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Original Research Article

Effect of Medium Components on Alkaline Protease Production using *Bacillus subtilis C3a-FIIRO-MW577298* as Fermentation Strain

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ABSTRACT

Alkaline protease is a bioprocessing aid that breaks protein polypeptide chains into monomers. The absence of industries producing enzymes in most sub-Saharan countries has created the opportunity to develop microbial-sourced alkaline protease as an industrial processing aid. To meet domestic demands, the effect of medium components on alkaline protease production using *Bacillus subtilis* C3a-FIIRO-MW577298 was investigated. In this study, *Bacillus subtilis* C3a-FIIRO-MW577298 was obtained, screened, and maintained on nutrient broth. The selection of fermentation time and the classical exclusion optimization approach were achieved through solid-state fermentation using rice bran as the fermentation substrate. The interaction effect of medium components was estimated by a statistical optimization technique (two-level factorial design). At an optimal fermentation time of 96 hours, the outcome of the classical exclusion optimization approach showed that the absence of ammonium sulfate ($(NH_4)_2SO_4$) and yeast extract affected the alkaline protease production compared to the exclusion of glucose, potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4$), and sodium carbonate (Na_2CO_3). Optimal production was achieved at 11 g/L yeast extract, 1 g/L glucose, 14 g/L KH_2PO_4, 1 g/L $MgSO_4$, 10 g/L Na_2CO_3, 1 g/L ($(NH_4)_2SO_4$, and a pH of 10, with alkaline protease activity increasing 1.79-fold (from 0.904 to 2.527 U/mL) significantly. The model's coefficients of determination were 99.9% for enzyme activity and 97% for protein concentration, indicating strong agreement between observed and predicted values. It was concluded that the main effect of yeast extract, and the interaction effect of glucose and $MgSO_4$, contribute significantly to alkaline protease production by *Bacillus subtilis* C3a-FIIRO-MW577298.

Keywords: Alkaline protease, Classical exclusion optimization approach, Regular two-level factorial design, *Bacillus subtilis* C3a-FIIRO-MW577298.

Introduction

A productive economy is mostly characterized by its ability to transform its indigenous resources into value-added products. [1] Most sub-Saharan countries include nations with a rapidly growing population and significant consumption potential for processed products. However, there is a notable absence of key players/manufacturers of processing aids such as enzymes. [2,3] This results in over-reliance on imports. The development of domestic production would alleviate costs, reduce pressure on foreign reserves, and lower production costs for enzyme-based goods. [2,3,4] Therefore, addressing the lack of local enzyme production is critical to supporting economic growth, especially in Nigeria and similar sub-Saharan countries. From a biochemical perspective, proteases are commercially exploited hydrolytic biocatalysts. [5-9] It targets peptide bonds, converting proteins into their monomers. [5-9]

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It holds a pivotal position in the industrial enzyme market segment valued at USD 2.76 billion in 2019. [5] Alkaline protease (EC 3.4.21) is valued for its function at alkaline pH and widespread applications, making it a significant contributor to the enzyme market. [5,6,9] This enzyme is used as an additive or processing aid in detergents, food and dairy industries, leather, pest management, medicine, animal feed, and waste management. [5-7] The phenomenal increase in alkaline protease applications stems from its ability to function outside its normal biological environment, high catalytic activity, substrate specificity, and potential to function as a green catalyst. [2,10] Microbial sources are valuable in enzyme production due to their ability to secrete these biocatalysts in considerable amounts within a short time, their generally recognized as safe (GRAS) status, the absence of ethical issues, and the ability of these catalytic components to function outside their natural environment. [2,5-7,11] Despite the exploitation of microorganisms in alkaline protease production, the enzyme market is dominated by the ubiquitous *Bacillus spp.* and *fungi spp*. [5,7] Unfortunately, the production of enzymes via industrial microbial cultivations, especially the *Bacillus* species, is not favorable enough to meet the upsurging demand for alkaline protease in diverse industries.[8] Thus, the need to increase its productivity to meet the rising demands consistently is essential.

Rice is valued as the most important staple food, the major source of calories in most sub-Saharan countries, and is ranked third in production after maize and wheat. [12,13] However, a major drawback in the production/primary processing of this staple grain in most sub-Saharan countries is the generation of rice-wastes.[13] One of the highly abundant waste residues with no commercial value generated during rice production in sub-Saharan countries such as Nigeria is rice bran.[2] Due to the high protein nutrients reported trapped within this bran, they are considered useful for the production of biochemicals that are beneficial

to man. [2,14] These trapped nutrients have been shown to function either as a source of macronutrients or solid support suitable for cultivating diverse microorganisms. [2,15,16] As a solid support, the offers of higher productivity by solid-state fermentation make it suitable and less expensive for enzyme production, in contrast to the sophisticated submerged fermentation. [15]

Medium optimization is recognized as one of the approaches employed to increase the productivity of biochemicals before large-scale production is considered. [17,18] The presence and concentration of nutrients in the fermentation medium have been reported to control the microbial metabolic activity, influencing the growth of the microbial strains as well as their potential to release/secrete enzymes. [17] As medium composition impacts the release of a specific end product of the fermentation process, identification, selection, and optimization of these components, which is usually oriented toward increasing productivity, is necessary. [17] While classical optimization serves as the basis of fermentation processes to lay the foundations for the production medium design, the use of statistical optimization techniques overcomes the limitations of time consumption, labor intensiveness, and the inability to quantify interaction effects found with the classical method. [17] As some components present in the medium do not contribute to enzyme production, it is of utmost importance that the significant contributing factors are identified. [16] The response surface methodology technique uses factorial design to provide an effective means for screening to find the significant factors critical to the enzyme production while permitting the simultaneous estimation of the interaction effect. [17] The gap between the industrial value alkaline proteases offer and the accessibility by the end users, most especially in sub-Saharan countries, has not only created an opportunity to optimize the production medium to favor higher yield, but it would result in easy accessibility of the enzyme with subsequent reduction in the dependence on importation and its consequences (in terms of cost). In order to meet the market demand of the end users in most sub-Saharan countries for alkaline protease, this study focused on investigating the influence of medium components on alkaline protease production using two different optimization approaches (classical exclusion approach and two-level factorial design). Solid-state fermentation was conducted to select a suitable medium and the optimal fermentation time. The classical exclusion optimization approach is a form of classical optimization approach, where each component of the medium is excluded one at a time, and its effect on alkaline protease production is evaluated. A regular two-level factorial design was employed to identify factors that significantly contribute to the alkaline protease production while simultaneously estimating interaction effects. Biochemical assays were used to measure the catalytic effect of the alkaline protease quantitatively under assay conditions.

