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Isolation, Characterization, and Lipase Production of Lipolytic Yeasts Isolated from Mt. Makiling Forest Reserve, Philippines

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Abstract

Mt. Makiling Forest Reserve (MMFR) is an untapped source of microorganisms that have great potential for the production of industrially important enzymes like lipases. In this study, yeasts were isolated from various forest materials by serial dilution and plating methods. Out of the 185 isolated putative yeasts, 54 showed lipolytic activity in the plate assay using a lipase production medium. These yeasts were secondarily screened by a liquid assay using basal mineral medium (BMM) with 2.0% (v/v) olive oil. Top putative yeasts based on measured cell density after incubation in BMM were molecularly identified as *Candida duobushaemulonis*, *C. jaroonii*, *C. quercuum*, *Vanrija humicola*, and *Wickerhamomyces* sp. by ITS-5.8S sequencing. Further screening of the identified lipolytic yeasts using non-enriched (with 0.5% peptone) and enriched (with 5.0% peptone) medium revealed that *Wickerhamomyces* sp. SS-2 was the top lipase-producing yeast, with the highest measured lipolytic activity of 0.62 U/mL after 48 h of enriched media fermentation. The performance of the select yeast was then tested on different fermentation mediums with different compositions. Medium composed of 0.2% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4.H_2O$, 0.01% $CaCl_2$, 0.01% NaCl, and 1.0% (v/v) olive oil, at pH 6.0, was found best for the select yeast's lipase productivity. Furthermore, olive oil as the main plant oil and the use of a 48h-old slant seed culture were found best for the lipase production of the select yeast *Wickerhamomyces* sp. SS-2.

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Introduction

Due to the interplay of unique environmental factors, forests are ecological zones that serve as a home to a variety of biological species including microorganisms. These microorganisms have vital roles in biomass decomposition, carbon and nutrient cycling, and linking plant and ecosystem functions (He *et al*., 2017). Since forest materials mainly plant biomass, some microorganisms, mostly fungi, are capable of degrading lignocellulosic materials with the help of three key enzymes, cellulase, laccase, and lipase (Haldar and Banerjee, 2022). Cellulase degrades cellulose, laccase, together with other ligninolytic enzymes, degrades lignin, and lipase serves as a catalyst by breaking down acids and resin that are formed during lignocellulose degradation (de Melo *et al*., 2018). These hydrolytic enzymes, specifically lipase and cellulase, have broad industrial applications.

Lipases (triacylglycerol ester hydrolases E.C. 3.1.1.3) catalyse the hydrolysis of water-insoluble esters such as triacylglycerols to produce di- and monoacylglycerols, free fatty acids, and glycerol (Singh *et al.,* 2019). Lipases are considered one of the most versatile biocatalysts as they are also capable of producing glycerides from glycerol and fatty acids by catalysing reverse reactions in non-aqueous conditions such as esterification, interesterification, and transesterification (Ramos-Sanchez *et al*., 2015). It has been used for organic syntheses, hydrolysis of fats and oils, flavour enhancement in food processing, resolution of racemic mixtures, and chemical analyses (Aravindan *et al*., 2007). In addition, lipases are also capable of catalysing acidolysis, alcoholysis, and aminolysis of triglycerides. In general, lipases have broad substrate specificity with optimal activity over a wide range of pH and temperature (Singh *et al*., 2019; Barriuso *et al*., 2016). Hence, a search for lipolytic yeasts is a necessity for the ever-growing need of various industries to discover yeast lipases with potential applications.

In the production of lipases, moulds and bacteria have been favoured, but there are also a number of yeasts that are known to produce these enzymes. Such yeast species belong to genus *Yarrowia*, *Candida*, *Rhodotorula*, *Torulaspora*, *Pseudozyma*, *Geotrichum*, *Debaryomyces*, *Pichia*, and *Sporidiobolus* (Arevalo-Villena *et al*., 2017; Sarmah *et al*., 2017; Ramos-Sanchez *et al*., 2015). Yeasts' lack of production of toxic secondary metabolites has made them desirable hosts of enzymes for food use (Arevalo-Villena *et al*., 2017). It is expected that with numerous species that are to be discovered in specific and unusual habitats, more yeasts will be found and will show physiological properties that will be promising and valuable for biotechnological exploitation.

Therefore, this study aimed at isolating and characterizing lipolytic yeasts in the long-term ecological plot in the Molawin-Dampalit watershed in MMFR, Philippines. The potential lipolytic activity of the yeasts was determined by fermentation in various lipase production mediums.

Material and methods

Yeast Isolation

Different forest materials like soil, tree bark and canopy leaves, and epiphytic plants were collected from the 2 ha long-term ecological plot at the Molawin-Dampalit watershed in MMFR, Philippines. Samples were serially diluted in saline-peptone water and spread plated onto dichloran rose bengal chloramphenicol (DRBC) agar, and incubated at 28 °C for 48 h. Unique colonies were picked and streaked for isolation in yeast extract peptone dextrose agar (YEPD) to purify the isolates.

