http://dergipark.org.tr/trkjnat
Trakya University Journal of Natural Sciences, 25(1): 41-54, 2024
ISSN 2528-9691
DOI: 10.23902/trkjnat.1317293

Research Article

Utilization of shea-nut cake for lipase production by thermophilic *Bacillus velezensis* EAC 9 isolated from hot compost and optimization of nutritional parameters

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Cite this article as:

Kazeem, M.O., Adegbemi E.A., Aisami A. & Onajobi I.B. 2024. Utilization of shea-nut cake for lipase production by thermophilic *Bacillus velezensis* EAC 9 isolated from hot compost and optimization of nutritional parameters. *Trakya Univ J Nat Sci*, 25(1): 41-54, DOI: 10.23902/trkjnat.1317293

Received: 20 June 2023, Accepted: 02 January 2024, Online First: 21 February 2024, Published: 15 April 2024

Abstract: Although the use of oily waste as a cost-effective substrate for lipase production has recently gained importance, shea-nut cake (SNC) remains under-explored in this regard. Lipases of thermophilic origin such as hot compost bacteria are of significant biotechnological and industrial importance due to favorable robust properties. This study describes the optimization of nutritional parameters for improving lipase production by a thermophilic lipase producing bacteria isolated from hot compost using Response Surface Methodology (RSM). The bacteria were isolated on tributyrin agar plate and used for lipase production on olive oil, SNC and their combination. Using Plackett-Burman Design (PBD) for screening and Central Composite Design (CCD) of RSM for optimization studies, factors influencing lipase production on SNC substrate were identified. One of the four most potent isolates, *Bacillus velezensis* EAC9, was identified using 16S rRNA and observed to show the maximum lipase activity on a mixture of olive oil and SNC (103.66 U/mL), which was higher than that of olive oil (65.22 U/mL) and SNC (41.72 U/mL) alone. The validity of the optimization model was confirmed, and an optimum medium containing olive oil and Tween 80 at 1.0% (v/v), sucrose at 1.0% (w/v), and $(NH4)_2SO_4$ at 0.1% (w/v) resulted in maximum lipase production at 200 U/mL, a 4.79-fold increase over the unoptimized medium. The findings suggest that SNC could be considered a cheap substrate for enhancing lipase production by the thermophilic *B. velezensis* EAC9 and suggest a model of nutritional parameters for optimal lipase production which could be scale up for industrial applications.

Özet: Yağlı atıkların lipaz üretimi için uygun maliyetli bir substrat olarak kullanılması giderek önem kazanmaktadır. Ancak shea tohumu özütü (SNC) bu bakımdan yeterince araştırılmamıştır. Sıcak kompost bakterileri gibi termofilik kökenli lipazlar, uygun ve dirençli özellikleri nedeniyle anlamlı biyoteknolojik ve endüstriyel öneme sahiptir. Bu araştırma, Yanıt Yüzey Yöntemi (RSM) kullanılarak sıcak komposttan izole edilen termofilik lipaz üreten bakteriler tarafından lipaz üretiminin iyileştirilmesi için beslenme parametrelerinin optimizasyonunu açıklamaktadır. Bakteriler tribütirin agar plakası üzerinde izole edildi ve zeytinyağı, SNC ve bunların kombinasyonlarında lipaz üretimi için kullanıldı. Tarama için Plackett-Burman Tasarımı (PBD) ve optimizasyon çalışmaları için RSM'nin Merkezi Bileşik Tasarımı (CCD) kullanılarak, SNC substratı üzerinde lipaz üretimini etkileyen faktörler belirlendi. En güçlü dört izolattan biri olan *Bacillus velezensis* EAC9, 16S rRNA kullanılarak tanımlandı ve zeytinyağı ve SNC karışımında (103,66 U/mL) maksimum lipaz aktivitesi gözlendi. Bu aktivite tek başına zeytinyağından (65,22) ve SNC'den (41,72 U/mL) daha yüksekti. Optimizasyon modelinin geçerliliği doğrulandı ve % 1.0 (v/v) zeytinyağı ve Tween 80, % 1.0 (w/v) sakaroz ve % 0.1 (w/v) $(NH4)_2SO_4$ içeren optimum ortam, maksimum lipaz üretimiyle. 200 U/mL'de optimize edilmemiş ortama göre 4,79 kat artış ile sonuçlandı. Bulgular, SNC'nin, termofilik *B. velezensis* EAC9 tarafından lipaz üretimini arttırmak için ucuz bir substrat olarak düşünülebileceğini ve endüstriyel uygulamalar için ölçeklendirilebilecek optimal lipaz üretimi için bir beslenme parametreleri modeli önerdiğini göstermektedir.

Edited by:
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Key words:
Optimization
Shea-nut cake
Lipase
Bacillus velezensis EAC9
Thermophilic

Introduction

Lipases EC 3.1.1.3 triacylglycerol hydrolase are serine hydrolases that catalyze the hydrolysis of triglycerides to fatty acid and glycerol (Almeida *et al*. 2019). They are capable of forming glycerides at the interface between the lipid and water due to its low water solubility (Pascoal *et al*. 2018). Although bacteria, fungi, plants and animals are all sources of lipases (Subramoni *et al*. 2010), microbial lipases are advantageous due to their high specificity and broad stability. Thermophilic bacteria are notable sources of thermostable enzymes in relation with their ability to survive in extreme environmental conditions (Ansari *et al*. 2018). Lipases with novel characteristics have been discovered from several extremophilic bacteria isolated from, hot springs, compost heaps and sugary environments (Duan *et al*. 2023, Leykun *et al*. 2023). Lipases from thermophilic bacteria display high activity and unchanged structure at elevated temperatures which is valuable for industrial applications. These lipases also exhibit remarkable resistance to chemical deformation and may be active at high acidic or alkaline pHs (Amin *et al.* 2018). The high activity and stability at alkaline pHs made lipases with this special feature important and valuable agent used in detergent industry. In addition, lipases derived from thermophiles are known to be functional in severe circumstances, providing valuable new properties for industrial bioprocessing (López-López *et al*. 2014). Baltaci *et al*. (2019) reported lipase production from the thermophilic *Bacillus licheniformis* A7 at 55°C and a pH value of 6.0 while Ovando-Chacon *et al*. (2020) reported lipase production from *Geobacillus stearothermophilus* at 71.6°C and pH 5.4.

