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Simultaneous Optimization of Protease and Biopesticide Productions: A Case Study with Industrial Perspective

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ABSTRACT

Bacillus sphaericus is a biopesticide that is highly effective for the control of mosquitos under harsh conditions such as polluted areas and UV light. In addition to its bioactivity, it produces proteases. In this present study, the composition of media comprising corn steep liquor (CSL) and molasses was optimised using response surface methodology (RSM) with a central composite design (CCD). Four different scenarios were arranged involving sole biopesticide and protease optimization (Scenarios 1 and 2), biopesticide optimization with protease as a by-product (Scenario 3) and protease optimization with biopesticide as a by-product (Scenario 4). The optimization of the simultaneous production of biopesticide and protease was not satisfactory to obtain large amounts of both products; however, when the production aim was a sole production with a by-product, optimal working conditions could be achieved. Also, according to industrial view, Scenario 1 is the only possible process for large scale systems.

Keywords: *Bacillus sphaericus*, Biopesticide, Protease, Response surface methodology (RSM), Molasses, Corn steep liquor (CSL)

Proteaz ve Biyopestisit Üretimlerinin Eşzamanlı Optimizasyonu: Endüstriyel Bakış Açısında Bir Durum Çalışması

ÖZ

Bacillus sphaericus biyopestisit olup, kirli alanlar ve UV ışık altı gibi zorlu koşullarda bile böcekler üzerinde yüksek etkiye sahiptir. Bu biyoaktivitesiyle beraber, proteaz enzimi de üretir. Bu çalışmada, mısır ıslatma şurubu (MIS) ve melastan oluşan ortam bileşenlerinin yüzey yanıt yönteminden (YYY) merkezi birleşik dizayn ile optimize edilmesi çalışılmıştır. Optimizasyon, dört farklı senaryo üzerinden yapılmıştır. Bunlar, sadece biyopesitisitin ve proteazın optimizasyonu (Senaryo 1 ve 2), proteazın yan ürün olduğu biyopesitist optimizasyonu (Senaryo 3) ve biyopesitisitin yan ürün olduğu proteaz optimizasyonu (Senaryo 4). Eşzamanlı optimizasyonlar, yüksek miktarlarda ürünlerin elde edilmesinde başarılı sonuçlar vermemiştir. Ancak, üretim hedefi yan ürünlü üretim şeklinde olursa, en uygun üretim koşullarına ulaşılabilir. Aynı zamanda, endüstriyel bakış açısıyla incelenecek olursa, Senaryo 1, büyük ölçekli üretim sistemi açısından uygulanabilir tek üretim yoludur.

Anahtar Kelimeler: *Bacillus sphaericus*, Biyopesitist, Proteaz, Yanıt yüzey yöntemi (YYY), Melas, Mısır ıslatma şurubu (MIS)

INTRODUCTION

Insecticides are commonly used for the protection of plant, animal and human health from pests. Chemical pesticides have approximately 90% of the market share; however, insecticides pollute the environment, disrupt ecosystems via the use of non-specific broad-spectrum chemicals, and lead to the development of insect resistance. Therefore, the use of biological insecticides is a promising and gradually increasing field [1].

Subspecies of *Bacillus thuringiensis* (Bt) and *Bacillus sphaericus* (Bs) are the most common biopesticides on the market. Although Bt and its subspecies have high activity on mosquitos, these species do not show killing activity in contaminated water environments and their activity is readily inhibited by UV light. Bs is a mesophilic and Gram-positive bacterium specific to target organisms and is frequently used to kill mosquito larvae. Bs is particularly used against *Culex* and *Anopheles* mosquitoes and has lower or no mosquitocidal activity against *Aedes* larvae [2]. Formulations of Bs are highly efficient and show a long time larvicidal activity, even in polluted environments [3, 4].

In the last three decades, new microbial insecticide production has advanced to the isolation of high activity strains that are more stable under different environmental conditions and more accurate against target organisms. Because of the economic importance of Bs as a biological control agent, particular attention has been paid to the production of Bs mosquitocidal toxins. Hence, the production costs of biopesticides must be reduced to reasonable economic production levels to replace chemical insecticides. The cost of raw materials is one of the major costs involved in overall biopesticide production and is approximately 30 to 40% of the total cost of biopesticides [5, 6]. Therefore, biopesticide production requires cheap and locally available sources to reduce transportation costs and ease preparation [7].