Materials and Methods

Collection and Preservation of Rice Bran Wastes
Rice bran was collected from the Federal Institute of Industrial Research in Oshodi (F.I.I.R.O.), Lagos, Nigeria, and preserved in an air-tight sterile container.

Inoculum Preparation of Bacillus subtilis C3a-FIIRO-MW577298
Bacillus subtilis C3a-FIIRO-*MW577298* was obtained from the culture bank present in F.I.I.R.O. A freshly prepared plate of *Bacillus subtilis* C3a-FIIRO-MW577298 was prepared by subculturing on nutrient agar. The inoculum was transferred from the freshly prepared plate onto the sterilized growth medium and allowed to grow in a constituted growth medium (0.3 % bacteriological peptone and 0.4 % yeast extract) for 24 h at 37 ºC and 150 rpm.[2]

Assay of Alkaline Protease
The enzyme sample (1 mL), borate buffer (1 mL of 0.2 M, pH 10), and casein solution (1 mL) were dispensed into test tubes, mixed, and incubated at 40 ℃ for 10 mins. After incubation, trichloroacetic acid (5 mL of 5 %) was dispensed into the mixture. The observed precipitate was separated using Whatman No.1 filter paper. The solution was neutralized with 5 mL of 0.5 M Na_2CO_3, and colour development was achieved using Folin's phenol reagent (1 mL of 1:3). After inversion, the enzyme mixture was subjected to incubation at 28 ºC for 30 mins. The resulting solution was subjected to spectrophotometric analysis at 600 nm. [2] A unit of alkaline protease activity was defined as the amount of alkaline protease that cleaved peptide bonds to release 1 µg of tyrosine per mL per minute under the stated assay conditions.

Determination of Protein Concentration
Crude alkaline protease extract (0.2 mL), diluent (0.8 mL), and alkaline solution (5 mL) were dispensed into tubes. The mixture was subjected to inversion and allowed to equilibrate for 10 mins. The Folin's phenol reagent (0.5 mL) was added to the mixture, inverted, and left to stand for 30 mins. The protein concentration (alkaline protease) was estimated spectrophotometrically (UV-6300PC, VWR, United States) at 600 nm. [2]

Alkaline Protease Production
Seven fermentation media were selected and modified for alkaline protease production by *Bacillus subtilis* C3a-FIIRO-MW577298 (Table 1). [2,19-21] The sterilized rice bran was moistened, inoculated with a grown fer=mentation strain, and incubated in a sterile environment at 28 ℃ for 96 h. [2]

Effect of Fermentation Time on Alkaline protease production
The effect of fermentation time on the alkaline protease production was determined for 5 days (120 h). At an interval of 24 h, aliquot amounts of the samples were collected. The catalytic effect of alkaline protease was analyzed.

Effect of Exclusion of Each Component on Alkaline protease production
The effect of the exclusion of each production medium component (yeast extract, Na_2CO_3, KH_2PO_4, $MgSO_4$, $(NH_4)_2SO_4$, and glucose) on alkaline protease production was determined.[17]

Statistical analysis
Yeast extract, Na_2CO_3, KH_2PO_4, $MgSO_4$, $(NH_4)_2SO_4$, glucose, and pH were identified as the medium components. They were subjected to a regular two-level factorial design to estimate the interaction effects. Each factor was represented at high and low levels (Table 2). The responses measured include protease activity and protein concentration. The experimental design and statistical analysis were conducted using the Stat-Ease Design-Expert package (Stat-Ease 23.1.0.0, USA). The model prediction was confirmed under the optimal conditions. [22]

The software package generated a polynomial equation (second-order) to model the relationship between the predictor and outcome variables.

$$Y = \beta_o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_6 F + \beta_7 G + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{15} AE + \beta_{16} AF + \beta_{17} AG + \beta_{23} BC + \beta_{24} BD + \beta_{25} BE + \beta_{26} BF + \beta_{27} BG + \beta_{34} CD + \beta_{35} CE + \beta_{36} CF + \beta_{37} CG + \beta_{45} DE + \beta_{46} DF + \beta_{123} ABC \quad \ldots\ldots(1)$$

In equation (1), Y is the dependent variable, βo represents the intercept, β_1, β_2, β_3, β_4, β_5, β_6 are regression coefficients for the corresponding factors, A, B, and C are independent variables. AB, AC, BC…EF are interactions between variables. The direction of the interaction between a predictor variable and the outcome was determined by the sign of each coefficient estimate.

The results were presented as mean ± standard deviation (SD). The data obtained were further subjected to Stat-Ease Design-Expert packages (Stat-Ease 23.1.0.0 (analysis) and Stat-Ease 23.1.8.0 (verification)) (Stat-Ease, Inc.; Minneapolis, USA).

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Table 1: Seven selected medium components for production of alkaline protease

Medium	Medium composition per litre	Medium composition references
A	Sodium carbonate 10 g	Control
B	Yeast extract 5.5 g, sodium carbonate 10 g, potassium dihydrogen phosphate 7 g, magnesium sulphate 0.5 g, ammonium sulfate 1g, and glucose 1g	[2]
C	Sodium carbonate 10 g, potassium dihydrogen phosphate 7.0 g, magnesium sulphate 0.5 g, ammonium sulfate 1g,	Modified [2]
D	Calcium carbonate 0.5 g, peptone 1 g, glucose 1 g, yeast extract 1g	Modified [17]
E	Potassium dihydrogen phosphate 2 g, magnesium sulphate 3 g, sodium carbonate 10 g, calcium chloride 3 g	Modified [18]
F	Sodium carbonate 5g, magnesium sulphate 10 g, potassium dihydrogen phosphate 10 g, calcium chloride 0.5,zinc sulfate 0.1 g	Modified [19]
G	Potassium dihydrogen phosphate 5 g, magnesium sulphate 5 g, calcium chloride 0.5 g, sodium carbonate 5 g, sodium chloride 0.5 g, peptone 5 g	Modified [19]

Table 2: Summary of design for production of alkaline protease

Factor	Name	Units	Minimum	Maximum	Co
A	Glucose	g/l	0.1000	1.0000	-1
B	KH_2PO_4	g/l	7.00	14.00	-1
C	Na_2CO_3	g/l	10.00	20.00	-1
D	$(NH_4)_2SO_4$	g/l	1.0000	2.00	-1
E	Yeast extract	g/l	5.50	11.00	-1 ↔ 5.50 +1 ↔ 11.00
F	$MgSO_4$	g/l	0.2500	1.0000	-1 ↔ 0.2500 +1 ↔ 1.00
G	pH		10.00	12.00	-1 ↔ 10.00 +1 ↔ 12.00