The production of lipase of bacterial origin is significantly affected by the cultural and nutritional parameters. Therefore, optimization of fermentation conditions and the medium components is essential in order to achieve high yield and cost-effectiveness. Over the years, the statistical modelling method of optimization has gained much relevance over the conventional one factor at a time (OFAT). The OFAT method does not display interactive effect amongst variables, it's laborious and time-consuming. These drawbacks have been overcome by the Response Surface Methodology (RSM). The RSM is a mathematical and statistical method for designing and modelling of multivariable experiment. It can be used to detect the effect of significant factors, factor interactions and optimum condition to maximize response. The Central Composite Design (CCD) of the RSM is commonly reported for optimizing parameters for enhanced lipase production (Amenaghawon *et al*. 2022, Fibriana *et al.* 2022). In this regard higher lipase yield could be achieved with the use of the statistical approach rather than the classical method (Isiaka & Olufolahan, 2018). The use of vegetable oil, olive oil, coconut oil, palm oil, sunflower oil and almond oil have been previously reported for lipase production (Geoffry & Achur, 2018, Suci *et al.* 2018). In addition to increasing the cost of production, the use of oil for lipase production could compete with food, pharmaceutical, cosmetic and other industries where their application is highly essential. Therefore, the use of waste biomass could circumvent these drawbacks. Some biomass waste reported for lipase production include bran (bran, soybean, wheat) (Putri *et al.* 2020, do Nascimento *et al.* 2021, Fatima *et al.* 2021), oil cakes (mustard, almond, palm kernel) (Oliveira *et al.* 2017, Sahoo *et al.* 2020), effluents (palm oil mill, sunflower mill) (Geoffry & Achur, 2018, Isiaka & Olufolahan, 2018) and bagasse (sugarcane) (Pinotti *et al.* 2017). The continuous demand for a low-cost media for lipase production necessitated the search for potential newer oily waste that can improve microbial growth and lipase yield. The shea-nut cake (SNC) is the sticky black/dark brown oily residue obtained after extraction and clarification of butter from shea-nuts. The anti-nutritional constituents in SNC such as tannin, saponin and theobromine have limited its wider application (Salihu *et al.* 2013). However, its biodegradable and oily nature suggests its high potential for use in the production of value added products such as lipase, but research on this potential is currently limited. Although, SNC was used for lipase production by *Aspergillus niger* (Salihu *et al.* 2013), we, in the present study, used SNC as a substrate for lipase production by the thermophilic *Bacillus velezensis* EAC9. Similarly, the high demand for the production of robust bacterial lipases for biotechnological and industrial applications has highlighted the need to explore extreme habitats, such as hot compost, for such bacteria. Such thermophilic bacteria have the innate potential to secrete thermostable lipase that can tolerate the harsh environment faced in industrial bioprocesses. Because of the remarkable characteristics and applications of lipase, it is critical to find highly robust bacteria. Accumulation of SNC in the mill causes series of environmental pollution. The bioconversion of SNC into value added product such as lipase could be one of the solutions to the aforementioned problem. With this in mind, the current study aimed to isolate thermophilic lipase producing bacteria from shea butter mill waste -based hot compost and optimize the nutritional component for maximum lipase yield using SNC as substrate which can be useful for future biotechnological applications.

Materials and Methods

Sample Preparation

Composting was prepared from a combination of SNC, shea butter effluent, sorghum-straw and chicken manure. The SNC and effluent were collected from a local shea butter industry at Apa Ola Ebu, Ilorin. The sorghum straw and chicken manure were obtained from a local farm at Oke-Odo, University road, Tanke, Ilorin, Kwara State, Nigeria. The sorghum straw was milled (local miller) to 1-2 mm particle size prior to use. All the samples were transported to the compost preparation site of the Microbiology Department, University of Ilorin. The composting was prepared in a mixture of sorghum straw (16 kg), chicken manure (8 kg) and SNC (5.3 kg) and then moistened with shea butter mill effluent to obtain a 70 %

moisture content (MC). The mixture was covered with polyethylene and jute bag to conserve heat and moisture. Continuous addition of shea butter mill effluent with frequent turning was done at 2 days interval while monitoring the temperature and MC.

Isolation of thermophilic lipolytic bacteria

Compost samples were collected during the thermophilic phase of the composting (40-68°C). Compost sample (5.0 g) was added to 100 mL of enrichment medium (EM) containing (w/v): olive oil 2 mL, NaCl 0.1 g, $MgSO_4.7H_2O$ 0.02 g, $MgCl_2$ 0.035 g, $CaCl_2$ 0.025 g, K_2PO_4 0.015 g, NH_4SO_4 0.025 g, and 0.01 mL trace element solution (0.026 g B, 0.05 g Cu, 0.05 g Mn, 0.006 g Mo and 0.07 g Zn) (Rahman *et al*. 2007). The medium was adjusted to pH 7.0 using 2M NaOH and then incubated at 50°C for 48 h at 150 rpm. Sample (1mL) from the EM was serially diluted and plated on Olive Oil Agar (OOA) containing 13 g/L nutrient broth, 15 g/L agar-agar and 1mL olive oil and incubated at 50°C for 24 h. Repeated streaking on OOA plate was done to obtain pure cultures and which were stored at +4°C until further use.

Primary Screening for lipolytic bacteria

The pure bacteria isolates were tested for lipase secretion on tributyrin agar plate containing (g/L) yeast extract 3.0, peptone 5.0, agar 15, tributyrin 10 mL, $MgCl_2$, 0.5 and $CaCl_2$ 0.3 (Carrazco-Palafox *et al*. 2018). Thereafter, 20 µL of the bacteria isolate previously standardized (OD_{600} =1.0) was transferred to a 5 mm well bored on the tributyrin agar plates and then incubated at 50°C for 72 h. Un-inoculated nutrient broth served as the control. Visible clear zone resulting from hydrolysis of tributyrin shows the presence of lipolytic activity.

Secondary screening for lipolytic activity

The lipolytic isolates were further subjected to secondary (quantitative) screening in a basal medium (g/L, v/v) containing $NaNO_3$ 7.0, K_2HPO_4 2.0, KH_2PO_4 1.0, KCl 0.1, $MgSO_4.7H_2O$ 0.5, $CaCl_2$ 0.01, $FeSO_4.7H_2O$ 0.012, yeast extract 1.0, trace element 0.01 %, olive oil 2 % supplemented with 40 µL tween 80. The medium pH was adjusted to 7.0 with 1 M NaOH or 1 M HCl (Rahman *et al*. 2007). A 3% (v/v) suspension from a 24 h old bacteria culture (OD_{600} =1.0) was inoculated into 100 mL basal medium and subjected to shaking (150 rpm) at 50°C for 24 h. Samples were transferred into a 2 mL tube followed by centrifugation (centrifuge 80-2 Maxmill medical) at 10000 x g at +4°C for a period of 10 min. The supernatant (crude lipase) was used to determine the lipase activity. The best lipase producing bacteria was selected for further studies.

Genomic DNA extraction

Fresh bacteria cultures were cultivated for 48 h at 37°C before being harvested for genomic DNA extraction. The pellet obtained following centrifugation at 4600 x g for 5 min was resuspended in 520 µL of TE buffer (10 mMTris-HCl, 1mM EDTA, pH: 8.0). Following this step, 15 µL of 20% SDS and 3 µL of proteinase K (20 mg/mL) were added and incubated for 1 h at 37°C before adding 100 µl of 5 M NaCl and 80 µL of 10% CTAB solution in 0.7 M NaCl and vortexed. The mixture was incubated for 10 min at 65°C before being placed on ice for 15 min. Equivalent volume of chloroform:isoamyl alcohol (24:1) was introduced, followed by 5 min of ice incubation and 20 min of centrifugation at 7200 x g. The aqueous phase was moved to a fresh tube, isopropanol (1:0.6) was added, followed by 16 h of DNA precipitation at -20°C. Following centrifugation at 13000 x g for 10 min, the DNA pellet was washed with 500 µL of 70% ethanol, air-dried at ambient temperature for about 3 h, and then dissolved in 50 µLof TE buffer (Trindade *et al.* 2007).

