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MEDIUM OPTIMIZATION FOR PROTEASE PRODUCTION VIA MODERATELY HALOTOLERANT *VIRGIBACILLUS PANTOTHENTICUS* USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Virgibacillus pantothenticus is an industrially promising, yet scarcely studied, moderately halotolerant microorganism (up to 10% NaCl) with high activity protease production potential. Following Response Surface Methodology, we employed a Central Composite Design for the experiments and constructed a second order polynomial model to represent the resulting data. For medium optimization for protease production we optimized the resulting model. 32 experiments (following the central composite design scheme) where, carbon (glucose), nitrogen (ammonium sulfate), potassium (potassium phosphate monobasic) and magnesium (magnesium sulfate) sources were studied in the media. Bacterial growth, residual glucose and protease activities were determined at the 48th hour of each experiment. The model was optimized and the concentrations were found for each parameter. Under the optimum conditions, the predicted protease activity is also experimentally verified and the model prediction was in very good agreement with experimental results. The interactions between medium components and their effect on cell growth and protease production are also sought. This work reports the improvements on protease production of a potentially interesting industrial host, *Virgibacillus pantothenticus.*

Keywords: *Virgibacillus pantothenticus*, Response Surface Methodology, Alkali Protease, Medium optimization

ILIMLI HALOFİLİK *VIRGIBACILLUS PANTOTHENTICUS*'TA PROTEAZ ÜRETİMİ İÇİN, YANIT YÜZEY YÖNTEMİ İLE BESİYERİ OPTİMİZASYONU

ÖZET

Virgibacillus pantothenticus endüstriyel olarak umut vadeden, halotolerant (%10'a kadar tuzu tolere edebilen), yüksek aktiviteli proteaz üreticisi olma potansiyeli olan bir organizmadır. Bu çalışmada, Yanıt Yüzey Yöntemi metodu takip edilerek, deney tasarımı için Merkezi Kompozit Tasarımı, model olarak da ikinci derece polinom kullanılarak, biyokütle ve proteaz üretimi amaç fonskiyonu ile besiyeri optimizasyonu gerçekleştirilmiştir. Yanıt yüzeyi modelinin parametrelerinin belirlenmesi için, 32 deneyde faklı karbon (glukoz), azot (amonyum sülfat), potasyum (potasyum fosfat monobazik) ve magnezyum (magnezyum sülfat) miktarları, besiyerlerinin hazırlanması için kullanılmıştır. Her bir deneyde, bakterilerin büyümesi, 48 saat sonunda kalan glukoz miktarı ve proteaz aktivitesi belirlenmiştir. Kurulan model optimize edilmiş ve her bir besiyeri bileşeninin optimum seviyesi belirlenmiştir. Bu koşullarda, model tahmini deneysel olarak çok yakın bir sonuçla teyit edilmiştir. Aynı zamanda, besiyeri bileşenlerinin karşılıklı etkileşimleri ve hücre büyümesi ile proteaz üretimi üzerindeki etkileri araştırılmıştır. Bu çalışma, proteaz üreticisi olarak *Virgibacillus pantothenticus*'un potansiyelini geliştirmiştir.

Anahtar Kelimeler: *Virgibacillus pantothenticus*, Yanıt Yüzey Yöntemi, Alkalen Proteaz, Besiyeri optimizasyonu

1. INTRODUCTION

The genus *Virgibacillus* was firstly isolated from the soils of Southern England, and reported as a member of *Bacillus* species by Proom and Knight in 1950; under the name of *Bacillus pantothenticus* [1]. They are halotolerant, mildly halophilic, rod shaped, gram positive, and endospore forming bacteria. It is imperative that thiamine, biotin and pantothenic acid (vitamin B5) are present for growth [2]. Further studies for the characterization of various strains of *Bacillus pantothenticus* by amplified rDNA restriction analysis and fatty acid methyl ester analysis indicated that they must have been another, novel genus; and the name

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Virgibacillus was proposed [3]. Since then, several species of *Virgibacillus* were isolated; mostly from soils as well as mural paintings, salt fields, fresh chicken meat, sea water, intestines of fish etc. [4-10].

