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Research Article

Optimization of Celullase Production by Molecular Identification Bacterial Isolated, PUA 18 by Using Response Simulation Modelling

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ABSTRACT
Cellulase enzymes are essential for converting cellulose into glucose, enabling sustainable industrial processes. Optimising cellulase production at an industrial scale requires refining microbial strains, growth medium components, and operating conditions. Traditional One-Factor-At-A-Time (OFAT) approaches are limited in efficiency, whereas Response Surface Methodology (RSM) provides a robust statistical tool for evaluating multiple variables simultaneously. This study improved cellulase production from *Bacillus subtilis* PUA-18, identified through 16S rRNA sequencing, using a combined Design of Experiment (DoE) approach. Screening and optimising were applied by using Plackett Burman Design (PBD) and Box-Behnken Design (BBD), respectively. Temperature was the most influential factor, followed by KNO_3, $FeSO_4$, and $CaCl_2$. The predicted maximum cellulase activity of 0.574 U mL^{-1} was achieved at 40 °C, 1.45 g L^{-1} KNO_3, 20 mg L^{-1} $FeSO_4$, and 50 mg L^{-1} $CaCl_2$. The DoE approach yielded a 293 % improvement in cellulase activity, demonstrating its superiority over traditional methods. These findings highlight *Bacillus subtilis* PUA-18 as a promising cellulase producer and emphasise the critical role of advanced optimisation strategies for enhancing enzyme production, paving the way for broader industrial applications.

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INTRODUCTION

Cellulase enzymes are pivotal in converting cellulose into monosaccharides like glucose, playing a critical role in advancing sustainable industrial processes (Annapure & Pratisha 2022). As the global demand for environmentally friendly chemicals surges, cellulases have emerged as key players in industries such as food and beverage, animal feed, detergents, textiles, and pulp production (Basak et al. 2021). This broad applicability underscores the need for efficient cellulase production systems to meet growing industrial demands.

Producing cellulases at an industrial scale requires optimizing multiple factors, including microbial strains, growth medium components, and operating conditions (Djekrif et al. 2024). Among these, the composition of the growth medium is particularly influential (Juturu & Wu 2014). While traditional One-Factor-At-A-Time (OFAT) approaches have been widely employed, they are time-intensive and fail to account for interactions between variables. In contrast, response surface methodology (RSM) has gained prominence as a robust statistical tool for process optimisation, enabling researchers to evaluate multiple variables simultaneously and to identify optimal conditions efficiently (Singh et al. 2017).

This study builds on the preliminary study by Alamsjah et al. (2024b), which optimised cellulase production using the OFAT method. The bacterial isolates used in that study were part of the collection maintained at the Biotechnology Laboratory of Andalas University. Among these, isolate PUA-18, originally isolated from the mangrove waters of the Mandeh region, exhibited the highest cellulase index (CI = 4.98) among 16 tested isolates. The study identified pH 6 and a temperature of 35 °C as the optimal conditions for cellulase production by PUA-18. To advance these findings, this research employs a Design of Experiments (DoE) approach to systematically identify significant factors and to optimise cellulase production conditions using response surface methodology. Key variables, including medium components, pH, temperature, inoculum concentration, and agitation, were analysed to provide deeper insights into their effects on enzyme activity. Additionally, the taxonomic classification of PUA-18 as a cellulase-producing bacterium will also be confirmed by molecular identification of the strain through 16S rRNA sequencing.

By combining advanced optimization techniques with molecular characterisation, this study contributes to improve cellulase production processes and underscores the potential of mangrove microorganisms as valuable enzymatic resources for industrial applications.

MATERIALS AND METHODS

Materials

The materials used comprised the PUA-18 isolate, ethanol, distilled water, 70 % alcohol, carboxymethyl cellulose (CMC), yeast extract, $MgSO_4 \cdot H_2O$, KNO_3, K_2HPO_4, $FeSO_4 \cdot H_2O$, $CaCl_2$, 3,5-dinitrosalicylic acid (DNS), phosphate buffer, NaOH, HCl, and Na_2SO_3. Additionally, the GeneJET Genomic DNA Purification Kit-Zymo Research, primers 16S rRNA-27F (forward) (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (reverse) (5'-GGTTACCTTGTTACGACTT-3'), and Promega PCR Master Mix were employed.

Methods

Molecular identification

Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit (Zymo Research) following the manufacturer's protocol. Bacteria were cultured in nutrient broth at 30 °C for 24 hours, collected by centrifugation at

10,000×g for 1 minute, and the DNA was eluted with DNase/RNase-free water. DNA amplification was conducted using PCR with Promega PCR Master Mix (12.5 µL; 0.05 U mL^{-1} Taq DNA polymerase, 0.4 mM dNTP, 4 mM $MgCl_2$), 1 µL 16S rRNA-27F forward primer (5' AGA GTT TGA TCC TGG CTC AG 3'), 1 µL 16S rRNA-1525R reverse primer (5' GGT TAC CTT GTT ACG ACT TT 3'), 1 µL genomic DNA, and nuclease-free water to a final volume of 25 µL. The PCR program included initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes. Amplified products were analysed on 1 % agarose gel stained with GelRed, visualised under UV light, and documented. Sequencing was performed bidirectionally at 1st Base Sequencing Services (Malaysia), and the sequences were assembled using DNA STAR software. The assembled sequences were compared to GenBank entries using BLASTn to validate bacterial identity. A phylogenetic tree was constructed using the Neighbour-Joining method in MEGA 10.2.6 software.

