Applied Biochemistry and Biotechnology (2021) 193:589–605
https://doi.org/10.1007/s12010-020-03431-6

# Sustainable Lipase Production by *Diutina rugosa* NRRL Y-95 Through a Combined Use of Agro-Industrial Residues as Feedstock

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Received: 3 August 2020 / Accepted: 28 September 2020 /
Published online: 12 October 2020


## Abstract

The potential use of alternative culture media towards the development of a sustainable bioprocess to produce lipases by *Diutina rugosa* is clearly demonstrated. First, a synthetic medium containing glucose, peptone, yeast extract, oleic acid, and ammonium sulfate was proposed, with lipase activity of 143 U/L. Then, alternative culture media formulated with agro-industrial residues, such as molasses, corn steep liquor (CSL), and olive mill waste (OMW), were investigated. An experimental design was conducted, and only CSL concentration was found to have a positive effect in lipase production. The highest lipase activity (561 U/L) was produced on a mixture of molasses (5 g/L), CSL (6 g/L), OMW (0.5% *v*/v), 0.5 g/L of ammonium sulfate, and 3 g/L of peptone at 24 h of cultivation. Lipase production was also carried out in a 1-L bioreactor leading to a slightly higher lipase activity at 24 h of cultivation. The semi-purified enzyme exhibits an optimum temperature and pH of 40 °C and 7.0, respectively. Finally, the media cost per unit of lipase produced (UPC) was influenced by the medium components, specially by the inducer used. The lowest UPC was obtained when the agro-industrial residues were combined and used at the improved concentrations.

**Keywords** Sustainability · Molasses · Corn steep liquor · Olive mill wastewater · *Candida rugosa* lipase (CRL) · *Diutina rugosa*

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## Introduction

The concept of circular economy and bio-economy has been gaining attention in the last years, not only to mitigate the environmental impacts of wastes, but also to promote the expansion and diversification of bio-based markets [1]. Recycling agro-industrial residues comprises an efficient resource exploitation, since these residues represent a large volume of biomass. These residues can be reused in new applications, such as in the formulation of culture media, providing nutrients for microbial growth or product formation. At the same time, the use of agricultural wastes and food processing by-products, in the production of high value-added products, requires the development of new technologies and bioprocessing schemes. The (bio)valorization of agro-industrial wastes using microbial technologies has been widely reported [2–4] as an alternative, not only to add value to the existing industries, but also to develop cheaper culture media [5, 6]. Furthermore, some recovery strategies describe the biotechnological transformation of by-products into other production systems, for instance, enzyme production, such as lipases [7–11]. Market analysis data clearly suggest that lipases are still important biocatalysts. The lipase market is projected to reflect a CAGR (compound annual growth rate) of 6.3 or 7.8% over the forecast period, 2016–2026 or 2017–2026, respectively, depending on the source [12, 13], and accounted for US$ 345 million in 2017 [14].

The production of lipase using submerged culture is the preferential process [15], since it allows a greater control of the growth conditions as compared with solid-state fermentation [16]. Several efforts have been conducted aiming at the optimization of the culture media for producing lipases, such as using different carbon, nitrogen, and inducer combinations and concentrations [5, 17–21]. Lipids are used as inducers in this process, and olive oil has been widely reported for this purpose [22–24]. Agro-industrial residues and/or by-products have a high nutrient concentration and can be used in the culture medium to grow microorganisms able to produce lipases. These have been reported as a means to decrease the production costs associated with raw materials [25, 26]. Besides, it is important to mention that some of these residues and/or by-products present risks to the environment; thus, any process that can add value and/or remove them from the environment is of utmost interest [26].

In this work, three agro-industrial residues have been evaluated as alternatives for the production of lipases, namely molasses, corn steep liquor (CSL), and olive mill wastewater (OMW). Molasses, a non-food raw material, is a waste from the sugar manufacturing process of sugarcane and an important carbon source that allows microorganism growth [27]. It contains a high concentration of carbohydrates (about 50%), as well as other valuable compounds such as vitamins [28]. Corn steep liquor (CSL) is a liquid by-product generated by the corn wet milling industry and contains 50% of water and corn components. It is rich in vitamins, minerals (Ca, K, Mg, P, S), amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tyrosine and valine), water-soluble proteins, and other natural organic acids (lactic acid) [19, 29], being an important source of nitrogen for many biotech processes [9, 28–30]. Those nutrients, especially amino acids [31, 32] and vitamins [19, 23], are reported to play an important role in the lipase production. Olive mill wastewater (OMW) is a dark-colored effluent originated by the use of hot water in the production of olive oil. In European countries, large amounts of OMW are produced, which is considered a large-scale environmental issue [15]. The main components of OMW, apart from water (83 to 94%), are lipids, carbohydrates, phenolic compounds, organic acids, tannins, pectin, and minerals [4]. The use of OMW to

produce lipases was previously reported [15, 33–35] for the production of lipases, because there are fractions of the oil residue that can induce the production of this enzyme.