Results and Discussion

Selection of Production Medium for Alkaline Protease Production

The exposure of fermentation strains to a specific inducer results in metabolic activities, which releases a specific biocatalyst into the culture medium at a higher concentration. The need for *Bacillus subtilis* C3a-FIIRO-MW577298 (Figure 1), a catalase-positive, Gram-positive, and rod-shaped bacterium, to facilitate alkaline protease production makes the selection of an appropriate nutrient medium essential. These nutrients furnish or initiate the synthesis of intermediate or precursors required for enzyme secretion.[17] Rice bran, a recognised residue generated during rice production, is reported to be enriched in nutritional values required to cultivate microorganisms as well as support diverse enzyme production.[13] In the presence of a fermentation substrate (rice bran), the alkaline protease production (1.830 U/mL ± 0.253) indicates that the rice bran protein was utilized. The intensity of the blue colour observed indicates the extent to which the alkaline protease secreted was able to hydrolyze the bonds present within the rice bran protein. [2] This hydrolytic process generates monomer units that were suggested to be assimilated/utilized as nutrients for bacterial growth. [2] Despite the high activity displayed by medium A (Figure 2), the low protein concentration led to the selection of medium B (Figure 3), whose activity was retained by a difference of 1.268%. The observed protein concentration, which favors medium B (92.118 mg/mL ± 0.004) by a difference of 25.14%, suggests the possibility of more monomers or the presence of proteins secreted along with alkaline protease.

Figure 1: *Bacillus subtilis* C3a-FIIRO-MW577298 on nutrient agar

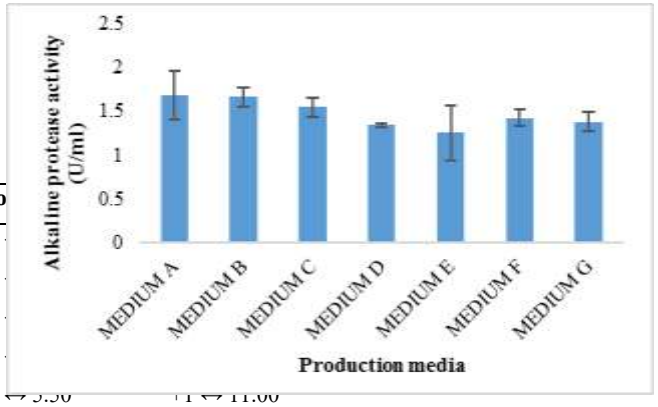

Figure 2: Alkaline protease activity obtained from the production media using *Bacillus subtilis* C3a-FIIRO-MW577298 as fermentation strain

A significant aspect of the enzyme-based fermentation processes is the fermentation time required for microorganisms to adapt to the medium components. As studies have shown, the optimal time for diverse *Bacillus spp.* to produce enzymes varies; the alkaline protease activity increased from 24 h and reached a peak at 96 h (Figure 4). After 96 h, a decline in activity by 45.3% was observed. In contrast to the increased production from 24 h to 96 h by 78.9%, studies have revealed maximum production at 24 h [23], 40 h [24] 48 h [25], and 120 h [26] using olive oil mill sols, corn cobs, wheat bran, and pomegranate peel as fermentation substrates. After 96 h incubation, the decline in enzyme production was suggested to be linked to the limitation of growth nutrients for the production strain, the limitation of a nutrient specific for the secretion of alkaline protease, and the possibility of by-product accumulation [20]

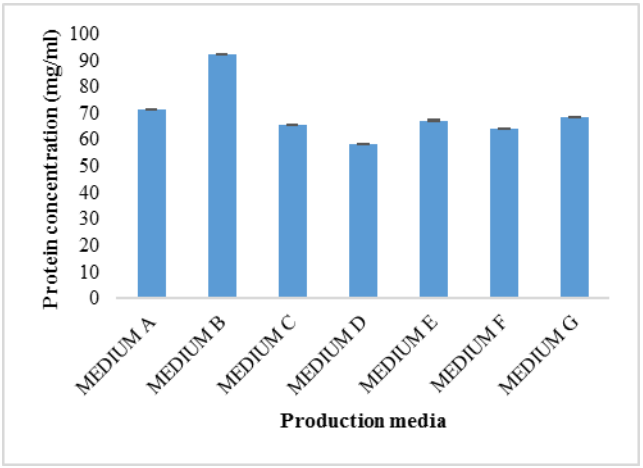

Figure 3: Protein concentration obtained from the production media using *Bacillus subtilis* C3a-FIIRO-MW577298 as fermentation strain

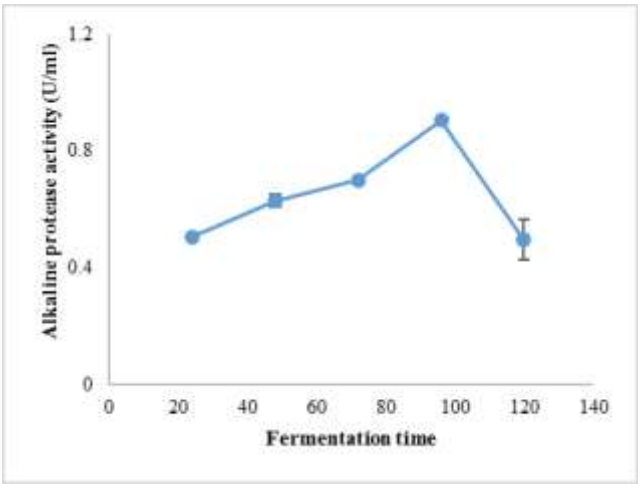

Figure 4: Effect of fermentation time on alkaline protease activity

Effect of Exclusion of Each Medium Component on the Alkaline protease production

As carbon sources are essential for microbial growth, nitrogen sources (organic and inorganic) are also crucial for enzyme production. Figure 5 shows that maximum activity was achieved when all the medium components were present. The absence of either nitrogen source, $(NH_4)_4SO_4$ or yeast extract, led to the lowest activity and protein concentration (Figure 5 and Figure 6).

Comparing medium B to media lacking $(NH_4)_2SO_4$, yeast extract, Na_2CO_3, and glucose, alkaline protease activity (medium B) increased by 78.9%, 73.4%, 66.08%, and 60.25%, respectively. By 44.1% and 42.3%, the absence of $(NH_4)_2SO_4$ and yeast extract resulted in a decrease in activity, respectively. In terms of protein concentration, medium B was higher than media lacking $(NH_4)_2SO_4$ and yeast extract by 73.08% and 71.46%, respectively. The role of yeast extract and inorganic nitrogen sources as stimulants for alkaline protease production has been reported by similar studies. [23,24,26] It is also believed that rice bran furnished an environment that enhanced the metabolic activity of *Bacillus subtilis* C3a-FIIRO-MW577298 in the absence of the synthetic nutrients, as shown in medium A, to yield alkaline protease. The lower protein concentration and low activity retention in medium A show the significance of micronutrients in fermentation processes, as the absence or deficiency of essential micronutrients could disrupt vital cellular processes, significantly impairing microbial metabolism and their functions, including enzyme secretion and stability.