Preparation of Cultivation Medium and Enzyme Extraction Procedure

The liquid medium was prepared with the following composition: CMC (carboxymethyl cellulose) 10 g L^{-1}, $MgSO_4 \cdot H_2O$ 0.2 g L^{-1}, KNO_3 0.75 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $FeSO_4 \cdot H_2O$ 20 mg L^{-1}, $CaCl_2$ 40 mg L^{-1}, yeast extract 2 g L^{-1}, and bacto-agar 1.5 g L^{-1}. The medium was inoculated with 1–2 loopfuls of bacterial culture and incubated at 35 °C with agitation at 150 rpm for 24 h. Subsequently, 5 mL of the inoculum was transferred into 95 mL of CMC broth in a 250 mL Erlenmeyer flask and incubated in an orbital shaker at 35 °C and 120 rpm for 72 h. The cultured medium was centrifuged at 12,000 rpm for 10 min at 4 °C to remove cells and debris. The resulting clear supernatant served as the crude enzyme source.

Cellulase assay

Cellulase activity was evaluated using a modified version of the DNSA method outlined by McKee (2017). To conduct the assay, a total of 250 µL crude enzyme extract was mixed with an equal volume of 1 % w v^{-1} CMC dissolved in a phosphate buffer solution. The reaction mixture was incubated at 30 °C for 30 minutes. After incubation, the enzymatic activity was halted by adding 500 µL of 3,5-dinitrosalicylic acid (DNSA) reagent. The mixture was then heated in a boiling water bath for 5 minutes, followed by rapid cooling in an ice water bath. The reducing sugars produced during the reaction were quantified using a UV–Vis spectrophotometer at 540 nm, with glucose serving as the standard. One international unit (IU) of cellulase activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute under the specified assay conditions (Ghose 1987).

Experimental design

The Plackett-Burman design was utilised to screen and assess the significance of various medium components and physical parameters in a 12-run experiment aimed at cellulase production. Each variable was assigned high (+) and low (-) levels, as detailed in Table 1. The nutrient factors evaluated included the concentrations of CMC, yeast extract, $MgSO_4$, KNO_3, K_2HPO_4, $FeSO_4$, and $CaCl_2$. In addition, physical parameters such as pH, temperature, agitation speed, and inoculum concentration were examined (Table 1). Statistical analysis of the experimental data was performed using Design Expert 13 software (Stat-Ease, Inc., Minneapolis, MN).

Data analysis

The Box-Behnken design (BBD) was employed to optimise the conditions for

Table 1. Chemical and physical parameters with their different variables applied in Plackett–Burman design for cellulase enzyme production

Factor	Name	Units	Minimum	Maximum
X1	CMC concentration	g L^{-1}	2.5	15
X2	Yeast extract concentration	g L^{-1}	0.5	4
X3	pH		6	8
X4	Temperature	C	25	45
X5	Agitation speed	RPM	100	200
X6	Inoculum concentration	%	2	8
X7	$MgSO_4.H_2O$	g L^{-1}	0.1	0.4
X8	KNO_3	g L^{-1}	0.375	1.5
X9	K_2HPO_4	g L^{-1}	0.25	1
X10	$FeSO_4$	g L^{-1}	0.01	0.04
X11	$CaCl_2$	g L^{-1}	0.02	0.08

cellulase production. The independent variables considered for optimisation included temperature (X6), KNO_3 concentration (X1), and a combination of $FeSO_4$ and $CaCl_2$ (X3), with their respective levels outlined in Table 3. This design was deemed suitable for developing a quadratic response surface and constructing a second-order polynomial regression model. The relationship between the actual and coded values was represented by the equation provided below:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \varepsilon \qquad (1)$$

In this equation, Y represents the cellulase activity (response), while X_i and X_j denote the independent variables. β_0 is the constant term in the model, and β_i, β_{ii}, and β_{ij} represent the linear, quadratic, and interaction effects, respectively. The term ε accounts for random error. Statistical analysis of the experimental data and the corresponding response surface graphs were performed using Minitab 21 Statistical Software (Minitab, Inc., State College, PA).

RESULTS AND DISCUSSION

Molecular identification

The phylogenetic analysis shown in Figure 1 was conducted by comparing various bacterial isolates identified through BLAST results using the Neighbour-Joining (NJ) method. A bootstrap value of 1000× was applied to estimate the confidence level of the phylogenetic tree, utilizing MEGA software version 10.2.6. The 16S rRNA sequence used for the phylogenetic tree analysis covered nucleotide positions 68 to 1455, out of a total sequence length of 1569 base pairs (bp).

The cellulase-producing bacterial isolate PUA-18 was found to be closely related to *Bacillus subtilis* strains DSM 10, JCM 1465, and NBRC 13719, with a similarity of 100 %. As a result, isolate PUA-18 was identified as *Bacillus subtilis*. The Neighbour-Joining (NJ) method was employed to construct the phylogenetic tree based on differences between sequences, with a bootstrap value of at least 80 % considered indicative of a robust phylogenetic tree.

Plackett-Burman Design for Screening and Evaluation of Factors

A two-level fractional factorial Plackett-Burman (PB) design was utilised to determine the key factors affecting cellulase production, as detailed in Table 1. This design included 12 experimental runs, with the resulting cellulase activities also shown in Table 1. Each experiment was conducted in triplicate, and the mean values were recorded. The influence of the evaluated variables is summarized in Table 2.

