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Received: 19 July 2023 | Accepted: 10 February 2024

DOI: 10.1002/bab.2570

ORIGINAL ARTICLE

Waste cooking oil and molasses for the sustainable production of extracellular lipase by *Saitozyma flava*

Silvia Donzella | **Andrea Fumagalli** | **Martina Letizia Contente** | **Francesco Molinari** | **Concetta Compagno**

Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Milan, Italy

Correspondence
Silvia Donzella, Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Via L. Mangiagalli 25, Milan 20133, Italy. Email: silvia.donzella@unimi.it

Funding information
Fondazione Cariplo; Cariplo Foundation (Italy), Grant/Award Numbers: ID 2020-1094, BioSurf

Abstract

Organic waste valorization is one of the principal goals of the circular economy. Bioprocesses offer a promising approach to achieve this goal by employing microorganisms to convert organic feedstocks into high value products through their metabolic activities. In this study, a fermentation process for yeast cultivation and extracellular lipase production was developed by utilizing food waste. Lipases are versatile enzymes that can be applied in a wide range of industrial fields, from detergent, leather, and biodiesel production to food and beverage manufacturing. Among several oleaginous yeast species screened, *Saitozyma flava* was found to exhibit the highest secreted lipase activity on *p*NP-butyrate, *p*NP-caproate, and *p*NP-caprylate. The production medium was composed of molasses, a by-product of the sugar industry, which provided nutrients for yeast biomass formation. At the same time, waste cooking oil was employed to induce and enhance extracellular lipase production. After 48 h of process, 20 g/L of yeast biomass and 150 mU/mg_{dw} of lipase activity were achieved, with a productivity of 3 mU/mg_{dw}/h. The purified lipase from *S. flava* showed optimal performances at temperature 28°C and pH 8.0, exhibiting a specific activity of 62 U/mg when using *p*-NPC as substrate.

KEYWORDS
circular economy, fermentation, lipase production, molasses, unconventional yeasts, waste cooking oil

1 | INTRODUCTION

Lipases (E.C. 3.1.1.3), defined as triacylglycerol-acyl-hydrolases, catalyze the hydrolysis of ester bond of tri-, di-, and mono-glycerides of long-chain fatty acids into fatty acids and glycerol. They belong to the super family of α/β-hydrolases, characterized by a catalytic triad usually formed by Ser, His, and Asp residues.[1,2] These enzymes have been applied in a wide industrial range because besides lipid hydrolysis, they catalyze reactions of esterification, of amino- and alcohol-lysis of a wide array of substrates making them widely used biocatalysts.[3–5]

Abbreviations: dw, biomass dry weight; gDNA, genomic DNA; *p*NPC, *p*-nitrophenyl caprylate; UVO, used vegetable oil; WCO, waste cooking oil; YE, yeast extract; YNB, yeast nitrogen base.

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Lipases can also synthesize esters from glycerol and long-chain fatty acids under low water activity conditions, such as in organic solvents as reaction medium in organic solvents.[5,6] Thanks to this versatility, lipases have been meeting needs for numerous industrial sectors such as biofuel production, food processing, leather manufacturing, and pharmaceuticals.[5,7] In particular, the obtainment of biosurfactants (as sugar fatty acid esters) through a biocatalytic approach using commercial or isolated lipases is a promising field in the green chemistry sector.

Microbial lipases are more available for industrial purpose in comparison to those derived from plants due to their regular supply, being not subjected to seasonal fluctuations. In addition, with respect to animal and plant lipases, they are usually considered more valuable due to their high yield production, and absence of seasonal fluctuations.[8,9] Furthermore, microbial species can easily adapt to extreme conditions, and natural environments provide a wide reservoir of extraordinary potential for lipases with specific features.[10–13] Microbial products have been gaining interest within the concept of biorefinery due to the opportunity to cultivate microorganisms in large scale on cheap media based on wastes. The actual valorization of wastes through their conversion into high-value products is based on the availability of (bio)technologies.[14,15] Therefore, the possibility to produce industrially relevant enzymes (such as lipases) from microorganisms grown on waste appears like highly attractive.[16]