Virgibacillus pantothenticus is the type species of its genus, studied mostly due its protease activity. Gupta et al. [7] isolated *V. pantothenticus* from chicken meat and reported thermostable alkaline protease activity at 50 °C. In another study, protease was produced in a conico-cylindirical flask via *V. pantothenticus*; increasing the production 22% by this design [11]. Thillaimaharani et al. [10] reported alkali protease activity of *V. pantothenticus* isolate from the intestines of tilapia, and suggested it could be used in the bioconversion of aquaculture feeds. Apart from protease production, extracellular lipase production by *V. pantothenticus* at moderately high temperatures (55 °C) was studied as well; it was shown that the purified lipase activity stayed the same at 35 and 55 °C [12]. In another study, ectT gene encoding the carrier protein EctT for ectoine transportation was cloned from *V. pantothenticus* into mutant *E. coli* cells that lack the ability to uptake osmoprotectants. They also showed that ectT gene is transcribed under high salinity conditions or at low temperatures [13]. A more recent study demonstrated that *V. pantothenticus* had plant growth promoter activity, its presence in the soil increased wheat yields by helping under salt stress [14].

Studying the genus *Virgibacillus* is promising due to its halotolerance. High NaCl concentrations promoted transcription of the gene responsible for ectoine biosynthesis by *V. pantothenticus* [15]. This halotolerance or mild halophilic nature makes *Virgibacillus* promising organism to be used in preparing fermented products, e.g. for fish-sauce fermentation [16].

Proteases belong the hydrolytic group of enzymes; they catalyze the breakdown of protein structures into peptides and amino acids by cleaving the peptide bonds. While intracellular proteases are essential in the metabolic pathways of the cell; extracellular proteases play a critical role in degrading the whole proteins for cell to absorb and utilize [17]. Therefore, extracellular enzymes have been used in several industries such as detergent, food, feed, leather and pharmaceutical [18]. Though various industrial enzymes exist; proteases constitute the 60% of the global enzyme market [19]. Microbial proteases are industrially critical in particular; since plant and animal derived proteases cannot meet the demand [20].

Proteases are used for diverse areas ranging from cheese production, brewing, baking to meat tenderization. Especially increasing awareness against processed food requires natural additives in this industry. In beer brewing, due to turbidity formation at low temperatures, microbial proteases are used as a solution [21]. Also, proteases are used in dairy products for partial hydrolysis of proteins into bioactive peptides; which become active and have various health advantages once ingested. They play part in regulating cardiovascular and gastrointestinal system and have antioxidant effects [22, 23].

Optimization of complex biological systems, involving numerous reactions and processes, for maximal performance (e.g. growth, production etc.) requires in depth knowledge of the biological components and the interactions among those, with which a detailed mechanistic model can be constructed, analyzed and optimized [24], referred as grey or white box approach. In case such detailed information is not available, black-box approach, whereby the inputs and the output(s) of the system are linked via a simple statistical model (e.g. a linear or polynomial model) whereby multiple parameters and their interactions are taken into account, not in a mechanistic way but from a phenomenological point-of-view. The approach is referred as "black-box" since what actually occurs within the system is not fully known (hence black-box).

Response surface methodology (RSM) is such a statistical approach for building models and calculating the optimum setup for practically any deterministic process [25, 26]. Conventional techniques for optimizing biological processes are typically based on one-factor-at-a-time approach where, all but one parameter remain constant [27]. Such an approach requires many experiments, and is inherently time consuming, costly, and most of all is not able to predict the potential interactions among the parameters [28]. By using response surface methodology, successfully predicting the response of the system is possible and it is more reliable than conventional method.

The two pillars of the RSM are: (i) the experimental design and (ii) the structure of the model to represent system. The experimental design should cover as broad range as possible for each parameter, but should also be compact enough for the assumption that the model will adequately represent the experimental results will hold. The experimental design should be balanced, e.g. it should have (roughly) equal number of experiments away from the central points to avoid bias. Lastly, the experimental design should contain few experiments. The model, in turn, should be as simple as possible yet should contain enough non-linearity to realistically represent the system under investigation.

For the experimental design, among the available designs (Box Behnken, Full Factorial, Taguchi, Placket-Burman etc.) Central Composite Design (CCD) provides few number of experiments and the changes in all independent variables are balanced. For the model, typically a 2nd order polynomial is used as the model for RSM studies, as it mimics the optimum point, somewhere (*a priori* unknown) within the experimental region and the parameters (and the diagnostics) can be estimated using multiple linear regression.

