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TB conceptualised the study, and conducted the experiments. TH, HI, AM, and MYB contributed to writing the manuscript. All authors read and approved the final version of the manuscript.

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Statistical Optimization and Scale-Up of Lipase Production Using Brassica Seed Cake in a Stirred Bioreactor

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Abstract:

Fungal lipases, particularly from *Aspergillus* species, are valued for their stability and high yield under optimized fermentation conditions. Agro-industrial residues such as Brassica seed cake provide a cost-effective and sustainable substrate for large-scale enzyme production. This study aimed to optimize and scale up lipase production from *Aspergillus sojae* using statistical approaches in a stirred bioreactor with emphasis on agro-waste utilization for potential industrial applications. A potent lipase-producing fungal isolate (*Aspergillus sojae* TB-MS) was cultivated. Preliminary trials were conducted in 250 mL Erlenmeyer flasks, followed by scale-up in a 5-liter stirred fermenter. Key substrates, including Brassica seed cake (complex carbon and nitrogen source), molasses (sucrose as a carbon source), sunflower oil (inducer and lipid substrate), sucrose (readily metabolizable carbon source), yeast extract (organic nitrogen source), and Tween 80 (emulsifier and lipase activity enhancer), were screened using a one-factor-at-a-time (OFAT) approach. Response Surface Methodology (RSM) was applied to evaluate the interactive effects of the components. Brassica seed cake and sunflower oil significantly enhanced lipase production, yielding 9.04 ± 0.251 IU/mL after 48 h. RSM optimization achieved a maximum extracellular activity of 12.5 IU/mL. Scale-up in a stirred fermenter produced peak extracellular (26.43 ± 0.057 IU/mL) and intracellular (28.04 ± 0.041 IU/g) activities after 40 h, with maximum glucose utilization and biomass formation at 8 h and 24 h, respectively. The study successfully optimized lipase production from *Aspergillus sojae* using Brassica seed cake as a low-cost agro-industrial substrate, demonstrating both high yield and scalability.

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INTRODUCTION

Enzymes are well thought out as nature's catalysts. Novo Nordisk in 1988 introduced the first commercially useful enzyme, known as Lipolase, which originated from the fungus *Humicola lanuginosa* (Krastanov *et al.,* 2008). Lipases act predominantly at oil–water interfaces, enabling them to process water-insoluble triglycerides by interfacial activation and emulsification (Kempka *et al.,* 2008).

Lipases are enzymes that hydrolyze triacylglycerides by releasing fatty acids and possess distinctive features such as region specificity, substrate specificity, and chiral selectivity (Park and Park, 2022). Fungi produce lipases both by solid-state and submerged fermentation techniques. The most commonly used constituents to enhance lipase production in a submerged fermentation system are yeast extract, peptone, beef extract, soy meal, and many different kinds of oils and the oil wastes residues (Gomes *et al.,* 2018; Ramani *et al.*, 2013; Lima *et al.*, 2019).

The fact that inducers such as soybean oil and glucose/peptone combinations are very effective in the production of fungal lipases in a submerged environment persists to date as suggested by recent studies (Dutta *et al.,* 2022; Cesário *et al.,* 2021; Abdullah *et al.*, 2018). It has been noted that commercially useful lipases are produced by fungi of the genera *Geotrichum*, *Penicillium*, *Rhizomucor*, *Aspergillus*, *Rhizopus*, and *Mucor* (Thakur, 2012). The availability of fungus-producing extracellular lipases, ease of production, and indeed their high substrate specificity and toleration of wide operating conditions owe much to their prevalence in industry (Kumar *et al.*, 2023). In addition, fungi require less moisture and thus they tend to perform more efficiently compared to other microorganisms (Mohseni *et al.*, 2012).

Fermentation is a biological process wherein a substance is broken down into simpler forms, aided by different microorganisms. There are two methods of fermentation used in the production of enzymes, namely: solid-state fermentation (SSF) and submerged fermentation (Souza *et al.*, 2025). Lipolytic activity and overall yield are highly dependent on the composition of the fermentation medium, including carbon sources (e.g. sugars, oils), nitrogen sources (organic/inorganic), and inducers such as vegetable oils or oily residues (Lanka and Latha, 2015). Fungal lipases have shown significant presence in the detergent, food, biodiesel, pharmaceutical, leather, paper, and cosmetic manufacturing industries, due to tolerance of wide pH, temperature, and solvent buffering, and the simplicity of downstream processing (Kumar *et al.*, 2023). Statistical analysis and response surface methodology are vital techniques for determining the optimum medium culture parameters and strategies for resolving issues regarding industrial fermentation (Dutta *et al.,* 2022; Riyadi *et al.,* 2017; Cesário *et al.*, 2021). Recent advances in bioengineering and statistical design with the use of agro-residues and other progressive bioprocessing strategies have up-scaled the enzyme production and made production systems more sustainable (Abdelaziz *et al.*, 2025).

