International Journal of Microbial Science, ISSN (online) 2582-967X, Volume 5, Issue 1, December 2024, pp 30-38
Available online at https://internationaljournalofmicrobialscience.com/index.php/archives/

Original Article

Optimization of Lipase Production with Response Surface Method using *Bacillus subtilis*

[1]Suyash Dusane, [2]Sudhakar Gutte

[1,2]Department of Microbiology, Mrs. Kesharbai Sonajirao Kshirsagar Alias Kaku Arts, Science and Commerce College, Beed, Maharashtra, India.

Article Info

Article history:
Received: December 1, 2024
Accepted: December 15, 2024
Published: December 22, 2024

Corresponding Author: Sudhakar Gutte
Email: dr.guttesl@gmail.com

Keywords: Lipase, Soil Bacteria, Response surface methodology

Abstract

The production and application of enzymes can be costly, largely due to the need for inducible production systems. This study aimed to optimize media components for cost-effective extracellular lipase production from soil microorganisms. By using Response Surface Methodology (RSM), the research focused on maximizing lipase yield and efficiency through systematic analysis of optimal parameters. Molasses (g/L), peptone (g/L), and palm oil (% v/v) were selected as key components in the culture medium. Central Composite Design (CCD) was used to optimize their concentrations and evaluate their effects on lipase yield. The experimental design included a three-factor, five-level setup with 20 runs. Statistical analysis involved the correlation coefficient (R), multiple correlation coefficient (R^2), and Fischer's F-statistic with its p-value. The highest lipase production, 145.86 U/mL, was achieved with 18 g/L molasses, 27 g/L peptone, and 0.1% v/v palm oil, with a predicted value of 160.24 U/mL. The model was statistically significant, with a p-value of 0.0143 and an F-value of 4.207578. The lack of fit was not significant (p-value of 0.3901), indicating a good model fit. This optimization strategy significantly improves microbial lipase production efficiency while offering a scalable industrial application model.

1. Introduction:

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are widespread enzymes present in animals, plants, fungi, and bacteria. They play a crucial physiological role by catalyzing the hydrolysis of triacyl glycerides into glycerides, monoglycerides, glycerol, and fatty acids at the lipid-water interface [1].

Lipases are highly versatile biocatalysts capable of facilitating a variety of bioconversion reactions, including hydrolysis, interesterification, esterification, alcoholysis, acidolysis, and aminolysis. Due to the catalytic abilities of hydrolyzing triglycerides, esterification, trans-esterification, and enantio-separation, triacylglycerol lipase (EC 3.1.1.3) finds widespread applications across industries such as detergents, pharmaceuticals, food (cheese and tea), pulp and paper, textiles, tanneries, cosmetics, biodiesel production, wastewater treatment, medicine, food, chemistry, textiles, and related industries [2-4]. Microbial lipases are particularly notable for their

extensive industrial applications, ease of cultivation, and scalability in production [5, 6]. Microbial lipases are primarily produced through submerged fermentation (SmF)a well-established method with fully developed engineering aspects.

However, solid-state fermentation (SSF) has demonstrated certain advantages over SmF for enzyme production, even on a commercial scale [4]. The traditional approach of optimizing media for the fermentation process by varying one variable at a time was often time-consuming, costly, and likely to misinterpretation. However, the high production costs of biocatalysts frequently limit their widespread application[7].

To address this challenge,explore various microorganisms, supplements and substrates to identify the most effective combinations for producing high-value lipases. This approach focuses on using substrates and operational conditions that can lower production costs on an industrial scale [8]. To enhance microbial lipase production, various culture parameters are typically examined, including carbon sources, nitrogen sources, initial pH, temperature, and aeration conditions [9].

The high cost of carbon sources contributes to the expense of lipase production. To mitigate this, low-cost substrates such as molasses[10], glycerol[11], olive pomace and wheat bran[12] have been explored as viable alternatives. Edible triacyl glycerides, including olive oil, palm oil, sunflower oil, and soybean oil, have also been used as carbon sources and inducers in lipase production[13].

