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Evaluation of Lipase from an Indigenous Isolated *Bacillus* Strain for Biodiesel Production

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ABSTRACT

Lipases are utilized in biodiesel production utilizing various types of substrates. The use of lipase in bioenergy production aims to reduce energy crises and environmental pollution. Lipase-producing indigenous bacteria *Bacillus licheniformis* (Accession no. OP56979) and *Bacillus rugosus* (Accession no. OP56980) were isolated from various oil-contaminated sites. The isolated potential lipolytic bacteria were screened for maximum lipase production. Then, the bacteria showing the highest lipolytic activity were subjected to identification using the 16s rRNA technique while other isolated were identified biochemically. Lipase [LipBL-WII(c)] from *Bacillus licheniformis* having the highest lipolytic activity expressed various characteristics. Characterization of crude LipBL-WII(c) expressed that it showed stability in a wide range of pH (4 to 10) with optimum lipolytic activity observed at pH 8. It was then found to be active at a temperature range from 20°C to 80°C with optimal at 50°C. Lipase activity was also stimulated in metal ions such as Ca^{+1}, Mg^{2+}, and Zn^{2+} the most. Furthermore, LipBL-WII(c) retained lipolytic activity in the presence of various organic solvents and surfactants. The kinetic parameters (Km and Vmax) for LipBL-WII(c) were ascertained using Lineweaver- Burk plot. LipBL-WII(c) showed a potential for biodiesel production using olive oil as a source. Lipase gave 84% yield of biodiesel production from olive oil. Thus, it could be employed as a potential candidate for green biodiesel production using oil sources.

INTRODUCTION

Lipases exist widely in nature while being available greatly. Having low production cost lipases from microbial sources are more favorable. The most common sources of lipases are bacteria and fungi, and the lipases produced by them are generally extracellular (Sarmah et al. 2018). Based on the total sales volume, lipases are considered the third biggest group of enzymes, followed by proteases and amylases. Lipase production is a business of billions of dollars due to its extensive range of applications (Zhao et al. 2021). Over the decade, various bacterial lipase has been stated from different species, including *Pseudomonas* spp., *Burkholderia* sp., *Bacillus* spp., *Streptococcus* sp., and *Staphylococcus* sp. (Bharathi & Rajalakshmi 2019, Priyanka et al. 2019, Rmili et al. 2019).

Industries showcase significant commercial applications performed by Lipases (E.C. 3.1.1.3). Hydrolysis of triacylglycerols into free fatty acid, and glycerol was stimulated by Lipases (Javed et al. 2018). Various applications of lipase include fat and oil hydrolysis, flavor enhancement in food processing, analysis of chemicals, racemic mixture resolution, fat modification, and synthesis of organic materials (Bento et al. 2017, Rios et al. 2018). Various habitats, like wastes from industries, dairies, oil-contaminated soil, etc., were found to be inhabited by lipase-producing microorganisms. Innovative technologies have been utilized on the microbial lipase for degrading and detoxifying the oil effluents (Gururaj et al. 2016).

Recently the interest of researchers has increased in enzymatic transesterification using lipase for biodiesel production. The capability of the lipase (EC 3.1.1.3, triacylglycerol hydrolases) to maintain notable catalytic activity in non-aqueous media made its usage possible in biodiesel production (Sarmah et al. 2018). In biodiesel production, compared to chemical catalysis, lipase uses various advantages like; easy separation of product, easy recovery of glycerol, minimal wastewater treatment requirements, and the absence of side reactions. Due to maintaining a high-temperature environment for alkaline catalysis, hydrolysis of triglycerides produces many free fatty acids (FFAs) in waste cooking oil, leading to various problems in alkaline catalytic biodiesel production (Tripathi et al. 2014). On the contrary, lipase can produce biodiesel

using high FFA feedstocks as it can convert triglycerides and FFAs into FAAEs.

The present work aimed at Isolating, and screening lipase-producing bacteria from various samples, identification of the isolates utilizing both biochemical and molecular methods (16s rRNA sequencing), characterization of the biochemical properties of the crude lipase along with the determination of kinetic parameters for crude lipase and the biodiesel producing potential of the crude lipase.

MATERIALS AND METHODS

Isolation of Bacterial Strains

Samples of wastewater, soil, and sludge rich in lipid sources were collected from different sites near Hisar district, Haryana, India were used after removing debris as listed in Table 1. 1mL or 1g of the samples were dissolved in 10 mL sterilized saline solution and were shacked evenly. Then, they were serially diluted from 10^{-1} to 10^{-6} in the saline solution. 1.0% suspension from all the dilutions was then used as an inoculum to be grown on the NA plates using the spread plate method for obtaining the bacterial colonies after the plates were subjected to 24 h of incubation at 37°C. The bacterial colonies appearing after 24 h of incubation were restreaked several times on the nutrient agar plates to obtain pure bacterial cultures.

Primary Screening for Lipase Production

The primary screening for lipolytic bacteria was done by streaking the pure bacterial cultures on TBA (Tributyrin Agar) plates. The TBA media comprised: 0.5% Peptone, 0.3% Beef extract, and 2.0% Agar supplemented with 1.0% Tributyrin as a carbon source at 7.5 pH. The media plates were kept at 37°C for 48 h of incubation. The bacterial cultures depicting visible hydrolysis zone around the colonies on the media were then further analyzed for the quantitative determination of lipase activity using agar well diffusion assay (cup well method). Solid media comprising different carbon sources like olive oil, Tween- 20, and Tween- 80 were assessed for lipolytic activity. The detection of enzymatic activity was done by observing visually and measuring the clear zones on the surface of the agar.

Table 1: Sample collection for bacterial strain isolation.