Screening for Lipolytic Activity

Plate Assay:Putative yeast isolates were point-inoculated onto lipase production medium (LPM) agar plates with the following components (per L): 10.0 g peptone, 5.0 g NaCl, 0.1 g $CaCl_2$, 1.0% Tween 80 (w/v) and 16.0 g agar. Plates were incubated at 28 °C for 72 h, and were observed for the zone of precipitation for positive lipolytic activity. Liquid Assay: Putative yeasts were grown in YEPD broth for 16 h, and were inoculated in basal mineral medium

(BMM) with olive oil, with starting OD_{660} of 0.1. BMM had the following components (per L): 4.0 g NH_4NO_3, 4.7 g KH_2PO_4, 0.119 g Na_2HPO_4, 1.8 g $MgSO_4.7H_2O$, 10.0 mg $FeSO_4.7H_2O$, 10.0 mg $CaCl_2.2H_2O$, 7.0 mg $MnSO_4$. H_2O, 0.1 g yeast extract, and 2.0% (v/v) olive oil, pH 5.5. Change in OD_{660} was measured after 72 h of incubation at 28 °C with shaking. The positive control yeast *Y. lipolytica* USDFST 49-49 used in this study was provided by the Phaff Yeast Culture Collection, UC Davis, California.

Molecular Identification

Genomic DNAs of putative yeasts were isolated using the Fungal/Bacterial DNA Mini Prep™ commercial DNA extraction kit (Zymo Research, California). The extracted DNA was used as the DNA template for PCR. 50 µL total reaction volume was used containing 1x PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.0 µM ITS1 forward primer, 1.0 µM ITS4 reverse primer, 1.25 U *Taq* DNA Polymerase, and 2.0 µL of the prepared DNA template (~100 ng/mL). The Thermocycling program used had the following settings: initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 2.5 min, and final extension at 72 °C for 5 min, with a total of 30 cycles. Primer pair ITS1 and ITS4 had the 5'-TCC GTA GGT GAA CCT GCG G-3' and 5'-TCC TCC GCT TAT TGA TAT GC-3' sequences, respectively. After PCR, amplicons were quality checked by agarose gel electrophoresis and were sent to 1st BASE, Malaysia for sequencing. The sequence data were then submitted to BLAST for BLASTN analyses, and the nucleotide sequences were subjected to multiple sequence alignment using MEGA software ver X for the generation of phylogenetic trees (Kumar *et al.*, 2018).

Lipase Production

Non-Enriched Versus Enriched Medium: Chosen putative yeasts were streaked onto YEPD agar slants and incubated at 28 °C for 48 h. After incubation, 10 mL of liquid medium (non-enriched: 0.5% peptone, 0.3% yeast extract, and 1.0% (v/v) olive oil, pH 6.0) was added in the agar slant and growth was scraped. The cell suspension was transferred to new Erlenmeyer flasks and incubated at 28 °C for 24 h with shaking. Seed culture was then transferred to 90 mL of both the non-enriched and enriched medium (5.0% peptone, 0.3% yeast extract, 0.1% $MgSO_4.7H_2O$, 0.1% $NaNO_3$, and 1.0% (v/v) olive oil, pH 6.0), and incubated at 28 °C for 48 h with shaking. Samples were taken every 24 h and lipolytic activity was determined by the titrimetric method.

Different Lipase Production Medium: The identified best lipolytic yeast was tested for lipase production using five different medium with different compositions based on literatures: Medium 1: 5.0% peptone, 0.1% $MgSO_4.7H_2O$, 0.1% $NaNO_3$, and 1.0% (v/v) olive oil, pH 7.0; Medium 2: 0.2% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, 0.01% NaCl, and 1.0% (v/v) olive oil, pH 6.0; Medium 3: 0.5% yeast extract, 1.0% KH_2PO_4, 0.1% $MgSO_4.7H_2O$, and 1.0% (v/v) olive oil, pH 6.8; Medium 4: 5.0% peptone, 0.3% yeast extract, 0.1% $MgSO_4.7H_2O$, 0.1% $NaNO_3$, and 1.0% (v/v) olive oil, pH 6.0; Medium 5: 0.2% glucose, 0.05% peptone, 0.1% KH_2PO_4, 0.3% K_2HPO_4, 0.2% Na_2SO_4, 0.01% $MgSO_4.7H_2O$, 0.1% gum arabic, 0.1% Tween 80, and 1.0% (v/v) olive oil, pH 7.0. All mediums were incubated at 28 °C for 48 h with shaking. Samples were taken every 12 h for measuring cell concentration and lipolytic activity.

Effect of Plant Oil Type and Inoculum Age: The effects of plant oil type and inoculum age were investigated in the production of lipase of the chosen yeast isolate using the determined best medium from the previous experiment. Commercial plant oils of olive and canola, and inoculum ages of 48 h and 96 h were used for lipase fermentation, with sampling every 12 h for 48 h. Samples were collected and subjected to cell concentration and lipolytic activity determination.