Amplification, sequencing and analysis of 16S rRNA

The amplification of the 16S rRNA of the lipase producing bacterial isolate was done using the primers 27F-5'-AGAGTTTGATCMTGGCTCAG-3'and -1525R,5′-AAGGAGGTGATCCAGCC-3′ and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µL with sterile distilled water, 8µl DNA template. The Polymerase Chain Reaction (PCR) was carried out in a Gene Amp 9700 PCR System Thermocycler (Applied Biosystem Inc., USA) at an initial denaturation at 94°C for 5 min, followed by 30 cycles consisting of 94°C for 30 s, annealing at 50°C for 60 s, extension at 72°C for 1 min 30 s, and a final termination at 72°C for 10 min and chilled at +4°C. Amplified products were checked by electrophoresis in a 1% Agarose gel at 120 v for 45 min. After staining with 3 µL of 0.5 g/mL ethidium bromide, it was visualized by ultraviolet trans-illumination. The resulting 16S rRNA gene sequence was compared with sequence in the GenBank database of the Nucleotide Basic Local Alignment Search Tool (BLAST N) software on NCBI (www.ncbi.nlm.nih.gov/BLAST). Alignment of the sequence was done using multiple sequence alignment tool, Clustal W. A, while Molecular Evolutionary Genetics Analysis tool (MEGA 7.0) (Kumar *et al.* 2016) was used in the construction of the phylogenetic tree using the neighbour joining method (Tamura *et al.* 2004).

Effect of carbon substrate and time on lipase production

The lipase production was carried out in basal medium used for the secondary screening (Rahman *et al*. 2007), but with some modifications. The basal media was modified by the addition of 3.0 g SNC, 1.0 mL olive oil and a combination of SNC and olive oil at 1.5 g and 0.5 mL, respectively. The media was sterilized at 121°C for 15 min. After cooling, a 3 % inoculum from a 24 h old bacteria culture (OD_{600} =1.0) was added and incubated at 50°C for 60 h under shaking at 150 rpm. A 2 mL sample was withdrawn at 12 h interval and centrifuged at 10,000 g for 10 min. The obtained cell free supernatant was used for the lipase assay.

Lipase Assay

Lipase assay was done according to Kwon & Rhee (1986). Olive oil emulsion [olive oil /50mM sodium

phosphate buffer (1:1 v/v) 2.5 mL] was mixed with 1 mL crude lipase and 20µL of 0.02 M $CaCl_2$ in water bath shaker at an agitation rate of 200 rpm. The reaction mixture was incubated at 50°C for 30 min using a shaking incubator. The reaction was halted with 1 mL of 6 M HCL and 5 mL of isooctane, followed by mixing for 30 sec and boiling for 5 min. The 4 mL of the fatty acid-containing upper isooctane layer was placed into a test tube and then agitated with 1 mL copper reagent for 30 sec. The absorbance was measured at 715 nm, and 1 unit of lipase activity was defined as the quantity of enzyme that released 1 mole of fatty acid per minute under the conditions of the experiment.

Plackett-Burman design

The PBD design (Plackett & Burman, 1946) was used to screen parameters that could influence lipase production. A total of eleven variables which comprises of ten nutritional components (A=Glucose, B= Sucrose, C= Yeast Extract, D=Peptone, E= Ammonium sulphate, F= Sodium nitrate, G= Magnesium Sulphate, H= Di-sodium hydrogen phosphate, J= Olive oil, K= Tween 80, and L = Temperature) were evaluated at two levels and concentration of each variable is illustrated in Table 1. The main effect of each factor was calculated as the difference between the means of lipase production measured using +1 and −1 concentrations. The choice of variables selected were based on nutritional factors affecting lipase production as highlighted in previous studies (Salihu *et al.* 2013, Ilesanmi *et al*. 2020). These variables were screened in duplicate to generate 24 runs of experiment using the Minitab 17 software (Mini-Tab LLC, Pennsylvania, USA). The media was prepared based on the design but with the addition of 3 % (w/v) of SNC and 3 % (v/v) inoculum (OD_{600} =1.0). The PB design based on the first order polynomial model is shown in Equation (1**)**

$$Y = \beta_0 + \sum \beta_i Xi$$

Where; γ is the response (Lipase production in U/mL)

β_0 is the model intercept

β_i is the linear coefficient and

Xi is the concentration of independent factors (i= 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11)

The magnitude of the coefficient β_i, whether it is positive or negative shows the impact of the corresponding factors on lipase production using SNC as substrate at 95% (p <0.05) confidence level for each factor. The significance of the model was calculated by ANOVA. The factors showing the highest effect from the Pareto chart were selected for optimization by RSM.

Optimization of lipase production using RSM

In this experiment, four independent variables (olive oil, tween 80, ammonium sulphate and sucrose that were significant from the PBD) were selected and optimized using CCD of RSM. Their effect on lipase production was studied and variables were tested at 5 code levels (-α, -1, 0, +1 and +α). Details of experimental plans are shown (Table 2). Using the MiniTAB software, 56 runs of experiment were generated with four center points. A second order polynomial equation generated is shown in Equation (2) below

$$\gamma = \beta0 + \beta1A + \beta2B + \beta3C + \beta4D4 + \beta11A2 + \beta22B2 + \beta33C2 + \beta44D2 + \beta12AB + \beta13AC + \beta14AD + \beta23BC + \beta24BD + \beta34CD$$

where γ is the predicted response, β0 is the model constant; A, B, C and D are independent factors (Olive oil, Tween 80, NH4SO4, Sucrose); β1, β2, β3 and β4 are linear coefficients; β12, β13, β14, β23, β24and β34 are cross product coefficients and β11, β22, β33 and β44 are the quadratic coefficients. In order to determine the significance of the model terms, the data obtained from the CCD were subjected to ANOVA.

Table 1. Screening of independent factors and their levels using PBD.

Factors	Symbol	Low level -1	High level +1
Glucose % (w/v)	A	0.2	1.0
Sucrose % (w/v)	B	0.2	1.0
Yeast extract % (w/v)	C	0.5	1.0
Peptone % (w/v)	D	0.5	1.0
$(NH_4)_2SO_4$ % (w/v)	E	0.1	0.6
$NANO_3$ % (w/v)	F	0.01	0.10
$MgSO_4$ % (w/v)	G	0.05	0.2
Na_2HPO_4 % (w/v)	H	0.1	0.6
Olive oil % (v/v)	J	0.2	1.0
Tween 80 % (v/v)	K	0.2	1.0
Temperature ˚C	L	50	60

Table 2. Experimental range of independent variables as used in CCD with their actual and code level

Independent Variable	Symbol	Actual Value of Code				
		+α	+1	0	-1	–α
Olive Oil % (v/v)	A	1.4	1.0	0.6	0.2	-0.2
Tween80 % (v/v)	B	1.4	1.0	0.6	0.2	-0.2
$(NH_4)_2SO_4$ % (w/v)	C	0.85	0.6	0.35	0.1	-0.15
Sucrose % (w/v)	D	1.4	1.0	0.6	0.2	-0.2

Results

Bacterial Isolation and screening

Among the 11 isolates screened for lipolytic activity on Tributyrin agar, 4 isolates (EAC4, EAC6, EAC9 and EAC10) showed positive activity, depicted by a clear zone formation around the agar well (Table 3). The zone of clearance around the isolate well is an indication of extra cellular lipase conversion of fat to water soluble butyric acid (Lanka & Latha, 2015). Isolate EAC 9 exhibited the highest lipolytic activity (1.9 mm) and was isolated at point where the compost temperature reached the highest at 68°C. This confirms that EAC 9 has the potential of secreting copious amount of lipase at very high temperature.