Molasses, a by-product of the sugar industry, provide an abundant raw material at low-cost. This waste can represent an economical and readily available carbon and nutrient source, as it contains 0.5%–0.9% nitrogen, and 10% (w/v) inorganic salts. Due to this fact, molasses is mainly used in fermentation processes[17] for production of products, such as bioethanol,[18,19] citric acid,[20] lactic acid,[21] and fructo-oligosaccharides.[22]

The use of oils as inducer to produce microbial lipase was reported, as sardine oil by *Cryptococcus* sp. and *Geotrichum*.[23,24] The amount of used vegetable oil (UVO) as industrial and urban waste has been estimated around 200 million tons per year.[25] If not properly discarded, UVO could lead to environmental damages, such as land or water contamination. Utilization of this waste for biodiesel production adsorbs nearly 90% of this raw material.[26,27] A recent review covers processes and technologies available so far for recycling waste cooking oil (WCO), which represents the main component of UVO.[28] Other applications focused on the recycle of WCO as substrate for microbial growth and metabolites production are now starting to be developed.[29,30] Waste of soybean frying oil has been recently used to produce cell-wall-associated lipases by *Yarrowia lipolytica*,[31] whereas *Y. lipolytica* W29 is able to grow on WCO simultaneously producing a lipid-rich biomass and high level of lipase activity.[32] Besides, *Y. lipolytica*[33] other oleaginous species have generally been reported to express lipases with interesting features.[34–36]

In this study, the oleaginous yeast *Saitozyma flava* was selected for its ability to secrete a lipase able to hydrolyze esters with different acyl chain length. In addition, we show that food wastes like molasses and WCO can be used for yeast cultivation and simultaneous lipase production. This represents a new waste-valorization bioprocess for the production of a new extracellular lipase, which can exhibit enzymatic peculiarity.

2 | MATERIALS AND METHODS

2.1 | Strains

The yeast strains studied in this work: *Cutaneotrichosporon oleaginosum* (ATCC 20509 and ATCC 20508), *Lipomyces lipofer* (ATCC 10742 and DBVPG 7048), *Lipomyces starkey* (DBVPG 6637), and *Cryptococcus albidus* (DBVPG 6110). Yeasts were stored in YPD 20% (vol/vol) glycerol stocks at −80°C.

2.2 | Media and cultivation

Yeasts were cultivated on different media. Yeast nitrogen base (YNB) (without amino acids and ammonium sulfate 0.17 g/L, Difco BD) supplemented with ammonium sulfate 5 g/L, 20 g/L glucose or sucrose; YNB supplemented with 2% Tween 80; YPD 10 g/L yeast extract (YE), 20 g/L peptone, and 20 g/L glucose.

Sugar beet molasses-based media were prepared by diluting molasses stock till to reach a concentration of 20 g/L sucrose with distilled water and adjusting to pH 6, before sterilization. Sugar beet molasses-based media were supplemented with 2% (vol/vol) Tween 80, with YE (1 or 10 g/L) and with 2% (vol/vol) oils (corn oil or WCO).

Yeast cells were cultivated at 28°C in a rotary shaker at 150 rpm in 1 L or 500 mL bluffed flasks containing 200 or 100 mL medium (medium–flask ratio 1:5).

Optical density was monitored at 600 nm (OD_{600}). Cells were pre-cultured for 24 h on YPD, harvested by centrifugation at 5000 rpm and washed tree times. Then they were used to inoculate the media at initial OD_{600} 1 (corresponding to 10^7 cells/mL approximately).

2.3 | Dry weight determination

For biomass dry weight (dw) measurements, samples from different culture conditions and time of growth were

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collected (in triplicate at each time). Cells were filtered through a glass microfiber GF/A filter (Whatman), washed with three volumes of deionized water, and dried at 100°C for 24 h.

2.4 | Identification of the strain and phylogenetic analysis

For strain identification, genomic DNA (gDNA) was isolated and purified following the method described.[37] gDNA was amplified with PHUSION taq polymerase employing universal primers for amplification on D1/D2 domain NL1: GCA TAT CAA TAA GCG GAG GAA AAG, NL4: GGT CCG TGT TTC AAG ACG G, 0.2 μM each, 200 μM DNTP, and $MgCl_2$ 2.5 mM. PCR amplification was carried out by denaturing at 98°C for 7 min, followed by 30 cycles of denaturing at 98°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The produced amplicon was purified and sequenced by Mycrosynth company.