Optimization of media components for every possible combination of required carbon, nitrogen sources and inducers was impractical due to the large number of required trials. In contrast, statistical experimental methods provide a more efficient and cost-effective way to simultaneously and systematically vary all components [14].

Response Surface Methodology (RSM) is an experimental approach used to determine the optimal conditions in a multivariable system. It is particularly effective for optimizing significant variables identified through factorial designs, such as the central composite design (CCD). By integrating factorial design and regression analysis, RSM evaluates the impact of various factors and their interactions. Advanced applications of RSM involve mathematical models to analyze experimental data and predict relationships between responses and variables, enabling the creation of predictive contours that facilitate faster and more efficient optimization with fewer experiments [15].

The main purpose of this study was to optimize the media components for cost-effective extracellular lipase production soil microorganisms. Utilizing the statistical optimization tool Response Surface Methodology (RSM), the study was conducted to enhance the efficiency and yield of lipase production. By systematically analyzing and refining the conditions, this approach aims to identify the optimal parameters that will maximize enzyme output and improve overall production processes.

2. Materials and Method:

2.1 Sample collection:

Soil sample was collected from agricultural fields from the Aurangabad district, at a depth of 5-6 cm. Using a sterile spatula, samples were placed in sterile 50 ml tubes to avoid contamination.

2.2. Isolation and screening:

For serial dilution, 1 g of soil sample was mixed with 10 ml of sterile distilled water in a 50 ml Erlenmeyer flask, then agitated at 120 rpm for 30 minutes at 37°C using a rotary shaker. The aqueous slurry was serially diluted up to 10^{-6} using 0.8% saline. Then, 100 µl from each dilution was spread on tributyrin agar plates containing 0.5% peptone, 0.3% yeast extract, 1% tributyrin, and 2% agar. The plates were incubated at 37°C for 24-72 hours, and lipolytic activity was assessed by observing zones of hydrolysis around bacterial colonies. The isolates showing maximum zone of clearance were selected for further analysis [16].

2.3.Morphological, Biochemical and Molecular identification of the isolates

The morphological and biochemical characterization of lipase-producing bacterial isolates was conducted following guidelines from Bergey's Manual of Systematic Bacteriology[17]. The confirmation of the

isolates was done by molecular identification using 16s rDNA sequencing.

2.4. Response surface methodology

To determine the optimal media component for the lipase production from the isolated bacteria molasses (g/L), peptone (g/L), and palm oil (%v/v) were selected as components of the culture medium. Central Composite Design (CCD) was then utilized to optimize the concentrations of these components and to evaluate their individual effects and interactions on lipase yield[18]. The experimental setup included a three-factor, five-level design (−α, −1, 0, +1, +α) with 20 experimental runs, detailed in Table 1. To develop the quadratic model and analyze the multinomial coefficients experiments were conducted using Design Expert v11.1.2.0 software, with lipase production measured in U/mL as the response variable (R1) [19].

2.5. Statistical analysis

Statistical analysis included the evaluation of the correlation coefficient (R), multiple correlation coefficient (R^2) representing the fit of the quadratic model, and Fischer's F-statistic along with its probability p(F). Response surface curves were generated based on the quadratic model[20]. The analysis was done using Statease Design Expert version 7.

3. Result

3.1. Isolation and screening

Colonies exhibiting maximumzone on clearance were selected for quantitative measurement of lipase activity, as colonies with a larger zone of clearance typically indicate higher enzyme activity. Among the isolates, one strain BS-L1 demonstrated the highest zone of clearance and was thus used for further identification and enzyme production and studies.

3.2. Identification of the isolate

The isolated strain was show gram-positive in nature**.** The selected lipolytic isolate was identified using sequencing techniques, PCR amplification of the 16S rDNA gene. The resulting sequence was then compared with those in GenBank using the BLAST program. As per the results of the isolated bacteria was identified as the *Bacillus subtills*.