Table 3. Potential lypolytic hot compost isolates.

Compost Temperature (°C)	Days	Bacteria Isolate	Hydrolysis Zone (mm)
45	1	EAC 1	Nil
45	1	EAC 2	Nil
55	9	EAC 3	Nil
63	2	EAC 4	1.1
63	2	EAC 5	Nil
65	3	EAC 6	1.5
65	3	EAC 7	Nil
53	14	EAC 8	Nil
68	4	EAC 9	1.9
68	4	EAC 10	0.9
68	4	EAC 11	Nil

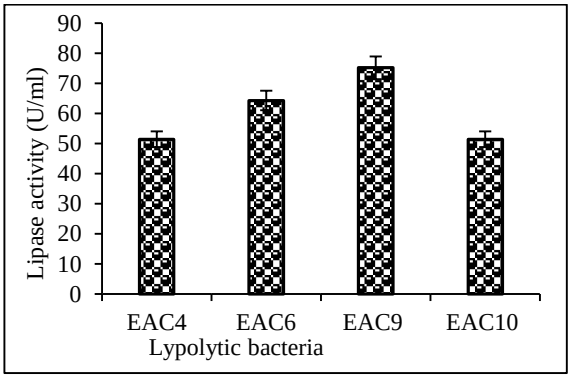

Fig. 1. Quantitative screening for lipase production.

All of the 11 isolates were further screened for lipase production (secondary screening) in basal liquid media, to validate the results from the primary (plate) screening. Interestingly, lipase activity was recorded from all 11 (100%) isolates (results not shown). However, EAC 9 exhibited the highest lipase activity (89.77 U/mL) out of the best four isolates (Fig. 1). The remaining 3 isolates produced some higher level of lipase activity with the least (51.44 U/mL) observed in both isolate EAC 4 and EAC 10.

Identification of the EAC9 Isolate

The analysis of the 16S rRNA suggests that the isolate EAC9 has 89% similarity with *Bacillus velezensis*. Hence, it was named *B. velezensis* EAC9. The relatedness of this isolate with other species of *Bacillus* is illustrated in the phylogenetic tree constructed using the neighbour joining method (Fig. 2).

Effect of carbon substrate and time on lipase production

In order to determine the best time and carbon substrate for lipase production, *B. velezensis* EAC9 was cultivated in basal medium containing SNC, olive oil and SNC-olive oil for 60 h. Optimal lipase production by *B. velezensis* EAC9 grown on SNC and olive oil was obtained at 41.72 U/mL and 65.22. U/mL, respectively (Fig. 3). From the results, lipase activity increased by 59.75% (SNC) and 37.08% (olive oil) when SNC was supplemented with olive oil, and a maximum activity of 103.66 U/mL was attained within 24 h.

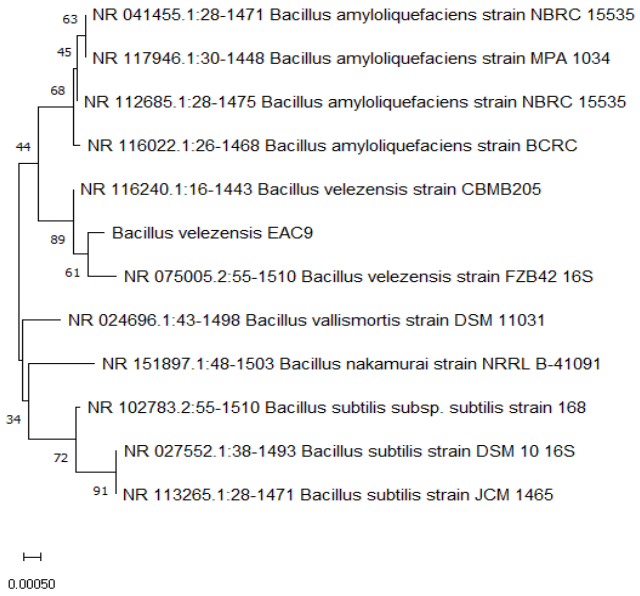

Fig. 2. Phylogenetic tree showing the relatedness of the isolate EAC 9 using the neighbour joining method.

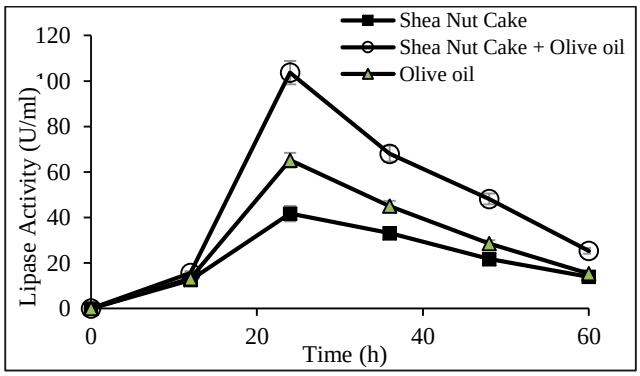

Fig. 3. Effect of carbon substrate and time on lipase production by *B. velezensis* EAC9.

Screening of parameters using PBD

The PBD was used to screen out factors contributing significantly to the production of lipase by *B. velezensis* EAC9 using SNC as the main substrate. Table 4 shows the distribution of the eleven variables according MiniTAB design version 17 and the results obtained.

The enzyme activity ranges from 42.22 U/mL to 142.00 U/mL in run 12 and run 6, respectively. The optimum activity at 142.00 U/mL was obtained at run 6. Result obtained from the PBD screening was used to form Pareto chart (Fig. 4) to determine the significant variables. Out of the eleven factor screened 8 were significant (B=Sucrose, E= NH_4SO_4, F= $NaNO_3$, G= $MgSO_4$, H= Na_2HPO_4, J= olive oil, K= tween 80 and L= temperature) while, three were not significant (A=glucose, C= yeast extract and D=peptone). However, the normal plot of the standardized effects (Fig. 5), shows that three of the factors (F=$NaNO_3$, G= $MgSO_4$, H= Na_2HPO_4) negatively affect lipase production. Hence, these factors were screened out from the optimization process using CCD of RSM.

Table 4. The PBD for screening of various variables for lipase production on SNC substrate.