Phylogenetic analysis was performed using Mega X 10.1.7 (www.megasoftware.net).

2.5 | Sugars determination

The concentrations of sugars during fermentation processes were determined by employing commercial enzymatic kits (K-GLUHK and K-SUFRG, Megazyme). All the assays were performed in triplicate and standard deviations varied between 1% and 5%.

2.6 | Enzyme assay

Lipase was assayed accordingly to Gupta et al.[38] with some modifications. *p*-Nitrophenyl caprylate (*p*NPC) was routinely used as substrate for measurement of lipase activity. Substrate solution was prepared as follows: 10 μL *p*NPC in 1 mL of absolute ethanol. For testing the other substrates, absolute ethanol was used with exception of *p*NP palmitate that was dissolved in acetonitrile. The enzyme assay mixture was composed by mixing 1 mL of K_2PO_4 0.2 M buffer (pH 8.0), 2% Triton, 10 μL of the substrate solution, suitably diluted enzyme sample, water to 2 mL. The released *p*-nitrophenol was measured at 410 nm by spectrophotometer. One unit of enzyme activity is defined as the amount of lipase releasing 1 μmol of *p*-nitrophenol per min under the standard conditions. Enzyme activity was expressed as mU/mg of biomass dw (mU/mg_{dw}).

2.7 | Lipase purification

Cell-free supernatants, obtained after centrifugation of the culture at 5000 g, were 20-fold concentrated by ultrafiltration, using the Vivaflow 200 apparatus (Sartorius). Then, the concentrated supernatants were purified, in order to eliminate media components, by diafiltration through a filter with a cut-off 10 kDa in buffer K_2PO_4 0.1 M pH 6.

2.8 | SDS–PAGE

The molecular mass of the purified lipase was determined by SDS–PAGE in a Mini Protean Tetra cell vertical electrophoresis unit (Bio-Rad) using a 10% (w/v) acrylamide gel. Samples were analyzed after staining with Coomassie Brilliant Blue (SIGMA), and molecular mass was estimated with reference to protein marker (BLUeye Prestained Protein Ladder, SIGMA).

2.9 | Enzyme characterization

Enzyme activity was assayed in the pH range of 6.0–9.0, using 0.1 M phosphate buffer, at 28°C. The optimal temperature was measured by performing the enzyme-substrate reaction at various temperatures (28–45–60–70°C) using 0.1 M phosphate buffer pH 8.0. Thermal and pH stability was determined after incubation of enzyme (in the absence of substrate) up to 24 h, and assaying the residual enzyme activity as described in the previous paragraph (enzyme assay).

2.10 | Protein quantification assay

Total protein content was quantified by Bradford protein assay in triplicate for each sample. Protein concentration was calculated following the Bradford reagent stock's calibration curve. Specific activity of purified lipase (mU/mg) was obtained by dividing activity assayed as described in the previous paragraph (enzyme assay) per total protein concentration obtained by the Bradford assay.

3 | RESULTS AND DISCUSSION

3.1 | Screening of yeast species for extracellular lipase activity

The following six strains of oleaginous yeasts were tested for lipase activity: *C. oleaginosum* (ATCC 20509 and ATCC

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TABLE 1 Screening of yeast lipase activity on different *p*-nitrophenyl esters.

	*p*NP-acetate	*p*NP-butyrate	*p*NP-caproate	*p*NP-caprylate	*p*NP-palmitate
Cryptococcus albidus ATCC 10742	81	196	246	120	246
Cutaneotrichosporon oleaginosum ATCC 20508	41	19	0	19	0
Cutaneotrichosporon oleaginosum ATCC 20509	20	16	0	0	0
Lipomyces lipofer DBVPG 6110	81	192	187	140	170
Lipomyces lipofer DBVPG 7048	56	628	1373	1513	83
Lipomyces starkey DBVPG 6637	59	10	0	16	0

Note: The activity was assayed on supernatants after 74 h of growth and expressed as mU/mL.