S.No.	Sample code	Source
1.	W(I)	Wastewater from Stainless Steel Industry, Hisar
2.	W(II)	Waste Water from Paper Mill, Panipat
3.	W(III)	Sweet Shop Drainage, Hisar
4.	W(IV)	Restaurant Drainage, Hisar
5.	W(V)	Restaurant Drainage, Hansi
6.	W(VI)	Wastewater from STP, Gangua
7.	W(VII)	Sewage discharge from random house, Hisar
8.	W(VIII)	Sewage water from GJU, Hisar
9.	S(A)	Sludge from local sewer hole, Sector-15, Hisar
10.	S(B)	Soil from a Local auto workshop, Hisar
11.	S(C)	Soil from Automarket, Hisar

Secondary Screening of Lipolytic Bacterial Strains Using Various Media

Phenol red agar: Phenol red agar media comprising: 1.0% Peptone, 0.5% NaCl, 0.01% $CaCl_2$, 0.01% Phenol red, 2.0% Agar and 1.0% Olive oil was prepared, and pH was set at 7.0. The bacterial cultures showing clear zones on TBA were fed into the sterile NB (nutrient broth) media and then incubated at 37°C for 24 h. 50 µL of the obtained new cultures supernatant was poured into the bored wells on plates prepared from phenol red agar media. Then incubation was done at a temperature of 37°C for 48 h. The sterile nutrient broth was employed as a control. The color change from red to yellow around the wells indicated lipase activity. All the experiments were performed in triplicates.

Tween- 20 agar: Tween-20 agar media comprising: 1.0% Peptone, 0.5% NaCl, 0.01% $CaCl_2$, 2.0% Agar and 1.0% Tween-20 was synthesized. 50 µL supernatant of the new bacterial cultures was added in the wells bored on plates prepared from agar media containing Tween- 20 and then subjected to incubation at a temperature of 37°C for incubation of 48 h. The control was used in the form of sterile nutrient broth. Noticeable precipitation around the wells indicated the existence of the activity of lipase. Experiments were performed in triplicates.

Identifying the Lipolytic Bacterial Strains

Physical and Biochemical Characterization

Physical characterization: Morphological and colony characteristics were studied from cultures grown on nutrient agar plates. The shape of the bacterial cells was seen by using Gram's staining method.

16srRNA sequencing: The Chromosomal DNA of the bacterial strain was hauled out by employing a spin column kit (HiMedia) (Clarridge 2004) and was amplified using PCR using a thermal cycler and Exo-SAP (Exonuclease I -Shrimp Alkaline Phosphatase) was utilized for the process of purification (Darby et al. 2005). The purified amplicons were sequenced using the Automated Sanger Sequencing method (ABI 3500xL genetic analyzer, USA). BLAST

was employed for scrutinizing the resulting sequences with flanking culture sequences recovered from the NCBI database that discovers the similarity regions locally amongst the sequences (Altschul et al. 1990). The program then relates the retrieved nucleotide sequences to sequence databases to calculate the match's statistical significance (Gertz 2005). The BLAST algorithms were employed to infer the relationships related to evolutionary and functional parameters amongst the sequences to find members of the gene families. BLASTN program was then primarily used to discover the potentially closely related strains (Altschul et al. 1990). The multiple sequence alignment tool, CLUSTAL W, was employed to align the sequences. MEGA (Molecular Evolutionary Genetics Analysis Version 6.0) was employed for the phylogenetic tree construction (Karlin & Altschul 1990).

Growth Kinetics and Lipase Production

The isolates showing significant zones on the above-mentioned media were inoculated in 15 ml of inoculums media comprising: 2.0% Glucose, 1.0% Yeast extract, 1.0% Peptone, 1.0% $CH_3COONa.3H_2O$, 0.009% $MgSO_4$, 0.003% $MnSO_4$, 0.15% $CuSO_4.5H_2O$, 0.05% KCl, 0.5% olive oil with 7.5 pH. The flasks of inoculums were then subjected to incubation at a temperature of 37°C overnight at 120 rpm. Then the submerged cultures were mixed with 100 ml of lipase production media: 0.2% Peptone, 0.25% NaCl, 0.04% $CaCl_2.2H_2O$, 0.1% $NH_4H_2PO_4$, 0.04% $MgSO_4.7H_2O$, and 2.0% Olive oil. The flasks were then further incubated at 37°C at 120 rpm for 120 h. 1.0M NaCl and 1.0M HCl were used to maintain the medium pH and adjusted to 7.2. The samples were drawn at 12-hour intervals from the production media to determine bacterial growth and lipase activity. The optical density at 600 nm was measured using a UV-Vis spectrophotometer to determine the cultures' bacterial growth. To determine the lipase activity of the samples, a refrigerated high-speed centrifuge was used at 10,000 rpm for 10 min at 4°C. The lipolytic activity of the crude enzyme was then assayed. The lipase activity was analyzed using a Colorimetric assay as distinct by Winkler & Stuckmann (1979).

Lipase Assay

The hydrolysis activity of lipase towards p-nitrophenyl palmitate (NPP) was recorded according to the colorimetric method with minor modifications (Gricajeva et al. 2019). The stock solution of the substrate pNPP (p-nitrophenyl palmitate, 20 mM) was prepared using isopropanol. The final reaction mixture was prepared by mixing 75 µL of pNPP of stock solution in 3 mL of Tris buffer (0.05 M, pH-8.0) and then subjected to incubation at 70°C inside a water bath for 10 minutes. After that, 25 µL of the crude enzyme was supplemented into the reaction mixture, followed by incubation at 35°C in a water bath for 30 minutes. After 30 min, adding 1 mL of stopping reagent (chilled acetone-ethanol (1:1)) halted the enzymatic reaction. In the control (blank) set, the addition of the crude enzyme step was skipped. The liberation of the yellow-colored compound (p-nitrophenol) in the reaction mixture was measured at the wavelength of 410nm alongside a reagent blank about the p- nitrophenol standard curve (2 to 20 $\mu g.mL^{-1}$ in 0.05M Tris HCl buffer (pH-8.0). The same assay was carried out in triplicates leading to the presentation of the mean values. One unit (U) of enzyme activity is defined as a micromole (µM) of the liberated p-nitrophenol by the p- nitrophenyl ester breakdown by 1.0 ml of the soluble enzyme every minute at a temperature of 35°C beneath the standard assay conditions.