3.3.Optimization of the media component by Response Surface Methodology

The experimental design focused on three variables: molasses, peptone, and palm oil, each tested at specific levels for lipase production using the CCD model. Molasses was assessed within a range of 2 to 18 g/L, with the extreme values of −α and +α set at -3.45434 and 23.45434 g/L, respectively. Peptone concentrations varied from 3 to 27 g/L, with −α and +α levels extending from -5.18151 to 35.18151 g/L. Palm oil was evaluated at concentrations ranging from 0.1% to 0.7% v/v, with −α and +α levels adjusted to -0.10454% and 0.904538%, respectively.

Table 2 and Figure 1, Figure 2 and Figure 3 provides a comprehensive overview of the experimental design and predicted responses for lipase production using Central Composite Design (CCD). The analysis highlights the highest and lowest actual and predicted values from the experiments. The highest actual lipase production occurred in Run 15, where 18 g/L molasses, 27 g/L peptone, and 0.1% v/v palm oil resulted in 145.86 U/mL, with a predicted value of 160.24 U/mL.

A similarly high production was observed in Run 19, which had the same conditions as Run 15, yielding 145.52 U/mL with a predicted value of 120.43 U/mL. Run 16, with 23.45 g/L molasses, 15 g/L peptone, and 0.4% v/v palm oil, achieved 141.89 U/mL compared to a predicted 127.27 U/mL. In Run 17, using 2 g/L molasses, 3 g/L peptone, and 0.7% v/v palm oil, the actual production was 140.80 U/mL, while the predicted value was 133.98 U/mL. Run 18, featuring 10 g/L molasses, 35.18 g/L peptone, and 0.4% v/v palm oil, resulted in 140.53 U/mL of lipase, with a predicted production of 132.61 U/mL.

On the lower end, run 13 recorded the lowest actual lipase production with 2 g/L molasses, 27 g/L peptone, and 0.7% v/v palm oil, yielding 49.62 U/mL, while the predicted response was 74.35 U/mL. Another lower production run was Run 3, which utilized 10 g/L molasses, -5.18 g/L peptone, and 0.4% v/v palm oil, resulting in an actual production of 98.48 U/mL and a predicted value of 108.25 U/mL.

Table 3 evaluated the impact of molasses, peptone, and palm oil, along with their interactions, on lipase production. The overall model was statistically significant, with a p-value of 0.0143 and an F-value of

4.207578, indicating that the combined effects of these variables and their interactions significantly influenced lipase production. Individually, molasses (A), peptone (B), and palm oil (C) didn't show significant effects on lipase production, as indicated by their respective p-values of 0.3492, 0.1597, and 0.6671.

However, the interaction between molasses and peptone (AB) was highly significant, with a p-value of 0.0073 and an F-value of 10.08944, demonstrating a strong synergistic effect on lipase production. Similarly, the interaction between peptone and palm oil (BC) was also significant, with a p-value of 0.0108 and an F-value of 8.830535, suggesting that these two factors together had a considerable impact on enzyme production. The interaction between molasses and palm oil (AC), although showing some effect, was not statistically significant, with a p-value of 0.1088.

The model's residuals, with a sum of squares of 3778.784 and a mean square of 290.6757, represented the unexplained variation, while the lack of fit test indicated that the model fit the data well, as the lack of fit was not significant (p-value of 0.3901). The total variation in the data was represented by the total sum of squares, which was 11117.03.