Enzymes have the largest market in the biotechnology industry. Food and pharmaceutical grade enzymes are produced from *Bacillus* sp., among other microorganisms, which produce significant amounts of extracellular enzymes and are more susceptible to protease production, particularly regarding yield, selectivity, and productivity. Protease synthesis has been well-studied in *Bacillus* sp., and the biosynthesis of protease is influenced by sporulation, media components and the carbon and nitrogen ratio [8-13]. Different operations have been performed to improve protease production [14].

Annually, large quantities of agricultural and food industrial wastes are generated through industrial processes [15-17]. Molasses and corn steep liquor (CSL) are traditionally used as the primary carbon and nitrogen sources, respectively, for microbial production [18]. Molasses is a waste product of sugar production. The composition of molasses varies depending on the source (sugar beet or sugar cane), source location and plant operations. CSL is the first wash of corn straws in a corn treatment plant; therefore, the nitrogen content is enriched in CSL. Response Surface Methodology (RSM) is a combination of mathematical and statistical techniques that is useful for modeling and analyzing problems influenced by some variables. The objective is to optimize this response [19]. The inherent advantages of RSM are fewer experimental numbers, suitability for multiple factor experiments, and ability to determine the relationship between factors, resulting in the most suitable conditions and estimation of the response [20-23].

The aim of the present study was to optimize the variables involved in the simultaneous production of biopesticides and extracellular alkaline proteases using response surface methodology under submerged fermentation in *Bacillus sphaericus*. This study is the first to examine the simultaneous production and optimization of biopesticides and proteases.

MATERIAL and METHODS

Preparation of Inoculum

Bacillus sphaericus MBI5 was used in the present study to produce both biopesticide and alkaline protease [24]. This strain is deposited in the Agricultural Research Service Culture Collection (United States) under accession number NRRL B-50199. The bacterium was maintained on agar slants and Petri dishes containing Luria-Bertani (LB) agar media at 30°C. The inoculum was prepared in LB medium in a 250-mL Erlenmeyer flask containing 50 mL of sterile medium (autoclaved at 121°C for 20 min) inoculated from the stock culture. After incubation for 16 h at 30°C with shaking at 150 rpm, 2% (v/v) of the culture was inoculated into 250-mL flasks containing 49 mL of sterile cultivation media.

Preparation of Media Components

In the fermentation media, two by-products were used: sugar beet molasses and CSL. To prepare the molasses solution, 100 g of raw molasses was diluted with 200 mL of distilled water. The diluted molasses was centrifuged at 8.000 rpm for 20 min. The supernatant was separated from the undesirable remains [25]. CSL was prepared at 2% (w/v), and the pH of the solution was adjusted to 7.0 using 1 M KOH. To obtain a clear solution, precipitated particles were removed by centrifugation at 4.000 rpm for 15 min. The media were sterilized at 121°C for 20 min and cooled to room temperature before use. In the optimization experiments, the concentration of the culture medium was changed according to the requirements [26]. The other components were minerals, such as $MgSO_4$ and $MnSO_4$, that are essential for sporulation and enzyme production.

Measurement of Cell Dry Weight

The biomass was collected by centrifugation at 10.000 rpm for 20 min. The supernatant was discarded, and each pellet was frozen at -80ºC overnight and lyophilized using freeze-dry techniques (Labconco Free

Zone 6). The cell dry weight was calculated and expressed as grams per liter. The same sample was used to determine the contents of the cell.

Protease Activity Assay

Proteolytic activity was determined based on the hydrolysis of casein [27]. The culture supernatant was centrifuged at 8.000 rpm and 4°C for 10 min. Subsequently, 5 mL of a 0.65% casein solution in 50 mM Glycine-NaOH buffer at pH 9.0 was mixed with 1 mL of cell-free supernatant. The solution was swirled and incubated at 37°C for precisely 30 minutes. The reaction was terminated by the addition of 5 mL of 100 mM trichloroacetic acid to each tube. The blank and test solutions were subsequently filtered using a 0.45-µm filter, and 2-mL aliquots of each filtrate were mixed with 5 mL of 0.5 M Na_2CO_3 and 1 mL of 0.5 M Folin-Ciocalteu reagent. The absorbance was measured at 660 nm. One unit of the protease was equivalent to the amount of enzyme required to release 1 µg of tyrosine/mL under standard assay conditions.

Statistical Analysis

The statistical software Design-Expert ver. 7.1 (Stat-Ease, Inc., Minneapolis, USA) was used for the regression analysis of the experimental data and to plot the response surface. ANOVA was used to estimate the statistical parameters.