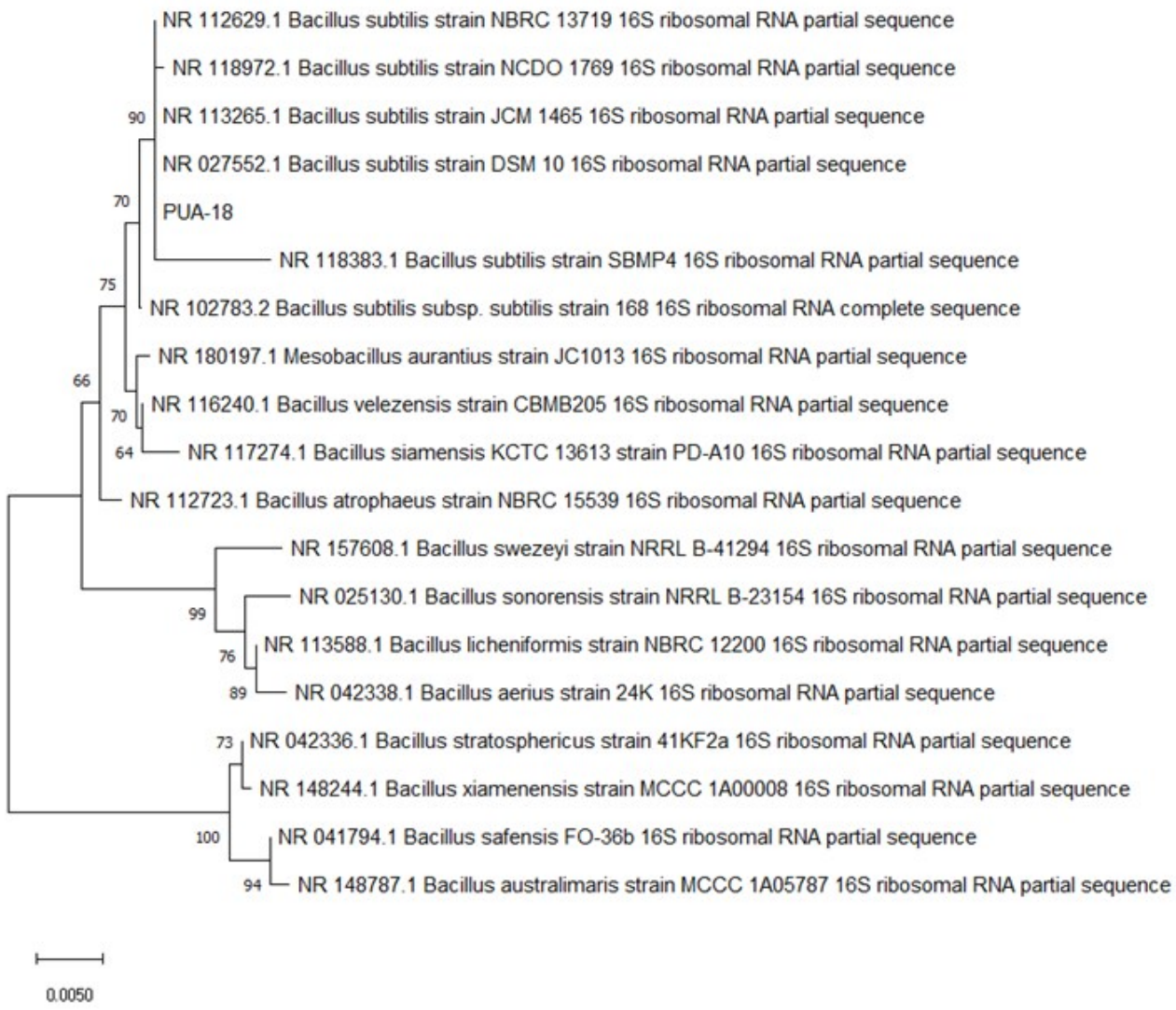

Figure 1. Phylogenetic analysis of PUA-18.

After conducting the Plackett-Burman design experiment, which evaluated 11 factors, the results indicated that temperature was the most significant variable, followed by KNO_3, $FeSO_4$, and $CaCl_2$. This conclusion was supported by the half-normal plot (Figure 2) and Pareto chart analysis (Figure 3).

Box-Behnken Design Optimisation

Building on the results of the Plackett-Burman Design (PBD), the experimental scope was expanded using the Box-Behnken design (BBD) for optimisation. The PBD analysis identified temperature (X4), KNO_3 (X8), $FeSO_4$ (X10), and $CaCl_2$ (X11) as significant variables influencing cellulase activity. To further investigate and to optimize these parameters, the BBD was employed. The experimental model, corresponding predicted, and observed values are provided in Table 3. Among the experimental runs, Run No. 28 demonstrated the highest cellulase activity (0.5412), achieved at 40 °C (X4), 1.5 % KNO_3 (X8), 20 % $FeSO_4$ (X10), and 0.125 % $CaCl_2$ (X11). Conversely, the lowest cellulase activity (0.1600) was recorded in Run No. 6, which was conducted at 30 °C (X4), 2 % KNO_3 (X8), 12.5 % $FeSO_4$ (X10), and 0.125 % $CaCl_2$ (X11). Using the experimental results, a quadratic polynomial equation (Equation 4) was developed to describe the relationships between the independent variables and the response in terms of actual values. A quadratic non-

Table 2. Plackett–Burman design for cellulase production.

Std	Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Cellulase Activity (U mL^{-1})		Residual
													Observed	Predicted	
3	1	15	0.5	8	45	100	8	0.4	1.5	0.25	0.01	0.02	0.0712	0.0845	-0.0134
2	2	2.5	4	8	25	200	8	0.4	0.375	0.25	0.01	0.08	0.1309	0.1298	0.0011
9	3	15	4	8	25	100	2	0.4	0.375	1	0.04	0.02	0.0873	0.0826	0.0047
6	4	2.5	0.5	6	45	100	8	0.4	0.375	1	0.04	0.08	0.0500	0.0519	-0.0019
12	5	2.5	0.5	6	25	100	2	0.1	0.375	0.25	0.01	0.02	0.0949	0.1063	-0.0114
5	6	2.5	0.5	8	25	200	8	0.1	1.5	1	0.04	0.02	0.1255	0.1150	0.0105
13	7	8.75	2.25	7	35	150	5	0.25	0.9375	0.625	0.025	0.05	0.1929	0.1929	0.0000
1	8	15	4	6	45	200	8	0.1	0.375	0.25	0.04	0.02	0.0312	0.0283	0.0028
8	9	15	4	6	25	100	8	0.1	1.5	1	0.01	0.08	0.1745	0.1623	0.0122
11	10	15	0.5	8	45	200	2	0.1	0.375	1	0.01	0.08	0.0803	0.0756	0.0046
4	11	2.5	4	6	45	200	2	0.4	1.5	1	0.01	0.02	0.0913	0.0845	0.0068
10	12	2.5	4	8	45	100	2	0.1	1.5	0.25	0.04	0.08	0.0854	0.0843	0.0010
7	13	15	0.5	6	25	200	2	0.4	1.5	0.25	0.04	0.08	0.1214	0.1386	-0.0171