The Model F-value of 4.21 implies the model is significant. There is only a 1.43% chance that a "Model F-value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, AB and BC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Following is the equation obtained after optimization of lipase production using Molasses, Peptone and Palm oil.

lipase Production = +125.4732157 - 4.133280508* Molasses+0.598093203*Peptone +24.34416869*Palm oil +0.199444324* Molasses* Peptone+4.324480234 * Molasses * Palm oil -4.975656267 * Peptone* Palm oil

4. Discussion:

This research successfully identified the most effective combinations of carbon sources, nitrogen sources, and inducers for enhancing lipase production by BS-L2. The central composite design (CCD) model, analyzed using ANOVA, revealed significant interactions, particularly between molasses and peptone (p = 0.0073) and between peptone and palm oil (p = 0.0108). These findings underscore the synergistic effects of these components in optimizing lipase production. Molasses, an economical and readily available carbon source, supports microbial growth due to its high sugar content and essential trace elements[21]. Peptone, rich in proteins, amino acids, and vitamins, provides a favorable environment for microbial cell growth and enzyme production [22]. The interaction between molasses and peptone not only enhances enzyme yield but also reduces production costs, making it a practical choice for large-scale applications. Similar findings were reported by Zhu, Liu [18], where the interaction of these two substrates significantly improved lipase activity (p = 0.0289).

The role of peptone combined with low concentrations of coconut oil in maximizing lipase production was highlighted by Patel, Ray [23], where the optimized medium achieved significant enzyme yield. Coconut oil, unlike palm oil, does not act as a strong inducer [24]. However, the cost limitations of ammonium oxalate as a nitrogen source in their study underscore the importance of finding cost-effective alternatives, such as the molasses-peptone combination explored in the present research.

Tanyol, Uslu [22] demonstrated optimal lipase production using peptone, ammonium sulfate, and sunflower oil cake. However, the rapid consumption of inorganic nitrogen sources like ammonium sulfate may lead to enzyme repression [25], a limitation that highlights the advantage of organic sources such as peptone in sustaining enzyme synthesis. Although promising, their reported enzyme yield of 10.8 U/mL was notably lower than the current study's maximum production of 160 U/mL.

Vasiee, Behbahani [26] reported a superior lipase activity of 343 U/mL using a medium containing coriander seed extract, yeast extract, olive oil, and MgCl2. Olive oil, as a less saturated lipid substrate compared to palm oil, likely served as a better inducer and substrate for lipase synthesis [27]. While the present study achieved lower enzyme activity, it

prioritized cost-effectiveness by utilizing low-cost substrates like molasses and palm oil. The choice of substrate underscores the trade-off between maximizing yield and maintaining economic viability. Future studies could explore alternative lipid sources and their potential to further enhance production.

Additionally, magnesium ions (MgCl2) were identified as essential cofactors in Vasiee et al.'s study, stabilizing enzyme structure and enhancing catalytic efficiency [28]. Incorporating such cofactors in subsequent optimizations could significantly improve the outcomes of this research.

5. Conclusion:

The optimization of the media components for extracellular lipase production by *Bacillus subtilis* isolated from soil using Response Surface Methodology (RSM)was successfully performed. The Central Composite Design (CCD) model demonstrated that molasses, peptone, and palm oil significantly influence lipase production, with a notable synergistic effect observed between molasses and peptone. The statistical analysis ANOVA confirmed that these interactions play a critical role in enhancing enzyme yield. The study findings suggest that utilizing molasses and peptone, both cost-effective and readily available substrates, can reduce production costs while maximizing lipase yield.

This optimization approach not only enhances the efficiency of microbial lipase production but also provides a scalable model for industrial applications. The significant interactions identified between the media components highlight the potential for further optimization and refinement in commercial enzyme production, offering a valuable framework for improving the economic viability of microbial lipase production processes.

Acknowledgement:

Authors are thankful to Dr. Kshirsager S.V., Principal, Mrs. Kesharbai Sonajirao Kshirsagar Alias Kaku Arts, Science and Commerce College, Beed, Maharashtra, India for providing the lab facilities for the research work.

Author contribution: SG: Developed an idea and verified the content, SD: Conducted experimental work and wrote the manuscript

Competing interest: Authors declare that no competing interest exists.

Ethical statement: Since the work does not violate ethical laws, no ethical permission required.