Run	A (w/v)	B (w/v)	C (w/v)	D (w/v)	E (w/v)	F (w/v)	G (w/v)	H (w/v)	J (v/v)	K (v/v)	L (°C)	Activity (U/mL)
1	0.2	0.2	1.0	1.0	0.6	0.01	0.20	0.6	0.2	1.0	50	60.889
2	0.2	1.0	0.5	0.5	0.1	0.10	0.20	0.6	0.2	1.0	60	60.889
3	0.2	0.2	0.5	1.0	0.6	0.10	0.05	0.6	1.0	0.2	60	68.111
4	1.0	1.0	0.5	1.0	0.6	0.01	0.20	0.1	0.2	0.2	60	54.222
5	0.2	0.2	0.5	1.0	0.6	0.10	0.05	0.6	1.0	0.2	60	71.444
6	0.2	1.0	1.0	0.5	0.6	0.01	0.05	0.1	1.0	1.0	60	142.000
7	1.0	0.2	1.0	1.0	0.1	0.10	0.05	0.1	0.2	1.0	60	63.667
8	1.0	1.0	0.5	1.0	0.1	0.01	0.05	0.6	1.0	1.0	50	121.444
9	0.2	0.2	1.0	1.0	0.6	0.01	0.20	0.1	1.0	1.0	50	58.111
10	1.0	0.2	0.5	0.5	0.6	0.10	0.20	0.1	1.0	1.0	50	95.889
11	1.0	0.2	0.5	0.5	0.6	0.10	0.20	0.1	1.0	1.0	50	88.667
12	0.2	0.2	0.5	0.5	0.1	0.01	0.05	0.1	0.2	0.2	50	42.222
13	1.0	0.2	1.0	0.5	0.1	0.01	0.20	0.6	1.0	0.2	60	68.111
14	1.0	0.2	1.0	1.0	0.1	0.10	0.05	0.1	0.2	1.0	60	67.556
15	1.0	0.2	1.0	0.5	0.1	0.01	0.20	0.6	1.0	0.2	60	67.000
16	0.2	1.0	1.0	1.0	0.1	0.10	0.20	0.1	1.0	0.2	50	67.000
17	0.2	1.0	1.0	0.5	0.6	0.01	0.05	0.1	1.0	1.0	60	138.111
18	1.0	1.0	1.0	0.5	0.6	0.10	0.05	0.6	0.2	0.2	50	49.778
19	1.0	1.0	0.5	1.0	0.1	0.01	0.05	0.6	1.0	1.0	50	118.667
20	0.2	1.0	1.0	1.0	0.1	0.10	0.20	0.1	1.0	0.2	50	78.667
21	1.0	1.0	0.5	1.0	0.6	0.01	0.20	0.1	0.2	0.2	60	52.556
22	1.0	1.0	1.0	0.5	0.6	0.10	0.05	0.6	0.2	0.2	50	47.556
23	0.2	1.0	0.5	0.5	0.1	0.10	0.20	0.6	0.2	1.0	60	62.000
24	0.2	0.2	0.5	0.5	0.1	0.01	0.05	0.1	0.2	0.2	50	45.333

A= Glucose, B= Sucrose, C= Yeast Extract, D=Peptone, E= $(NH_4)_2SO_4$, F=$NaNO_3$, G= $MgSO_4$, H= Na_2HPO_4, J= Olive oil, K= Tween 80, L= Temperature

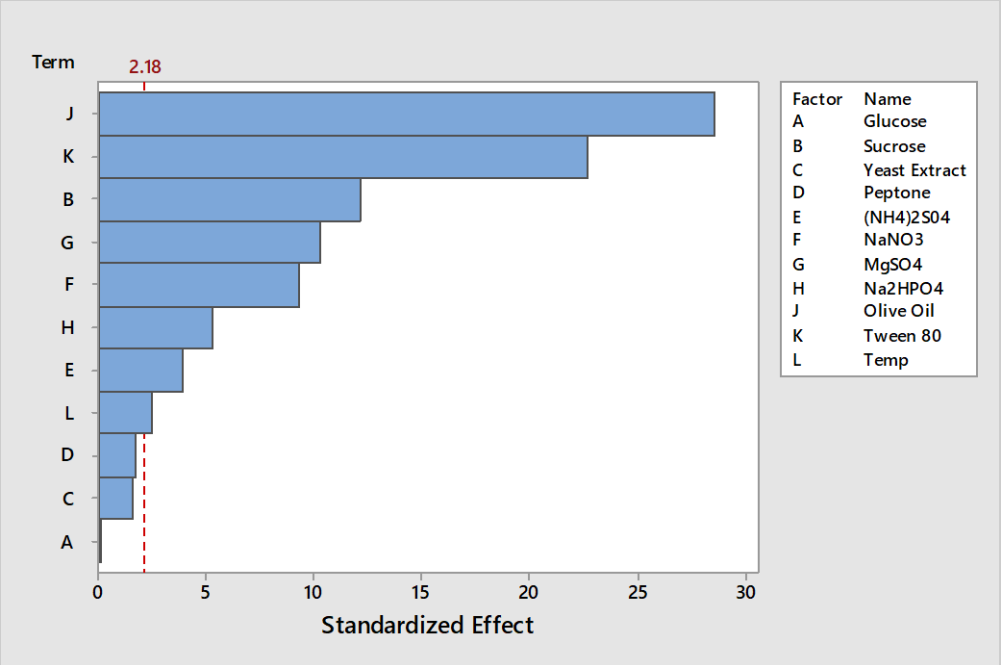

Fig. 4. Pareto chart of the standardized effects of screened factors on lipase activity (U/mL) at α= 0.05.

Table 5. The CCD for lipase production.

Run	Olive Oil %(v/v)	Tween 80 % (v/v)	(NH4)2SO4 % (w/v)	Sucrose % (w/v)	Lipase Activity (U/mL)
1	0.6	0.6	0.35	1.4	121.44
2	0.6	0.6	0.15	0.6	122.55
3	0.6	0.6	0.35	1.4	120.88
4	0.6	1.4	0.35	0.6	124.77
5	0.6	0.6	0.35	0.6	114.22
6	1.4	0.6	0.35	0.6	131.44
7	0.2	0.6	0.35	0.6	61.44
8	0.6	0.6	0.15	0.6	123.66
9	0.6	0.6	0.85	0.6	60.88
10	0.6	1.4	0.35	0.6	137.55
11	0.6	0.6	0.35	0.6	119.77
12	0.6	0.6	0.35	0.2	79.22
13	0.2	0.6	0.35	0.6	66.44
14	0.6	-0.2	0.35	0.6	73.11
15	0.6	-0.2	0.35	0.6	73.66
16	1.4	0.6	0.35	0.6	133.66
17	0.6	0.6	0.35	0.6	119.22
18	0.6	0.6	0.35	0.2	78.111
19	0.6	0.6	0.85	0.6	61.444
20	0.6	0.6	0.35	0.6	114.77
21	1.0	1.0	0.60	0.2	96.44
22	0.2	0.2	0.60	1.0	62.00
23	0.6	0.6	0.35	0.6	119.77
24	0.2	1.0	0.10	1.0	124.22
25	0.6	0.6	0.35	0.6	119.22
26	1/0	1.0	0.60	1.0	134.22
27	1.0	1.0	0.6	0.2	97.55
28	1.0	1.0	0.10	0.2	152.55
29	0.2	0.2	0.10	0.2	057.55
30	0.2	0.2	0.10	0.2	057.55
31	1.0	0.2	0.60	0.2	66.44
32	0.2	1.0	0.60	1.0	84.22
33	0.2	0.2	0.10	1.0	59.77
34	0.6	0.6	0.35	0.6	119.22
35	0.2	1.0	0.10	0.2	72.55
36	1.0	1.0	0.10	1.0	200.33
37	0.2	1.0	0.10	1.0	121.44
38	1.0	0.2	0.60	0.2	67.55
39	1.0	1.0	0.10	0.2	152.00
40	1.0	0.2	0.10	0.2	73. 11
41	0.2	0.2	0.60	0.2	44.22
42	1.0	0.2	0.10	1.0	127.00
43	1.0	0.2	0.60	1.0	58.66
44	1.0	1.0	0.60	1.0	132.55
45	0.2	0.2	0.6	1.0	60.33
46	1.0	0.2	0.10	1.0	125.88
47	0.2	1.0	0.60	0.2	054.22
48	1.0	0.2	0.60	1.0	60.333
49	0.6	0.6	0.35	0.6	119.22
50	0.2	0.2	0.10	1.0	058.66
51	1.0	1.0	0.10	1.0	197.55
52	0.2	1.0	0.60	0.2	055.33
53	0.2	1.0	0.10	0.2	074.22
54	0.2	0.2	0.60	0.2	044.77
55	1.0	0.2	0.10	0.2	073.11
56	0.2	1.0	0.60	1.0	085.33