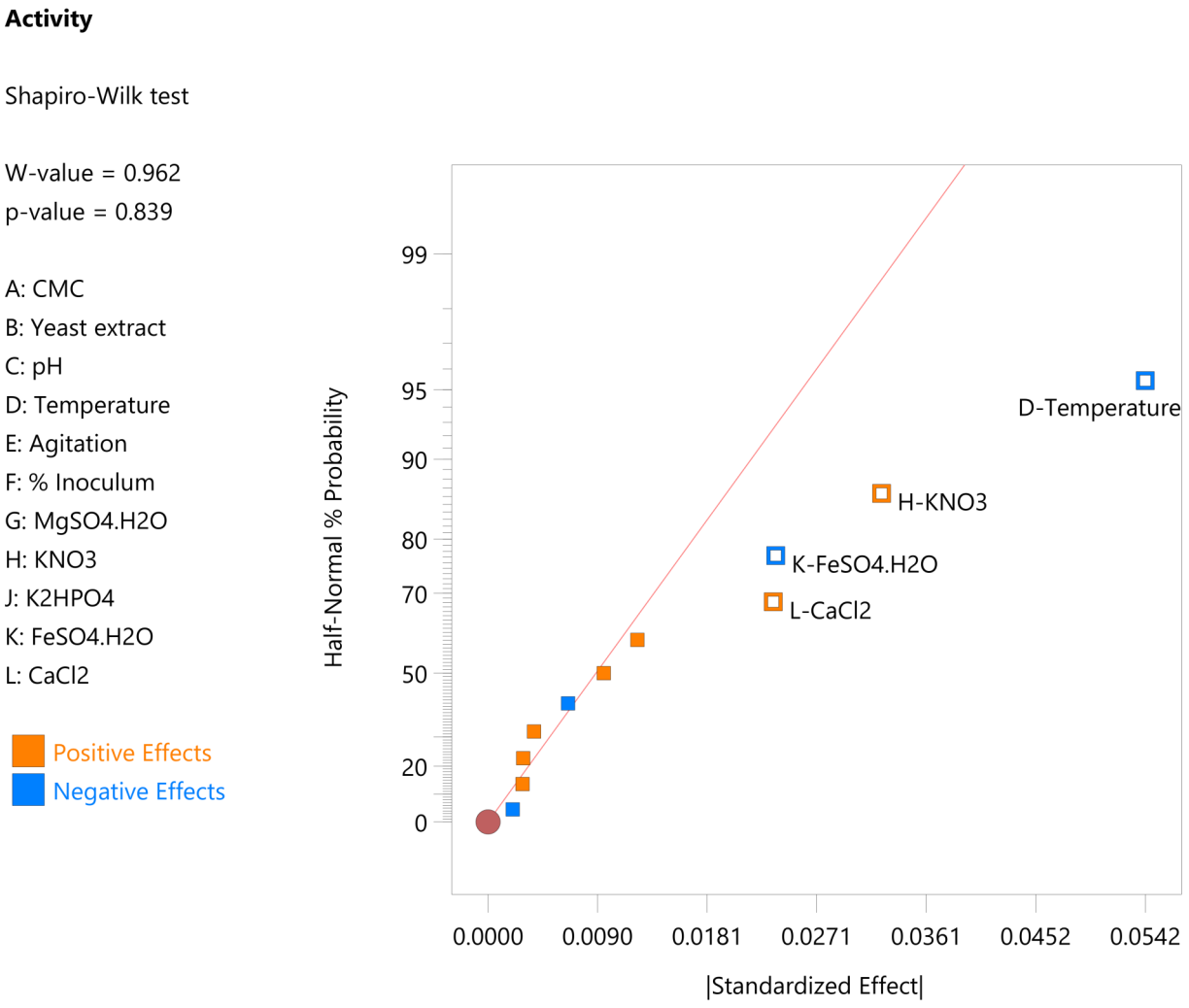

Figure 2. Half-Normal Plot of significant variables for cellulase production.