The enzyme activity ($U\ mL^{-1}\ min^{-1}$) was calculated using equation 1.

$$\text{Lipase activity (Units } mL^{-1}min^{-1}) = \frac{\mu \text{ moles of p-nitrophenol liberated}}{Volume\ of\ enzyme\ (0.025mL) * Incubation\ time\ (30)} \quad \ldots(1)$$

Specific activity is the enzyme activity per mg of the total protein (expressed in µ mol min^{-1} mg^{-1}) using equation 2.

$$\text{Specific enzyme activity } (Umg^{-1}) = \frac{\text{Enzyme activity (U/mL)}}{\text{Total protein content (mg/mL)}} \quad \ldots(2)$$

Determination of Protein content: protein content

The protein concentration was estimated using Bradford's method, in which BSA (Bovine Serum Albumin) is a standard protein (Bradford 1976).

Characterization of Crude Lipase

Effect of Temperature: The optima of temperature for crude lipase has been estimated by incubating crude enzyme aliquots at various temperatures ranging from 20-80°C for 24 h at 200 rpm, maintaining pH at 8 using Tris-HCl buffer. The maximum activity was considered 100% at the beginning of incubation.

Effect of pH: The optima of pH for crude lipase was estimated after the incubation of LipBL-WII(c) in various buffers (50mM): pH- 2 (glycine- HCl), pH 3-5 (acetate-acetic acid), pH 6-7 (phosphate), pH 8- 9 (Tris-HCl), pH 10.0 (glycine- NaOH), pH 11.0 (phosphate- NaOH) and pH 12 (KCl- NaOH). The maximum LipBL-WII(c) activity was established as 100% relative lipolytic activity.

Effect of organic solvents: For determining the impact of organic solvents (ethanol, isopropanol, hexane, chloroform, and methanol) on the enzyme activity, LipBL-WII(c) was

incubated with different organic solvents at a concentration of 50% (v/v) for 6 h at 37°C at 150rpm. The lipolytic activity of control (in the absence of organic solvent) was considered 100.0%.

Effect of metal ions: Crude lipase, LipBL-WII(c) was incubated with metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, and Zn^{2+}) of 1mM concentration at 37°C temperature with 150 rpm for 6 h to assess the impact of metal ions on enzyme activity. Control was devoid of the metal ion solution. The change in the lipase activity compared to the control's activity was referred to as the relative activity.

Effect of surfactants: Lipase was incubated in the occurrence of various surfactants (Tween- 20, 80, SDS, and Triton X-100) at a concentration of 1.0% for 6 h at 37°C temperature with 150 rpm to assess their effect on the enzyme activity of lipase. No surfactant was used in the sample solution used as a control. The change in the lipase activity compared to the control's activity was referred to as the relative activity.

Kinetic Parameters of Crude Lipase

The velocity of reaction for crude lipase was studied utilizing p- NPP (p-nitrophenyl palmitate) as a substrate at a variable concentration of (0.1-10mM) in buffer Tris- HCl (50 mM, pH 8.0). The Michaelis constant (Km) and maximum velocity (Vmax) were estimated utilizing the Lineweaver-Burk plot.

Biodiesel Producing Potential of Crude Lipase

Biodiesel production was planned to refer to (Yang et al. 2009) with fewer modifications. A 7.89 ml of Olive oil was mixed with 0.99 mL of methanol and kept in glass tubes with screw caps, then added 2.6 mL of crude lipase and incubated at 40°C temperature along with shaking at 220 rpm for 48 h. After the incubation period, 200 μL of the sample was withdrawn from the reaction's mixture and then diluted using 1.0 mL of n-hexane for 2min. Afterward, the centrifugation of samples was done at 10000 rpm for 15 min, and 10 μL of the upper layer was subjected to a Thin Layer Chromatography (TLC) plate. Methyl oleate was utilized as a reference for biodiesel in TLC. After the development of the plate in 90:10:1 (n-hexane/ ethyl acetate/ acetic acid), then iodine vapor was used for visualizing the spots for a short period of time. The yield of the biodiesel production was calculated using Equation 3.

$$\text{Yield} = \frac{Volume\ of\ Biodiesel\ produced}{Volume\ of\ oil} \times 100 \quad \ldots(3)$$

RESULTS AND DISCUSSION

Isolation, Screening and Identification

Lipid-rich wastewater, soil, and sludge samples were collected from sites near Hisar district, Haryana, India. The sites were chosen rationally to offer a good atmosphere for the lipolytic bacteria to thrive. 36 isolates of bacteria were isolated on NA (nutrient agar) medium. The purified isolated bacterial strain is shown in Fig. 1. All 36 strains were screened for lipase activity by streaking them onto a Tributyrin agar medium (TBA). Out of 36 bacterial isolates, only 17 exhibited clear zones around the colonies on the Tributyrin agar (TBA) medium. The clear zones around

Fig. 1: Purified isolated WII(c).

Table 2: Zone production by lipase-producing bacterial isolates.