Determination of Lipolytic Activity

Titrimetric assay by Freire *et al.* (1997) was used for measuring the lipolytic activities of the collected crude enzymatic extracts. Olive oil/arabic gum

emulsion (5.0% (v/v) olive oil and 5.0% arabic gum) in 100 mM sodium phosphate buffer (pH 7.0) was prepared by homogenization in a blender for 3 min. The enzymatic reaction was started by adding 1 mL of the crude enzymatic extract into 19 mL of the prepared olive oil/gum arabic emulsion, and incubated for 30 min at 37 °C with shaking.

The reaction was stopped by adding 20 mL of acetone-ethanol 1:1 (v/v) solution, and the released fatty acids were titrated with 0.05M NaOH solution until a stable pink color of the solution was observed. One unit of lipase activity was defined as the amount of enzyme that released 1 µmol of fatty acid per minute under the described conditions, and was computed according to the following equation:

$$LA = \frac{(Va - Vb) \times M \times 100}{t \times v}$$

Where: LA is the lipolytic activity (U/mL)
Va is the volume of NaOH used for the sample (mL)
Vb is the volume of NaOH used for the blank (mL)
M is the molarity of the NaOH solution
t is the reaction time (min)
v is the volume of enzymatic extract (mL)

Result and discussion

Yeast Isolation and Screening

Various soil and tree materials were collected and processed for yeast isolation. Using DRBC agar plate, a total of 185 putative yeasts were isolated, suggesting that the surfaces of leaves and barks, and soils collected in MMFR present a good habitat for the yeasts to thrive in. These putative yeasts were then screened for lipolytic activity using the LPM agar plate. Twenty-nine percent (54/185) of the putative yeasts showed positive activity for lipase production (Fig. 1).

These potential lipolytic yeasts were then subjected to liquid assay using BMM supplied with 2.0% (v/v) olive oil, and change in OD_{660} was measured after 72 h of incubation at 28 °C with shaking (Table 1).

Table 1. Cell density of lipolytic putative yeasts grown in BMM supplied with 2.0% (v/v) olive oil after 72 h of incubation at 28 °C with shaking (starting OD_{660} of 0.1).

OD_{660} value	Number of isolates	Isolate codes
X ≥ 5	2	NEL-6, BUS-3, *Y. lipolytica* UCDFST 49-49 (positive control)
4 ≤ x < 5	3	NFL-12, BGS32-3, SS-2
3 ≤ x < 4	5	NMR-3, NFL-1, NFL-9, NUC-6, BGLB22-2
2 ≤ x < 3	6	NFR-1, NFR-2, NFR-10, NLC-7, BGS32-4, SS-3
1 ≤ x < 2	16	NOR-4, NOR-6, NFR-6, NFR-16, NFR-18, NFL-19, NFL-21, NLC-5, NLC-8, NLC-13, NLC-20, NUC-7, NUB-3, NUB-4, BGS32-6, BGS32-7
0 ≤ x < 1	22	NMR-1, NMR-4, NOR-7, NOR-8, NFR-17, NFR-22, NFL-2, NFL-20, NEL-10, NLC-1, NLC-3, NLC-9, NLC-10, NLC-14, NUC-4, NUC-5, NUB-7, NUB-8, NUB-9, BGLB22-1, BGVL22-1, BGOL22-1

Lipolytic yeasts produce lipase enzymes to hydrolyse the triglycerides content of olive oil, to make it an energy source and support their biomass production (Pereira-Meirelles *et al.*, 1997). Most of the tested isolates had poor growth in BMM, where 91.0% (49/54) had OD_{660} values of less than 4.0. The remaining isolates had better growths with OD_{660} values of 4.0 to 9.0. The positive control strain *Y. lipolytica* UCDFST 49-49 had an OD_{660} value of 5.32, of which some of the test isolates were comparable including isolates NFL-12, BGS32-3, SS-2, NEL-6, and BUS-3. Isolate BUS-3 had a notable OD_{660} value of 9.06 after 72 h of incubation, which was almost 2-folds compared to the control strain *Y. lipolytica* UCDFST 49-49. *Y. lipolytica* is a model microorganism that is capable of breaking-down hydrophobic substrates, such as plant oils, through specific metabolic pathways, that are used for the production of cell biomass, lipases, and other metabolites (Fickers *et al.*, 2004).

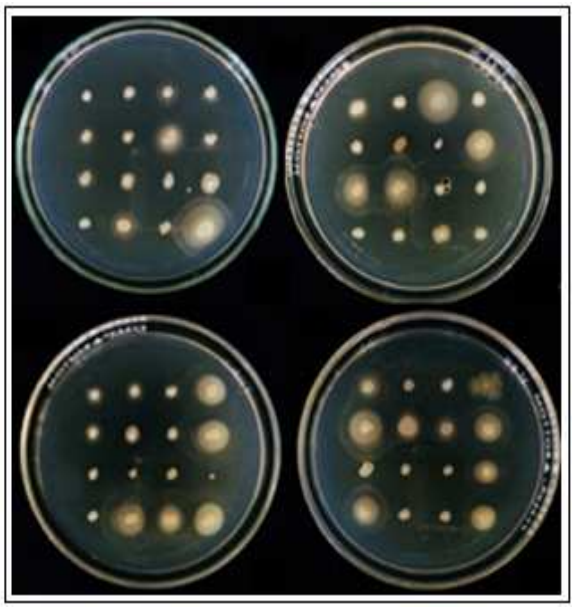

Fig. 1. LPM agar plates showing putative yeasts with lipolytic activity, exhibiting zone of precipitation around colonies after incubation at 28 °C for 72 h.