20508), *L. lipofer* (ATCC 10742, and DBVPG 7048), *L. starkey* (DBVPG 6637), and *C. albidus* (DBVPG 6110). Nonionic detergents, such as Tween 80, are reported to induce extracellular lipase activity in yeast.[39] Therefore, yeasts were initially cultivated on mineral synthetic medium (YNB) containing Tween 80 as sole carbon source to induce lipase activity, excluding any possibility of negative effects on its expression due to the presence of other carbon sources. Substrates (*p*-nitrophenyl esters) of varying chain lengths ranging from 2C to 16C were used to evaluate lipase activity in the culture supernatants (Table 1). Three strains, *C. albidus* (DBVPG 6110) and *L. lipofer* (DBVPG 7048, ATCC 10742), showed lipase activity on all substrates. *C. oleaginosum* (ATCC 20508), and *L. starkey* (DBVPG 6637) exhibited activity on *p*NP-acetate, on *p*NP-butyrate and *p*NP-caprylate. *C. oleaginosum* (ATCC 20509) exhibited activity only on *p*NP-acetate and on *p*NP-butyrate. In particular, the screening led to the selection of *L. lipofer* DBVPG 7048 as the strain secreting lipase with the highest activity on *p*NP-butyrate, *p*NP-caproate, and *p*NP-caprylate.

As the most interesting lipase activity was found in the supernatants of the culture of *L. lipofer* DBVPG 7048, we decided to check the identity of this strain by using a molecular approach. By the alignment of 26S rDNA D1/D2 domain sequences, the strain *L. lipofer* DBVPG 7048 showed 99.48% identity with *S. flava* (CBS 331), previously classified as *Cryptococcus flavus*.[40]

3.2 | Optimization of growth conditions and lipase expression

Yeast growth and lipase activity are highly dependent on the kind of carbon and nitrogen sources; for example, bioactive peptides derived from peptone and tryptone casein-hydrolysates as well as the presence of fatty acids have been reported to modulate lipase production, also enhancing its secretion, in *Y. lipolytica*.[41,42] With this in mind, in order to optimize conditions for *S. flava* growth and lipase production, batch cultivations on media containing different carbon and nitrogen sources were carried out.

On synthetic media (YNB) containing glucose or sucrose as carbon source and ammonium sulfate as nitrogen source, a similar amount of yeast biomass (around 8 g/L of dw) was produced after 48 h of cultivation. The lipase activity detected in the supernatant of both cultures after 24 h of growth (Table 2) was found to be higher on glucose than on sucrose, being 19 and 5 mU/mg_{dw}, respectively. After 48 h, the extracellular lipase activity observed with the yeast growing on glucose increased to 31 mU/mg_{dw}, whereas it became undetectable in the presence of sucrose. The addition of Tween 80 to glucose medium caused an induction of lipase activity to 60 mU/mg_{dw} after 48 h of cultivation (Table 2). However, this activity halved after 72 h. When *S. flava* was cultivated on YPD (Table 2), a similarly high lipase activity was detected (66 mU/mg_{dw}) after 48 h, and it was stably maintained until to 72 h before decreasing to 22 mU/mg_{dw} after 96 h.

In conclusion, these experiments demonstrate that in this yeast species, a basal activity of extracellular lipase is expressed, but it can be induced by the presence of specific substrates, as Tween 80 or compounds contained in YE and peptone. Fickers et al.[41] highlighted tryptone as the best nitrogen source for lipase production in *Y. lipolytica*. Recently, Nunes et al.[31] reported that the combination of YE and peptone yielded the best results for production of lipase by *Y. lipolytica*.

3.3 | Lipase production by the recycle of food-industrial waste

To develop processes based on the recycle of food wastes, media containing sugar beet molasses were employed for yeast cultivation. Under these conditions, molasses provided carbon and nitrogen source for yeast growth, resulting in the production of 11 g/L of biomass dw after 72 h (Figure 1, light blue line). Lipase activity (40 mU/mg_{dw}) was detected after 48 h from the inoculum, with a slight increase in the next 24 h up to 45.5 mU/mg_{dw}. As observed

TABLE 2 Lipase activity (expressed as mU/mg_{dw}) assayed on supernatants at different times of growth on different media.

Condition	24 h	48 h	72 h	96 h
YNB sucrose (20 g/L)	5	0	0	0
YNB glucose (20 g/L)	19	31	30	ND
YNB glucose (20 g/L) + Tween 80 (2%)	ND	60	30	ND
YPD	8	66	60	22

Abbreviations: ND, not detected; YNB, yeast nitrogen base.