Although there have been numerous investigations on the fungal lipases, they have mostly been on well-studied genera such as *Aspergillus niger*, *Rhizopus*, and *Penicillium* fungi, with little being done in investigating *Aspergillus sojae* as a prospective fungal source of lipase. Moreover, there are few studies that deeply explore the intracellular and extracellular production of lipase subjected to the best submerged fermentation conditions. The lack of an integrated technique employing a combined technique of conventional (OFAT) and statistical (RSM) optimization techniques to produce an increased yield of lipase with novel fungal strains is also a fact. The study fills these by screening *A. sojae* as a producer of lipase, optimizing the medium and scale-up of the strategy to industrial scale.

This study aimed to check the interactive effect of critical medium components on lipase production using RSM and its scale up trial.

MATERIALS AND METHODS

Isolation and maintenance of fungal culture

A fungal isolate was obtained from oil-roasted bread and cultured on malt extract agar (MEA), and the strain was purified by repeated sub-culturing and designated. Preliminary identification as *Aspergillus* sp. was performed through microscopy and micrometry, and molecular identification was subsequently confirmed by commercial sequencing services (Malaysia).

For maintenance, the fungal isolate was sub-cultured on 4% MEA slants and stored at 4 °C, as described by de Souza Rabello et al. (2022). Spore suspensions were prepared from 5–7-day-old cultures by adding 10 mL of sterile distilled water to each slant, scraping with a sterile inoculating needle, and vortexing to obtain a homogeneous suspension (Senanayake *et al.*, 2022).

Molecular analysis

Genomic DNA was extracted from fungal mycelium and used for PCR amplification of the ITS region with ITS1 and ITS4 primers. The amplified product was sequenced, and BLAST analysis followed by phylogenetic evaluation (MEGA 7.0) confirmed the isolate as *Aspergillus sojae* (Khan *et al.*, 2020).

Lipolytic assay

Lipase activity was determined titrimetrically with minor modifications of Kempka et al. (2008) by adding 1 mL of culture supernatant to 17 mL of assay mixture containing 5 mL phosphate buffer, $CaCl_2$ (2 mL 0.6%), and 10% gum acacia emulsion in 10% (v/v) olive oil (10mL), followed by incubation at 30 °C with shaking at 150–200 rpm for 1 h, 10 mL of acetone: ethanol (1:1, v/v) were added, and the liberated free fatty acids were titrated against 0.1 N NaOH using phenolphthalein as an indicator. A unit (U) of lipase activity was expressed as the amount of enzyme catalyzing 1 µmol of fatty acids/minute of assay.

$$\text{Lipase activity (U/mL)} = \frac{\Delta V \times N \times 1000}{V \times 60}$$

ΔV = volume of NaOH used (mL), N = normality of NaOH, V = volume of enzyme used (mL).

Estimation of dry cell mass

A previously weighed Whatman No. 1 filter paper was used to filter culture broth (10mL) to estimate the dry biomass. At 60°C, the residual biomass was dried until its weight remained unchanged. The difference between the final and first filter weights was used to compute the dry cell mass (Lee *et al.*, 2021).

Optimization and fermentation methodology

Fermentation (SmF) was initially performed in shake flasks containing basal medium adapted from Aickel et al. (2010): sucrose 5.0 g/L, molasses 1.0 g/L, K_2HPO_4 0.5 g/L, KH_2PO_4 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, and yeast extract 2.0 g/L (pH 8.0, 30 °C, 150 rpm). The medium composition was modified in the present study by supplementing 0.5 g Brassica meal per batch, in addition to the reported constituents. Brassica seed cake (oil-extracted residue from Brassica seeds) was incorporated at a fixed concentration of 0.5 g per 100 mL of medium and served as a basal substrate throughout the fermentation process. The seed cake was collected from a local oil mill, oven-dried at 60 °C, powdered, and sieved before use.