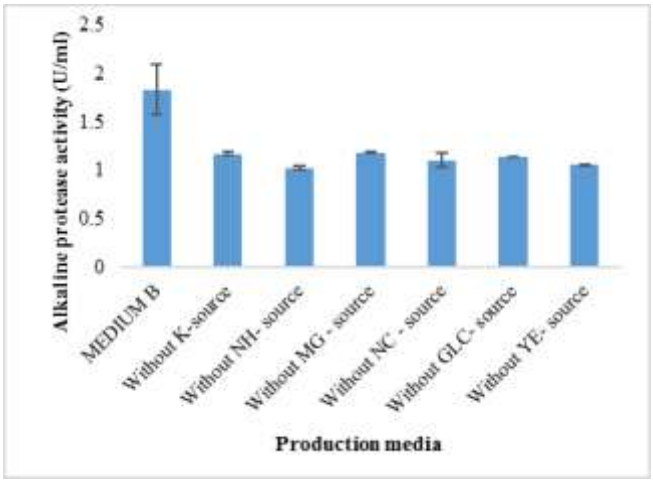

Figure 5: Effect of absence of each component on alkaline protease activity [K: Potassium sources; NH: Ammonium sulfate; Mg: Magnesium ion source; NC: Sodium carbonate; GLC: Glucose; YE: Yeast extract]

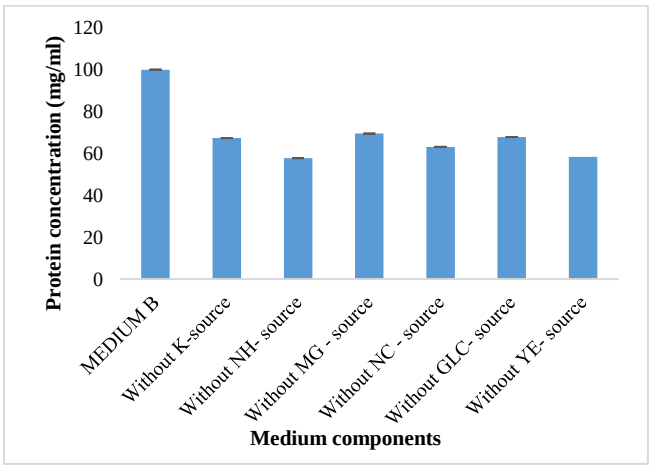

Figure 6: Effect of absence of each component on alkaline protease protein concentration [K: Potassium sources; NH: Ammonium sulfate; Mg: Magnesium ion source; NC: Sodium carbonate; GLC: Glucose; YE: Yeast extract]

Main Effect of Medium Components on the Alkaline Protease Production

The half-normal plot and the Pareto plot were used to guide the identification, selection, and separation of significant effects from insignificant ones (Figure 7 and Figure 8). The selection of significant

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effects was based on the two t-limits on the plots, the t-critical value, and the lower limit. The effect above the Bonferroni limit and above the standard t-critical value is considered important, and below the t-critical value is discouraged.

The interaction between glucose and $MgSO_4$ (12.08%) contributed significantly to alkaline protease activity, followed by yeast extract (11.18%) as a single factor. For protein concentration, it was shown that yeast extract (15.6%) contributed the most, followed by interaction between KH_2PO_4/$(NH_4)_2SO_4$ (15.528%) and $(NH_4)_2SO_4$/yeast extract (15.5%).

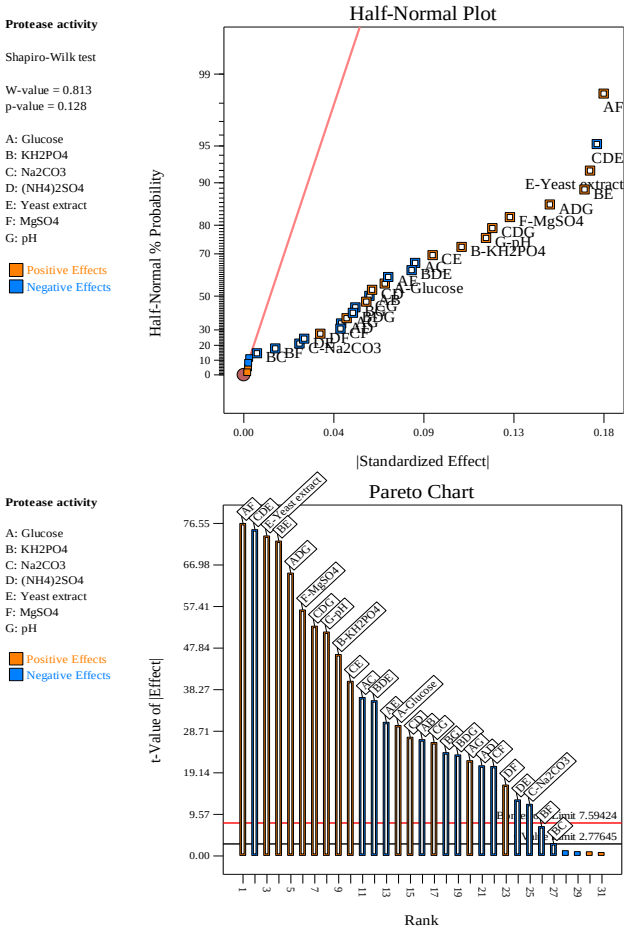

Figure 7: Half plot and Pareto plot for alkaline protease activity

Analysis of alkaline protease activity and protein concentration, as presented in Table S1.2- Table S1.5, reveals a significant p-value with result less than 0.05, the insignificant lack of fit, the reasonable agreement between predicted R^2 and adjusted R, and the adequate precision (greater than 4). This indicates that model predictions could be achieved.

The model's coefficient of determination for the activity at 99.9% and protein concentration at 97% indicates a very good agreement between the outcomes. The response plots, with the possibility of three-level interactions, as depicted by the cube plots (Figures 9- Figure 13), reveal the interaction effects of the medium components on the responses.

The observed values for responses and confirmation of the model prediction are presented in the supplementary data (Table S1 and Table S1.1).