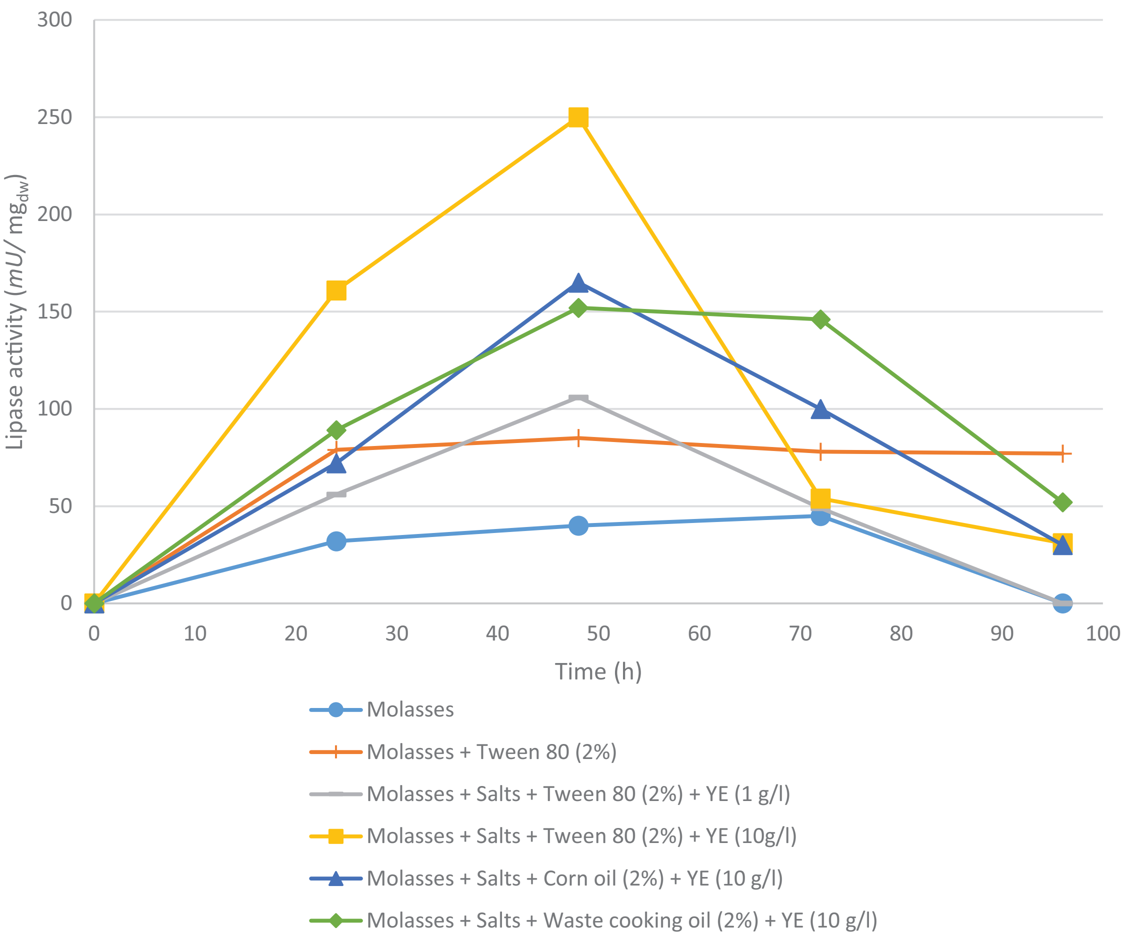

FIGURE 1 Lipase activity (expressed as mU/mg_{dw}) assayed on supernatants at different times of growth on different waste-derived media.

in cultivation on synthetic media, the supplementation of Tween 80 induced lipase expression that reached the level of 85 mU/mg_{dw} after 48 h (Figure 1, orange line). However, the enrichment of the medium with YE clearly overstimulated lipase production, and this positive effect was directly correlated to its concentration: Activities of 106 and 250 mU/mg_{dw} were obtained by supplementing YE at 1 or 10 g/L, respectively (Figure 1, grey and yellow line). The highest specific lipase production (250 mU/mg_{dw}) along with the highest concentration of biomass (21 g/L dw) was obtained after 48 h of cultivation by supplementing Tween 80 2% and YE 10 g/L (Figure 1, yellow line). However, 24 h later (72 h of cultivation), a drastic decrease (fivefold) of lipase activity occurred. At the same time, the culture reached pH 9.0 (initial pH 7.0). A similar situation has been reported in *Y. lipolytica*, in which the secreted lipase activity dramatically decreased when the pH of the cultivation medium reached alkaline values, due to proteolytic degradation caused by activation of alkaline extracellular proteases.[43]