ITS-5.8S Region Sequencing

The top yeast isolates that grew well in BMM with 2.0% (v/v) olive oil were molecularly identified by ribosomal ITS-5.8S sequencing. The gel-electrophoresed PCR amplicons of the five yeasts had sizes of approximately 600 bp except for isolate NFL-12, which only had an estimated size of 400 bp. BLAST analyses resolved the identities of the three isolates at the species level as *Candida duobushaemulonis*, *Candida jaroonii*, and *Vanrija humicola* for isolates NFL-12, NEL-6, and BUS-3, respectively. BGS32-3 had a low percent similarity of 93.0% with a type strain of *C. quercuum*, while SS-2 had two relevant hits at 98.0% similarity to type strains of *C. silvicultrix* and *Wickerhamomyces ciferrii*. Phylogenetic tree analyses inferred using the Neighbor-Joining Test confirmed the initial identification of the yeast isolates as they clustered with the same species of the type strain yeasts (Fig. 2).

Some yeast species belonging to the *Candida* and *Wickerhamomyces* genera have been reported to be capable of producing lipase. *C. quercuum* isolated in an alkali aquatic environment showed a high growth rate at pH 3.0 to 9.0 (Urano *et al.*, 2019). A paper by Shimizu *et al.* (2020), reported the capability of *C. quercuum* to produce lipase enzyme at low temperature using agar plates with olive oil. These findings show the potential of this yeast species to be a source of other industrially important enzymes that can be tolerant to extreme conditions.

C. jaroonii has been isolated in various sources like flowers, insect frass, mushroom, and fruit exudate. It has also been found as a culturable yeast in the phylloplane of rice (Limtong and Kaewwichian, 2015), and in the culture-independent analysis of cacao bean fermentation (Grondin *et al.*, 2016).

There is no related literature that can be found on the lipase production of this yeast species. *C. duobushaemulonis*' taxon synonym is *C. duobushaemulonii*. *C. duobushaemulonii* is an emerging rare pathogenic yeast that has been classified as part of the *C. haemulonii* complex, comprised of *C. haemulonii* and *C. haemulonii* var *vulnera*. This group of *Candida* species is known to be multiresistant human pathogenic yeasts (Chow *et al.*, 2018; Jurado-Martin *et al.*, 2020). Hence, this isolate was omitted for the succeeding fermentation experiments. *W. ciferrii* is common on pods and exudates of trees like *Acacia*.

This species of yeast has been biotechnologically utilized for the production of the biomedically important tetraacetylphytosphingosine (Kurtzman *et al.*, 2011). *V. humicola*, originally *Torula humicola*, synonyms with *C. humicola* and *Cryptococcus humicola*, is a common soil-borne yeast that can be isolated in consortium with other yeasts (Yurkov, 2018). There is also no report on the lipolytic activity of this yeast species.

Non-Enriched and Enriched Medium Fermentation

Wickerhamomyces sp. SS-2 had the best enzymatic activity when cultivated on both non-enriched and enriched mediums, with measured lipolytic activities of 0.24 U/mL to 0.62 U/mL, respectively, after 48 h of fermentation time (Fig. 3). This showed that the increase in peptone concentration favoured the select yeast isolate's enzyme productivity.