S.No.	Isolate No.	Zone Diameter (in mm)	
		Phenol Red Agar	Tween-20 Agar
1.	SA(b)	20	26
2.	SA(c)	18	26
3.	SA(d)	20	24
4.	SB(a)	14	26
5.	SB(d)	18	32
6.	SB(e)	12	14
7.	SC(d)	14	16
8.	WI(a)	20	22
9.	WII(a)	14	30
10.	WII(c)	30	66
11.	WIII(a)	18	24
12.	WIV(a)	14	32
13.	WIV(c)	18	34
14.	WV(a)	14	24
15.	WVI(a)	14	16
16.	WVII(a)	24	20
17.	WVIII(a)	24	16

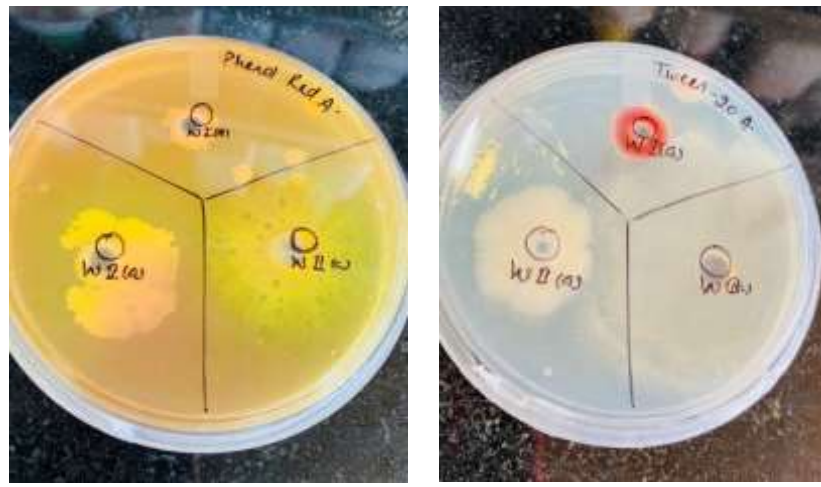

Fig. 2: Zone of hydrolysis around the wells by various isolated strains.

the bacterial colonies depicted the degradation of tributyrin into butyric acid by the lipolytic activity of the bacteria. As shown in Table 2, all 17 strains of bacteria were then monitored for the quantitative analysis of enzyme production employing solid media like phenol red and Tween- 20 agar media. In phenol, red, and tween-20 agar media, strain WII(c) exhibited the highest lipase production. At first, the strains were screened using a chromogenic approach by using the dye phenol red as an indicator and olive oil as an inducer substrate. The pH indicator was Phenol red, with a pH of 7.3-7.4 as the endpoint. The lipolytic reaction of olive oil ends in releasing fatty acids. In turn, the dye pH decreases, exhibiting yellow color zones around the wells indicating the lipase activity of the tested strain of bacteria (Patel & Desai 2018).

Further, Tween- 20 agar media consisted of tween- 20 being an inducer substrate. The lipolytic activity of the strains was depicted by the development of a big visible zone of precipitates of calcium salt surrounding the well, resulting from the reduction of tween-20 into fatty acids. Promoting ideal interaction between the enzyme or cells and substrates makes tween- 20 the commonly used substrate for detecting the lipolytic activity of bacteria. Fig. 2 shows the hydrolysis zone depicted by various isolated strains on different media.

Identification

The bacterial strain was initially identified by morphological characterization like Gram staining, which showed maximum lipolytic activity. Studies on the physiological characteristics of all the isolates were performed. The biochemical characteristics (Catalase, Oxidase, Indole, Methyl red, Voges- Proskauer, Nitrate reduction, Citrate utilization, Hydrogen sulfide production, gelatin hydrolysis) and tests of sugar fermentation were also carried out using standard reference biochemical tests for identification of bacteria genus as per Bergey's manual of systematic bacteriology as described in Table 3. Two strains, WII(c) and SA(c) were selected for further identification using 16SrRNA sequencing due to their exhibition of top-most lipolytic activity amongst all the other strains. BLAST search analysis of the strain WII(c) revealed that it exhibited close homology (99%) with *Bacillus licheniformis,* and SA (c) exhibited closed homology (99.92%) with *Bacillus rugosus* and multiple species. The study impacted the strain WII(c) as B. licheniformis and SA(c) as *Bacillus rugosus*. The 16S rRNA gene sequence was deposited in Gene Bank and obtained accession no. for *Bacillus licheniformis* (Accession no. OP56979) and *Bacillus rugosus* (Accession no. OP56980). Fig. 3 illustrates the constructed tree of phylogeny showing the evolutionary relationships of the strain WII(c) and SA(c) to other lipolytic *Bacillus sp*. The strains WII(c) and SA(c) came from the same genus. Hence, the strain with the maximum lipolytic activity, i.e., WII(c), was used for further study.

Growth Kinetics and Lipase Production

B. licheniformis WII(c) was grown using the basal medium for 120 h to determine the true growth phase, depicting the highest extracellular lipase production. Lipase production and bacterial growth were assayed on samples taken at every 12 h interval. A parallel relationship between lipolytic activity and cell growth was recorded, depicting lipase production as growth linked. The early log phase recorded the initiation of lipase production, and the optimum was reached throughout the late exponential growth phase. Maximum production of lipase (50 $U^{-1}.mL^{-1}.min^{-1}$) was detailed at 72 h of the incubation period (Fig. 4). This was

Table 3: Biochemical characterization of isolated lipolytic strains.