Grant Support Details: The authors did not receive funding for this work from any agency.

References:

1.Gupta R, Gupta N, Rathi P. Bacterial lipases: an overview of production, purification and biochemical properties. Applied microbiology and biotechnology. 2004;64:763-81.

2.Hasan F, Shah AA, Javed S, Hameed A. Enzymes used in detergents: lipases. African journal of biotechnology. 2010;9(31):4836-44.

3.Ribeiro BD, Castro AMd, Coelho MAZ, Freire DMG. Production and use of lipases in bioenergy: a review from the feedstocks to biodiesel production. Enzyme research. 2011;2011(1):615803.

4.Jamilu H, Ibrahim AH, Abdullahi SZ. Isolation, optimization and characterization of lipase producing bacteria from abbatoir soil. Int J Adv Sci Technol. 2022;3:2708-7972.

5.Sood A, Kaur M, Gupta R. Lipases and their Applications in Biomedical Field. Current Biotechnology. 2023;12.

6.Andualema B, Gessesse A. Microbial Lipases and Their Industrial Applications: Review. Biotechnology(Faisalabad). 2012;11:100-18.

7.Snellman EA, Sullivan ER, Colwell RR. Purification and properties of the extracellular lipase, LipA, of Acinetobacter sp. RAG-1. European Journal of Biochemistry. 2002;269(23):5771-9.

8.Rigo E, Ninow JL, Di Luccio M, Oliveira JV, Polloni AE, Remonatto D, et al. Lipase production by solid fermentation of soybean meal with different supplements. LWT - Food Science and Technology. 2010;43(7):1132-7.

9.Baloch KA, Upaichit A, Cheirsilp B. Use of low-cost substrates for cost-effective production of extracellular and cell-bound lipases by a newly isolated yeast Dipodascus capitatus A4C. Biocatalysis and Agricultural Biotechnology. 2019;19:101102.

10.Gutarra ML, Godoy MG, Maugeri F, Rodrigues MI, Freire DM, Castilho LR. Production of an acidic and thermostable lipase of the mesophilic fungus Penicillium simplicissimum by solid-state fermentation. Bioresource technology. 2009;100(21):5249-54.

11.Fabiszewska AU, Kotyrba D, Nowak D. Assortment of carbon sources in medium for Yarrowia lipolytica lipase production: A statistical approach. Annals of microbiology. 2015;65:1495-503.

12.Oliveira F, Salgado JM, Abrunhosa L, Pérez-Rodríguez N, Domínguez JM, Venâncio A, et al. Optimization of lipase production by solid-state fermentation of olive pomace: from flask to laboratory-scale packed-bed bioreactor. Bioprocess and biosystems engineering. 2017;40:1123-32.

13.Domínguez A, Deive FJ, Sanromán MA, Longo MA. Effect of lipids and surfactants on extracellular lipase production by Yarrowia lipolytica. Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology. 2003;78(11):1166-70.

14.Ruiz C, Javier Pastor F, Diaz P. Isolation and characterization of Bacillus sp. BP-6 LipA, a ubiquitous lipase among mesophilic Bacillus species. Letters in Applied Microbiology. 2003;37(4):354-9.

15.Rajendran A, Thangavelu V. Sequential optimization of culture medium composition for extracellular lipase production by Bacillus sphaericus using statistical methods. Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology. 2007;82(5):460-70.