RSM is frequently used to optimize enzyme production from both natural and recombinant biological sources. One study by [29] reported the optimum pH, temperature and inoculum size for polygalacturonase production by *Aspergillus niger* fermentation using mango peels as the substrate; and another optimized the temperature and NH_4Cl concentration for endo- and exo-pectinase production from orange peels by *Penicillum oxalicum* [30]. Another study increased the cellulase production by optimizing the fermentation medium of *Penicillum janthinellum* mutant (EU2D-21); results were 1.87 times higher for β-glucosidase and 1.67 for FPase after the optimization with RSM [31]. Sathishkumar et al. [32] used RSM for the protease production from *Virgibacillus halodenitrificans* using marine waste, shrimp shell powder, in the medium and another study reported approximately 3 fold (from 298.34 U/ml to 982.68 U/ml) enhancement in protease production from a *Marinobacter* sp. isolate by optimizing the culture conditions and the medium composition [33]. *Virgibacillus* sp. was also studied for NaCl-tolerant protease production by optimizing the salt concentration in the medium using RSM [34]. Saxena & Singh [35] performed two step statistical approach for the simultaneous production of amylase and protease by Solid state fermentation via *Bacillus megaterium.* Their work revealed that using 20% mustard oilseed cake, an agroindustrial waste, as substrate with 45% moisture and 84h incubation time increased the protease production by 2.95 folds and amylase by 2.04.

In this paper, we report the experimental design, resulting data on enzyme production and growth and optimum chemically defined medium composition for the mildly halotolerant *Virgibacillus pantothenticus* for its growth and protease production. In doing so, we aim not only to optimize the medium composition but also to delineate possible interactions among medium constituents.

2. MATERIALS AND METHODS

2.1. Culture Media

The basal medium (BM) is formulated based on preliminary screening studies. BM is used as the center point in listing the designed experiments. The composition is given in Table 1. K_2HPO_4 and Yeast Nitrogen Base are kept constant in each experiment.

Table 1. The composition of the basal medium

Medium component	**Concentration (g/L)**	ΔX **(g/L)**
Glucose	10	5
$(NH_4)_2SO_4$	5	2.5
KH_2PO_4	4	1.5
$MgSO_4$	1.5	0.5
K_2HPO_4	3	-
YNB	1.7	-

All cells are kept at -80°C in glycerol stocks. The culture media is optimized in 4 components, namely glucose as the carbon source, $(NH_4)_2SO_4$ as the N-source, and KH_2PO_4 as the P source as well as pH buffer (together with K_2HPO_4) and $MgSO_4$ as the Mg^{2+} source. Together with ammonium sulfate, magnesium sulfate serves also as the S-source for the bacteria. Additional to these essential components, vitamins and trace metals are provided to the culture media, by using Yeast Nitrogen Base (YNB) without amino acids and ammonium sulfate (Sigma, Y1251). Ingredients of Yeast Nitrogen Base are (in µg/L: Biotin 2, Copper sulfate 40, Calcium pantothenate 400, Potassium iodide 100, Folic acid 2, Ferric chloride 200, Niacin 400, Manganese sulfate 400, p-Aminobenzoic acid 200, Sodium molybdate 200, Pyridoxine HCl 400, Zinc sulfate 400, Riboflavin 200, Thiamine HCl 400, Boric acid 500, Inositol 2000. Additionally, the following salts are also available in YNB (in g/L) Magnesium sulfate 0.5, Sodium chloride 0.1, Potassium phosphate monobasic 1, Calcium chloride 0.1. In each experiment, the final amounts of both $MgSO_4$ and KH_2PO_4 are prepared taking into account the already available amounts in YNB.

2.2. Experimental Design

Central composite design (CCD) has been adopted for the experiments due to its symmetry and low number of experiments. In each experiment, the value of each medium component is coded as:

$$x_{coded} = \frac{X_{real} - X_{center}}{\Delta X}$$

where, x_{coded} represents the coded value, X_{real} represents the actual concentration of the medium component, X_{center} is the concentration at the center point and ΔX represents the increase in concentration. The ΔX values for each medium component, is provided in Table 1. The complete list of experiments are provided in Table 2.