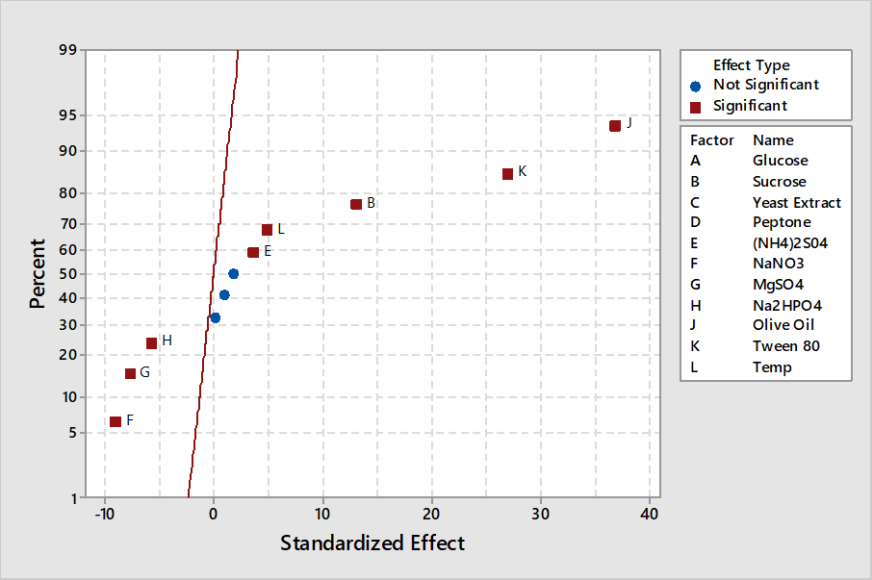

Fig. 5. Normal plot of the standardized effects of screened factors on lipase activity (U/mL) at α= 0.05.

Table 6. ANOVA test results for lipase production.

Source	Sum of Squares	Df	Mean square	F value	*p* value
Model	72269.8	15	4818.0	65.00	0.000
A (Olive Oil)	20076.4	1	20076.4	270.86	0.000
B (Tween 80)	19311.3	1	19311.3	260.54	0.000
C $(NH_4)_2SO_4$	11459.8	1	11459.8	154.61	0.000
D (Sucrose)	8312.6	1	8312.6	112.15	0.000
A^2	1788.6	1	1788.6	24.13	0.000
B^2	1370.2	1	1370.2	18.49	0.000
C^2	2817.7	1	2817.7	38.02	0.000
D^2	1649.5	1	1649.5	22.25	0.000
AB	2740.1	1	2740.1	36.97	0.000
AC	2194.5	1	2194.5	29.61	0.000
AD	155.6	1	155.6	2.10	0.155
BC	1082.4	1	1082.4	14.60	0.000
BD	929.2	1	969.2	13.08	0.001
CD	805.6	1	805.6	10.87	0.002
Lack of Fit	2653.5	10	265.4	25.57	0.000

p <0.05 were considered to be significant R^2 = 0.9606, R^2 (adj) =0. 9458, R^2 (pred) = 0.9196

Optimization of the medium components using CCD

Four factors (Olive oil, Tween 80, ammonium sulphate and sucrose) that positively influence lipase production by *B. velezensis* EAC 9 from the PBD experiment were selected. The CCD was used to determine their optimum concentration and interaction between the variables. A total of 56 experimental runs were generated as presented in Table 5. The highest lipase activity (200 U/mL) was achieved at run 36 and the lowest lipase activity (44.222 U/mL) was obtained at run 41.

The regression equation of the CCD design for lipase production by *B. velezensis* EAC9 generated using MiniTAB is presented as follows (Equation 3):

$Y=4.0+83.1A+55.4B+139.0C+65.7D-38.28\ A^2-33.50\ B^2-123.0\ C^2-36.76\ D^2+57.83\ AB-82.8\ AC+13.78AD-58.2BC+34.40BD-50.2CD$

where Y is the lipase activity (U/mL) produced as a function of the coded levels of olive oil concentration (A), Tween 80 (B), ammonium sulphate (C) and Sucrose (D), respectively.

The level of significance of the model terms is shown in the ANOVA table (Table 6). The F value of 65.00 and *p* value of $p<0.0000$ of the model indicated that the selected model was significant and suitable for lipase production. The p values of all the coefficients (A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, BC) were all significant except for AD (0.155). This implies that the variables in the model interact significantly to influence the overall production of lipase greatly. Olive oil has the strongest influence on lipase production, while sucrose showed least pronounced effect based on the F values of the main variables studied.