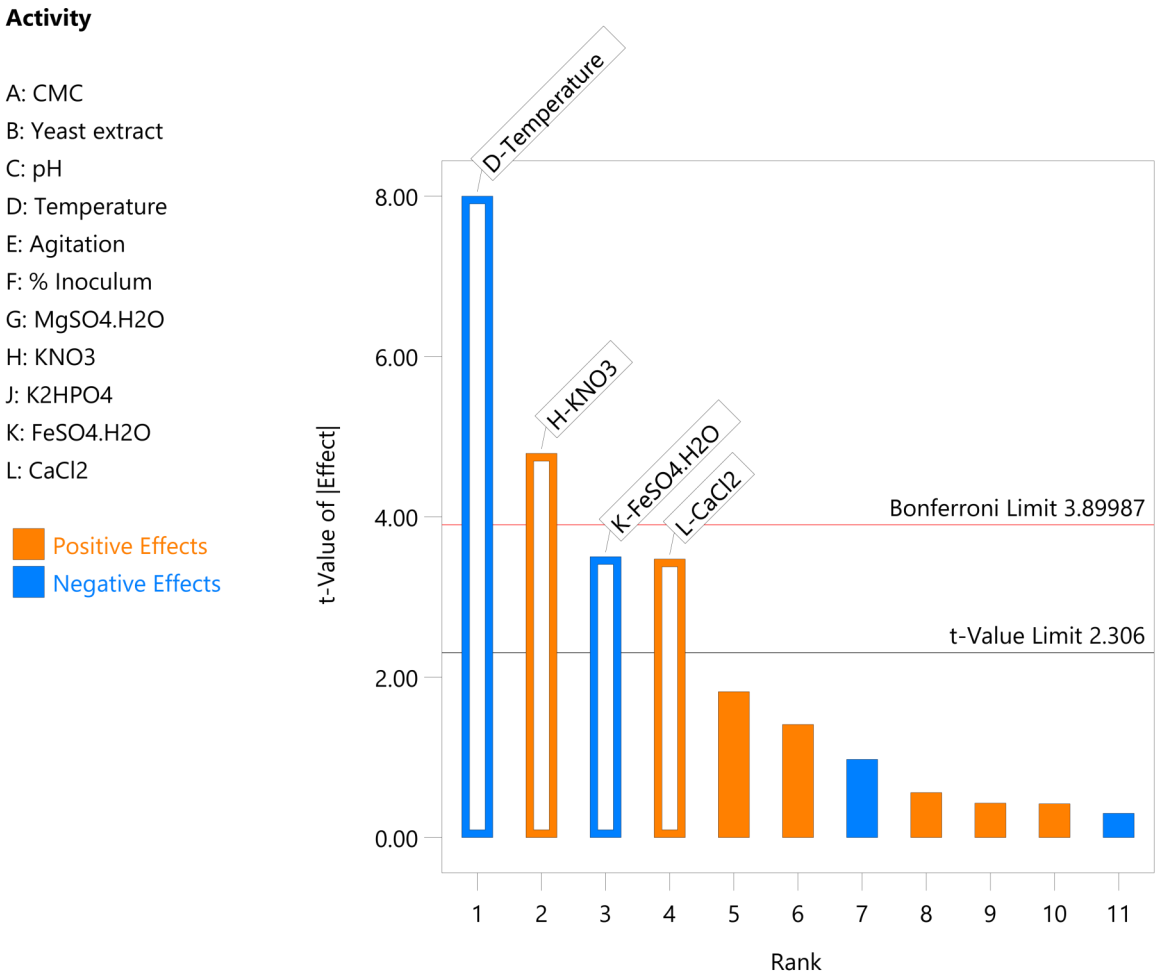

Figure 3. Pareto chart of significant variables for cellulase production.

linear polynomial equation (1) was developed based on the experimental results and the independent variables in terms of actual values.

A quadratic non-linear polynomial equation (1) was developed based on the experimental results and the independent variables in terms of actual values.

$$\text{Activity} = 2.373 - 0.1536\ \text{Temperature} + 0.935\ KNO_3 - 0.05947\ FeSO_4 + 0.001499\ CaCl_2 + 0.001950\ \text{Temperature*Temperature} - 0.3336\ KNO_3*KNO_3 - 0.000162\ FeSO_4*FeSO_4 + 0.000001\ CaCl_2*CaCl_2 + 0.00576\ \text{Temperature}*KNO_3 + 0.002151\ \text{Temperature}*FeSO_4 + 0.000003\ \text{Temperature}*CaCl_2 - 0.00719\ KNO_3*FeSO_4 - 0.001104\ KNO_3*CaCl_2 - 0.000023\ FeSO_4*CaCl_2$$

Equation averaged over blocks. The model's adequacy and fitness were evaluated using ANOVA and Fisher's F-test, as shown in Table 4. The high F-value (69.82) and low p-value ($p < 0.05$) confirmed the model's significance in explaining the response variation. Temperature was the most influential factor ($F = 475.21$), with significant linear and square effects observed for KNO_3 and temperature ($p < 0.05$). Interaction effects, such as KNO_3*$CaCl_2$ ($F = 30.65$), were also significant, while others, like temperature*$CaCl_2$ ($F = 0.02$), were not.

The lack-of-fit test ($p = 0.977$) indicated a good model fit, with an R^2 of 98.85 %, adjusted R^2 of 97.43 %, and predicted R^2 of 95.39 %. The low error variance ($S = 0.0149559$) further demonstrated the model's reliability and precision. These results validated the model as robust and were suitable for

Table 3. Box–Behnken design for cellulase production.

STD	Block	Run	X4	X8	X10	X12	Activity (U mL^{-1})	
							Observed	Predicted
4	1	1	40	2	12.5	0.125	0.3821	0.3815
8	1	2	35	1.5	20	0.2	0.2937	0.3065
1	1	3	30	1	12.5	0.125	0.2872	0.2855
6	1	4	35	1.5	20	0.05	0.3617	0.3607
5	1	5	35	1.5	5	0.05	0.3785	0.3634
3	1	6	30	2	12.5	0.125	0.16	0.1645
7	1	7	35	1.5	5	0.2	0.3633	0.3620
2	1	8	40	1	12.5	0.125	0.4517	0.4449
10	1	9	35	1.5	12.5	0.125	0.3741	0.3538
9	1	10	35	1.5	12.5	0.125	0.3244	0.3538
14	2	11	40	1.5	12.5	0.2	0.4712	0.4687
13	2	12	30	1.5	12.5	0.2	0.2788	0.2781
11	2	13	30	1.5	12.5	0.05	0.3026	0.3083
20	2	14	35	1.5	12.5	0.125	0.3446	0.3351
12	2	15	40	1.5	12.5	0.05	0.4903	0.4941
18	2	16	35	2	20	0.125	0.1669	0.1549
19	2	17	35	1.5	12.5	0.125	0.3381	0.3351
16	2	18	35	2	5	0.125	0.2341	0.2379
17	2	19	35	1	20	0.125	0.3017	0.3010
15	2	20	35	1	5	0.125	0.2611	0.2762
25	3	21	35	1	12.5	0.05	0.2831	0.2839
27	3	22	35	1	12.5	0.2	0.3456	0.3389
30	3	23	35	1.5	12.5	0.125	0.359	0.3452
28	3	24	35	2	12.5	0.2	0.1655	0.1639
29	3	25	35	1.5	12.5	0.125	0.3278	0.3452
23	3	26	30	1.5	20	0.125	0.1985	0.1955
22	3	27	40	1.5	5	0.125	0.4106	0.4128
24	3	28	40	1.5	20	0.125	0.5412	0.5450
21	3	29	30	1.5	5	0.125	0.3906	0.3859
26	3	30	35	2	12.5	0.05	0.2686	0.2745

exploring the experimental design space.