Response Surface Methodology (RSM) was used to study optimization with the concentrations of sucrose, molasses, yeast extract, sunflower oil, and Tween 80. A statistical method using Response Surface Methodology (RSM) was used to maximize the culture medium composition. The BoxBehnken Design was used to organize the experimental trials, and each of the independent factors was tested at three levels (−1, 0, +1) to measure the linear, quadratic, and interactive effects. The design matrix and the model fitting were created with the help of Design-Expert version 10.0.3.1 (Stat-Ease Inc., Minneapolis, USA), and CoStat software was used to provide additional

statistical confirmation. A 10-L stir fermenter (7-L working volume) was used to produce on a pilot scale (Colla *et al.*, 2016). The Bradford technique was used to measure the concentration of proteins in the culture media using bovine serum albumin as the standard (Song *et al.*, 2023). Each and every assay was done in triplicate to ensure accuracy and reproducibility of the procedures.

RESULTS

A fungal isolate was obtained from oil-roasted bread and designated as TB-MS. Microscopic and micrometric analyses indicated characteristics of the genus *Aspergillus*. Molecular identification based on ITS rDNA sequencing confirmed the isolate as *Aspergillus sojae* (TB-MS). The 18S rDNA sequence was aligned against NCBI GenBank sequences using BLASTn, and phylogenetic analysis clustered the isolate with *A. sojae* reference strains (Figure 1).

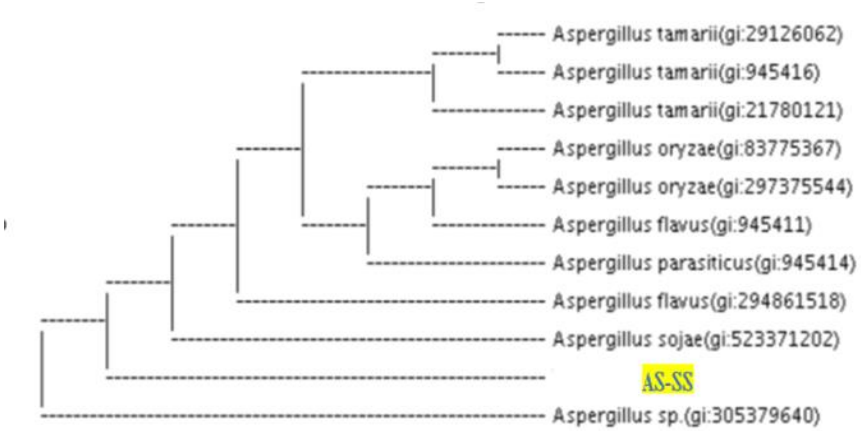

Fig. 1. Phylogenetic tree showing the relationship of *Aspergillus sojae* with closely related strains based on ITS sequences.

Using the OFAT approach under submerged fermentation, five medium components were tested, and their effects on lipase production by *A. sojae* (TB-MS) were determined. Sunflower oil was tested at different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0% v/v) to assess its effect on enzyme production. After 48 hours of fermentation, lipase activity increased with sunflower oil up to 1.5% (v/v), after which no further improvement was observed. Consequently, this concentration was selected for subsequent experiments (Figure 2).

Lipase production was evaluated by testing different concentrations of Tween 80 (0.5, 1.0, 1.5, 2.0, and 2.5% v/v), and the highest enzyme activity of 7.91 ± 0.65 IU/mL was recorded at 0.5% after 48 hours of fermentation; this concentration was subsequently used for further optimization, with all experiments conducted in triplicate and results presented as mean values (Figure 3).