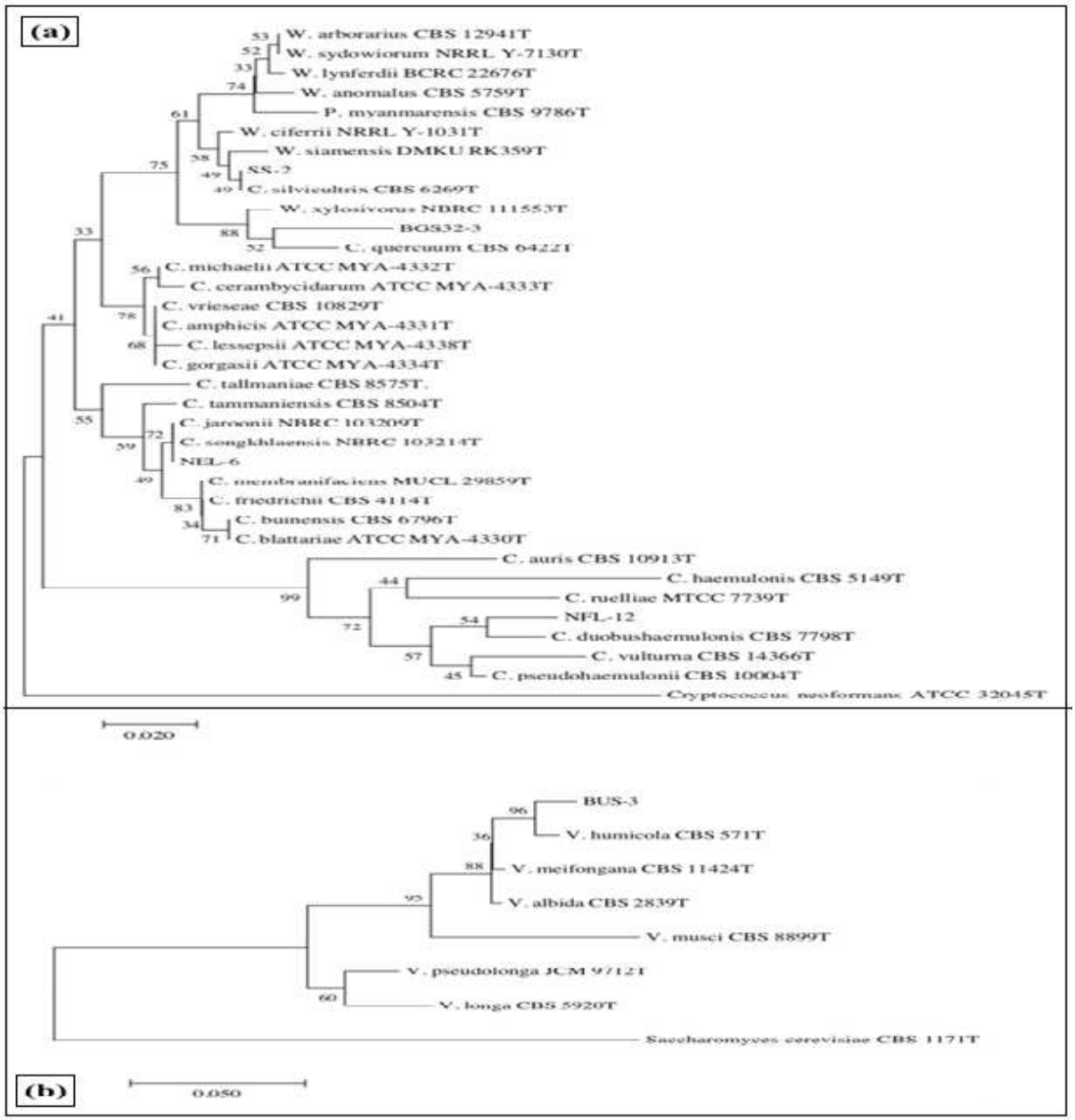

Fig. 2. Phylogenetic analyses based on the ITS-5.8S sequences inferred using Neighbor-Joining of unknown isolates (a) NFL-12, NEL-6, BGS32-3, and SS-2, and (b) BUS-3.

The measured values were relatively higher compared to the other isolates tested and the positive control *Y. lipolytica* UCDFST 49-49, which presented lipolytic activities of only 0.09 U/mL and 0.04 U/mL under the same conditions. *Candida* sp. BGS32-3 and *V. humicola* BUS-6 had very low to zero activities using the enriched media.

Observations on the low production of the enzyme after fermentation could be observed in most yeasts tested, which possibly was associated with the release of proteases during the cell growth phase (Dalmau *et al.*, 2000). A similar finding was reported by Bussamara *et al.* (2010), who evidenced the presence of proteases in the medium resulting in the reduction in enzymatic activity of *Pseudozyma hubeiensis* after 19 h of fermentation. The reduction of activity of *Candida rugosa* lipase by proteases was also observed in a study by Puthli *et al.* (2006). Therefore, *Wickerhamomyces* sp. SS-2 was selected from the initial 54 lipolytic yeasts. This isolate showed considerable levels of activity in both the cultivation media used compared to the other yeast isolates tested.