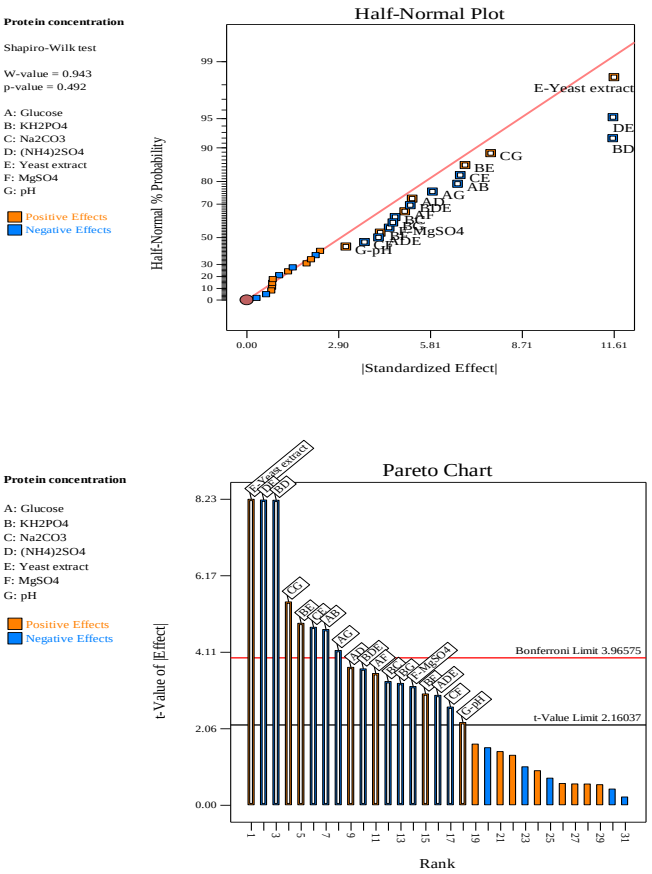

Figure 8: Half plot and Pareto plot for alkaline protease protein concentration

The equations below describe the interaction between the variables and the responses in terms of coded factors (Factorial model: non-hierarchical factorial model):

Protease activity $= 1.97 + 0.0346 \text{ x A} + 0.0533 \text{ x B} - 0.0137 \text{ x C} + 0.0847 \text{ x E} + 0.0652 \text{ x F} + 0.0593 \text{ x G} - 0.0308 \text{ x AB} - 0.0420 \text{ x AC} - 0.0239 \text{ x AD} - 0.0354 \text{ x AE} + 0.0881 \text{ x AF} + 0.0252 \text{ x AG} - 0.0033 \text{ x BC} + 0.0834 \text{ x BE} - 0.0078 \text{ x BF} - 0.0274 \text{ x BG} + 0.0314 \text{ x CD} + 0.0463 \text{ x CE} - 0.0237 \text{ x CF} + 0.0300 \text{ x CG} - 0.0148 \text{ x DE} + 0.0188 \text{ x DF} + 0.0749 \text{ x ADG} - 0.0411 \text{ x BDE} - 0.0267 \text{ x BDG} - 0.0864 \text{ x CDE} + 0.0609 \text{ x CDG}$

[A-Glucose, B-KH_2PO_4, C-Na_2CO_3, D- $(NH_4)_2SO_4$, E- Yeast Extract, F-$MgSO_4$, G-pH]

[p-value < 0.0001; F-value 1796.84; R^2:0.9999, Adjusted R^2:0.9994, Predicted R^2: 0.9947, Adeq Precision: 151.1672]

Protein concentration$= 58.73 + 5.81 \text{ x E} - 2.25 \text{ x F} + 1.57 \text{ x G} - 3.34 \text{ x AB} + 2.62 \text{ x AD} + 2.50 \text{ x AF} - 2.94 \text{ x AG} - 2.35 \text{ x BC} - 5.79 \text{ x BD} + 3.45 \text{ x BE} + 2.11 \text{ x BF} - 2.31 \text{ x BG} - 3.38 \text{ x CE} - 1.86 \text{ x CF} + 3.86 \text{ x CG} - 5.79 \text{ x DE} - 2.09 \text{ x ADE x} - 2.59 \text{ x BDE}$

[A-Glucose, B- KH_2PO_4, C-Na_2CO_3, D- $(NH_4)_2SO_4$, E- Yeast Extract, F-$MgSO_4$, G-pH]

[p-value: < 0.0001 F-value: 23.34: R^2: 0.9700, Adjusted R^2: 0.9284, Predicted R^2:0.8182, Adeq Precision: 19.9253]

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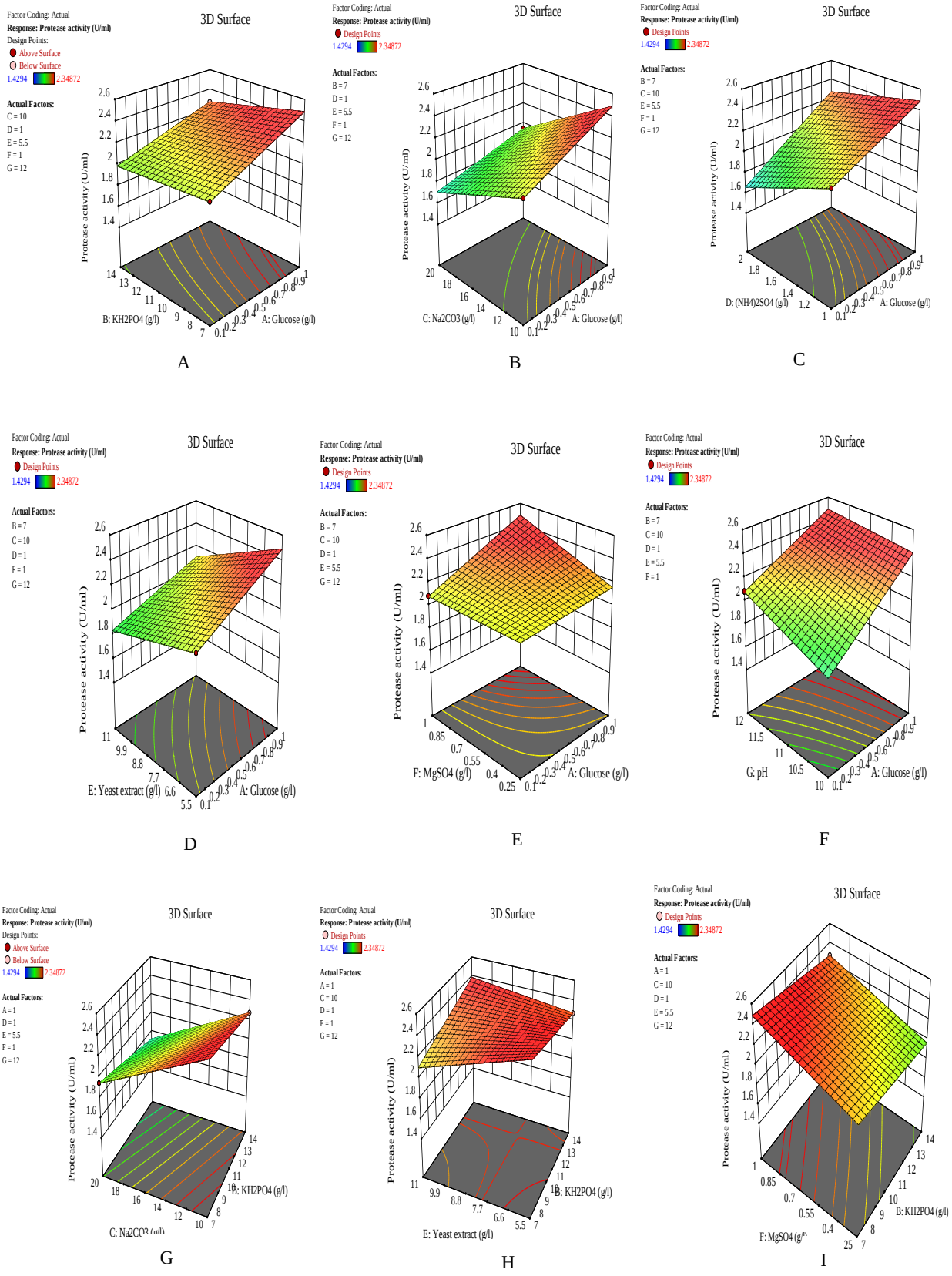