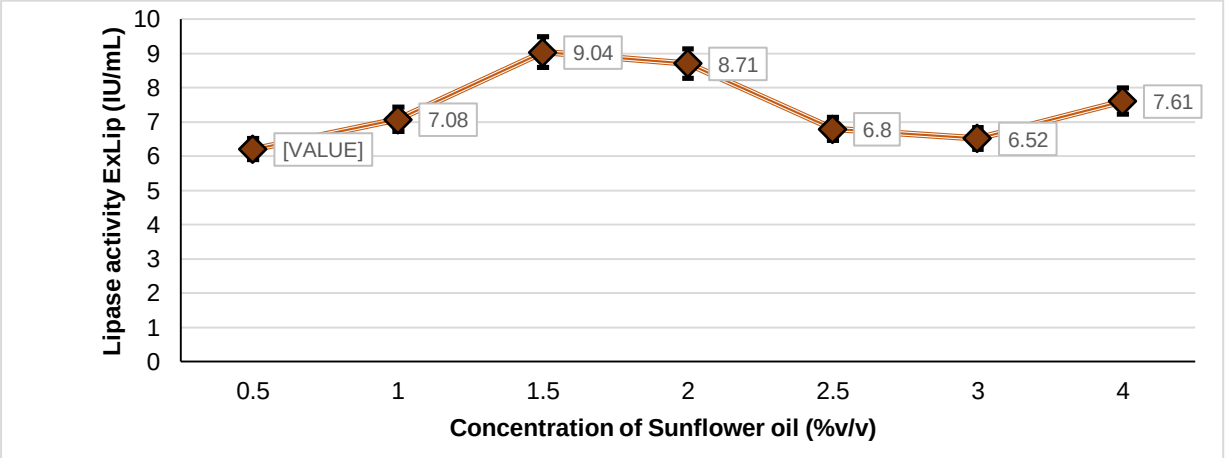

Fig. 2. Effect of concentration of sunflower oil on extracellular lipases production by *Aspergillus sojae* using SmF.

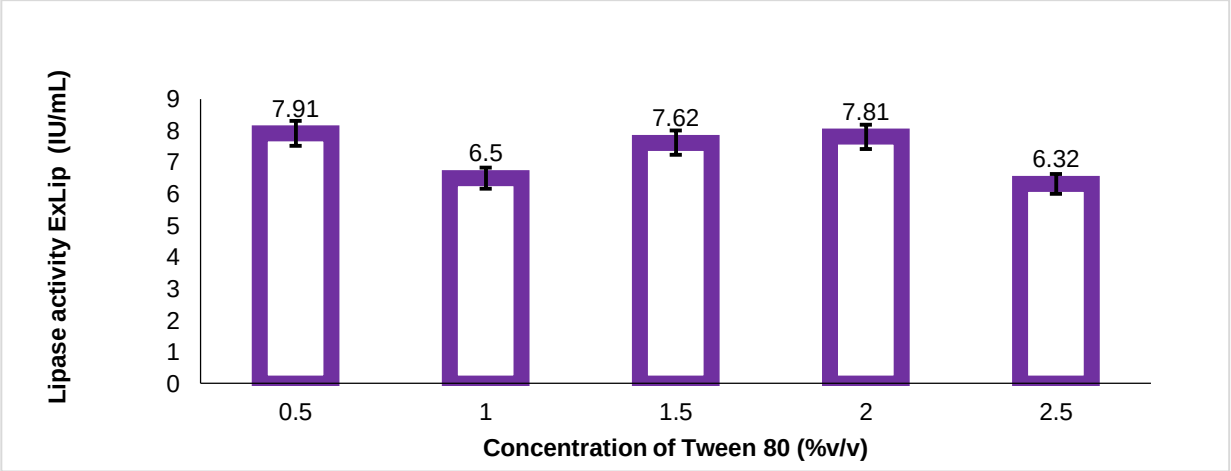

Fig. 3. Effect of concentration of Tween 80 on extracellular lipases production by *Aspergillus sojae* using SmF.

Sucrose was tested at concentrations of 1–5 g/L, increasing in 1 g/L, to evaluate its influence on lipase production in submerged fermentation. The highest enzyme activity (6.61 ± 0.40 IU/mL) was observed at 3 g/L after 48 hours, and this concentration was chosen for subsequent experiments (Figure 4).

Yeast extract was evaluated at 0.5, 1.0, 1.5, 2.0, and 2.5 g/L to determine its impact on lipase production. After 48 hours of submerged fermentation, the highest lipase activity (7.4 ± 0.22 IU/mL) was recorded at 1.0 g/L, which was subsequently chosen as the optimal concentration for further studies (Figure 5).

Molasses was supplemented at concentrations ranging from 1 to 5 g/L (in 1 g/L increments) to investigate its effect on lipase production during submerged fermentation. After 48 hours, the maximum lipase activity (6.39 ± 0.28 IU/mL) was obtained at 3 g/L molasses. Therefore, 3 g/L was selected as the optimized concentration for further studies (Figure 6).