Although each of the three agro-industrial residues alone have been reported for the production of enzymes, the novelty of this work focuses on the combined use of the residues to establish an inexpensive culture medium, which has not yet been reported. Therefore, the aim of the present study was to assess the suitability of OMW, CSL, and molasses as a growth medium for the production of microbial lipases by the yeast *Diutina rugosa* NRRL Y-95, which was previously known as *Candida rugosa* [36] and was recently reclassified. In sum, the aim was to develop a bioconversion processes based on the circular economy and bio-economy concepts.

## Materials and Methods

### Microorganism and Inoculum Preparation

*Diutina rugosa* (former *Candida rugosa* NRRL Y-95) from the ARS Culture Collection (Agricultural Research Service), reported as a lipase producer [37], was kindly provided by the US Department of Agriculture. All cultures were activated in media containing sabouraud dextrose agar (SIGMA) at 30 °C for 48 h. Cultures were prepared in media containing 40 g/L of glucose and 10 g/L of peptone, supplemented with glycerol (30% *v*/*v*), and the cultures were further stored in a cryogenic freezer at − 80 °C.

The inoculum was obtained in 250-mL Erlenmeyer flasks by inoculating 50 mL of culture medium with a single colony, taken from an agar plate, and incubated in an orbital shaker for 20 h at 30 °C and 170 rpm. The culture medium was composed of glucose (10 g/L), peptone (10 g/L), yeast extract (4 g/L), and oleic acid (0.5% *v*/*v*) in 50 mM potassium phosphate buffer, pH 7.0. An inoculum size equal to 5% (*v*/*v*) of the final volume was used in all assays.

### Agro-Industrial Residues

Sugarcane molasses were kindly provided by Refinarias de Açúcar Reunidas, S.A (RAR, Portugal), and contains around 490 g/L of carbohydrates and 0.6 g/L of protein [38]. CSL was supplied by Companhia Portuguesa de Amidos, S.A. (COPAM, Portugal), and contains 75 g/L of carbohydrates and 5 g/L of protein [28, 38]. The OMW was obtained from an olive oil factory located in northern Portugal, composed of water and residual oil: 11.9% (*v*/*v*) palmitic acid, 3.0% (*v*/ *v*) stearic acid, and 78.5% (*v*/*v*) oleic acid [38].

### Shake Flask Batch Cultures

Shake flask experiments were conducted in 250-mL Erlenmeyer's flasks containing 50 mL of culture medium at 30 °C and 170 rpm of agitation for 24 or 48 h. Samples of 2 mL were collected under aseptic conditions from each flask to determine the cell growth, carbohydrate consumption, pH, and enzyme activity. Cells were removed from the culture broth by centrifugation (5,000×g for 20 min at 4 °C), and the cell-free culture supernatant was stored

for further analysis. The assays were performed in triplicate, and the results are expressed as the mean ± standard deviation.

### Screening of Agro-Industrial Residues for Lipase Production by *D. rugosa* NRRL Y-95

Shake flask cultures were used to evaluate the potential of the agro-industrial residues in the production of lipase by *D. rugosa* NRRL Y-95 at 30 °C for 24 h. Initially, a synthetic medium was studied as control, with the following composition: glucose (20 g/L), peptone (3 g/L), $KH_2PO_4$ (1 g/L), ammonium sulfate (0.5 g/L), yeast extract (4 g/L), and oleic acid (1% *v*/*v*). Afterwards, four agro-industrial residues were evaluated as alternative nutrients in the formulation of the culture medium (Table 1). In these alternative media, the amount of molasses used was set according to an estimated glucose content corresponding to a concentration of 10 g/L. The amount of CSL used was set according to the concentration of yeast extract used in the synthetic media (4 g/L). The amount of OMW used was 1% (*v*/*v*). The alternative media also contained 0.5 g/L ammonium sulfate and 3 g/L peptone. All culture media were sterilized at 120 °C for 15 min.

### Fractional Factorial Design to Evaluate the Effect of the Agroindustrial Residue Concentration in Lipase Production

A fractional factorial design ($2^{3-1}$) with 3 factors (molasses, CSL, and OMW concentrations) and 3 repetitions at the central point was conducted in a total of 7 trials. The concentration of the different agroindustrial residues in culture medium 4 (Table 1) was defined as the independent variable of the design, and the lipase activity was the response variable. Concentrations of molasses were varied between 5 and 15 g/L, CSL between 2 and 6 g/L, and OMW between 0.5 and 1.5% *v*/*v*. The response variable was the extracellular lipase activity. Cultures were grown in shake flasks for 24 h.