Table 2. Experimental design, and the results as protease activity, residual glucose and growth.

Exp #	Glucose (-)	$(NH_4)_2SO_4$ (-)	KH_2PO_4 (-)	$MgSO_4$ (-)	Protease Act (U/mL)	Growth (OD)	Residual glc (g/L)
1	1	1	1	1	0.777	1.974	5.36
2	1	1	1	-1	0.964	1.877	6.31
3	1	1	-1	1	1.043	1.964	7.64
4	1	1	-1	-1	1.183	2.013	8.87
5	1	-1	1	1	1.134	1.818	8.13
6	1	-1	1	-1	1.218	1.890	5.28
7	1	-1	-1	1	1.078	1.789	7.08
8	1	-1	-1	-1	0.604	1.913	8.00
9	-1	1	1	1	0.782	1.901	1.63
10	-1	1	1	-1	1.071	1.814	1.71
11	-1	1	-1	1	0.872	1.784	1.79
12	-1	1	-1	-1	0.947	1.732	1.79
13	-1	-1	1	1	1.234	1.718	1.84
14	-1	-1	1	-1	0.841	1.791	1.90
15	-1	-1	-1	1	1.052	1.826	1.90
16	-1	-1	-1	-1	1.463	1.741	1.91
17	1.4	0	0	0	1.837	1.904	3.53
18	-1.4	0	0	0	1.159	0.397	0.38
19	0	1.4	0	0	1.856	1.929	2.30
20	0	-1.4	0	0	2.227	1.956	2.23
21	0	0	1.4	0	1.837	1.964	2.19
22	0	0	-1.4	0	1.974	1.836	2.46
23	0	0	0	1.4	1.800	1.567	2.41
24	0	0	0	-1.4	1.977	1.601	3.51
25	0	0	0	0	1.825	1.526	2.78
26	0	0	0	0	1.974	1.954	2.62
27	0	0	0	0	1.461	1.589	2.63
28	0	0	0	0	1.809	1.751	2.57
29	0	0	0	0	2.033	1.737	2.83
30	0	0	0	0	1.727	1.879	2.59
31	0	0	0	0	1.817	1.912	2.56
32	0	0	0	0	1.709	1.846	2.54

Second order polynomial is used to capture the effect of medium components to the growth and protease production. The structure is provided by the equation:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_1^2 + \beta_6 X_2^2 + \beta_7 X_3^2 + \beta_8 X_4^2 + \beta_9 X_1 X_2 + \beta_{10} X_1 X_3 + \beta_{11} X_1 X_4 + \beta_{12} X_2 X_3 + \beta_{13} X_2 X_4 + \beta_{14} X_3 X_4$$

where, y represents the response (either protease production or growth), X_i are the medium components and each β is the corresponding coefficient. In its matrix form the model is presented in the following form, where y is the response matrix composed of measurements, X is the matrix composed of X_i's and β represents the coefficient matrix:

$$y = X \cdot \beta$$

The parameters then, being linear in the model, can be obtained using linear regression as, whereX^T is the transpose of matrix X:

$$\beta = (X^T X)^{-1} X^T y$$

The confidence interval of the parameters (with critical level of α) for the linear model is provided by

$$CI(\beta_j) = \beta_j \pm t_{\frac{\alpha}{2}, n-2} \cdot diag\left(\sqrt{s^2 (X^T X)^{-1}}\right)$$

where, $t_{\frac{\alpha}{2}, n-2}$ is the critical value of t statistics for α level of confidence and $n-2$degrees of freedom, $diag(\ \ )$ refers to the diagonal of the matrix and s^2 is the variance-covariance matrix, calculated from measurements.