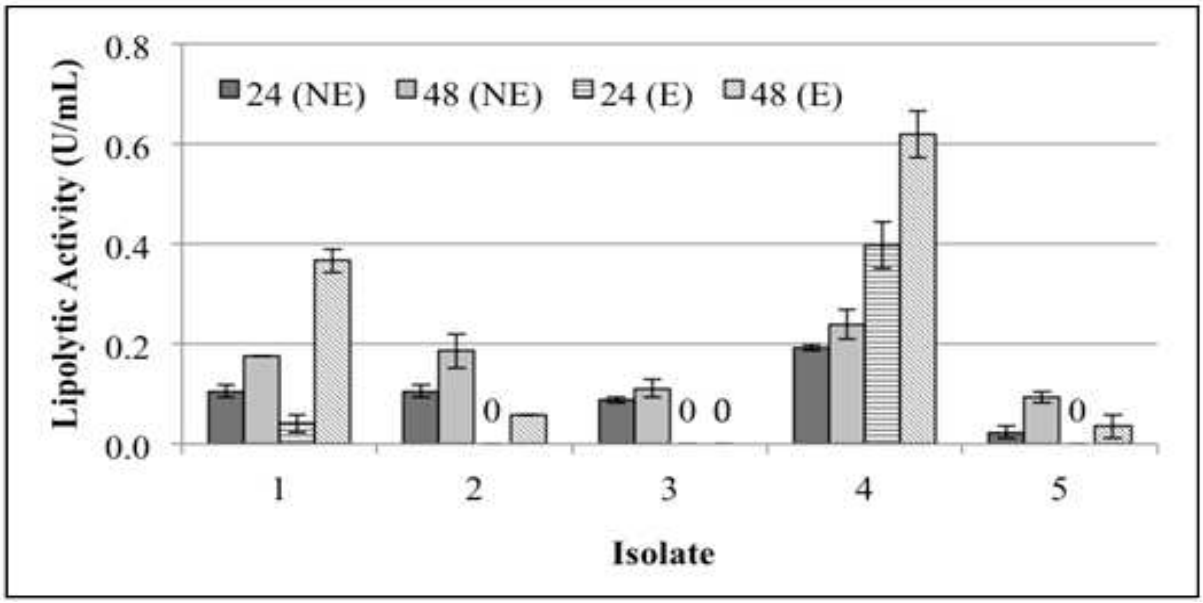

Fig. 3. Lipolytic activity of yeasts using non-enriched (NE) and enriched (E) medium; Isolate 1 – *Candida* sp. NEL-6; Isolate 2 – [*C.*] *quercuum* BGS32-3; Isolate 3 – *Vanrija humicola* BUS-6; Isolate 4 – *Wickerhamomyces* sp. SS-2; Isolate 5 – *Y. lipolytica* UCDFST 49-49.

Fermentation in Medium with Different Compositions

Fig. 4 shows the lipolytic activity and cell concentration of *Wickerhamomyces* sp. SS-2 is cultivated in five different culture mediums with different compositions. The maximum lipolytic activity of each culture medium occurred at times 36, 48, 48, 36, and 0 h for Medium 1, 2, 3, 4, and 5, respectively. Medium 2 showed the highest recorded lipolytic activity of 0.53 U/mL after 48 h of fermentation. In addition, Medium 2 already had the highest lipolytic activity (0.35 U/mL) after 24 h of cultivation. In all culture media, except for Medium 2, significant variations in activity were not observed.

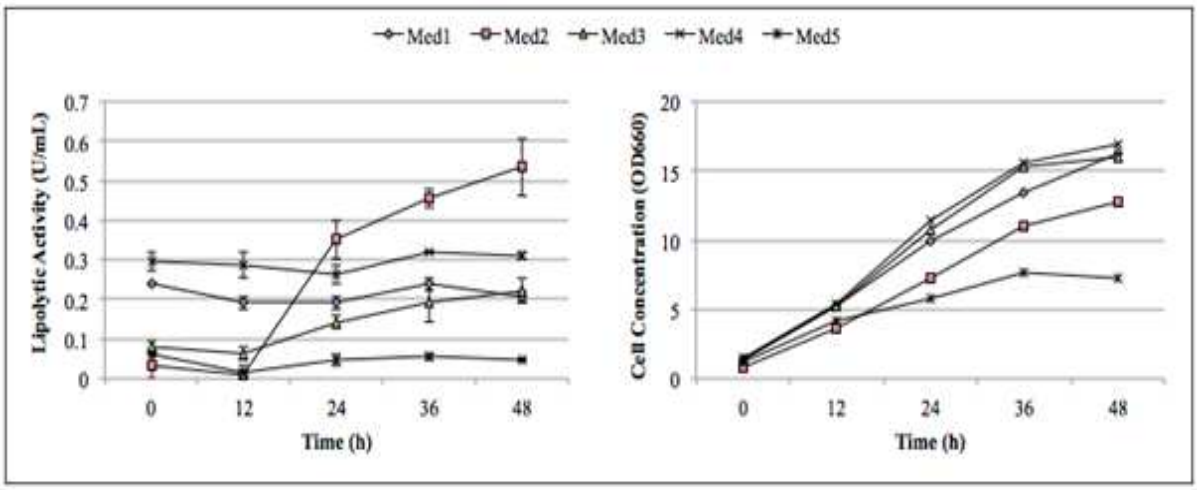

Fig. 4. Lipolytic activity and cell concentration of *Wickerhamomyces* sp. SS-2 in different fermentation medium; Med 1: 5.0% peptone, 0.1% $MgSO_4.7H_2O$, 0.1% $NaNO_3$, and 1.0% (v/v) olive oil, pH 7.0; Med 2: 0.1% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, 0.01% NaCl, and 1.0% (v/v) olive oil, pH 6.0; Med 3: 0.5% yeast extract, 1.0% KH_2PO_4, 0.1% $MgSO_4.7H_2O$, and 1.0% (v/v) olive oil, pH 6.8; Med 4: 5.0% peptone, 0.3% yeast extract, 0.1% $MgSO_4.7H_2O$, 0.1% $NaNO_3$, and 1.0% (v/v) olive oil, pH 6.0; Med 5: 0.2% glucose, 0.05% peptone, 0.1% KH_2PO_4, 0.3% K_2HPO_4, 0.2% Na_2SO_4, 0.01% $MgSO_4.7H_2O$, 0.1% gum arabic, 0.1% Tween 80, and 1.0% (v/v) olive oil, pH 7.0.