Central Composite Design (CCD)

The levels of five significant factors and the interaction effects between various media constituents that significantly influence biomass and protease production were analyzed and optimized using a response surface methodology with a CCD design. A small CCD with four variables was used to optimize the response. The selected variables were molasses, CSL, $MnSO_4$ and $MgSO_4$ concentrations. Each variable was analysed at five levels coded as: - α, -1, 0, +1 and + α (Table 1). A CCD of 30 runs included 16 runs for factorial design, 8 runs for axial points and 6 runs for replication of the central points. The working parameters and responses in the experimental design are shown in Table 2.

Table 1. The levels of the variables for the central composite experimental design.

Independent variables	Symbols	Units	Levels				
			-α	-1	0	+1	+α
Molasses	A	%, v/v	3.787	10	25	40	46.213
CSL	B	%, v/v	3.787	10	25	40	46.213
$MnSO_4$	C	%, w/v	0.0515	0.3	0.9	1.5	1.7485
$MgSO_4$	D	%, w/v	0.0515	0.3	0.9	1.5	1.7485

RSM was used to define the optimal levels of key factors after the optimal region of each significant variable was determined. Therefore, the predicted response can be calculated using a second-degree polynomial (Eq. 1) that includes all of the interaction terms.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i<j}^{n} \beta_{ij} x_i x_j + \sum_{j=1}^{n} \beta_{jj} x_j^2 \qquad \text{Eq (1)}$$

where *Y* is the predicted response. In the present study, four variables were involved; hence, *n* = 4. Thus, Eq. 2 becomes:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \qquad \text{Eq (2)}$$

where *Y* is the predicted response variable, β_0 is the constant term, and $X_1, X_2, X_3, \text{and } X_4$ represent the codded values of molasses, CSL, $MnSO_4$, and $MgSO_4$, respectively. $\beta_1, \beta_2, \ldots\ldots, \beta_{34}$ are the model coefficients.

The statistical significance of the model equation and the model terms were evaluated in Design Expert software using Fisher's test. According to the correlation coefficients R and R^2, which explain the quality of fit of the regression model, the contour plots were determined as a two-dimensional graphical representation that generated response surface curves.

RESULTS and DISCUSSION

Statistical Analyses and Models of Biomass and Alkaline Protease Production

The CDW response was transformed using power to obtain a significant model at –1.46. The transformation was conducted according to Box-Cox [28]. Prior to Box-Cox transformation, the predicted-R^2 value was 0.4432, a value lower than the adjusted model, as shown in Table 3. In addition, high probability values (data not shown), such as 0.532, 0.214, 0.491, and 0.984 for *AD* (Molasses x $MgSO_4$), *BD* (CSL x $MgSO_4$), *CD* ($MnSO_4$ x $MgSO_4$), and C^2 ($MnSO_4$), respectively, were removed from the model equation to sustain the model equation. Strnad et al. [22] performed Box-Cox transformation,

and R^2 and adjusted-R^2 were calculated as 0.739 and 0.691, respectively, for the cultivation conditions in erythropoietin production, and these data and transformation were approved for bioprocess optimization. The protease activity response was obtained as a linear model equation using none-transformation (lambda=1.0). However, $MgSO_4$ was determined to be an insignificant parameter in these experiments. Therefore, $MgSO_4$ was eliminated from the model equation. Before the elimination of this variable, the *P*-value of *D* ($MgSO_4$), adjusted-R^2 and predicted-R^2 values were 0.8246, 0.6619 and 0.6086, respectively, and these values were lower than the adjusted model (Table 3). Even *B* (CSL), *C* ($MnSO_4$), and *D* ($MgSO_4$) were not statistically important for the CDW, multiplied and square forms of these parameters, as *AB*, *AC*, *BC*, and D^2 were relatively significant. Therefore, these variables remained in the ANOVA results.

Table 2. The composition of various experiments of the central composite design and corresponding results on Bs growth (CDW) and protease production.