Isolate No.	Shape	Gram's Character	Biochemical Characteristics													Probable identity
			Indole	MR	VP	Citrate	H_2S production	Nitrate Red.	Catalase	Oxidase	Urease	Glucose	Lactose	Fructose	Xylose	
SA(b)	Rod-shaped	-	-	-	-	+	-	-	+	+	+	+	+		+	*Pseudomonas* sp.
SA(c)	Bacillus	+	-	-/+	-/+	-	-	+	+	-	-	+	+	+	-	*Bacillus* sp.
SA(d)	Coccus	+	-	+	+	-	-	+	+	-	+	+	+	+	-	*Staphylococcus* sp.
SB(a)	Coccus	+	-	-	+	-	+	+	+	-	+	+	+	+	-	*Staphylococcus* sp.
SB(d)	Bacillus	+	-	-/+	-/+	-	-	+	+	-	-	+	+	+	-	*Bacillus* sp.
SB(e)	Coccus	+	-	-	-	+	-	-	+	-	-	-	+	+	-	*Staphylococcus* sp.
SC(d)	Coccus	+	-	-	+	-	-	+	+	-	+	+	+	+	-	*Enterococcus* sp.
WI(a)	Coccus	+	-	-	-	+	-	-	+	-	-	+	+	+	-	*Micrococcus* sp.
WII(a)	Coccus	+	-	-	-	+	-	-	+	-	+	-	+	+	+	*Staphylococcus* sp.
WII(c)	Bacillus	+	-	-/+	+	+	-	+	+	-	+	+	-	+	-	*Bacillus* sp.
WIII(a)	Coccus	+	-	-	-	-	-	-	+	-	-	+	+	+	+	*Micrococcus* sp.
WIV(a)	Coccus	+	-	-	+	-	-	+	+	-	-	-	+	+	-	*Staphylococcus* sp.
WIV(c)	Coccus	+	-	-	-	+	-	-	+	-	-	+	+	+	-	*Staphylococcus* sp.
WV(a)	Coccus	+	-	-	+	+	-	-	-	-	-	+	+	+	-	*Enterococcus* sp.
WVI(a)	Coccus	+	-	-	+	-	-	+	+	-	+	-	+	+	-	*Staphylococcus* sp.
WVII(a)	Bacillus	+	-	-	-/+	+	-	+	+	-	-	+	-	+	-	*Bacillus* sp.
WVIII(a)	Bacillus	+	-	-	+	-	-	-/+	+	-	-	+	+	+	-	*Bacillus* sp.

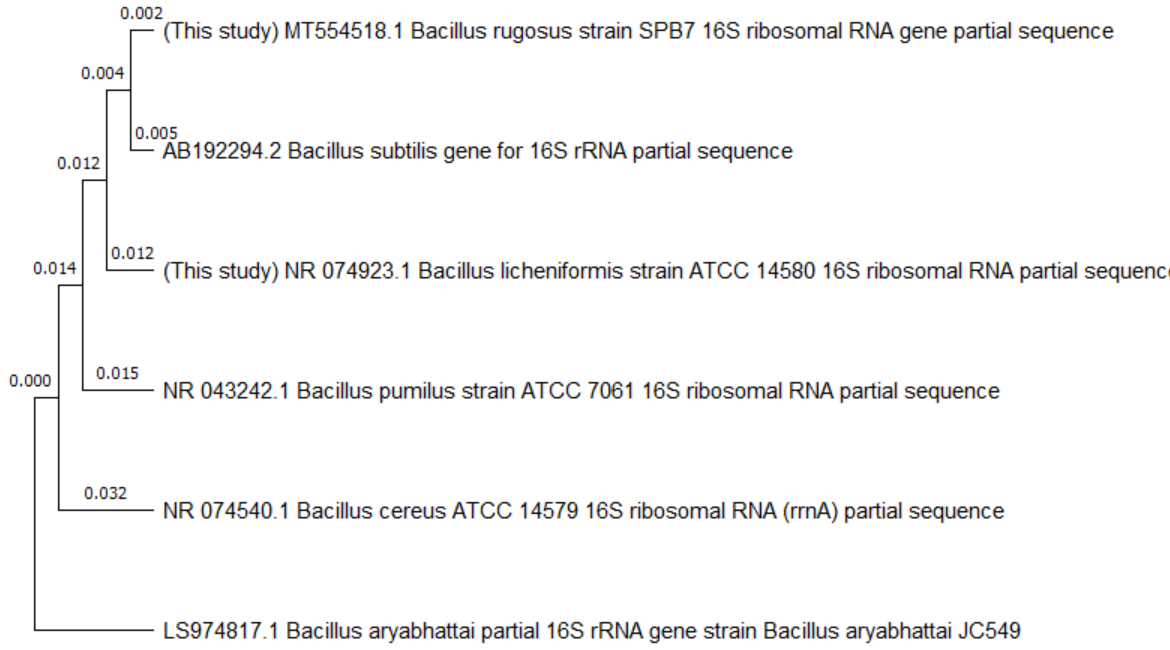

Fig. 3: Phylogenetic tree based on 16S rRNA gene sequences of WII(c) and SA(c) showing their similarity to other lipolytic *Bacillus* sp. strains.