The consistently measured low lipolytic activities may be due to the secretion of proteases during the fermentation process (Dheeman *et al.*, 2010). Medium 2, which showed the highest lipase productivity, exhibited low biomass concentration compared to the other medium tested. High cell concentration did not equate to high enzymatic activity. This may be attributed to the metabolism of the yeast, since productivity and lipase activity is affected by genetic and environmental factors (Liu *et al.*, 2015). Thus, Medium 2, composed of 0.2% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, 0.01% NaCl, and 1.0% (v/v) olive oil, pH 6.0, was selected for the continuity of the work. Further fermentation screening aimed to increase the production of extracellular lipase of the select yeast *Wickerhamomyces* sp. SS-2.

Effect of Plant Oil Type and Inoculum Age

The effect of plant oil type on the lipase production of *Wickerhamomyces* sp. SS-2 was investigated using olive oil and canola oil. The fermentation profile is shown in Fig. 5. *Wickerhamomyces* sp. SS-2 grew well in both mediums containing different plant oils.

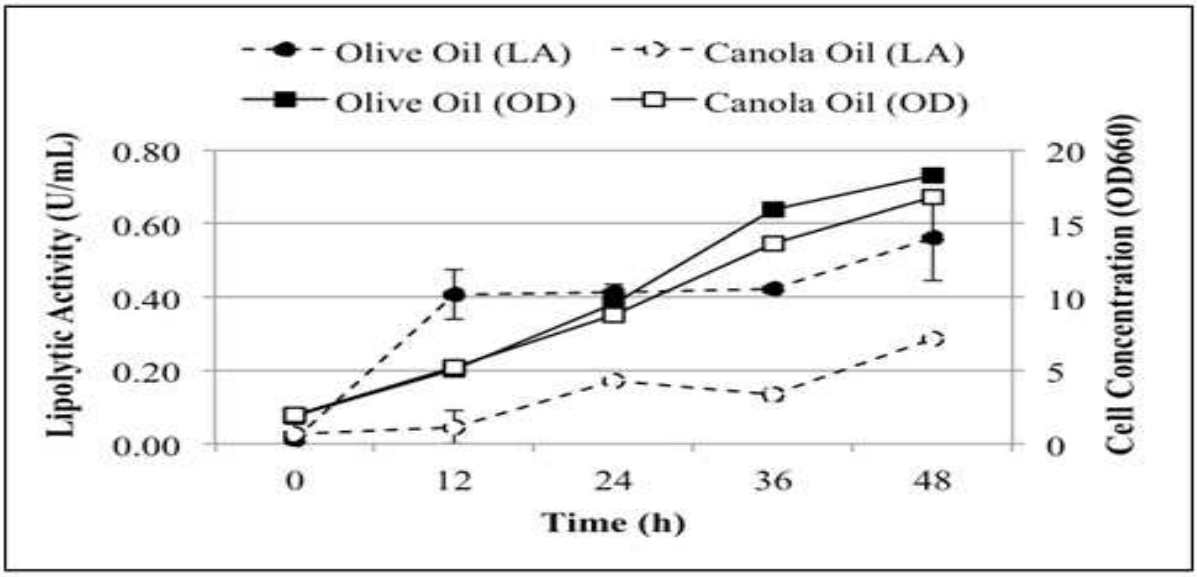

Fig. 5. Lipolytic activity and cell concentration of *Wickerhamomyces* sp. SS-2 in fermentation medium with olive oil or canola oil as main carbon source.

The maximum cell concentration in both plant oils was observed to have no significant difference, but a significantly higher lipase activity of 0.56 U/mL was observed in the medium containing olive oil, compared to 0.29 U/mL in the medium supplied with canola oil.

Olive oil has been found as the best inducer in the lipase production of many microorganisms. Darvishi *et al.* (2009) determined olive oil as the best inducer for lipase production in *Y. lipolytica*. This was also observed for other microorganisms like *Apergillus niger*, *Candida rugosa*, *Pseudomonas* sp., *Mucor hiemalis*, *Penicillium restrictum*, *Rhizopus homothallicus*, *R. oryzae*, and *Geotrichum candidum*, that among vegetable oils, olive oil has been one of the best inductors of lipase production (Zarevucka, 2012).