	Factors				Responses	
Standards	A:Molasses (%, v/v)	B:CSL (%,v/v)	C:$MnSO_4$ (%, w/v)	D:$MgSO_4$ (%, w/v)	CDW (g/L)	Protease activity (mU/mL)
1	10.00	10.00	0.30	0.30	4.409	200.734
2	40.00	10.00	0.30	0.30	1.987	540.322
3	10.00	40.00	0.30	0.30	8.511	383.151
4	40.00	40.00	0.30	0.30	3.411	575.717
5	10.00	10.00	1.50	0.30	5.582	336.371
6	40.00	10.00	1.50	0.30	2.800	597.993
7	10.00	40.00	1.50	0.30	2.813	567.549
8	40.00	40.00	1.50	0.30	5.784	678.435
9	10.00	10.00	0.30	1.50	4.713	210.882
10	40.00	10.00	0.30	1.50	2.258	364.622
11	10.00	40.00	0.30	1.50	7.298	495.275
12	40.00	40.00	0.30	1.50	3.866	605.913
13	10.00	10.00	1.50	1.50	7.849	372.260
14	40.00	10.00	1.50	1.50	3.160	559.133
15	10.00	40.00	1.50	1.50	2.402	608.636
16	40.00	40.00	1.50	1.50	4.293	622.002
17	3.79	25.00	0.90	0.90	6.693	391.567
18	46.21	25.00	0.90	0.90	3.704	606.408
19	25.00	3.79	0.90	0.90	7.096	446.020
20	25.00	46.21	0.90	0.90	3.978	620.269
21	25.00	25.00	0.05	0.90	3.869	433.149
22	25.00	25.00	1.75	0.90	2.980	532.154
23	25.00	25.00	0.90	0.05	2.562	576.459
24	25.00	25.00	0.90	1.75	2.618	553.688
25	25.00	25.00	0.90	0.90	3.767	275.730
26	25.00	25.00	0.90	0.90	2.533	572.251
27	25.00	25.00	0.90	0.90	3.058	535.867
28	25.00	25.00	0.90	0.90	2.898	505.918
29	25.00	25.00	0.90	0.90	3.016	558.391
30	25.00	25.00	0.90	0.90	3.296	585.617

The values for CDW were the best fit using a second-order polynomial equation, and the protease activity response was calculated using a first-degree polynomial equation. The following equations (Eq. 3) of the models based on the coded values:

Eq. 3:

a) $((CDW\ (g/L))^{-1.46} = 0.19+0.038\times A-0.011\times B+0.00156\times C-0.00347\times D-0.052\times A\times B-0.043\times A\times C+0.040\times B\times C-0.033\times A^2-0.038\times B^2+0.039\times D^2$

b) $Protease\ activity\ (mU/mL) = 497.08 + 83.66\times A+80.04\times B+55.29\times C$

where A is molasses, B is CSL, C is $MnSO_4$, and D is $MgSO_4$.

The responses were verified using ANOVA for each factor (Table 3). The *P* values (*P*<0.1, statistically significant) indicated that the models were significant at a high confidence level for each response. The lack of fit values (0.4212 for CDW and 0.9910 for protease activity) was not significant with respect to the corresponding pure error.

Table 3. Statistical analysis of the following experiments: (a) analysis of variance (ANOVA), (b) statistical calculations of CDW, and (c) statistical calculations of protease.

(a)

Model	CDW (g/L)			Protease activity (mU/mL)		
Transformation	Power (Lambda:-1.46)			None		
ANOVA for RSM	Reduced Quadratic			Reduced Linear		
Source	Sum of Squares	F Value	p-value Prob > F	Sum of Squares	F Value	p-value Prob > F
Model	0.16	9.59	< 0.0001	329200	21.01	< 0.0001
A-Molasses	0.029	16.66	0.0006	140000	26.80	< 0.0001
B-CSL	0.0025	1.44	0.2446	128100	24.53	< 0.0001
C-MnSO4	0.000049	0.028	0.8680	61137.16	11.71	0.0021
D-MgSO4	0.00024	0.14	0.7118			
AB	0.043	24.92	< 0.0001			
AC	0.029	17.06	0.0006			
BC	0.026	15.00	0.0010			
A2	0.011	6.19	0.0223			
B2	0.014	8.16	0.0101			
D2	0.014	8.44	0.0091			
Residual	0.033			135800		
Lack of Fit	0.025	1.28	0.4212	68405.66	0.24	0.9910
Pure Error	0.0071			67388.37		
Cor Total	0.20			465000		

(b)

Std. Dev.	0.034	R-Squared	0.8346
Mean	0.12	Adj R-Squared	0.7476
C.V. %	28.24	Pred R-Squared	0.5523
PRESS	0.070	Adeq Precision	10.876

(c)

Std. Dev.	0.006	R-Squared	0.7080
Mean	0.027	Adj R-Squared	0.6473
C.V. %	22.14	Pred R-Squared	0.6358
PRESS	0.0014	Adeq Precision	16.597

F-value – Calculated value from a hypothesis test; P-value – Probability level;
PRESS – Prediction error sum of squares; C.V. % – Coefficient of Variance %.