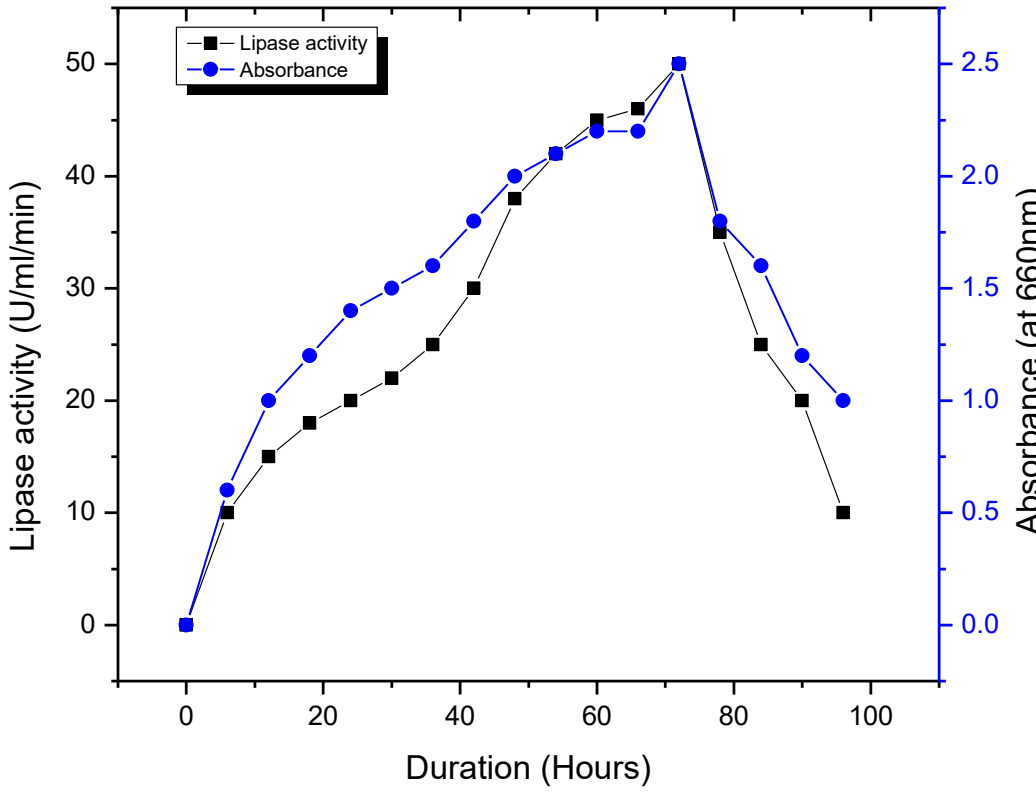

Fig. 4: Growth curve (■) and Lipase production curve (●) by *Bacillus licheniformis* WII(c).

comparable to the conclusions of Tripathi et al. (2014) and Zhao et al. (2021) showing the optimum production of lipase by a *micro bacterium* and *Staphylococcus caprae* through the late exponential phase of growth. The lipolytic activity was reduced after 72 h of incubation, possibly due to proteases in the fermentation media.

Characteristics of LipBL-WII(c)

Effect of temperature: The optimal temperature for LipBL-WII(c), a crude lipase from *Bacillus licheniformis*, was 55°C (Fig. 5 (a)). On increasing the temperature beyond 50°C, lipase activity decreased, indicating thermal destruction of the tertiary enzyme structure. The optimal activity of the lipase from *Anoxybacillus flavithermus* was at the temperature of 50°C and was detected to be firm from 25°C to 50°C for incubation of 24 h (Bakir & Metin 2016). The optimal temperature for the enzyme activity was 40˚C with decent stability at 10-30˚C for the lipase produced by the bacteria identified as *Acinetobacter haemolyticus* (Sarac & Ugur 2015). Saraswat et al. 2017 reported the alkaline and thermotolerant lipase (BSK-L) production from the strain *Bacillus subtilis*. Lipase has optimum activity at 37°C and exhibits active activity of lipase at the temperature range of 30°C to 60°C.

Effect of pH: The optimal pH of LipBL-WII(c) was estimated to be 8 and exhibited stability in pH from 4 to 10 (Fig. 5 (b)). Lipase from *Cohnella sp*. A01 showed optimum activity at pH 8.5 and was ideally stable at pH 8.5–10 for the incubation of 180 minutes (Golaki et al. 2015). The pH optima for the purified lipase produced by *Bacillus subtilis* was pH 8 (Mazhar et al. 2016). Rmili et al. (2019) reported that lipase SCL produced by *Staphylococcus capitis* retained greater than 60% of its initial activity over extensive pH values ranging from 5 to 11 at room temperature. Sarac and Ugur (2015) reported that the lipase produced by *Acinetobacter haemolyticus* showed the pH optima at 9 with good stability at pH ranging between 5.0- 11.0.

Effect of organic solvents: LipBL-WII(c) from *B. licheniformis* WII(c) was incubated in the presence of 50% (v/v) organic solvents for 6 h and observed notable retaining of relative activity with hexane, ethanol, methanol, and isopropanol while moderately tolerant to chloroform as shown in (Fig. 5 (c)) stating that this lipase unveiled decent stability with the utmost of the organic solvents. Similarly, *Bacillus sp*. was tolerant to methanol, ethanol, and acetonitrile, making the strain produce a solvent-tolerant lipase enzyme, rendering it a likely nominee of the solvent-tolerant lipase for a variety of industrial applications