To better comprehend the interactions between variables and their impact on cellulase activity, both three-dimensional response surface graphs (Figure 4) and contour plots were generated. In the three-dimensional graphs, cellulase activity is represented on the z-axis against two selected parameters, while the remaining variables are fixed at their central values. Meanwhile, the six contour plots illustrate how temperature, KNO_3, $FeSO_4$, and $CaCl_2$ affected cellulase activity, with each plot showing the relationship between two variables and the corresponding enzyme activity. The color gradients ranged from dark blue (low activity) to dark green (high activity), indicating that the optimal combination of variables lies in the green zone. The first row of plots compares each variable with temperature, whereas the second row displays interactions among the variables without temperature. In these plots, the hold values (temperature 35 °C, KNO_3 1.5 g L^{-1}, $FeSO_4$ 12.5 mg L^{-1}, and $CaCl_2$ 125 mg L^{-1}) were used to keep the other variables constant while varying one or two parameters. The results clearly illustrated significant interactions among the independent variables. Based on data modelling, the optimal cellulase activity of 0.574 U mL^{-1} was predicted under the following conditions: a temperature of 40 °C, KNO_3 concentration of 1.45 g L^{-1}, $FeSO_4$ concentration of 20 mg L^{-1}, and $CaCl_2$ concentration of 50 mg L^{-1}.

Discussion

The cellulase-producing bacterial isolate PUA-18, obtained from the mangrove waters of the Mandeh Region in West Sumatra, was identified as *Bacil-*

Table 4. Analysis of variables for cellulase activity.

Analysis of Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	16	0.249863	0.015616	69.82	0.000
Blocks	2	0.001758	0.000879	3.93	0.046
Linear	4	0.136651	0.034163	152.73	0.000
Temperature	1	0.106295	0.106295	475.21	0.000
KNO_3	1	0.025503	0.025503	114.01	0.000
$FeSO_4$	1	0.002538	0.002538	11.34	0.005
$CaCl_2$	1	0.002316	0.002316	10.35	0.007
Square	4	0.074127	0.018532	82.85	0.000
Temperature*Temperature	1	0.016291	0.016291	72.83	0.000
KNO_3*KNO_3	1	0.047705	0.047705	213.27	0.000
$FeSO_4$*$FeSO_4$	1	0.000570	0.000570	2.55	0.134
$CaCl_2$*$CaCl_2$	1	0.000084	0.000084	0.38	0.550
2-Way Interaction	6	0.037327	0.006221	27.81	0.000
Temperature*KNO_3	1	0.000829	0.000829	3.71	0.076
Temperature*$FeSO_4$	1	0.026034	0.026034	116.39	0.000
Temperature*$CaCl_2$	1	0.000006	0.000006	0.02	0.878
KNO_3*$FeSO_4$	1	0.002905	0.002905	12.99	0.003
KNO_3*$CaCl_2$	1	0.006856	0.006856	30.65	0.000
$FeSO_4$*$CaCl_2$	1	0.000697	0.000697	3.12	0.101
Error	13	0.002908	0.000224		
Lack-of-Fit	10	0.001165	0.000116	0.20	0.977
Pure Error	3	0.001743	0.000581		
Total	29	0.252771			

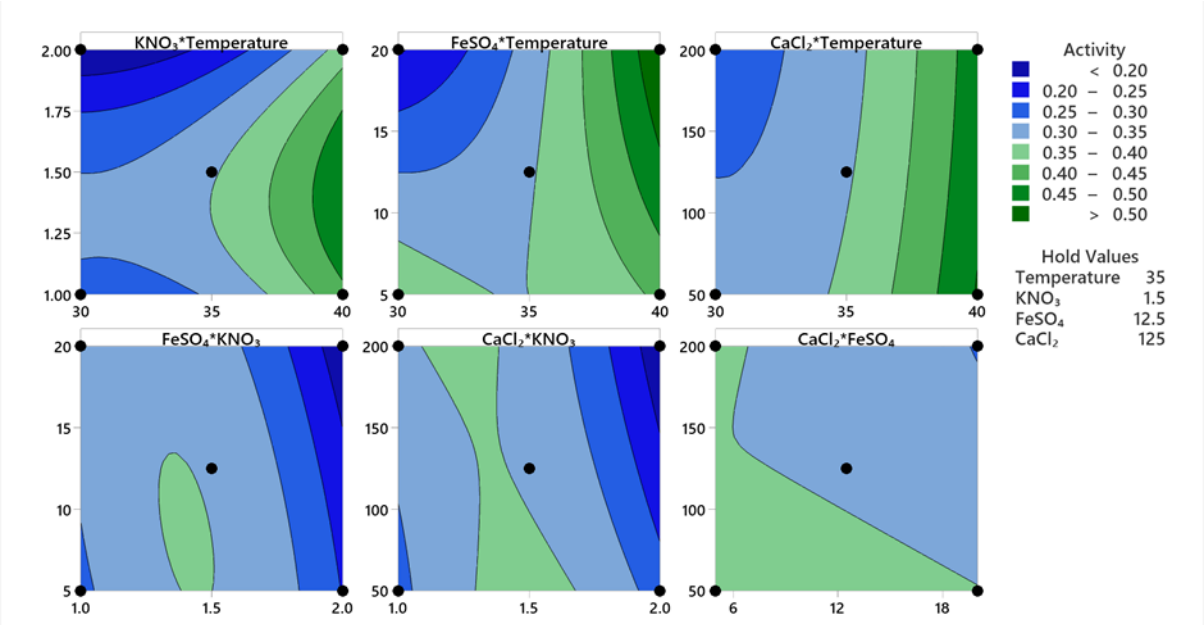

Figure 4. Three dimensional response surface plots showing effects of variables and its interaction on cellulase activity.

lus subtilis through 16S rRNA sequencing, showing 100 % similarity to strains DSM 10, JCM 1465, and NBRC 13719. This finding aligns with previous studies highlighting the prevalence of *Bacillus subtilis* in mangrove ecosystems. Dewiyanti et al. (2024) identified *Bacillus* as the most common genus among cellulolytic bacteria in the mangrove soils of Aceh Province, Indonesia, with two isolates confirmed as *Bacillus subtilis*. Similarly, Naresh et al. (2019) reported that *Bacillus subtilis* as one of the thermophilic cellulolytic bacteria is present in the mangrove soils of northern Malaysia. Collectively, these studies emphasised the ecological significance and cellulolytic potential

of *Bacillus subtilis* in mangrove environments in Southeast Asia.