Figure 9: Response surface plot showing the effect of medium components on protease activity [A: Glucose and KH_2PO_4; B: Glucose and Na_2CO_3; C: Glucose and $(NH_4)_2SO_4$; D: Glucose and Yeast extract; E: Glucose and $MgSO_4$; F: Glucose and pH; G: KH_2PO_4 and pH; H: KH_2PO_4 and Yeast extract; I: KH_2PO_4 and $MgSO_4$

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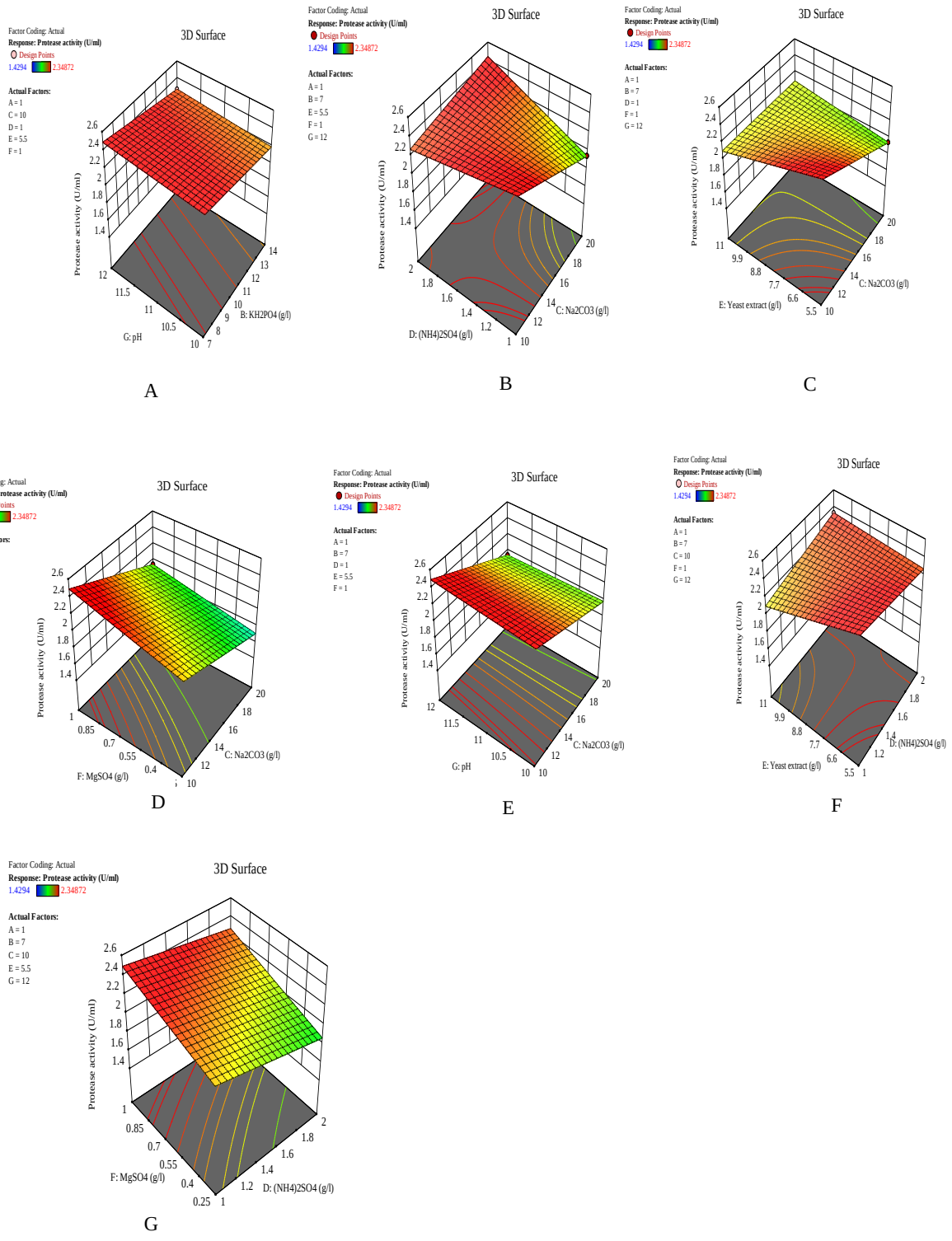

Figure 10: Response surface plot showing the effect of medium components on protease activity [A: KH_2PO_4 and pH; B: Na_2CO_3 and$(NH_4)_2SO_4$; C: Na_2CO_3 and Yeast extract; D: Na_2CO_3 and $MgSO_4$; E: Na_2CO_3 and pH; F: $(NH_4)_2SO_4$ and Yeast extract; G: $(NH_4)_2SO_4$ and $MgSO_4$]

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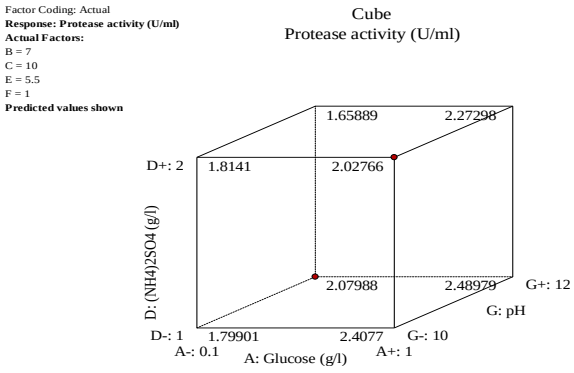