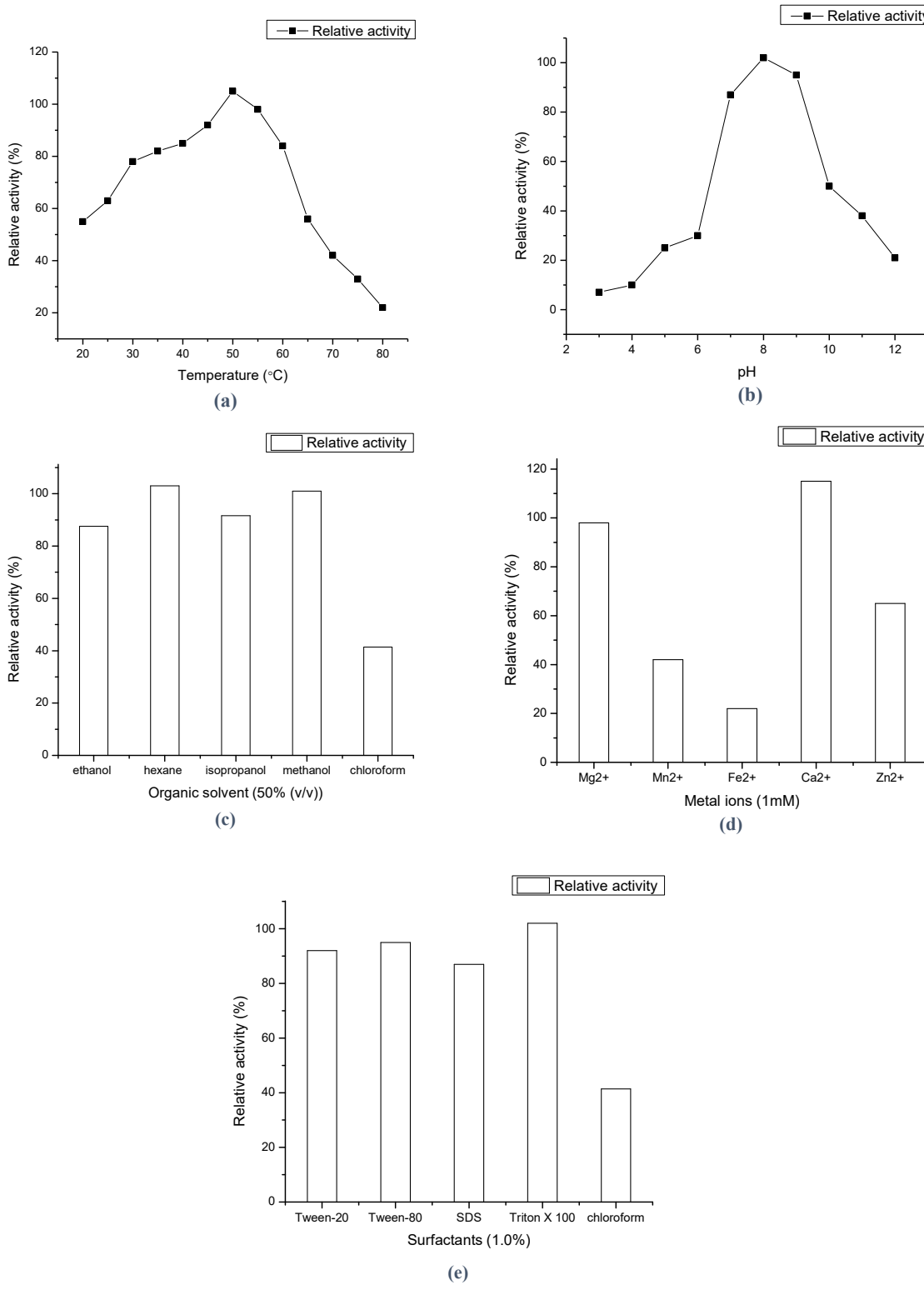

Fig. 5: Effect of various characteristics on the lipolytic activity of LipBL-WII(c). (a) Temperature (b) pH (c) organic solvents (d) metal ions (e) surfactants.

(Jaiganesh & Janganathan 2016). Lipases tolerant to organic solvents are crucial for the production of biopolymeric, fine chemicals, and biodiesel material production (Javed et al. 2018). The lipase produced by *Aureobasidium pullulans* exhibited outstanding stability in some 30% (v/v) organic solutions, including hexane, DMSO (dimethyl sulfoxide), n-propanol, and isopropanol (Li et al. 2019).

Effect of metal ions: LipBL-WII(c) was incubated with 1mM molarity of metal ions for six h and perceived a decent tolerance in the occurrence of Ca^{+1}, Mg^{2+}, and Zn^{2+} (Fig. 5(d)). Likewise, augmentation of the lipolytic activity in the presence of K^{+} and Ca^{2+} ions was conveyed by (Wang et al. 2012). A decrease in the lipase activity on treatment with EDTA (a metal ion chelator) along with Ca^{2+} (71.5%) and Mn^{2+} (62.8%), as metal ions assist in the structural and functional maintenance of the enzyme was described by Golaki et al. (2015). While lipase activity of the extracellular lipase from *Azospirillum sp*. was decreased by adding Mg^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} metal ions (Lestari et al. 2016). Li et al. (2019) showed that Ca2+ heightened the lipase catalytic activity produced from Aureobasidium pullulans and was marginally inhibited by Zn^{2+} and Mn^{2+} at a concentration of 10 mmol.L^{-1}.

Effect of surfactants: The effect of different surfactants on the LipBL-WII(c) was examined by incubating the lipase with various surfactants for six h. The relative activity was upgraded by adding Triton X-100 while it was retained by Tween 20, Tween 80, and SDS (Fig. 5(e)). On the other hand, Golaki et al. (2015) stated that the bio-catalytic activity of the enzyme was considerably reduced to 16.2% by the surfactant SDS. Non-ionic detergents cause disintegration by decreasing the hydrophobic interactions amongst the lipase enzyme, stabilizing the enzyme activity. At the same time, SDS affects the enzyme's denaturation by destroying the disulfide linkages (Sajna et al. 2013). Kaur et al. (2016) reported that the enzyme produced by *Bacillus licheniformis* could preserve its activity in the occurrence of different detergents (Tween- 20, 40, SDS and Triton X-100). Li et al. (2019) described that the lipase from *Aureobasidium pullulans* was stimulated by the non-ionic surfactants (Tween- 80, Triton X- 100, and Tween 20) and the anionic surfactant SDS. Still, it was severely repressed by the cationic surfactant CTAB.

Kinetic Parameters of Lipase

Lineweaver-Burk plot was used to determine the kinetic parameters (Km and Vmax) of the enzyme lipase. The values of Km and Vmax for lipase by utilizing pNPP (p-nitrophenyl palmitate) as a substrate were determined to be 2.05 mM and 6.02 $mmoles^{-1}min^{-1}mg^{-1}$, respectively (Fig. 6). A