Cultures of *S. flava* were carried out on molasses-based media added with corn oil and WCO to investigate the growth and lipase induction. When the medium was supplemented with corn oil (Figure 1, blue line), a

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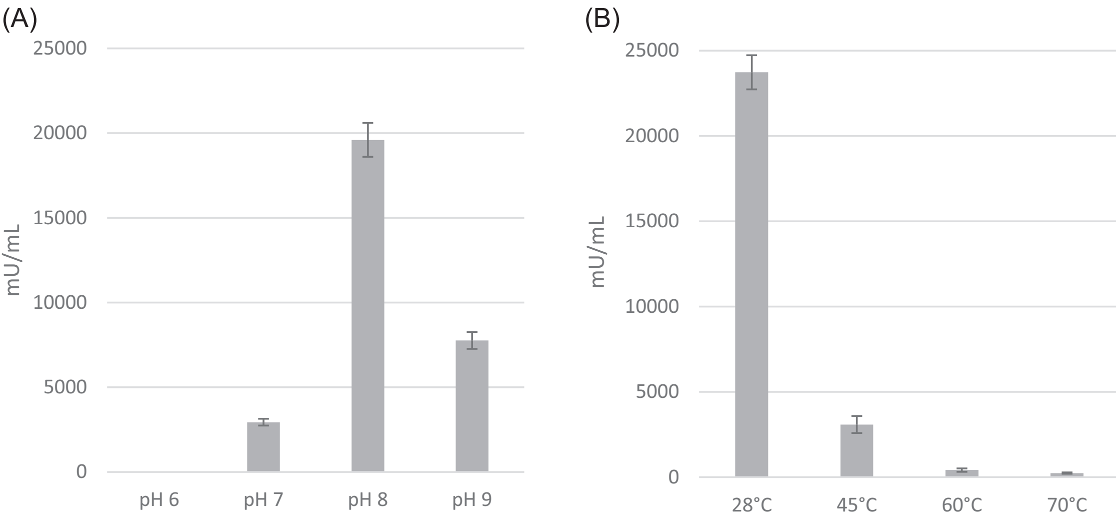

FIGURE 2 Lipase activity (mU/mL) detected at different pH values (A) and at different temperatures (B).

biomass concentration of 22 g/L dw and a lipase activity of 165 mU/mg_{dw} were obtained after 48 h, corresponding to a productivity of 3.4 $mU/mg_{dw.}/h$. When the medium was supplemented with WCOs (Figure 1, green line), after 48 h of cultivation, a productions of biomass corresponding to 20 g/L dw and a lipase activity of 150 mU/mg_{dw} were obtained, with a productivity of 3.1 $mU/mg_{dw.}/h$. The enzyme activity was maintained for at least 24 h more, when the pH reached 9.0. These results indicate that *S. flava* can use not only molasses as carbon and nitrogen sources, but also that WCOs can replace Tween 80 as inducer, leading to high biomass and lipase production. We cannot exclude the possibility that the presence of some inhibitors in the oil waste could negatively affect the production compared to Tween 80. However, also the fatty acid composition of different oils can affect lipase induction. In *Y. lipolytica*, a process for lipase production was optimized by feeding tryptone and olive oil.[44] The activity and productivity values obtained in this work (3 U/mL, corresponding to 152 U/mg) are in line with those assessed in other relevant studies where WCOs were employed for lipase production by yeasts.[31,45]

In conclusion, the conditions for *S. flava* growth and extracellular lipase production were substantially enhanced by consecutive optimization of the basal medium. Food-industrial wastes, like sugar beet molasses and WCOs, can be used to supply carbon and nitrogen sources for yeast *S. flava* biomass production and for lipase induction and secretion. By scaling-up the process in an industrial reactor under controlled conditions, it will be possible to maintain the optimal pH. In this way, avoiding the activation of proteases, a process with high lipase productivity can be performed.