The simultaneous effect of all of the factors on responses was compared using perturbation plots. CDW was affected after changing the substrates to low concentrations of molasses and CSL and high concentrations of $MgSO_4$, although a high *P*-value was observed. Manganese had no effect on CDW, but low and high concentrations of magnesium were associated with a slight difference in CDW (Fig. 1a). However, magnesium showed no effect on protease activity. The effect of molasses and CSL was higher than that of manganese (Fig. 1b).

The interactions between the factors visualized in the surface plots graphs showed a statistically significant effect on at least one of the responses (Fig. 2). The low concentrations of molasses and CSL generated high CDW values (Fig. 2a). However, based on biochemical reactions, biomass production is enhanced by increased the levels of carbon and/or nitrogen sources until substrate inhibition. The results presented in Fig. 2a also show a high CDW at elevated levels of molasses and CSL, while the highest CDW was observed with minimum levels of these substrates. Consistently, Fig. 2b plot also supported low levels of molasses. Notably, Fig. 2c showed that the increasing CSL level positively affected biomass generation. However, the protease activity increased with increasing molasses and CSL concentrations (Fig. 2d). The combination of CSL and molasses was important for protease production, reflecting enriched protein and energy sources, respectively. Afify et al. [29] reported results consistent with these data. These authors used fodder yeast for protease production, showing that the highest protease activity was observed using fodder yeast as the sole medium component. The amount and type of nitrogen source are important for *B. sphaericus* protease production.

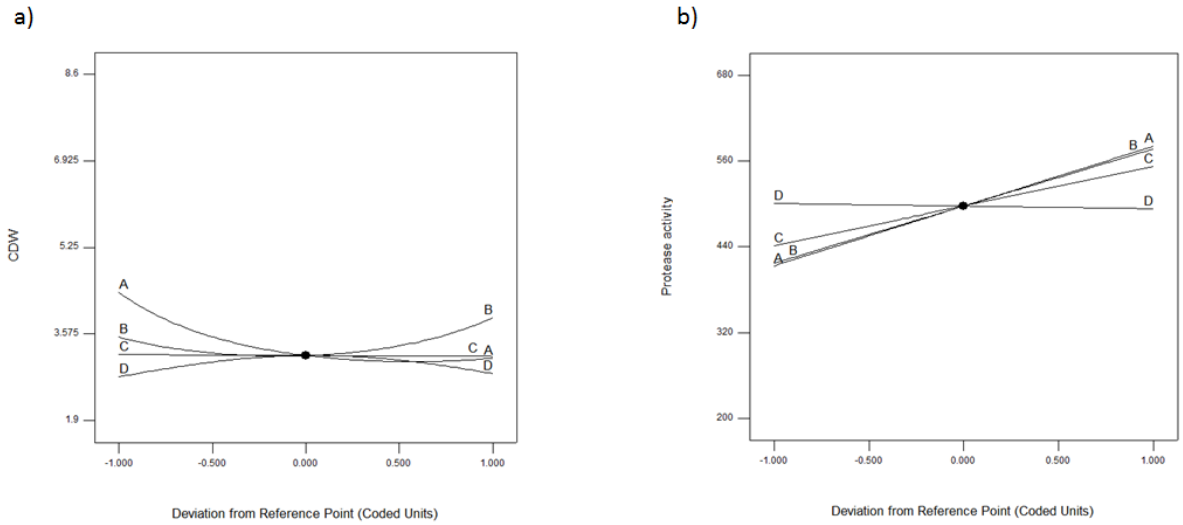

Figure 1. Perturbation graphs of CDW and protease activity showing the effect of the variables on responses (A is molasses at 25%; v/v, B is the CSL at 25%; v/v, C is the $MnSO_4$ at 0.9%; w/v, and D is the $MgSO_4$ at 0.9%; w/v).