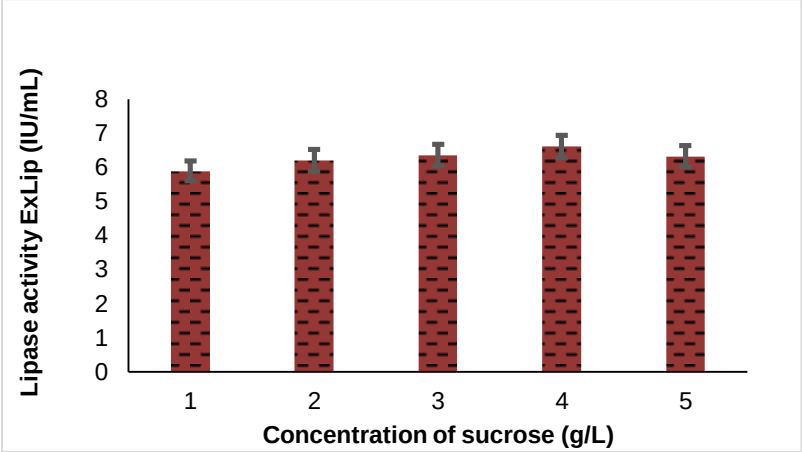

Fig. 4. Effect of concentration of sucrose on extracellular lipases production by Aspergillus sojae using submerged fermentation.

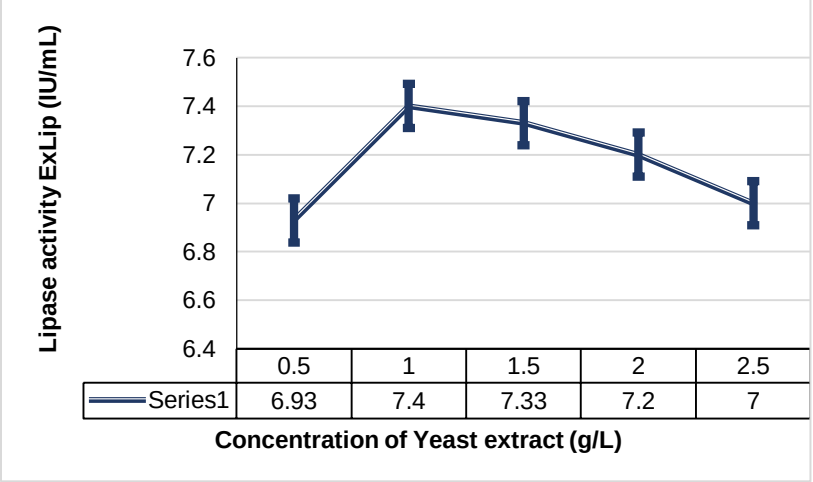

Fig. 5. Effect of concentration of Yeast extract on extracellular lipases production by *Aspergillus sojae* using SmF.

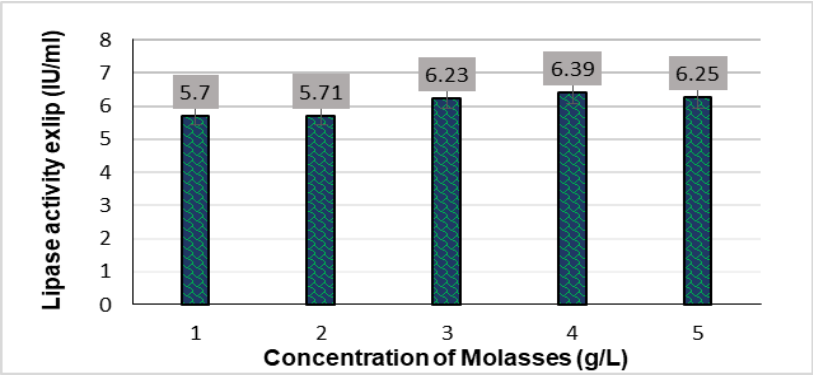

Fig. 6. Effect of concentrations of Molasses on extracellular lipases production by *Aspergillus sojae* using SmF.

A Box–Behnken design comprising 46 experimental runs was used to assess the interactive effects of independent variables (sucrose, yeast extract, sunflower oil, molasses, and Tween 80) on lipase production. The quadratic model was statistically significant (F = 2.10; p = 0.0417), demonstrating an adequate fit to the data. The model equation describing lipase activity (Y_1) was: Y_1(U/mL) =7.68+0.24A+0.55B+0.55C−0.50D−0.044E+0.28AB−0.025AC+0.43AD+0.000AE+1.50BC−1.02BD+1.10BE−0.17CE+0.42DE−0.079A2+0.50B2+0.43C2+0.23D2+0.0039E2. The coded factors used in the study were sucrose (A), molasses (B), yeast extract (C), sunflower oil (D), and Tween-80 (E (Table 1).