3.4 | Enzyme purification and characterization

Lipases are known to be diversified in their activity properties, and therefore, it is important to characterize them. Most extracellular yeast lipases are monomeric proteins with molecular weight ranging from 30 to 40 kDa.[46,47] First, we proceeded with the recovery and the purification of the *S. flava* lipase from the cultivation medium. To facilitate the purification process and reduce the presence of proteins/peptides in the medium, yeast cells were cultivated on synthetic medium YNB supplemented with Tween 80. The cell-free supernatants were concentrated 20-fold by ultrafiltration and purified by diafiltration in buffer K_2PO_4 0.1 M at pH 6. The SDS–PAGE profile revealed a single protein band of molecular mass between 30 and 40 kDa (Figure S1).

Lipases display activity that is dependent on pH and temperature. Considering its possible application in the synthesis of biosurfactants, the optimal pH and temperature of *S. flava* lipase were determined. The activity of the purified enzyme was measured over a pH range from 6.0 to 9.0, and at temperatures ranging from 28 to 70°C, by using *p*-NPC as substrate.

No activity was detected at pH 6.0, whereas the highest lipase activity was observed in alkaline conditions at pH 8.0 (Figure 2A), and 40% was retained at pH 9.0. In *Y. lipolytica*, extracellular lipase exhibits highest activity at pH 7.0–7.5.[48,49]

Concerning temperature, the optimum for *S. flava* lipase activity was found at 28°C, tested at pH 8.0 (Figure 2B). The activity remained stable when stored at this temperature up to 24 h. Low activity was detected at higher

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temperatures, with a 7.6-fold decrease at 45°C, 60-fold at 60°C, and 100-fold at 70°C (Figure 2B). In the latter case, the activity was further fivefold lower after 1 h of incubation.

The purified *S. flava* enzyme, tested at 28°C using *p*-NPC as substrate, showed a specific activity of 62 U/mg.

4 | CONCLUSIONS

A new waste-valorization bioprocess and a new source of microbial extracellular lipase were studied. Among several oleaginous yeast species screened, *S. flava* was selected for its ability to secrete high lipase activity on most of the tested substrates (*p*NP-butyrate, *p*NP-caproate, and *p*NP-caprylate). The conditions for yeast growth and lipase production were optimized. Media based on sugar beet molasses and WCOs were demonstrated to provide carbon and nitrogen sources for biomass production, as well as for lipase induction and secretion. After 48 h of cultivation, 20 g/L of biomass (cell dry weight) and 150 mU/mg_{dw} lipase were achieved, with a productivity of 3 $mU/mg_{dw}/h$. The optimal condition of temperature and pH for purified *S. flava* lipase activity was 28°C and pH 8.0.

These findings provide valuable insights into the enzymatic properties of *S. flava* lipase and its potential application in various industrial processes, in particular for the synthesis of biosurfactants starting from renewable resources.[50] Overall, this study demonstrates the potential of utilizing food-industrial wastes for the sustainable production of lipase, contributing to waste valorization and resource optimization in food industry. Further scale-up of the process in controlled industrial reactors can enable the maintenance of optimal pH conditions, preventing protease activation and enhancing lipase productivity.

AUTHOR CONTRIBUTIONS

Conceptualization: Concetta Compagno and Francesco Molinari. *Methodology; investigation and data curation*: Andrea Fumagalli, Martina Letizia Contente, and Silvia Donzella. *Writing*: Concetta Compagno and Silvia Donzella. *Revision*: Francesco Molinari and Martina Letizia Contente. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in the manuscript and the supplementary information.

CONSENT TO PARTICIPATE AND CONSENT TO PUBLISH

No individual was used as a case study in this work; thus, no consent to participate and no consent to publish were necessary.

ORCID

Silvia Donzella https://orcid.org/0000-0003-4134-2560
Martina Letizia Contente https://orcid.org/0000-0002-3885-1375
Francesco Molinari https://orcid.org/0000-0003-1958-423X

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How to cite this article: Donzella S, Fumagalli A, Contente ML, Molinari F, Compagno C. Waste cooking oil and molasses for the sustainable production of extracellular lipase by *Saitozyma flava*. Biotechnol Appl Biochem. 2024;1–9. https://doi.org/10.1002/bab.2570

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