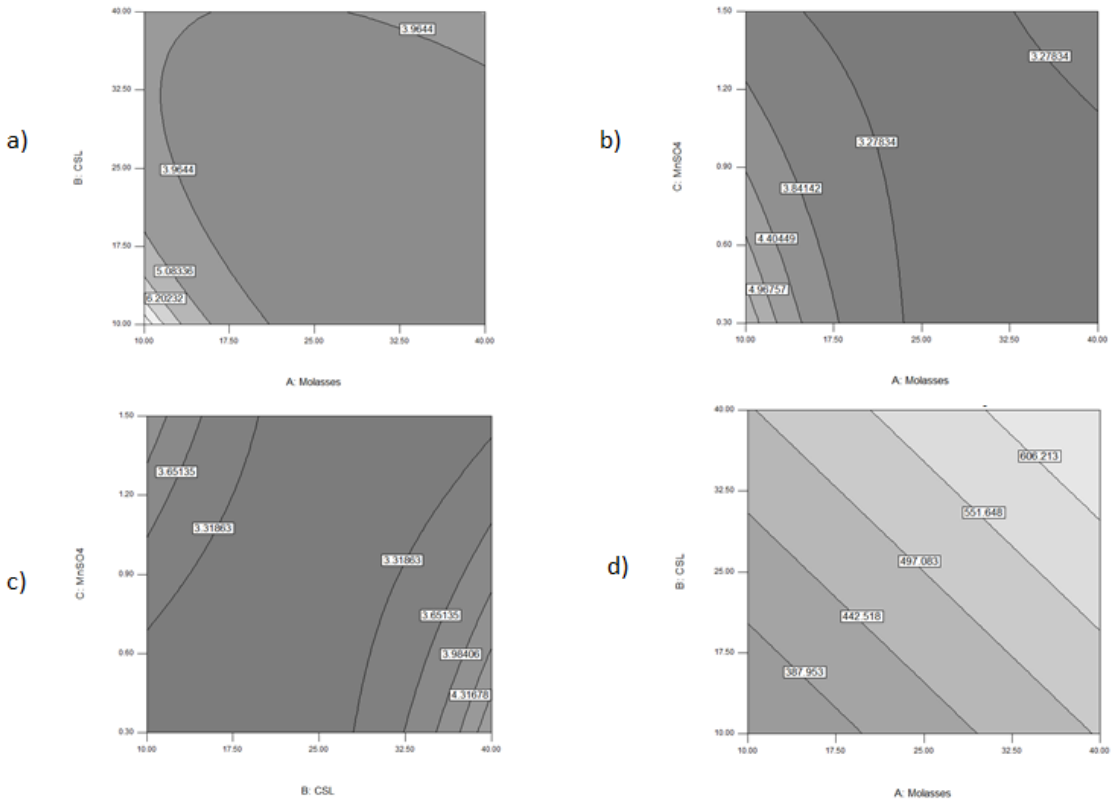

Figure 2. Contour plots of the following interactions for protease activity: molasses x CSL (a), molasses x MnSO4 (b), CSL x MnSO4 (c) for CDW, and molasses x CSL (d).

Optimization of Simultaneous Productions of Biomass and Protease

There are two studies concerning the simultaneous production of biopesticides and proteases [29, 30]. Surendran et al. [30] used *B. sphaericus* for protease production to investigate the enzyme activity under different carbon sources and working conditions. However, these authors did not report data concerning biomass generation during protease production. Afify et. al [29] also used *B. sphaericus* to examine protease production and larvicidal activity in fermenter production. In the present study, we investigated and optimized the appropriate media composition for the individual and simultaneous production of biopesticides and proteases. Numerical optimisation was performed to set goals in different perspectives, such as sole biopesticide production (Scenario 1), sole protease production (Scenario 2), biopesticide as the primary product and protease as the by-product (Scenario 3), and protease as the primary product and biopesticide as the by-product (Scenario 4) (Table 4).

Table 4. Potential optimized values and simultaneous optimization values for biomass and protease production

Scenario	Variables				Predicted values	Observed values
	Molasses (%)	CSL (%)	$MnSO_4$ (g/L)	$MgSO_4$ (g/L)		
1	11.04	39.99	0.31	0.99	8.799	8.652
2	39.01	38.32	1.48	0.71*	700.198	684.451
3	11.61	40.00	0.30	0.98	8.511 for CDW; 447.162 for Protease	8.598 for CDW; 412.874 for Protease
4	40.00	40.00	1.49	0.93	5.234 for CDW; 715.596 for Protease	5.441 for CDW; 692.149 for Protease

*: Not significant.