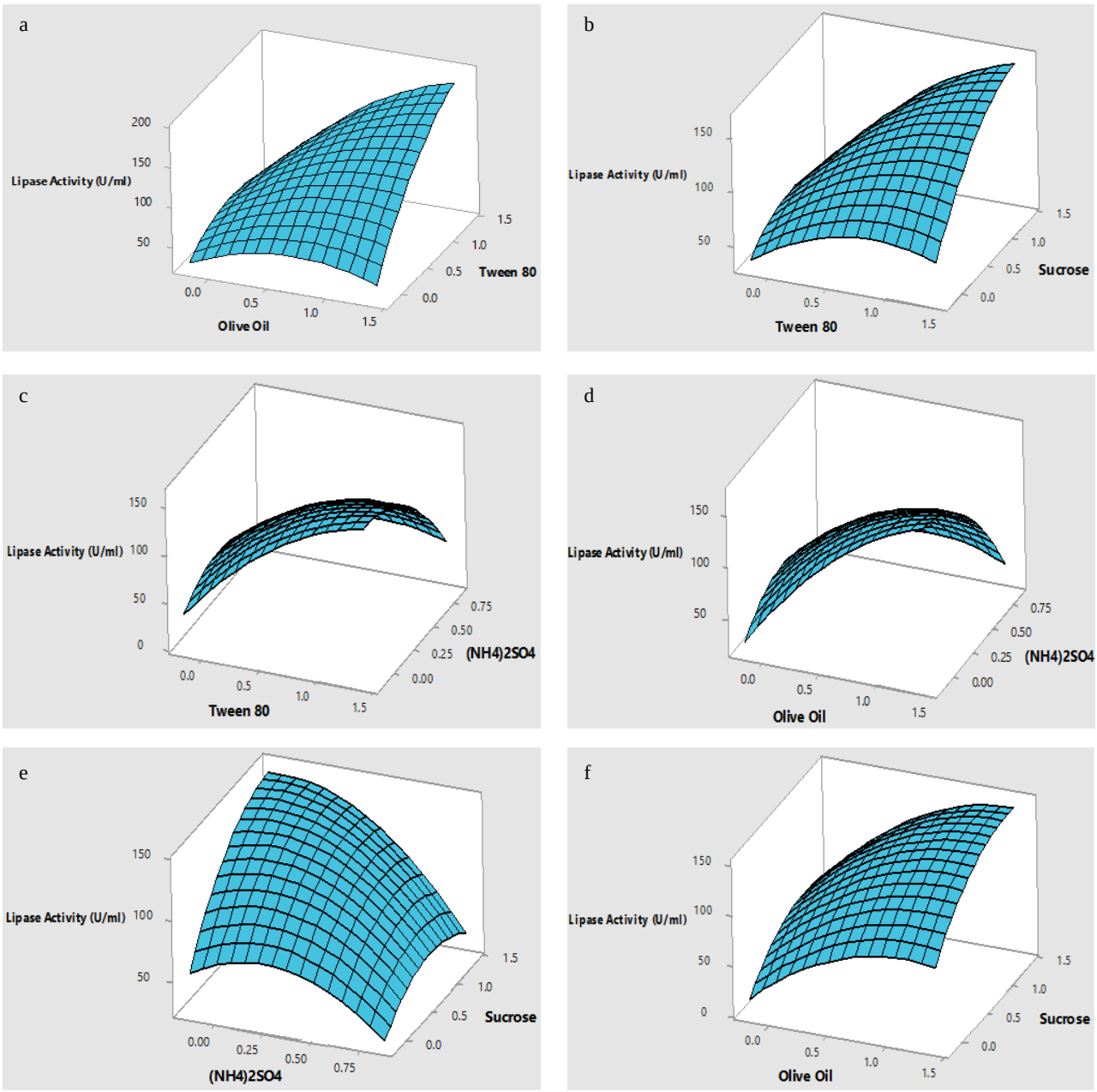

Fig. 6. The 3D surface plot for interaction effect between olive oil and Tween 80. **a.** Tween 80 and sucrose, **b.** ammonium sulphate and Tween 80, **c.** ammonium sulphate and olive oil, **d.** ammonium sulphate and sucrose, **e.** sucrose and olive oil, **f.** for lipase production.

The coefficient of determination R^2 suggested that 96.06% of the variation to response could be explained by the model and 3.94% cannot be explained by the model. Hence, predicted R^2 showed a reasonable agreement with the R^2 and the adjusted R^2, emphasizing that the model was highly significant and appropriate for predicting the individual and interactive effect of the parameters on lipase production by *B. velezensis* EAC9.

Using the regression equation, three dimensional response surface plots were constructed to investigate the interaction between the variables and to deduce the maximum concentration of each variable for highest yield of lipase (Figs 6 a-f). Here, two parameters were estimated while keeping other variables at mid-point. The 3D plot (Fig. 6a) shows that both olive oil and Tween 80 has significant influence on lipase production by *B. velezensis* EAC9. At low level of olive oil (1%) and a high level of tween 80 (1%), lipase activity was at maximum level (200 U/mL). Fig. 6b shows that the interaction between tween 80 and sucrose moderately affect lipase production. At 1.0 % they both gave optimum lipase activity but tween 80 had more distinct impact on lipase yield when compared with sucrose. The 3D plot of Tween 80 and ammonium sulphate (Fig. 6c) showed that optimum yield of lipase was obtained at high level of Tween 80 (1.0%) and low concentration of ammonium sulphate (0.1%), beyond which a reduced

lipase activity was detected. The interaction between ammonium sulphate and olive oil on lipase production (Fig. 6d) showed that the concentration of ammonium sulphate at 0.1% and olive oil at 1.0 % contribute significantly towards lipase production by *B. velezensis* EAC9. However, increasing ammonium sulphate and keeping the concentration of olive oil constant will decrease lipase production. The interaction between ammonium sulphate and sucrose (Fig. 6e) shows that optimum lipase activity was reached at 0.1 % ammonium sulphate and 1.0 % sucrose. Increase in the concentration of ammonium sulphate and decrease in sucrose level resulted in decline in lipase yield which further revealed the significance of the interaction between ammonium sulphate and sucrose. The optimized condition predicted by the RSM was validated by carrying out the experiment in shake flask using the condition predicted to confirm the accuracy of the model. Lipase production showed an increase up to 204 U/mL which was in close proximity to the predicted 200 U/mL