The identification of PUA-18 as cellulolytic *Bacillus subtilis* underscores its enzyme production capabilities, as evidenced by high cellulolytic activity and the production of cellulase. These enzymes play a pivotal role in breaking down cellulose materials into simpler, usable forms, which has been demonstrated in several studies. For example, *Bacillus subtilis* strains have achieved over 114 % hydrolysis efficiency on carboxymethyl cellulose agar (Nwagala et al. 2024), while specific strains like RLI2019 have shown remarkable enzymatic versatility (Liu et al. 2023). These findings highlighted the broad applicability of cellulase production by *Bacillus subtilis*, from bioconversion processes to industrial enzyme applications and enzyme-based innovations.

This study explored eleven factors influencing cellulase activity, revealing both positive and negative contributors. Nitrogen source concentration, pH, agitation, inoculum concentration, KNO_3, K_2HPO_4, and $CaCl_2$ were identified as positively influencing cellulase production, while carbon source concentration, temperature, $MgSO_4$, and $FeSO_4$ negatively impacted it. Among these, temperature emerged as the most significant factor, followed by KNO_3, $FeSO_4$, and $CaCl_2$. However, discrepancies with previous research suggested variability in cellulase production, influenced by experimental setups, microbial strains, or environmental conditions. For instance, while this study found $MgSO_4$ to negatively affect cellulase activity, others Ghazanfar et al. (2022) and Neesa et al. (2020) emphasized its importance. Similarly, variables like CMC, inoculum size, and pH, reported as critical in other studies (Shajahan et al. 2017), were less significant here. These differences highlight the need for tailored optimisation approaches to account for specific conditions and strains.

Building on the foundational work of Alamsjah et al. (2024b), which utilized the One Factor at a Time (OFAT) approach to optimize PUA-18 cellulase production, achieving a 145 % increase in activity, this study adopts a more advanced optimization strategy. While the OFAT method demonstrated its utility in improving enzyme production, as seen in another study by Alamsjah et al. (2024a) where protease activity from mangrove-derived bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 improved by an average of 21.86 %, its inherent limitations in addressing interactions between variables are evident.

In comparison, Shah and Mishra (2020) combined OFAT with Box-Behnken Design (BBD) and achieved a 55.6 % improvement in cellulase activity, while (Fouda et al. 2024) reported a significant 296.9 % increase using a similar integration of OFAT and BBD under different conditions. The current study surpassed these prior efforts by employing a more comprehensive Design of Experiment (DoE) approach. By integrating Plackett-Burman Design (PBD) for factor screening and BBD for responsible optimisation, cellulase activity in PUA-18 was enhanced by an impressive 293 %. These findings underscore the effectiveness of advanced statistical methodologies in enzyme production optimization and highlight the transformative potential of tailored experimental designs in achieving substantial improvements.

The advantages of Design of Experiments (DoE) methodologies, such as PBD and BBD, extend beyond optimizing enzyme yield. These approaches enable the systematic evaluation of multiple factors and their interactions, reducing resource consumption, enhancing precision, and accelerating the optimisation's process. DoE has been shown to maximise production yield while minimizing uncertainties in parameter estimation (Yu et al. 2015, 2019). Its application in diverse enzyme systems, including cellulase and trypsin assays (Jänsch et al. 2019; Onyeogaziri & Papaneophytou 2019), further underscores its practicality. Fractional factorial designs within DoE have proven particularly effective, identifying key factors in days compared to

weeks required by traditional OFAT methods.

While the complexity of implementing advanced statistical designs may discourage widespread adoption, the demonstrated benefits in this study underscore their value. The integration of PBD and BBD not only achieved significant improvements in cellulase activity but also illustrated the broader potential of DoE methodologies for advancing enzyme production systems. Future research should prioritise adopting these robust techniques to enhance efficiency and to expand the applicability of cellulase production across industrial, agricultural, and environmental domains.

CONCLUSIONS

In conclusion, this study establishes *Bacillus subtilis* PUA-18, isolated from Mandeh Region in West Sumatra, as a potent cellulase producer, identified through 16S rRNA sequencing with 100 % similarity to established strains. Advanced optimisation using Plackett-Burman Design (PBD) and Box-Behnken Design (BBD) achieved a 293 % improvement in cellulase activity, surpassing traditional methods like OFAT. Key factors such as temperature, nitrogen sources, and agitation significantly influenced production, underscoring the importance of tailored optimisation strategies. These findings highlighted the ecological and industrial significance of *Bacillus subtilis* in enzyme production and demonstrate the value of Design of Experiments (DoE) methodologies in enhancing efficiency and scalability for biotechnological applications.

AUTHOR CONTRIBUTION

F.A. conceived the original idea for the study, contributed to the design and planning of experiments, provided guidance and supervision throughout the research, reviewed and provided critical feedback on the manuscript, and drafted the initial manuscript. D.R.A. designed the experimental approach and methodology, conducted experiments, and collected data. A.A. reviewed and revised the manuscript for clarity and coherence and provided necessary resources and equipment for the study.

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CONFLICT OF INTEREST

This research was conducted without any conflicts of interest, and all authors declare no competing interests related to this study.

