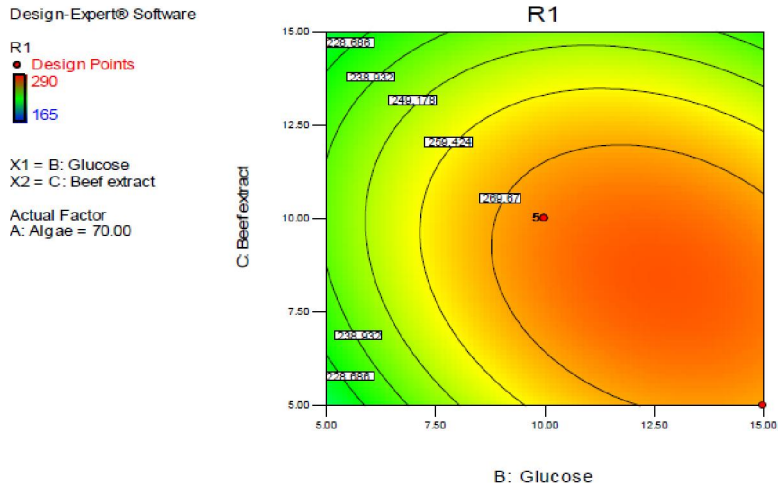


**Figure 8.c : Contour interaction between beef extract and glucose with algae protein as constant**

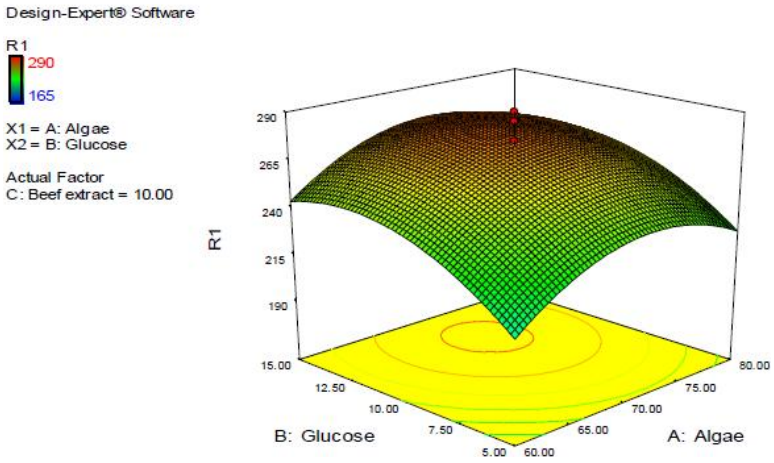


**Figure 9.a : Response surface curves of protease production from *enterococcus hirae* showing interactions between glucose and algae protein**

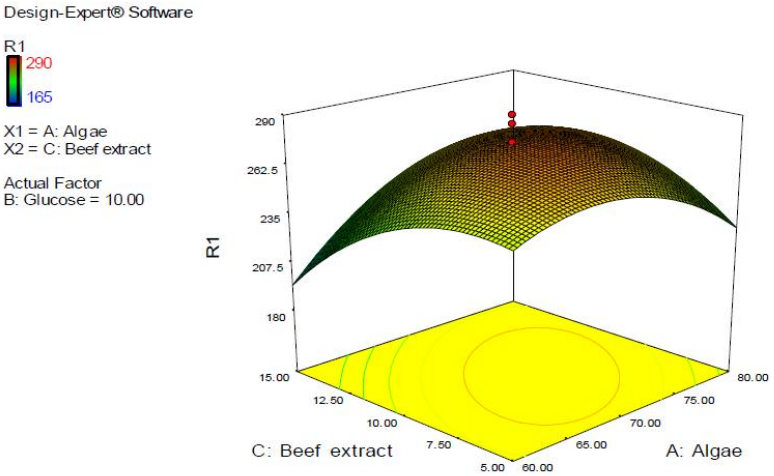


**Figure 9.b : Response surface curves of protease production from *enterococcus hirae* showing interactions between algae protein and beef extract**

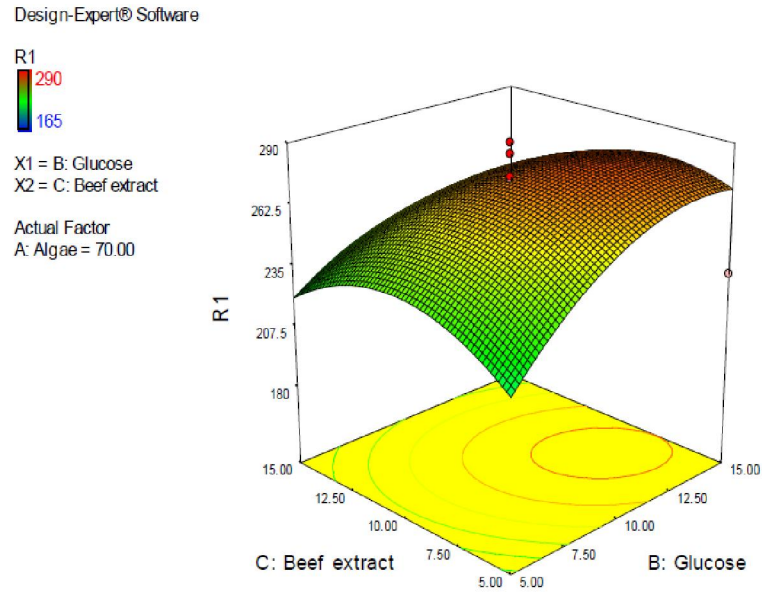


**Figure 9.c : Response surface curves of protease production from *enterococcus hirae* showing interactions between beef extract and glucose**

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two parameter responses were observed keeping the third parameter as constant (Figure 8b & 8c).