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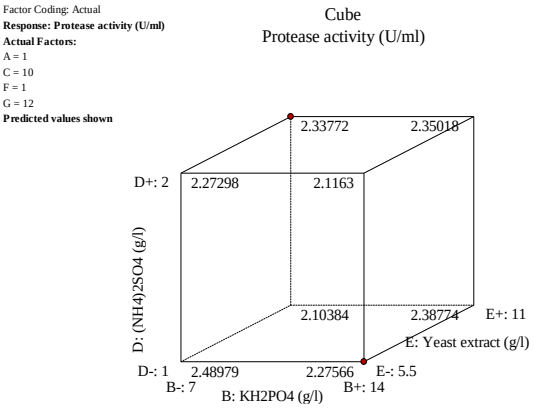

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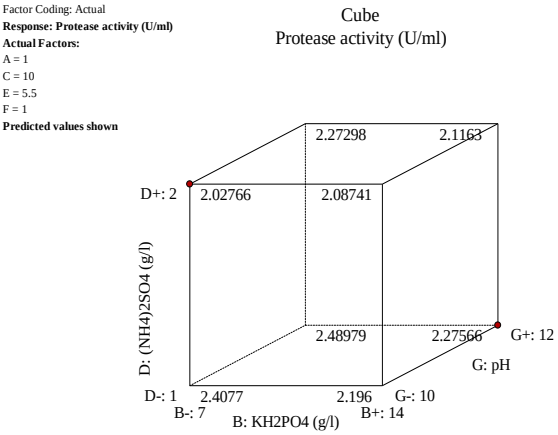
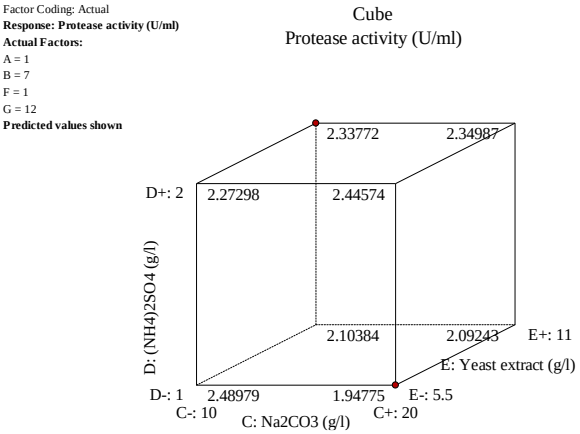
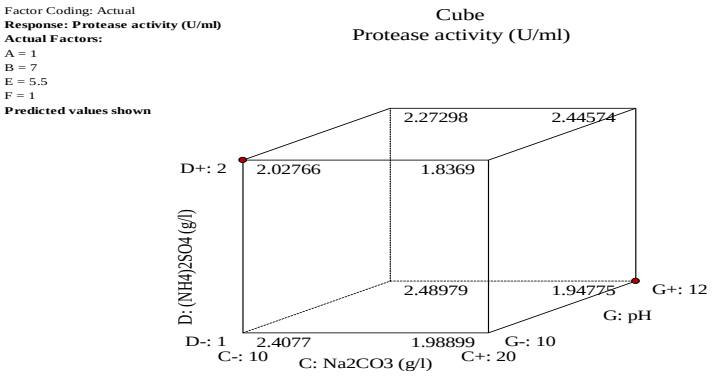


C



D



E

Figure 11: Cube plot showing the effect of three medium components on protease activity [A: Glucose, $(NH_4)_2SO_4$ and pH; B: KH_2PO_4, $(NH_4)_2SO_4$ and Yeast extract; C: KH_2PO_4, $(NH_4)_2SO_4$ and pH; D: Na_2CO_3, $(NH_4)_2SO_4$ and Yeast extract; E: Na_2CO_3, $(NH_4)_2SO_4$ and pH]

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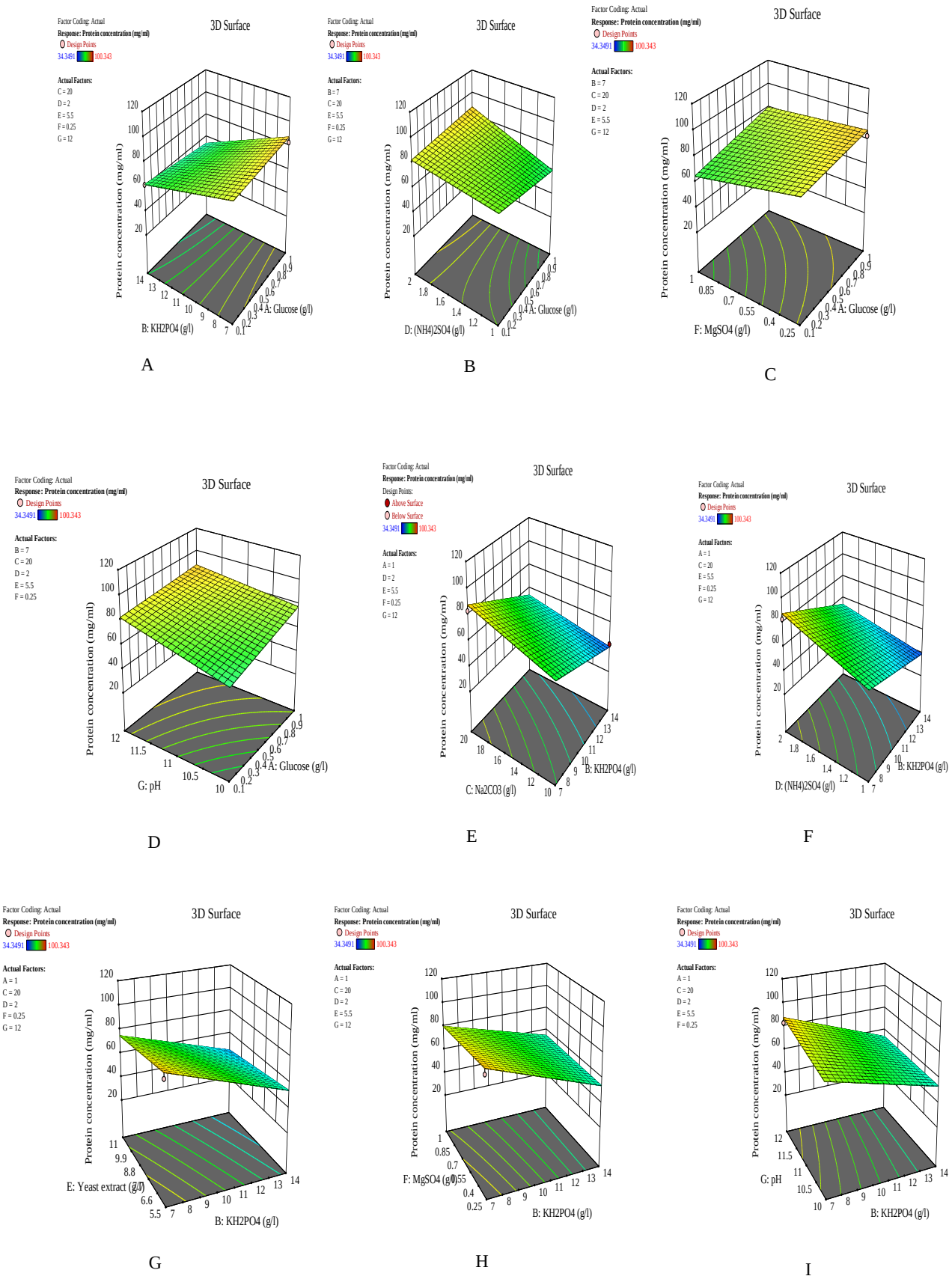