Table 1. Analysis of variance (ANOVA) for the quadratic model developed using response surface methodology.

Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F
Model	37.13	20	1.86	2.10	0.0417
A-Sucrose	0.95	1	0.95	1.08	0.3096
B-Molasses	4.84	1	4.84	5.49	0.0278
C-Yeast extract	3.06	1	3.06	3.47	0.0747
D-sunflower oil	3.56	1	3.56	4.04	0.0559
E-Tween 80	0.028	1	0.028	0.031	0.8612
Residual	21.17	24	0.88		
Lack of Fit	19.02	19	1.00	2.33	0.1774
Pure Error	2.15	5	0.43		
Cor Total	58.30	44			

The 42nd run demonstrated the maximum enzyme activity (12.5 IU/mL). This peak response was achieved when sucrose was fixed at 3 g/L and Tween-80 at 1.5% (v/v), while higher levels of molasses (5 g/L) and yeast extract (2.5 g/L) were combined with sunflower oil supplementation of 2.25% (v/v) (Figure 7).

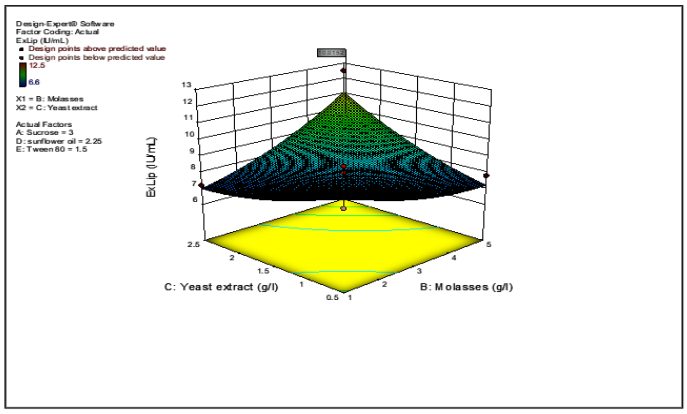

Fig. 7. Response Surface 3D Curve of maximum lipase activity showing the Interactive effects of molasses and yeast extract concentration.

According to the response surface plot (Figure 8), an enzyme activity of 8.4 IU/mL was obtained in the 35th run when sucrose was adjusted near 3 g/L in combination with a lower sunflower oil concentration (~0.5% v/v), while molasses, yeast extract, and Tween-80 were maintained at 5 g/L, 1.5 g/L, and 1.5% (v/v), respectively (Figure 8).

Analysis of the response surface highlighted a narrow set of favorable conditions for enzyme synthesis. These included maintaining the carbon sources at low levels, particularly sucrose and molasses (around 1.0 g/L each), a moderate supply of nitrogen through yeast extract, and balancing the hydrophobic phase by supplementing sunflower oil together with Tween-80 (2.25% v/v; 1.5% v/v) (Figure 9).

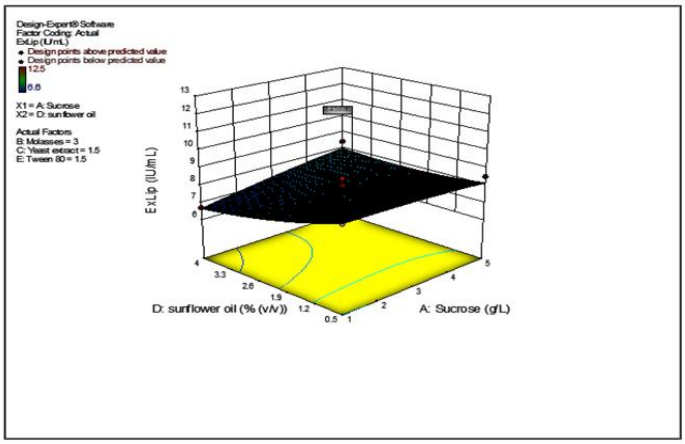

Fig. 8. Response surface 3D curve of intermediate lipase activity showing interactive effects of sucrose and sunflower oil concentration. The experiment revealed that the 28th run corresponded to the minimum lipase yield of 7.22 IU/mL.