The result showed that the protease production was increased when all factors are at the centre levels after optimization by using response surface methodology. The 3D representation of Response surface methodology depicted in Figure 9a, 9b & 9c.

The optimum conditions for the protease production were proposed to be 70ml/l Algal protein, 10g/l of Glucose, 10 g/l of Beef extract. The maximum amount of protease produced was 290 µg/ml was predicted by the model. The suggested medium composition was repeated thrice. The validation of the experiment showed that the experimentally determined production values were in close agreement with the statistically predicted ones, confirming the model authenticity. The *Enterococcus hirae* produced 290 µg/ml of protease under optimum conditions.

## CONCLUSION

*Enterococcus hirae* was found to be most prominent in protease production comparatively with the other bacterial species. This present study has explored additional substrate like algal protein that plays a major role in protease production. Chlorella sp. has high protein content, and so its potentiality as substrate for protease production was explored and it is confirmed with the statistical analysis. From various literature studies, it has been discovered that humic acid increases the biomass and nutritional content of the algae by increasing the uptake of nutrients by algae[4]. By incorporating this study into increasing the protein content of the algae, this algae has been made into a potential contender for protease production. Primarily the protein was isolated from algae using various chemical and physical methods. This protein is used in media cultivation for protease production.

Protease production is confirmed by protease assay. The application of statistical design for screening and optimization of culture conditions allows quick identification of important factors and interactions between them. The eventual objective of RSM is to determine the optimum operating conditions for the system to determine a region of the factor space in which operating specifications are satisfied. This work has demonstrated the use of central composite design by determining conditions leading to the maximum enzyme production. The CCD method allowed to study and explore culture conditions supporting changes in concentrations of medium component in 20 experimental runs. Algae from polluted water can be used as media component for protease production, this marks the importance of this work in the environmental perspective. In further studies we wish to provide concrete applications for it, it may include the use of protease in tablets for clots, in recovery of silver from x-ray film, in leather industries etc.

## ACKNOWLEDGEMENT

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## REFERENCES

**[1]** M.V.Sankar; Ramasubramanian; 'Biomass production of commercial algae chlorella vulgaris on different culture media', E-Journal of Life Science, **1(1)**, 56-60 **(2012)**.

**[2]** A.Boulus, E.Spaneir, M.Friedlander; 'Effect of outdoor conditions on growth rate and chemical composition of Gelidium crinale in Culture', J.App.l Phycol.,' **19**, 471-478 **(2007)**.

**[3]** P.Prakash, Karthik Raja, N.Namasivayam, K.Niveditha, Vishnu Tejaswini; 'Optimization of humic acid by trichoderma viridi and it's effect on sorghum plant', Journal of Biopesticides, **3(1)**, 155 – 157 **(2010)**.

**[4]** P.P.K.Prakash, B.Dhanalakshmi; Anusha; 'Effect of humic acid on Spirulina plantisis production and analysis of nutrient contents', Recent Research in Science and Technology, **3(1)**, 87-89 **(2011)**.

**[5]** Chandran E.Masi, J.Balaji, C.Vigneshwar, M.Chandramohan, Fazil Ahmed, N.Parthasarathy, 'Isolation and production of proteolytic enzyme by bacterial strains by using agrowates as substrate', Asian Journal of Chemistry, **26(7)**, 2182 – 2184

**(2014)**.

**[6]** Chandran P.Masi, Vivek, J.Kotteshwari, C.Mithun, A.Uma Maheswaran, N.Parthasarathi; 'Cloning, Expression and characterization of serine proteasegene from Entrococcus hirae', BioTechnology: An Indian Journal, **11(9)**, 328- 334 **(2015)**.

**[7]** Qasim Khalil Beg, Vikram Sahai, Rani Gupta; 'Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor', Process Biochem, **39**, 203- 209 **(2003)**.

**[8]** S.Mehrotra, P.K.Pandey, R.Gaur, N.S.Darmwal; 'The production of alkaline protease by a bacillus species isolate', Bioresour Technol., **67**, 201-203 **(1999)**.

**[9]** Seyedeh Faranak, Ghaemi Oskouie, Fatemeh Tabandeh, Bagher Yakhchali, Fereshteh Eftekhar; 'Response surface optimization of media composition for alkaline protease production by Bacillus clausii', Biochem.Eng.J., **39**, 37-42 **(2008)**.