2.3. Determination of Growth, Protease Activity, Glucose Level

Microbial growth was determined by measuring the optical density of the samples at 580 nm. Residual glucose concentrations were determined using DNS method as described by Miller [36]. Protease activity was measured according to Sigma's colorimetric tyrosine detection method [37, 38], using Folin-Ciocalteu reagent. Culture samples were centrifuged at 4°C and 10000 rpm to obtain crude enzyme. 0.65% (w/v) casein solution in 0.2M Glycine-NaOH buffer at pH 9.00 was used as the substrate. 130 µL casein solution and 25 µL crude enzyme were mixed and incubated at 37°C for 10 minutes; the reaction was stopped by adding 130 µL 110 mM trichloroacetic acid (TCA) and incubated for 20 minutes at 37°C. Samples were then centrifuged for 5 minutes at 10000 rpm to get rid of aggregated proteins. 50 µL of the supernatant, 125 µL of the 500 mM sodium carbonate and 25 µL diluted Folin-Ciocalteu reagent were mixed. After 30 minutes of incubation at 37°C, optical densities were measured at 660 nm. A calibration line is plotted with known concentrations of tyrosine, correlating optical density to the concentration of tyrosine. Amount of tyrosine in the samples were determined and the activity is calculated using the following formula. 1 unit of enzyme is defined as the µmoles of tyrosine released from casein per minute.

$$Proteaseactivity\left(\frac{\text{units}}{\text{mL}}\right) = \frac{\mu\text{mole of tyrosine} \times \text{reaction volume (mL)}}{\text{sample volume (mL)} \times \text{reaction time (min)} \times \text{volume assayed (mL)}}$$

It should be noted that comparison among published literature for protease activity should be carried with care, since the definition of activity changes from one study to another. Several authors adopt the definition based on µg Tyrosine released while, others (also this paper) do report activities based on µmol Tyr released, which can be converted to each other by using the molecular weight of Tyrosine (MW_{Tyr} = 181 g/mol).

3. RESULTS AND DISCUSSION

This paper reports the results of a statistical optimization study of halotolerant *Virgibacillus pantothenticus* cells for optimum medium composition for growth and protease production. Considering that proteases constitute the major part of the enzyme market (up to 60%) and are used in several industries from food to feed, medical, and leather as well as textile industry, finding optimum conditions for improved production is of high value. For this, alternative well-characterized hosts, optimum production conditions, medium composition and scalable approaches are always in demand in industrial processes.

Being a halotolerant (up to 10% NaCl in culture media) and producer of highly active protease and as well as other industrially relevant enzymes, *V. pantothenticus* represents interesting potential for industrial biotechnology applications with its larger portfolio of proteases, ability to be cultivated at open ponds or under aseptic conditions [3].

The results of the 32 experiments performed are reported in Table 2. The experimental results consisting of protease activity measurements, final optical density and residual glucose concentration at the 48th hour are separately fitted to the 2nd order polynomial model using multiple linear regression. The resulting parameters for each model are provided in Table 3. The parity plot for all experimental values and the corresponding model prediction is provided in Figure 1, overall the model fits the data well with relatively high R^2 value (0.71 for protease production), indicating that the model explains 71% of the total variation, which is a success considering the broad range of medium components. The R^2 value is found to be 0.56 for growth and 0.88 for residual glucose.

Table 3. The estimated model parameters using experimental data in **Table 2**.

Parameter	Value for Activity model (M1)	Value for Growth model (M2)	Value for Residual glucose model (M3)
β_0	1.9497	1.7131	2.2147
β_1	0.0344	0.1527	2.3401
β_2	-0.0756	0.0269	-0.0419
β_3	-0.0208	0.0101	-0.3613
β_4	-0.0284	-0.0022	-0.0984
β_5	-0.3859	-0.2259	0.2932
β_6	-0.1083	0.1782	0.4489
β_7	-0.1780	0.1565	0.4804
β_8	-0.1865	-0.0047	0.8047
β_9	0.0532	0.0164	0.0195
β_{10}	0.0369	-0.0163	-0.3882
β_{11}	0.0277	-0.0187	-0.0077
β_{12}	-0.0425	0.0078	-0.2093
β_{13}	-0.0665	0.0232	-0.2574
β_{14}	-0.0009	0.0047	0.2447