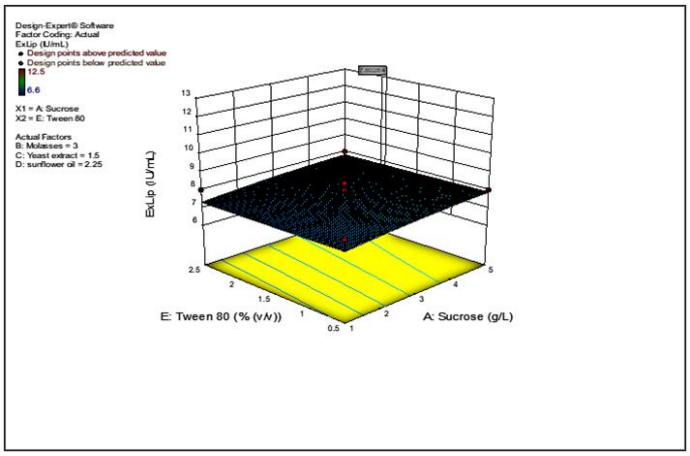

Fig. 9. Response surface 3D curve, minimum lipase activity showing interactive effects of Tween 80 and sucrose concentration.

Kinetics of Lipase activity in stirred fermenter

In a stirred fermenter, the synthesis of intracellular and extracellular lipases was studied, with measurements taken every 8 hours. Maximum extracellular and intracellular lipase activities of 26.43 ± 0.041 IU/mL and 28.04 ± 0.057 IU/mL, respectively, were

observed after 40 hours of fermentation, while the highest dry cell mass was recorded at 24 hours for both types of lipases (Figure 10), (Table 2).

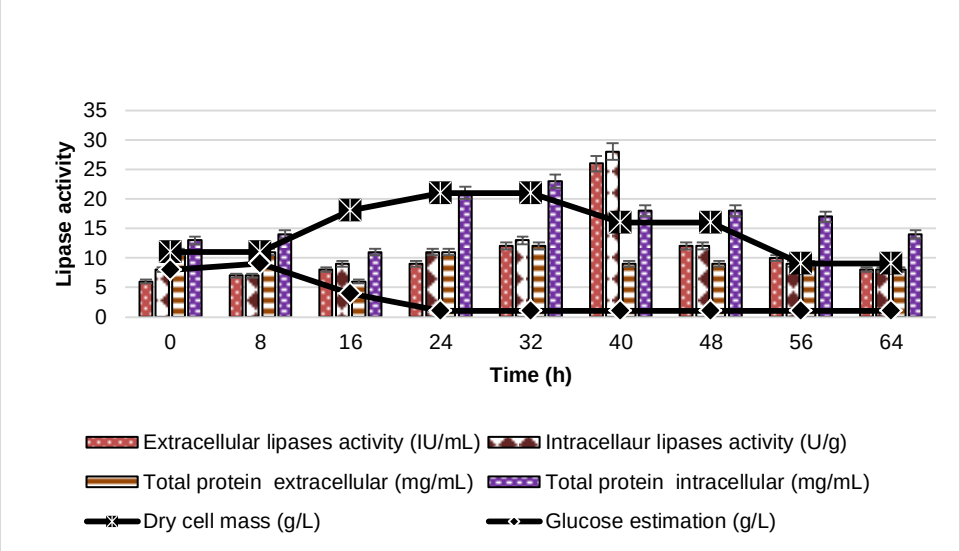

Fig. 10. Effect of critical medium components on lipase production by *Aspergillus sojae* using a Fermenter.

Table 2. Optimization of medium components and fermentation conditions for lipase production

Parameters / Conditions	**Optimized Value**	**Lipase Activity (IU/mL)**
Sunflower oil (% v/v)	1.5	9.04 ± 0.25a
Tween 80 (% v/v)	0.5	7.91 ± 0.65[a]
Sucrose (g/L)	3.0	6.61 ± 0.40[a]
Yeast extract (g/L)	1.0	7.40 ± 0.22[a]
Molasses (g/L)	3.0	6.39 ± 0.28[a]
Box–Behnken optimized medium	Yeast extract 2.5 (g/L), Sunflower oil 2.25 (% v/v), Sucrose 3.0 (g/L), Molasses 5.0 (g/L), Tween-80 1.5 (% v/v)	12.55
Fermenter kinetics (40 h)	24h	Extracellular lipase: 26.43 ± 0.04[a] Intracellular lipase: 28.04 ± 0.05[a]