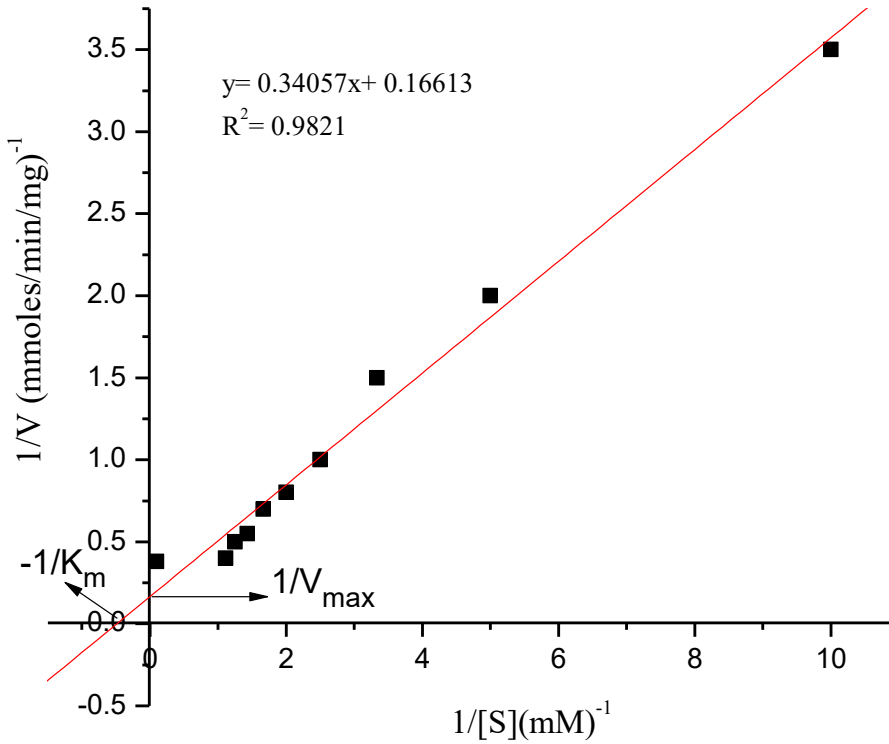

Fig. 6: Line weaver-Burk plot of LipBL-WII(c) from *Bacillus licheniformis*.

low value of Km implies a greater affinity of the enzyme towards its substrate. In comparison, a high Km implies less affinity. Tripathi et al. (2014) stated the values of Km and Vmax being 3.2 mM and 0.005 $mmoles^{-1}min^{-1}mg^{-1}$ for lipase from *Microbacterium sp*. while which depicted the interaction of enzymes toward its substrates. Sarac and Ugur (2015) described the lipase production from *Acinetobacter haemolyticus* depicting lipase's Km and Vmax as 0.8 mM and 3.833 $mmoles^{-1}min^{-1}mg^{-1}$, respectively. Km and Vmax values of the lipase production by the native *B. subtilis* strain Kakrayal_1 (BSK-L) were perceived to be 2.2 mM and 6.67 $mmoles^{-1}min^{-1}mg^{-1}$, respectively (Saraswat et al. 2018). Sharma and Kanwar (2015) verified the Vmax and Km of the purified lipase of *Bacillus licheniformis* SCD11501 were to be 2.27 and 0.43 $mM^{-1}mmol^{-1}ml^{-1}min^{-1}$, respectively, for the breakdown of p-NPP. The values of Vmax and Km of purified lipase for isolates G14, B10, and OI were 17.6, 24.4, and 135 $mmoles^{-1}min^{-1}mg^{-1}$ and 1.3, 1.6, and 0.681 mM, respectively (Shart & Elkhalil 2020).

Biodiesel Production Potential of Crude Lipase

LipBL-WII(c) catalyzed biodiesel production in the occurrence of olive oil and methanol. The production of biodiesel was confirmed by the TLC plate assay (Fig. 7). The separation of compounds in TLC is based on the competition of solute and mobile phase for the binding place on the stationary phase. As the biodiesel sample being separated was colorless, iodine vapors were used as a general non-specific color reagent. The biodiesel sample separated at the same retention factor (R_f of 0.8) as the standard used (methyl oleate). The yield of the biodiesel produced using olive oil was 84%. LipBL-WII(c)'s characteristics, properties, and stability amongst organic solvents make them useful for the trans-esterification process for biodiesel production as a bio-catalyst. Lipase-mediated biodiesel production depicts various merits like; the requirement of low energy and water for isolation of product, moderate reaction conditions, and less alcohol required during the reaction. The essential reaction time for different lipases is 5–72 h (Ugur et al. 2014).

CONCLUSION

In the stated investigation, 17 indigenous lipase-producing strains were isolated and biochemically identified, out of which 2 were identified using the 16srRNA sequencing method. The two strains were: *Bacillus licheniformis* and *Bacillus rugosus*. Lipase produced from WII(c) had the highest lipolytic activity; hence, it was characterized and evaluated for biodiesel production efficiency. Microbes growing indigenously are found to be economical, better adapted to biotic and abiotic stress, and stable. Characterization of crude LipBL-WII(c) divulged that it showed stability in a wide pH range of (4 to 10) with optimum

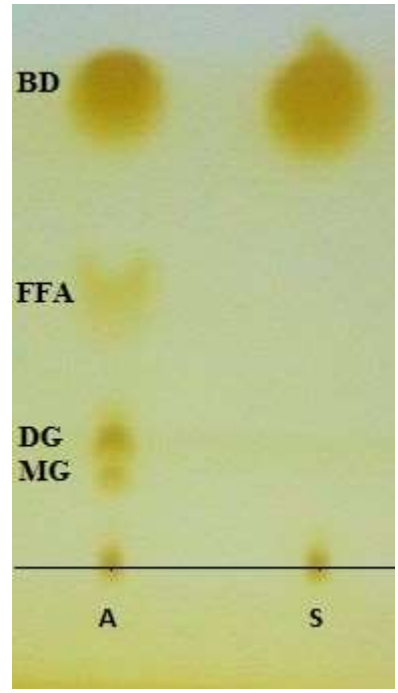

Fig. 7: LipBL-WII(c) catalyzed biodiesel production, TLC plate. S: standard (methyl oleate), A: sample. BD: biodiesel, DG: diglyceride, FFA: free fatty acid, MG: monoglyceride.

lipolytic activity observed at pH 8. LipBL-WII(c) was then found to be lipolytic at a temperature range from 20°C to 80°C, having 50°C as optima. Lipase activity was also stimulated in metal ions like Ca^{+1}, Mg^{2+}, and Zn^{2+} the most.

Furthermore, LipBL-WII(c) retained its lipolytic activity in various organic solvents and surfactants. The kinetic parameters (Km and Vmax) for LipBL-WII(c) were ascertained using Lineweaver- Burk plot. LipBL-WII(c) showed a potential for biodiesel production using olive oil as a source. Thus, it can be employed as a potential candidate for green biodiesel production using oil sources. However, further work is required to optimize lipase production, its purification, and the characterization of purified LipBL-WII(c) for more efficient biodiesel production using oil sources.

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