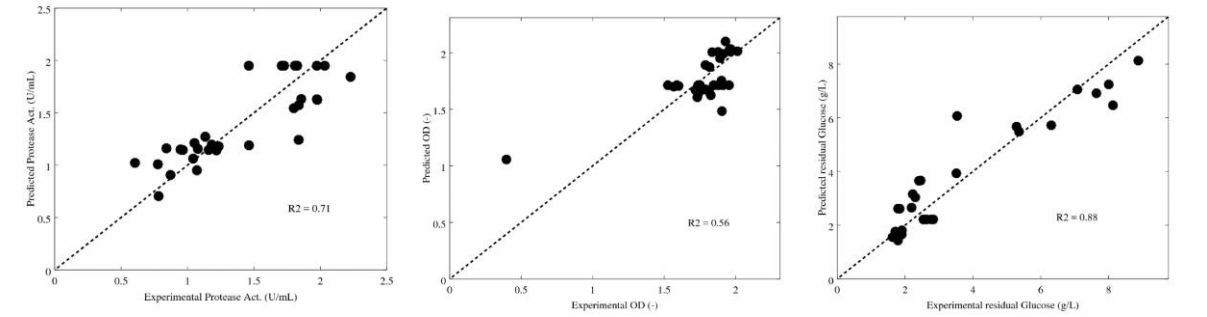

Figure 1. Parity plots representing the model fits for each of the responses (left panel: protease, middle panel: growth, right panel: residual glucose). The dashed line is the 45 degree line for each plot.

Analyzing the resulting model M1, in particular the negative values for all the parameters β_5 to β_8 indicate that the response surface has a downward concave shape and points to a maxima within the experimental region considered. Among these parameters, β_5 is found to be statistically significant, indicating that the negative effect at elevated glucose concentrations on protease production is pronounced. This is expected, since the enzyme production typically occur when the host cells are in stress [39, 40], yet glucose-rich conditions is expected to induce little-to-no stress for the cells.

After studying the main effects, the interactions among the variables are sought. The importance of any interaction is assessed via the value of the corresponding model parameter (β_i) and the significance of that parameter. The parameters β_9 to β_{11} being positive (albeit the absolute value being low) points to a positive synergistic effects of each medium component with glucose. This is in line with our expectation, since an efficient balanced growth and production would require the increase (or decrease) of each medium components in concert. The interactions among the parameters are also illustrated in the surface plots (Figure 2). Interestingly, the interactions among medium components are different for protease and for growth. For example, magnesium and carbon sources effect positively around the center and away from the center these two synergistically decrease the enzyme activity. However, the same couple has almost no interaction during growth, exhibited as almost parallel contour lines. Similarly, the interaction of nitrogen source with carbon source exhibits a centrally-located-optimum point for the protease activity while it exhibits a minimax or a saddle point for the growth. These interactions illustrate the different culture media need either task, which needs to be taken along while considering the further design of larger scale enzyme production process.