The high concentration of oleic acid, at 55.0 to 87.0%, as the main fatty acid in olive oil has been proven to highly influence the lipase production in many microorganisms (Garcia-Gonzalez *et al.*, 2009). Since canola oil also has a high oleic acid content of about 55.0 to 60.0%, it was the other plant oil tested for the lipase production of *Wickerhamomyces* sp. SS-2 (Barthet, 2016; Darvishi *et al.*, 2009; Kostik *et al.*, 2013). Studies have shown that canola oil is also a good plant oil for the production of lipase in *Y. lipolytica* (Kamzolova *et al.*, 2011; Darvishi *et al.*,

2009). Canola-oil seed cake or rapeseed cake as an agro-industrial waste has also been utilized for the production of extracellular lipases of strains of *B. amyloliquefaciens*, *Penicillium camemberti*, and *Pleurotus ostreatus* (Chen *et al.*, 2019; Rehman *et al.*, 2019). Waste frying canola oil has also been utilized for lipase production in strains of *Geotrichum candidum* (Rywinska *et al.*, 2008). Other plant oils with good oleic acid contents like soybean oil and palm oil can be investigated for the production of extracellular lipases in *Wickerhamomyces* sp. SS-2. For the effect of inoculum age, the fermentation profile is shown in Fig. 6. Inoculum age also did not have a drastic effect on the growth pattern of *Wickerhamomyces* sp. SS-2, but had a significant effect on the measured lipolytic activity.

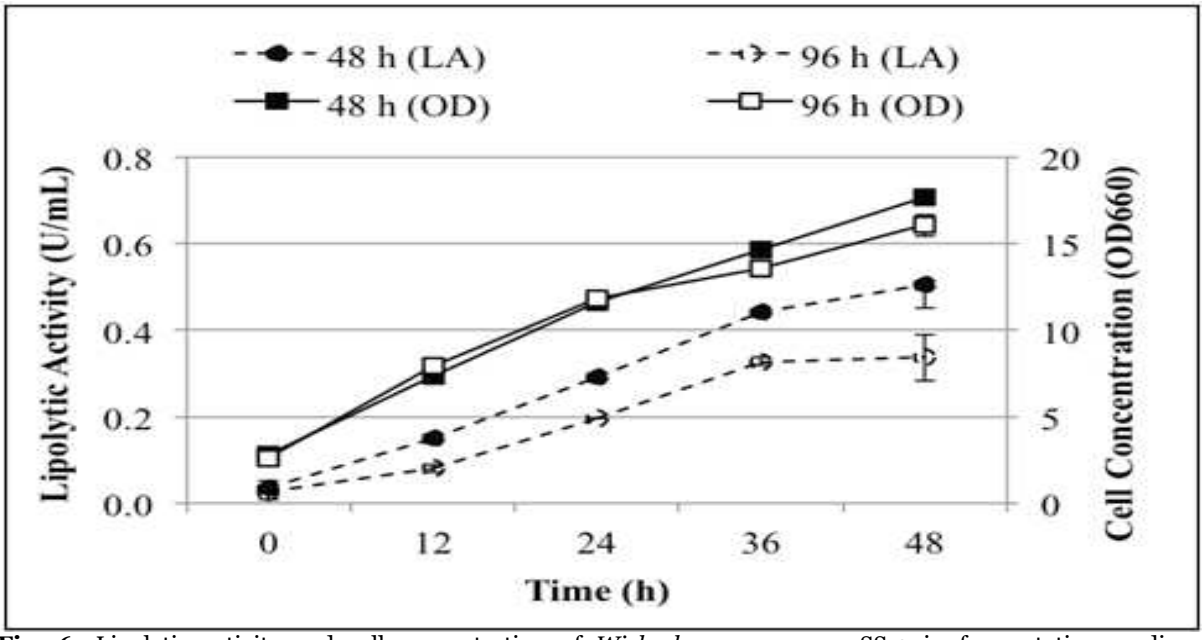

Fig. 6. Lipolytic activity and cell concentration of *Wickerhamomyces* sp. SS-2 in fermentation medium inoculated with 48 h- and 96 h-old inoculum.

The desired age of inoculum is highly variable depending on the process, cultivation conditions, medium composition, and the microorganism (Maldonado *et al.*, 2014). The experiment is very important so as to reduce the variability of the inoculum quality that is to be used for the fermentation process. Inoculum age is a physical factor that can have a significant influence on lipase production, which may also affect the fungal growth and enzyme production of *Wickerhamomyces* sp. SS-2.

The use of old cultures as inoculum can cause a decrease in lipase production as microorganisms may undergo late adapting to the fermentation medium (Demirkan *et al.*, 2021). Inoculum age is an important factor that has an influence on the growth as well as the lipase production of a strain (Banoth *et al.*, 2021).

Conclusion

From a total of 185 putative yeasts isolated from various soil and forest materials, 54 showed potential lipolytic activities. Top isolates were molecularly identified as members of the yeast genus *Candida*, *Vanrija*, and *Wickerhamomyces*. Fermentation in low and high nitrogen medium revealed that isolate, *Wickerhamomyces* sp. SS-2 was the best lipolytic yeast. Subsequent experiments showed that olive oil and the use of 48h-old inoculum were found best for the lipase production of the select yeast in medium composed of 0.2% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, 0.01% NaCl, and 1.0% (v/v) olive oil, pH 6.0. All the data gathered from this study can be used as base information for further optimization studies using experimental designs to further increase the lipase production of the select yeast.

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