DISCUSSION

In this study, Brassica seed cake was used at a constant concentration of 0.5 g per 100 mL as a basal substrate. The presence of residual proteins, lipids, and carbohydrates in the seed cake likely contributed to sustained fungal growth and enzyme induction. Although not optimized as a variable, its supplementation provided a cost-effective base medium that supported lipase biosynthesis by *Aspergillus sojae*. This study identified *A. sojae* (TB-MS) as a promising lipase producer, with enzyme synthesis strongly influenced by carbon, nitrogen, lipid, and surfactant supplementation. Sucrose (3 %) was the best source of carbon (6.61 ± 0.40 U/mL). This coincides with the findings of Iftikhar et al. (2011), but contrasts with the findings of other researchers who reported the use of glucose as the favorable substrate (Iftikhar *et al.,* 2012; Salihu *et al.,* 2012). The difference can be due to species-specific differences in sugar uptake and regulation of lipase-encoding genes. Molasses

was also found to be efficient as another source of carbon, with 3% being the most active source of lipolytic activity. This is due to the variety of its sugar content (sucrose, glucose, fructose) and micronutrients, which favor fungi growth and enzyme activation. The same results were detected in other microbial systems in which lipase yield was improved with the addition of molasses (Zhu *et al.,* 2018). The addition of sunflower oil was revealed to be an effective lipid inducer with peak activity at 1.5% supplementation. Previous studies indicated the effect of vegetable oils on the secretion of lipase in *Aspergillus niger* (Aickel *et al.,* 2010). Sunflower oil could be a good inducer, even though it was hypothesized that it is not invariably a potent inducer, due to the high degree of unsaturated fatty acids that most likely allow the oil to undergo emulsification and the subsequent production of enzymes in *A. sojae* (Facchini *et al.,* 2015). Other oils like sunflower and palm have been found to complement sugar-containing substrates to stimulate fungal lipase production (Alabdalall *et al.,* 2020).

The supplementation with Tween 80 also increased lipase production, with the peak of the activity being at 0.5% (v/v). Tween 80 in low concentrations seems to enhance the emulsification of the substrate and also causes a rise in the membrane permeability, thus allowing the release of the enzyme. Similar results were also demonstrated by Salihu et al. (2011), who demonstrated that Tween 80 enhanced the lipase activity in *Candida cylindracea*, but very high concentrations were inhibitory. Response surface methodology (RSM) was used, and it was found that there were major interactive effects among medium components. It is important to note that the synergistic effect on the production of lipase was achieved by adding molasses (3 g/L) and yeast extract (2.25 g/L). The same trends have been reported in other microbial systems, in which the carbon and nitrogen sources balance promoted the production of enzymes (Polat *et al.,* 2022; Zhu *et al.,* 2018). Similarly, the interaction of the presence of both sucrose (3 g/L) and sunflower oil (2.5% v/v) was very strong, which is in line with the research that indicates that combinations of sugar-rich substrates and oils favor greater lipase production (Donzella *et al.,* 2024; Sakpuntoon *et al.,* 2023). Interactions of Tween 80 were also observed, with moderate concentrations stimulating activity, whereas the higher ones resulted in activity inhibition, probably because of the stress on fungal cells that surfactants cause (Sipiczki *et al.,* 2024). The experiments of scale-up in a stir-tank fermenter showed that the maximum extracellular and intracellular lipase activity was reached at 40 h, which was earlier than that at 48 h of shake flask culture. This shorter fermentation time can be explained by better control over environmental factors like temperature, pH, and agitation. The same has been observed in *A. oryzae* (Iftikhar *et al.,* 2024) and *A. niger* (Salvatierra *et al.,* 2021), in which fermenter-based cultures were more productive than flask cultures. The outcomes of this study indicate that the potential of *A. sojae* (TB-MS) to be used as an industrial source of lipase is evident since the results of the shake flask were successfully translated into a bioreactor scale.

CONCLUSION

This paper is the first to indicate the maximum extracellular production of lipase by a new isolate, *Aspergillus sojae,* implying its great industrial interest. *A. sojae* exhibited high reproducible enzyme activity with scalable conditions, and thus it is specifically useful in biodiesel production, the development of detergents, and biological catalysis in the food and pharmaceutical industries. The combined application of OFAT and RSM techniques not only provided the exact medium optimization but also provided a cost-effective and sustainable strategy in the large-scale production of lipase. The findings have made a very new starting point in the study of industrial enzymology and have great potential for biotechnological prospects in terms of the exploitation of *A. sojae*.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors regarding this publication.

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