REFERENCES

Alamsjah, F., Ananta, Y. & Agustien, A., 2024a. Optimization of Temperature and pH for Protease Production from Some Bacterial Isolates of Mangrove Waters. *OnLine Journal of Biological Sciences*, 24(4), pp.911–918. doi: 10.3844/ojbsci.2024.911.918.

Alamsjah, F., Aswan, D.R. & Agustien, A., 2024b. pH and Temperature Optimization of Several Bacterial Isolates from Mangrove Waters in the Mandeh Area to Produce Cellulase Enzyme. *OnLine Journal of Biological Sciences*, 24(3), pp.367–373. doi: 10.3844/ojbsci.2024.367.373.

Annapure, U.S. & Pratisha, N., 2022. Psychrozymes: A novel and promising resource for industrial applications. In *Microbial Extremozymes: Novel Sources and Industrial Applications*. Academic Press, pp.185–195. doi: 10.1016/B978-0-12-822945-3.00018-X.

Basak, P. et al., 2021. Cellulases in paper and pulp, brewing and food industries: Principles associated with its diverse applications. In *Current Status and Future Scope of Microbial Cellulases*. Elsevier, pp.275–293. doi: 10.1016/B978-0-12-821882-2.00003-X.

Dewiyanti, I. et al., 2024. Analyzing cellulolytic bacteria diversity in mangrove ecosystem soil using 16 svedberg ribosomal ribonucleic acid gene. *Global Journal of Environmental Science and Management*, 10(1), pp.51–68. doi: 10.22034/gjesm.2024.01.05.

Djekrif, S.D. et al., 2024. Production Optimization, Partial Characterization, and Gluten-Digesting Ability of the Acidic Protease from Clavispora lusitaniae PC3. *Fermentation*, 10(3), 139. doi: 10.3390/fermentation10030139.

Fouda, A. et al., 2024. Synthesis, Optimization, and Characterization of Cellulase Enzyme Obtained from Thermotolerant Bacillus subtilis F3: An Insight into Cotton Fabric Polishing Activity. *Journal of Microbiology and Biotechnology*, 34(1), pp.207–223. doi: 10.4014/jmb.2309.09023.

Ghazanfar, M. et al., 2022. Cellulase Production Optimization by Bacillus Aerius Through Response Surface Methodology in Submerged Fermentation. *Cellulose Chemistry and Technology*, 56(3–4), pp.321–330.

Ghose, T.K., 1987. Measurement of cellulase activities. *Pure and Applied Chemistry*, 59(2), pp.257–268. doi: 10.1351/pac198759020257.

Jänsch, N. et al., 2019. Using design of experiment to optimize enzyme activity assays. *ChemTexts*, 5, 20. doi: 10.1007/s40828-019-0095-2.

Juturu, V. & Wu, J.C., 2014. Microbial cellulases: Engineering, production and applications. *Renewable and Sustainable Energy Reviews*, 33, pp.188–203. doi: 10.1016/j.rser.2014.01.077.

Liu, G. et al., 2023. Whole genome sequencing and the lignocellulose degradation potential of Bacillus subtilis RLI2019 isolated from the intestine of termites. *Biotechnology for Biofuels and Bioproducts*, 16(1), 130. doi: 10.1186/s13068-023-02375-3.

McKee, L.S., 2017. Measuring Enzyme Kinetics of Glycoside Hydrolases Using the 3,5-Dinitrosalicylic Acid Assay. *Methods in Molecular Biology (Clifton, N.J.)*, 1588, pp.27–36. doi: 10.1007/978-1-4939-6899-2_3.

Naresh, S. et al., 2019. Isolation and Partial Characterisation of Thermophilic Cellulolytic Bacteria from North Malaysian Tropical Mangrove Soil. *Tropical Life Sciences Research*, 30(1), pp.123–147. doi: 10.21315/tlsr2019.30.1.8.

Neesa, L. et al., 2020. Optimization of Culture Conditions and Reaction Parameters of β-glucosidase From a New Isolate of Bacillus subtilis (B1). *Journal of Applied Biotechnology Reports*, 7(3), pp.152–158. doi: 10.30491/jabr.2020.109990.

Nwagala, P. et al., 2024. Evaluation of the probiotic ability of Cellulolytic Bacillus subtilis SBMP4 (FSP20) isolated from waste-dump site in FIIRO, Lagos Nigeria. *ASFI Research Journal*, 1(1), e13512. doi: 10.70040/asfirj-e5vc-e54k.

Onyeogaziri, F.C. & Papaneophytou, C., 2019. A General Guide for the Optimization of Enzyme Assay Conditions Using the Design of Experiments Approach. *SLAS Discovery*, 24(5), pp.587–596. doi: 10.1177/2472555219830084.

Shah, F. & Mishra, S., 2020. In vitro optimization for enhanced cellulose degrading enzyme from Bacillus licheniformis KY962963 associated with a microalgae Chlorococcum sp. using OVAT and statistical modeling. *SN Applied Sciences*, 2, 1923. doi: 10.1007/s42452-020-03697-9.

Shajahan, S. et al., 2017. Statistical modeling and optimization of cellulase production by Bacillus licheniformis NCIM 5556 isolated from the hot spring, Maharashtra, India. *Journal of King Saud University - Science*, 29 (3), pp.302–310. doi: 10.1016/j.jksus.2016.08.001.

Singh, V. et al., 2017. Strategies for Fermentation Medium Optimization: An In-Depth Review. *Frontiers in Microbiology*, 7, 2087. doi: 10.3389/fmicb.2016.02087.

Yu, H. et al., 2019. Integration of Optimal Experimental Design and Process Optimisation to An Enzyme Reaction System. *2019 Chinese Automation Congress (CAC)*, pp.3615–3620. doi: 10.1109/CAC48633.2019.8997445.

Yu, H., Yue, H. & Halling, P., 2015. Optimal Experimental Design for an Enzymatic Biodiesel Production System. *IFAC-PapersOnLine*, 48(8), pp.1258–1263. doi: 10.1016/j.ifacol.2015.09.141.