16.Sagar K, Bashir Y, Phukan MM, Konwar B. Isolation of lipolytic bacteria from waste contaminated soil: A study with regard to process optimization for lipase. Int J Sci Technol Res. 2013;2(10):214-8.
17.Farrokh P, Yakhchali B, Karkhane AA. Cloning and characterization of newly isolated lipase from Enterobacter sp. Bn12. Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]. 2014;45(2):677-87.
18.Zhu J, Liu Y, Qin Y, Shen N, Li Y, Liang G, et al. Optimization of a molasses based fermentation medium for lipases from Burkholderia sp. Bps1 based on response surface methodology. Food Science and Technology Research. 2018;24(5):757-65.
19.Ebrahimpour A, Rahman RNZRA, Ean Ch'ng DH, Basri M, Salleh AB. A modeling study by response surface methodology and artificial neural network on culture parameters optimization for thermostable lipase production from a newly isolated thermophilic Geobacillus sp. strain ARM. BMC biotechnology. 2008;8:1-15.
20.Batra J, Beri D, Mishra S. Response surface methodology based optimization of β-glucosidase production from Pichia pastoris. Applied biochemistry and biotechnology. 2014;172:380-93.
21.Paterson Beedle M, Kennedy JF, Melo FAD, Lloyd LL, Medeiros V. A cellulosic exopolysaccharide produced from sugarcane molasses by a Zoogloea sp. Carbohydrate Polymers. 2000;42(4):375-83.
22.Tanyol M, Uslu G, Yönten V. Optimization of lipase production on agro-industrial residue medium by Pseudomonas fluorescens (NRLL B-2641) using response surface methodology. Biotechnology & Biotechnological Equipment. 2015;29(1):64-71.
23.Patel H, Ray S, Patel A, Patel K, Trivedi U. Enhanced lipase production from organic solvent tolerant Pseudomonas aeruginosa UKHL1 and its application in oily waste-water treatment. Biocatalysis and Agricultural Biotechnology. 2020;28:101731.
24.Yele VU, Desai K. A new thermostable and organic solvent-tolerant lipase from Staphylococcus warneri; optimization of media and production conditions using statistical methods. Applied biochemistry and biotechnology. 2015;175:855-69.
25.Iftikhar T, Naiz M, Afzal M, Rajoka MI. Maximization of intracellular lipase production in a lipase-overproducing mutant derivative of Rhizopus oligosporus DGM 31: a kinetic study. Food technology and biotechnology. 2008;46(4):402-12.
26.Vasiee A, Behbahani BA, Yazdi FT, Moradi S. Optimization of the production conditions of the lipase produced by Bacillus cereus from rice flour through Plackett-Burman Design (PBD) and response surface methodology (RSM). Microbial Pathogenesis. 2016;101:36-43.
27.Greco-Duarte J, de Almeida FP, de Godoy MG, Lins U, Freire DMG, Gutarra MLE. Simultaneous lipase production and immobilization: morphology and physiology study of Penicillium simplicissimum in submerged and solid-state fermentation with polypropylene as an inert support. Enzyme and Microbial Technology. 2023;164:110173.
28.Albayati SH, Masomian M, Ishak SNH, Mohamad Ali MSb, Thean AL, Mohd Shariff Fb, et al. Main Structural Targets for Engineering Lipase Substrate Specificity. Catalysts. 2020;10(7):747.

Cite this article as

Dusane S, Gutte S. Optimization of Lipase Production with Response Surface Method using *Bacillus subtilis*. Int. J. Micro. Sci. 2024; 5(1), 30-38.

APPENDICES

Table 1: Variables and their levels for response surface methodology

Name	Units	-1 Level	+1 Level	-alpha	+alpha
Molasses	(g/L)	2	18	-3.45434	23.45434
Peptone	(g/L)	3	27	-5.18151	35.18151
Palm oil	(% v/v)	0.1	0.7	-0.10454	0.904538

Table 2: Experimental design and Predicted responses using CCD for lipase production.