The preferable concentrations of molasses and CSL were different for biopesticide and protease activity. Higher values of biopesticide and protease activity were observed at lower and higher levels of molasses and CSL, respectively. Mineral concentrations strongly influenced biopesticide and protease activity. The optimized values of Scenario 1 aggregated in three different regions: low levels of molasses and CSL, high concentrations of molasses and CSL, and low concentration of molasses with a high concentration of CSL (Fig. 3a). As discussed above, high substrate concentrations until substrate inhibition are needed for biomass production. Therefore, proposed designs with low concentrations of molasses and CSL were discarded, although a high biomass concentration was observed in Fig. 3a. According to these data, high molasses concentrations inhibit cell growth. The proposed optimization system supported the fitted model, but not the exact structure. Hence, we focused on a design involving a low concentration of molasses and a high concentration of CSL for biopesticide media optimization (Table 4). Compared with the calculated data in Scenario 1, the error was + 1.6, which was highly acceptable. In Scenario 2, optimum values for protease production were obtained at high concentrations of substrates, including manganese. The 3-D plot for protease production is shown in Fig. 3b. We concluded that molasses have significant importance for the production of proteinaceous substances. Bioprocess plant facilities are typically established to produce one specific product. To create a cost-benefit facility, the recovery, and utilization of the by-product is an essential step for engineers. The value obviously differs depending on the biomass and protease, but the aim is to reach a minimum 5 g/L and 500 mU/mL for biomass and protease production, respectively [5, 12]. Scenario 3 served biopesticide production facilities with obtaining protease as a by-product, obtaining similar optimum values compared with sole biopesticide production (Scenario 1), as shown in Table 3. To observe the restrictions of the working conditions, biomass growth and protease production were set at 5.5 – 8 g/L and 450 – 550 mU/mL, respectively, with 0.3 g/L of $MnSO_4$ and 40% CSL (Fig. 4a). The precise combination of molasses and magnesium should be adjusted for Scenario 3. Protease production and biomass growth parameters were set at 600 – 700 mU/mL and 4.5 – 5.5 g/L, respectively, with 0.9 g/L of $MgSO_4$ and 40% CSL for Scenario 4 (Fig. 4b). In this case, high concentrations of molasses were essential for protease production. As shown in Table 4, in both cases, biopesticide and protease activity decreased 41 and 37% based on sole productions, respectively. Therefore, it is not possible to produce both products in high amounts. The optimised values for biopesticide production were 11% molasses, 40% CSL, 0.31% $MnSO_4$ and 0.99% $MgSO_4$, to yield 8.799 g/L CDW. For protease activity of 700.198 mU/mL, the optimized values were 39% molasses, 38% CSL, and 1.48% $MnSO_4$.

Scenarios with Industrial Perspective

Turkey's detergent and agricultural markets were chosen as models. The calculations were summarized in Table 5. The total detergent (both heavy duty liquid and powder detergent) purchase of Turkey is around 565,082 ton/year, and hence, the protease is consumed as 2,610 ton/year in detergent formula [31]. If the market share of the detergent protease was aimed to 5%, the need for one fermenter volume is higher than 30 million liters with our enzyme activity and process time. Consequently, the protease activity in the industrial area should be higher than 60 U/mL for plant size and applicable one fermenter volume that is maximum at 300,000 liters. Therefore, Scenario 2 and 4 were eliminated. If the market share of the detergent protease was aimed to 0.5% that was the by-product approach, fermenter volume was still enormous as 3,000,000 liters as for microbial production. The most likely production in the industrial case was Scenario 1 that was sole biopesticide production. The total pesticide consumption of Turkey is around 50,000 ton/year and 3% of this one (1,500 ton/year) was biopesticide itself. The ratio of a microorganism to fertilizer was traditionally adjusted to 1/1000 in formula [32]. If the market share of biopesticide was aimed to 5%, the total working volume of fermenter was found approximately 100 L when 8.65 g/L of microorganism concentration was achieved with optimized media composition and 90 batches per year.