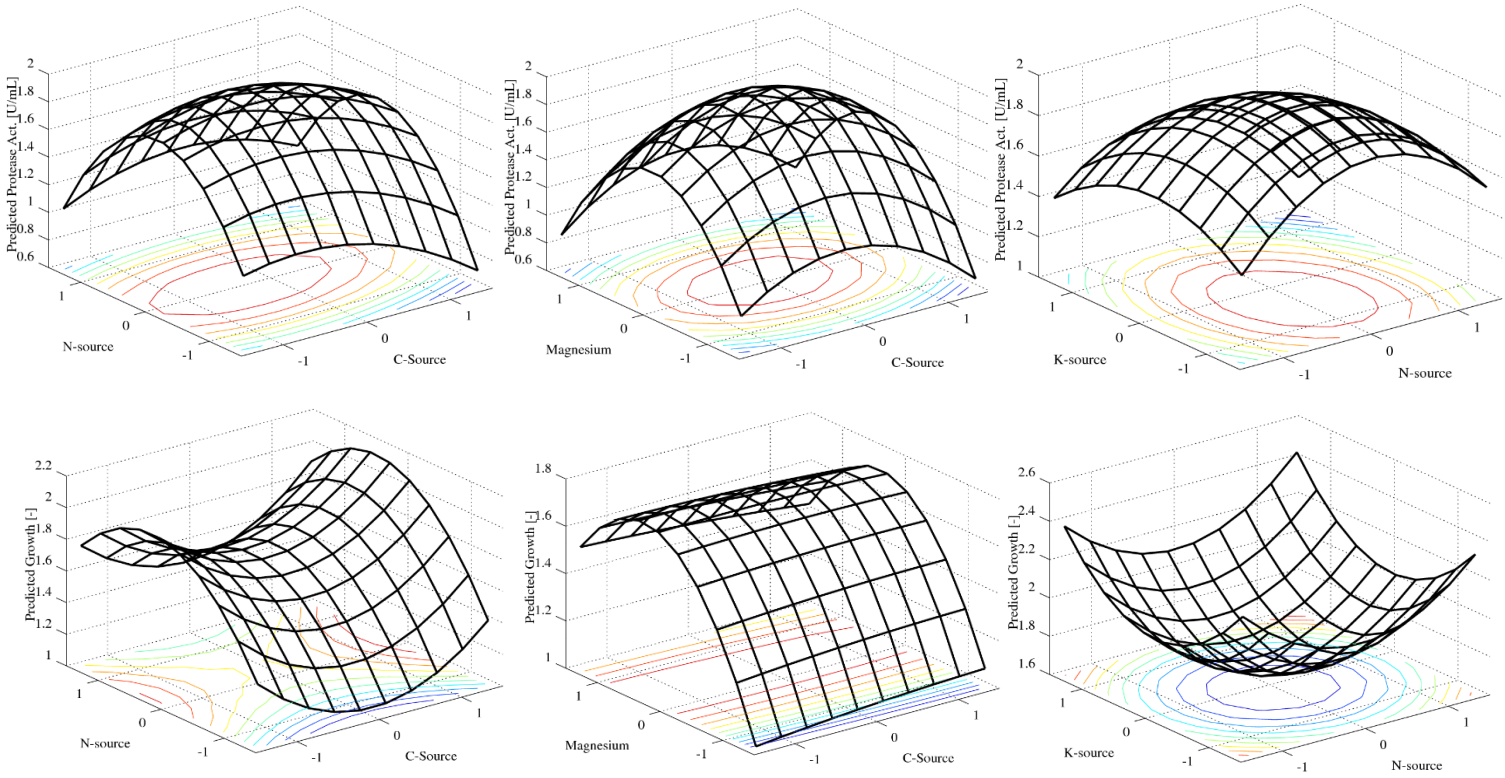

Figure 2. Interaction plots for the protease and growth models (M1 and M2 respectively). The upper panel presents the interactions for the protease activity, Nitrogen-Carbon, Magnesium-Carbon, Potassium-Nitrogen from left to right. The lower panel represents the interactions for the growth.

3.1. Optimization of Medium Composition for Protease Production

Focusing on the protease production, M1 model has been maximized using Nelder-Mead simplex method to find the medium composition that would result in most protease activity. The optimum medium composition for this case is found as 0.02 for glucose, -0.34 for $(NH_4)_2SO_4$, -0.02 for KH_2PO_4 and -0.01 for $MgSO_4$, given as coded variables and the model prediction at this point is found to be 1.96 U/mL. A new experiment has been carried out at this optimum condition to verify the model prediction and the measured activity was found to be 1.98 U/mL, which is in very good agreement with the model prediction. Through medium optimization, we increased the protease activity by 10.6% with respect to center point. Such an improvement is often seen in other medium optimization studies as well (for two recent examples see [41] for prokaryotic and [42] for eukaryotic organisms).

Despite all the appealing features, the amount of scientific literature on the growth and production conditions is highly scarce for *V. pantothenticus*, including optimization studies. Among the limited literature available, Fitriani, and Güven reports isolation of halophilic bacteria with protease activity, showing high resemblance to *V. pantothenticus* according to gram staining and ribotyping results [43]. Authors claim that the crude protease activity of this microorganism is 0.12 U/mL, and 1.06 U/mL (upon adjusting for the same protease activity definition, see Materials and Methods) after ammonium sulfate precipitation. Sinha and Khare reported protease activity of approximately 1.49 U/mL for *Virgibacillus* sp. EMB13 with complex media [44], and Thillaimaharani et al reported approximately 10 U/mL protease activity with complex media [10]. Lastly, Gupta et al. reports protease activity of 18.2 U/mL under optimized conditions in a complex medium [7]. In this work, the reported optimum activity of 1.98 U/mL for unpurified crude enzyme in chemically defined medium which is very promising as the activity is expected to further increase with complex media.

Overall, an industrially promising host, halotolerant *Virgibacillus pantothenticus* cells are evaluated for their growth and protease production capacity, with a large experimental dataset and accompanying model. Further studies focusing on mechanistic properties (e.g. characteristics/types of different proteases by the organism, in depth dynamics of production, downstream and scale-up for further production) will provide more information on this promising host. Studies will alleviate the potential scale-up problems.

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