Run	Molasses (g/L)	Peptone (g/L)	Palm oil (% v/v)	Actual responses (U/mL)	Predicted responses (U/mL)
1	10	15	0.4	126.0847	120.4309027
2	18	3	0.1	88.8071	71.6337116
3	10	-5.181513966	0.4	98.47786	108.2472113
4	10	15	0.4	98.40901	120.4309027
5	18	27	0.7	140.2274	141.5405228
6	10	15	0.904537849	135.1034	117.3759794
7	10	15	0.4	116.4857	120.4309027
8	-3.454342644	15	0.4	126.5568	113.588399
9	10	15	0.4	116.3874	120.4309027
10	10	15	0.4	115.04	120.4309027
11	18	3	0.7	106.5917	124.5830053
12	2	3	0.1	116.2989	122.5473728
13	2	27	0.7	49.61758	74.35255347
14	10	15	-0.104537849	114.9318	123.485826
15	18	27	0.1	145.8629	160.2406794
16	23.45434264	15	0.4	141.8895	127.2734064
17	2	3	0.7	140.7979	133.9816562
18	10	35.18151397	0.4	140.5323	132.6145941
19	10	15	0.4	145.5187	120.4309027
20	2	27	0.1	144.9974	134.5677203

Table 3: Analysis of variance

Source Model	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Model	7338.244	6	1223.041	4.207578	0.0143	Significant
A-Molasses	274.1396	1	274.1396	0.943111	0.3492	
B-Peptone	646.7362	1	646.7362	2.224941	0.1597	
C-Palm oil	56.29722	1	56.29722	0.193677	0.6671	
AB	2932.755	1	2932.755	10.08944	0.0073	
AC	861.748	1	861.748	2.964637	0.1088	
BC	2566.822	1	2566.822	8.830535	0.0108	
Residual	3778.784	13	290.6757			
Lack of Fit	2575.102	8	321.8878	1.337097	0.3901	Not Significant
Pure Error	1203.682	5	240.7364			
Cor Total	11117.03	19				