Discussion

The SNC and shea butter mill effluent composted with sorghum straw and chicken manure provided a good environment for the growth of lipase producing bacteria. Likewise, the EM supplemented olive oil stimulated the proliferation of the target bacteria. Lipase producing bacteria isolated from oil mill waste water (Mobarak-Qamsari *et al.* 2011, Oshoma *et al.* 2021) and domestic waste compost (Purkan *et al.* 2020) have been reported. The findings of this study add to the list of putative thermophilic sources of lipolytic bacteria that are currently available in the literature. The quantitative screening (secondary screening) is usually carried out to avoid the problem of false positive or negative results. As reported in a study by Stathopoulou *et al.* (2013), 17% of 101 isolates showed lipolytic activity during primary screening while the percentage increased to 74% with the secondary screening. However, contrary to this, the percentage of lipolytic positive isolates increased to 100% upon secondary screening. In the current study, the most potent isolate (EAC 9), isolated on the 4th day of composting at thermophilic temperature of 68°C was identified as *Bacillus velezensis.* Results from this study is similar to that reported by Roslan *et al.* (2021), who discovered lipase producing *B. paralicheniformis* and *B. velezensis* from pre-thermophilic and thermophilic stage of a food waste digester. Recently, thermophilic halotolerant lipase producing *Bacillus* sp. was isolated from a compost manure pit at 60°C (Nomwesigwa *et al.* 2023). Girdhari & Pathak (2022), also isolated lipase producing *B. licheniformis* from hot spring of Ganeshpuri. In another recent study, thermophilic multi enzyme producing *Bacillus* and *Geobacillus* species have been implicated in the aerobic thermophilic biodegradation of food waste (Ji *et al.* 2023). To provide a cheaper and more cost-effective substrate for lipase production, SNC was used as a carbon source. The increase in lipase activity as a result of olive oil supplementation could be attributed to the fact that olive oil is high in Oleic acid, which most lipases prefer (Dwi *et al.* 2021). Some researchers, notably Alex *et al.* (2017) found an increase in lipase activity from 31.43 u/gds to 157.33 u/gds when olive oil was utilized to substitute poultry fat during *Candida viswanathii* lipase synthesis. Several researchers have reported lipase production from edible oil such as palm oil, sun flower oil, soy bean oil (Barbosa *et al.* 2011, Isiaka Adetunji & Olufolahan Olaniran, 2018, Ramani *et al.* 2013). However, due to food competition, this strategy is not sustainable. Lipase production from non-edible fatty sources reduces food competition and ensures its long-term viability. The capability of *B. velezensis* to utilize SNC as substrate for lipase production will reduce the environmental menace caused by the accumulation of SNC on the mill. Lipase from the EAC 9 isolate could also be applied in shea mill effluent treatment before being discharged to the water bodies. Lipase production by *Stenotrophomonas maltophilia* from neem cake and coconut cake was 58.737 U/mL and 61.991 U/mL, respectively (Neethu *et al.* 2015), which was higher than the 41.7 U/mL reported for SNC in this investigation. However, the addition of olive oil increased lipase production to 103 U/mL, above that of Neethu *et al.* (2015). Bacteria have distinct growth patterns that allow for maximum enzyme release. In the current study, a decline in lipase activity beyond the 24 h, which could probably be due to the depletion of nutrient and build-up of other metabolites was observed for all three substrates. According to Purkan *et al.* (2020), *Proteus* sp. produce lipase optimally at 19 h with the activity of 1.771 U/mL at 45°C. While Dwi *et al.* (2021) reported a lesser time of 18 h optimum for lipase production by *Bacillus* sp. isolated at the mesophilic phase of composting process of domestic waste. The ability of *B. velezensis* EAC9 to produce higher enzyme activity within 24 h is remarkable. This ability of *B. velezensis* EAC9 is useful in industrial applications where higher lipase activity is required to boost productivity and minimize production costs. The RSM is an important tool for increasing production while lowering costs and improving system performance. The RSM statistical methods are useful for refining, evolving, and optimizing procedures in which a response of interest is adjusted by a variety of objectives and variables in order to optimize this response (Baş & Boyacı, 2007). In the current study, RSM was used as a step-by-step and exploratory technique to establish interactions between four independent variables, including Tween 80, ammonium sulphate, sucrose, and olive oil concentration, and to discover the ideal conditions for maximum lipase production. The improvement in lipase activity due to higher levels of olive oil and Tween 80 is agreement with the findings of Ameri *et al.* (2019) who reported significant effect of olive oil (5%) on lipase production by *Bacillus atrophaeus* FSHM2. More so, Putri *et al.* (2020) observed that the presence of 1% olive oil, 0.5% tween 80 and 1% NaCl produced maximum yield of lipase (282 U/mL). Several other studies have reported olive oil having significant effect on lipase production (Balaji *et al.* 2020, Ilesanmi *et al.* 2020). However, Geoffry and Achur (2018) reported that olive oil has a repressive effect on

extracellular lipase production by *Fusarium solani* strain NFCCL 4084. Addition of sucrose revealed moderate improvement in lipase production upon interaction with Tween 80. Previous studies revealed that sucrose and other disaccharides like maltose and galactose have positive influence on lipase production (Abdel-Fattah *et al.* 2012, Ameri *et al.* 2019). Lipase production was improved by low concentration of ammonium sulphate. There are several accounts on the influence of ammonium sulphate on lipase production. Patel *et al.* (2021) in a study on optimization of media and culture conditions for the production of lipase by *Streptomyces tsukubaensis* reported that ammonium sulphate significantly affected lipase yield. In addition Kebabci & Cihangir (2022) reports on the effect of various nitrogen sources on lipase production by *Candida tropical* showed that only ammonium sulphate increased lipase production whereas urea, peptone and casein did not show a distinct effect. In contrast, Geoffry & Achur (2018) reported that ammonium sulphate had a negative influence on lipase production by *Fusarium solani* NFCCI 4084. In addition, the findings of Akhter *et al.* (2022) on the effect of nitrogen sources on lipase production by *Bacillus cereus* NC7401 showed that only tryptone increased lipase production whereas ammonium nitrate, yeast extract and peptone suppressed the growth of the bacteria. Meanwhile, casein influenced maximum lipase production by a new *Actinomycetes* among other nitrogen sources tested (Patel *et al.* 2021). Therefore, lipase response to organic nitrogen sources varies with preference from different bacteria. Lipase production was improved by 4.79-fold at optimal level of 1.0% (v/v) of olive oil, Tween 80 1.0% (v/v), sucrose 1.0% (w/v) ammonium sulphate 0.1% (w/v). It is interesting to note that the thermophilic EAC9 isolate requires lower nutritional concentrations to produce maximal lipase. This distinguishing feature is beneficial for biotechnological and commercial applications since lower nutrient requirements minimize production costs. As a result, in lipase production studies, it is critical to look for bacteria with minimal nutrient requirements. The current findings are limited since they solely address nutritional parameters and ignore the physical parameters. However, the promising results in this study has sparked interest in further research into the effects of physical parameters such as temperature, pH and agitation on lipase synthesis by bacteria. According to Lau *et al.* (2023), a 1.6 –fold increase in lipase production from waste engine oil was recorded using RSM. Also using RSM, a 1.15-fold increase in lipase production from *Lactobacillus fermentum* was reported (Fathi *et al.* 2022). The design of experiment and analysis were effective in revealing the parameters significant for optimum conditions for maximum lipase production. Improvement of lipase production by the thermophilic *B. velezensis* EAC9 using CCD-RSM was considerably higher than that of *B. amylolequifacience* 41.20 U/mL on soybean extract (Mazhar *et al.* 2023), thermotolerant *Bacillus* sp. 8.88 U/mL (Kaur & Gupta, 2023), mutant *B. licheniformis* MLP 52.22 U/mL (Lakshmi & Dhandayuthapani 2022) and *Bacillus cereus* 51 U/mL (Abdelkader *et al.* 2023). The potential of lipase from *B. velezensis* EAC9 can be fully explored by studying its thermostability, pH stability, tolerance to metal ions and chemicals which is suggested for future studies.

Conclusion

In the current study a total of 11 thermophilic bacteria isolated from hot compost were screened for lipase production capacity. Thermophilic *B. velezensis* EAC9 produced highest lipase activity at 65.22 U/mL on olive oil media which was increased to 103 U/mL when supplemented with SNC. The RSM optimization studies revealed that olive oil, Tween 80, sucrose and ammonium sulphate significantly affected lipase production by *B. velezensis* EAC9 on the SNC substrate. Overall lipase production was increased by 4.79-fold from the optimization of nutritional parameters. The study further reiterates the potential of SNC as a low-cost substrate for lipase production and the improvement in lipase production by the CCD which can provide a baseline cost-effectiveness for industrial production of lipase by *B. velezensis* EAC9.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: All data are available within the study.

Author Contributions: Concept: M.O.K., Design: M.O.K., E.A.A., Execution: M.O.K., E.A.A., Material supplying: M.O.K., E.A.A., Data acquisition: E.A.A., Data analysis/interpretation: E.A.A., I.B.O., A.A., Writing: M.O.K., E.A.A., Critical review: I.B.O., A.A.

Conflict of Interest: The authors have no conflicts of interest to declare.

Funding: The authors declared that this study has received no financial support.

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