Figure 12: Response surface plot showing the effect of medium components on protein concentration [A: Glucose and KH_2PO_4; B: Glucose and $(NH_4)_2SO_4$; C: Glucose and $MgSO_4$; D: Glucose and pH; E: KH_2PO_4 and Na_2CO_3; F: KH_2PO_4 and $(NH_4)_2SO_4$; G: KH_2PO_4 and yeast extract; H: KH_2PO_4 and $MgSO_4$; I. KH_2PO_4 and pH]

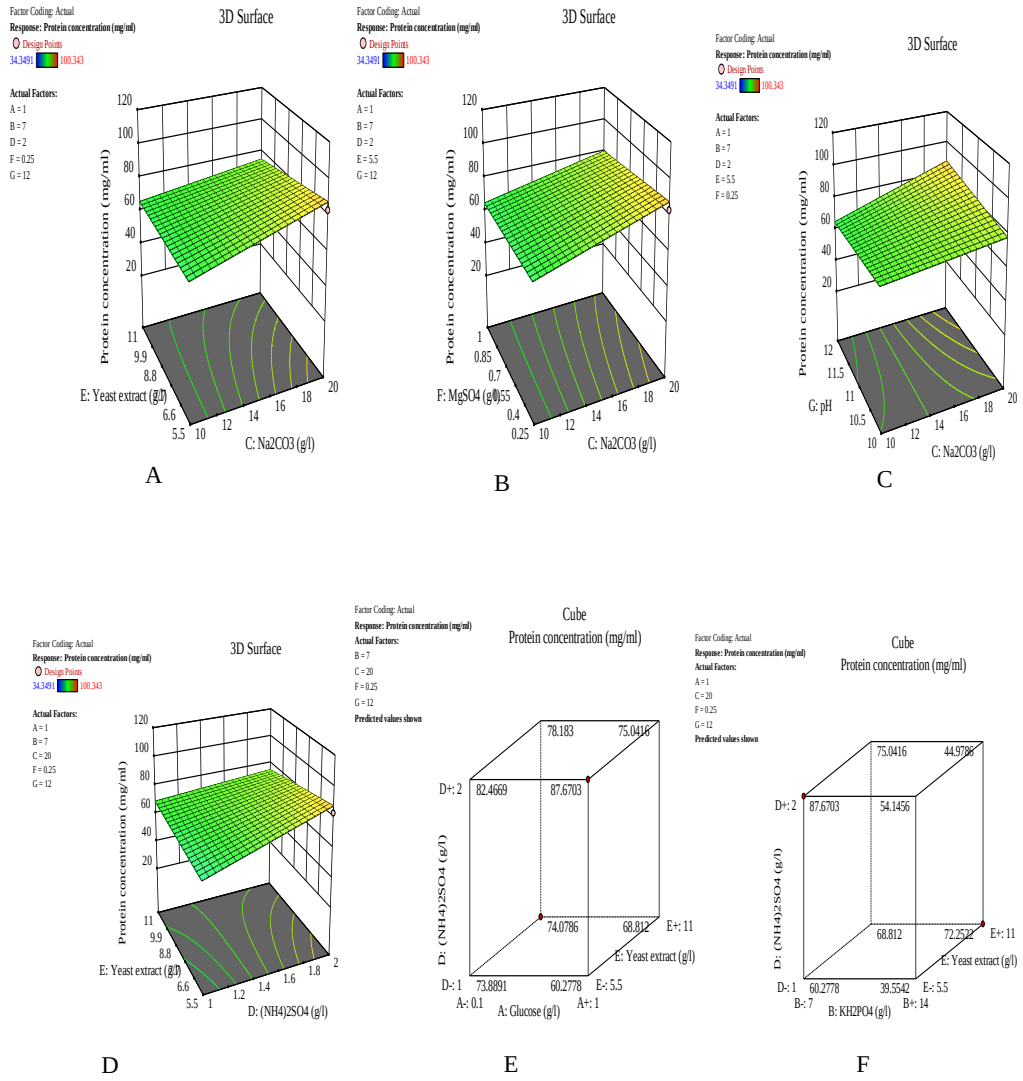

Figure 13: Response surface plot and cube plot showing the effect of medium components on protein concentration [A: Na_2CO_3 and yeast extract; B: Na_2CO_3 and $MgSO_4$; C: Na_2CO_3 and pH; D: $(NH_4)_2SO_4$ and yeast extract; E: Glucose, $(NH_4)_2SO_4$ and yeast extract; F: KH_2PO_4, $(NH_4)_2SO_4$ and Yeast extract]

Conclusion

The present study shows that the microbial alkaline protease production was influenced by medium constituents at alkaline pH. The suitability of production medium B was based on its potential to retain its activity longer and its high protein concentration. At an optimal fermentation time of 96 hours, the absence of each nitrogen source (ammonium sulfate and yeast extract) affected the alkaline protease production compared to other components. Using a regular two-level factorial design, alkaline protease activity increased by 1.79-fold (from 0.904 to 2.527 U/ml). It was concluded that the main effect of yeast extract, and the interaction effect of glucose and $MgSO_4$, contribute significantly to alkaline protease production by *Bacillus subtilis* C3a-FIIRO-MW577298. Hence, the combination of the classical exclusion optimization approach and the two-level factorial design was useful in identifying components of the production medium that contribute to alkaline protease production.

Most studies on alkaline protease in sub-Saharan Africa have not moved beyond their publications. However, a crucial factor that has strong potential to promote alkaline protease applications is its characterization, which determines its suitability and integration into any industrial setting.

The introduction of the characterized enzyme into various industrial processes would ascertain the safety of the enzyme and give end users or manufacturers of diverse processed products control over the characteristics of their products, with a positive impact on the environment and human health.

From a commercial perspective, this would further enhance the industrial profile of the enzyme-producing nations, increase confidence in the accessibility of the enzyme, and reduce over-reliance on its importation. Hence, the need to continuously prioritize research on processing aids in sub-Saharan countries that are heavily dependent on importation cannot be overemphasized.

Conflicts of interest

The authors declare no conflicts of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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