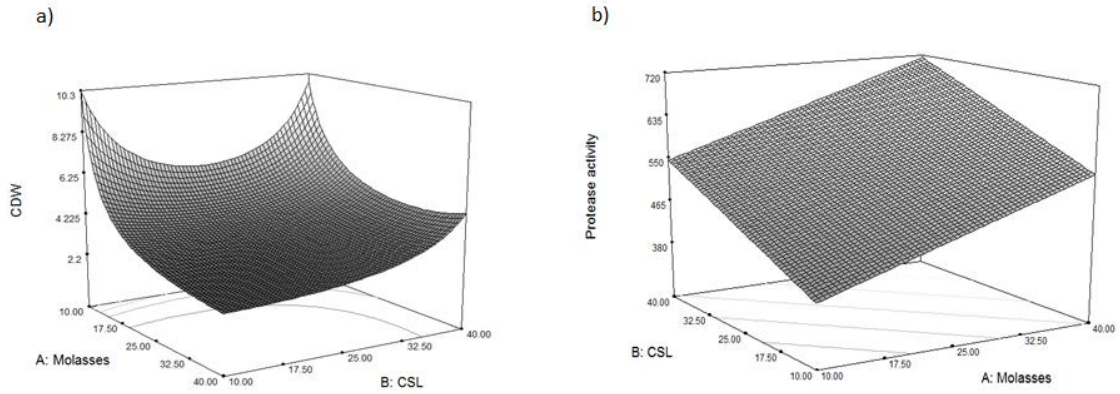

Figure 3. Numerical sole optimization graphs of CDW as Scenario 1 (a) and protease activity as Scenario 2 (b) based on molasses and CSL.

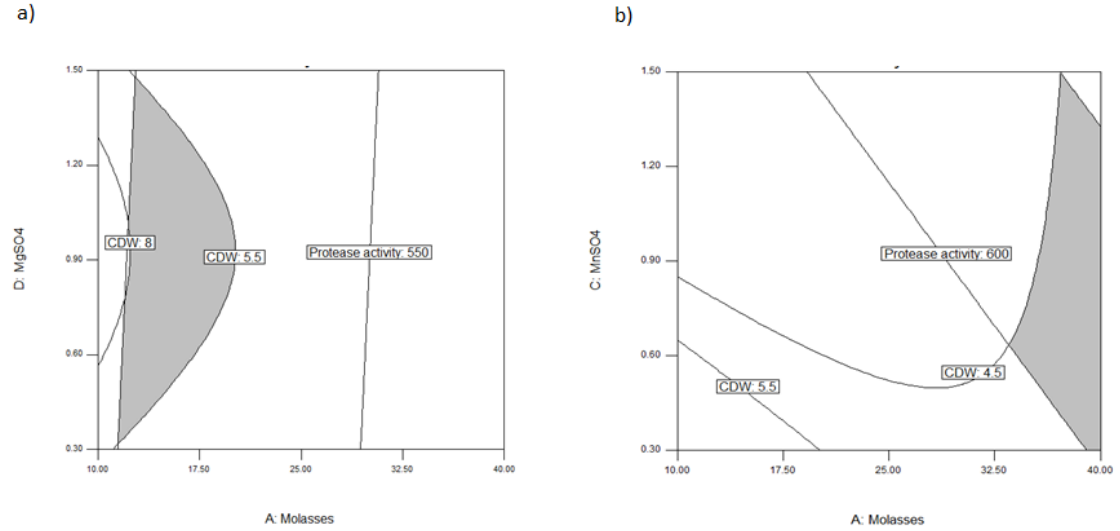

Figure 4. Graphical simultaneous optimization for Scenario 3 (a) and Scenario 4 (b).

Table 5. Process evaluation of protease and biopesticide production.

Items	Protease	Biopesticide
Consumption rate of material in Turkey (ton/year)	565082 (detergent)	50000 (pesticide)
Consumption rate of targeted active material in Turkey (ton/year)	2610 (protease)	1500 (biopesticide)
Targeted market size (%)	5	5
Production capacity (ton/year)	131	75
Fermentation time (days)	3	3
Capacity ratio of plant (%)	90	90
Working days in year	300	300
Production times (Batches/year)	90	90
Enzyme concentration (U/g)	15000	-
Enzyme activity in formulation (U/100 g detergent)	60	-
Protease production volume annually (U/year)	1.96×10^{12}	-
Enzyme unit per batch	2.18×10^{10}	-
Enzyme activity (U/L)	700	-
Necessary batch volume for enzyme production (L)	3.1×10^{7}	-
Biopesticide concentration in fermenter (g/L)	-	8.65
Biopesticide to fertilizer ratio	-	0.001
Biopesticide production annually (g)	-	75000
Biopesticide per batch (g/batch)	-	833
Necessary batch volume for biopesticide production (L)	-	96

CONCLUSIONS

There are no reports describing the optimization of biomass and alkaline protease production in *B. sphaericus* using a CCD and RSM. This statistical approach showed significant results for optimizing the process parameters for biomass and alkaline protease production under submerged fermentation. Results of this present study demonstrated that simultaneous production of biopesticide and protease was inefficient compared with the sole production. However, if the aim is to obtain by-product, then optimal working parameters could be obtained. As industrial perspective, sole production of biopesticide is applicable even with a large pilot plant fermentation facility as a starter company.

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