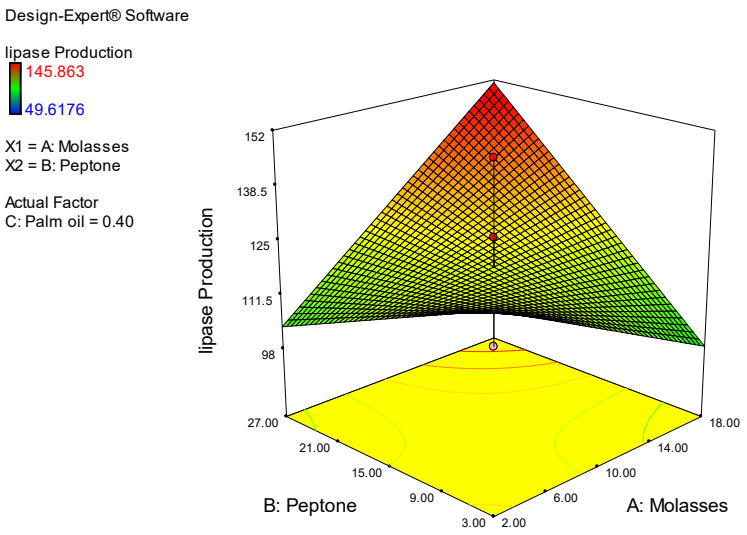

Figure 1: Molasses and peptone at a fixed level of Palm oil

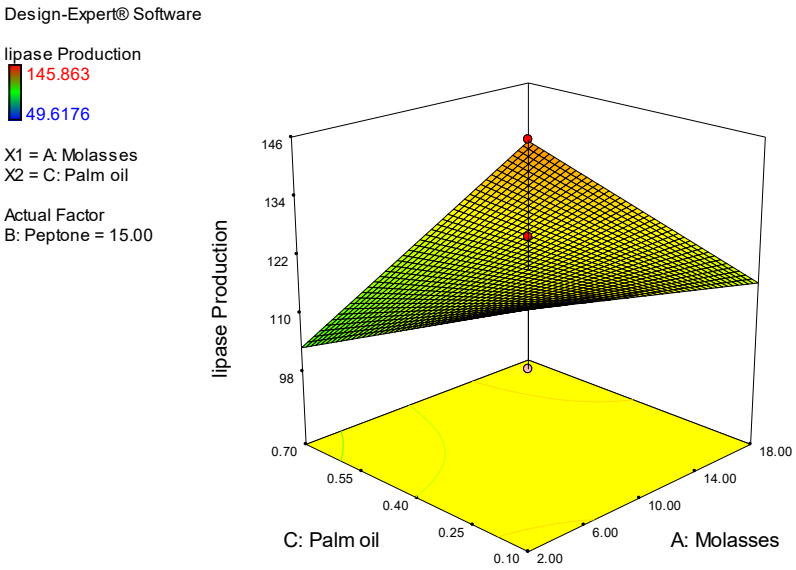

Figure 2: Palm oil and molasses at a fixed level of peptone

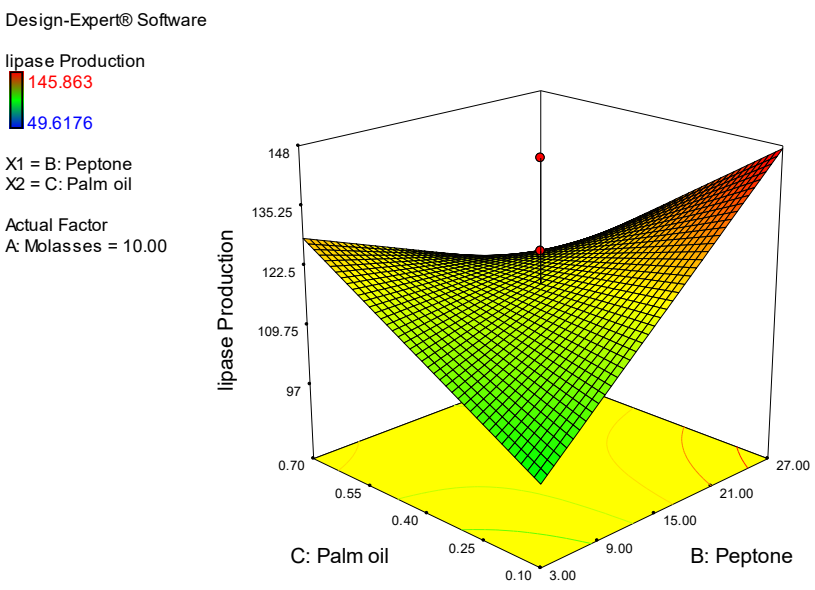

Figure 2: Palm oil and molasses at a fixed level of peptone

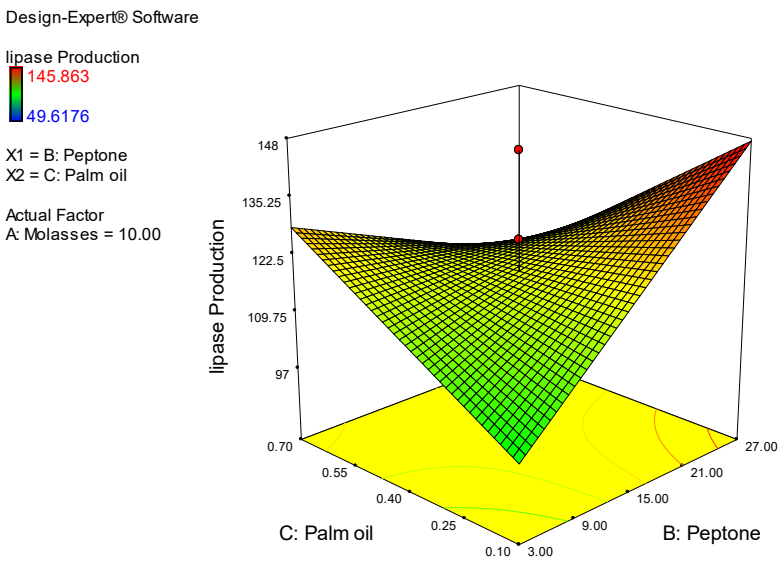

Figure 3: Peptone and palm oil at a fixed level of molasses