### Influence of CSL Concentration on Lipase Production by *D. rugosa*

The impact of changing the concentration of CSL on lipase production was investigated using the modified medium 4 (medium 4*), with the following composition: molasses (5 g/L carbon source), CSL (6–20 g/L nitrogen source), OMW 0.5% (*v*/*v*) (inducer), 0.5 g/L ammonium sulfate, and 3 g/L peptone. Molasses concentration was kept constant while the CSL concentration was varied. Assays were conducted in shake flasks, as previously described in "Shake flask batch cultures" section, for 48 h. The carbon-to-nitrogen (C/N) ratio was estimated, and for that, the amount of carbon and nitrogen present in the residues used was retrieved from the

**Table 1** Synthetic and alternative culture media containing agro-industrial residues used for the production of lipase by *D. rugosa* NRRL Y-95. The remaining media components were kept constant as follows: 0.5 g/L ammonium sulfate and 3 g/L peptone prepared in 50 mM of potassium phosphate buffer at pH 7.0

| Medium | Carbon source (10 g/L) | Nitrogen source (4 g/L) | Inducer 1% (*v*/*v*) |
|---|---|---|---|
| Synthetic (control) | Glucose | Yeast extract | Oleic acid |
| 1 | Molasses | Yeast extract | Olive oil |
| 2 | Glucose | CSL | Olive oil |
| 3 | Glucose | Yeast extract | OMW |
| 4 | Molasses | CSL | OMW |

literature [4, 28, 38]. Some authors reported that molasses contain 490 g/L and 0.6 g/L of carbohydrates and protein, respectively, and CSL contains 75 g/L and 5 g/L of carbohydrates and protein, respectively [28]. OMW is mainly composed of water (83 to 94%) and residual oil [4]. Oleic acid (78.5%) and palmitic acid (11.9%) are the major components in its fatty acid composition [38].

### Bench-Scale Stirred Tank Bioreactor Cultures

Lipase was produced in a 1-L bioreactor containing 750 mL of modified culture medium 4 (medium 4*) at 30 °C and 300 rpm for 48 h. Cells (corresponding to 5% inoculum) were aseptically transferred to the bioreactor containing molasses (5 g/L), CSL (6 g/L), OMW (0.5%), ammonium sulfate (0.5 g/L), and peptone (3 g/L) in 50 mM of potassium phosphate buffer at pH 7.0. The pH was controlled at 6.5 with NaOH 1.5 M. Samples (2 mL) were collected under aseptic conditions to determine cell growth, carbohydrate consumption, and enzyme activity.

### Effect of pH and Temperature on Lipase Activity

The effect of pH and temperature on the lipase activity was determined. Cell-free supernatants from culture broth (crude extracts), containing the extracellular lipase produced by *D. rugosa* NRRL Y-95 obtained at 24 h of cultivation, were used.

To evaluate the pH effect on the enzyme activity, samples of the crude extract were incubated at pH values between 4.0 and 9.0 at a constant temperature of 40 °C. The following buffer systems were used at a concentration of 50 mM: sodium acetate buffer, pH 4.0–5.0; potassium phosphate buffer, pH 6.0–7.0; and sodium bicarbonate buffer, pH 8.0–9.0. Afterwards, the lipase activity was determined using a standard assay [22].

The effect of temperature on the enzyme activity was determined at a constant pH value of 7.0 incubating samples of the crude extract at varying temperatures ranging from 30 to 70 °C. Afterwards, lipase activity was determined using a standard assay [22].

### Culture Medium Costs

An estimate of the culture medium cost (MC) was determined by relating the components used in the medium formulation, considering a total volume of 1 L (Eq. (1)). In order to compare the different culture media studied, the unit production cost (UPC) of lipase was defined as shown in Eq. (2):

$$\mathrm{MC} = \left(\sum_{k=1}^{n} C_k Pc_k\right) \tag{1}$$

$$\mathrm{UPC} = \frac{MC}{P_P} \tag{2}$$

where MC (culture medium cost) was expressed in dollar cents per liter (¢/L), $C_K$ (concentration of component $k$) in the medium (kg/L), $Pc_K$ (purchase cost of component $k$) in the medium in dollar cents (¢/kg), UPC (unit production cost of lipase) in dollar cents per liter (¢/U), and $P_P$ lipase activity (U/L) in the reactor medium.

The prices of the medium components were estimated from the literature and e-commerce platforms (Table 2) in order to better represent industrial market prices [39].

## Analytical Methods

### Reducing Sugar Concentration

Reducing sugar concentration was determined by the Miller method described elsewhere [40]. The standard curve was prepared from a glucose solution at concentrations between 0.2 g/L and 3 g/L. A volume of 100 μL of sample was stirred with 100 μL of 3,5-dinitrosalicylic acid (DNS) reagent and then boiled at 100 °C for 5 min. Afterwards, 1 mL of distilled water was added, and the mixture was cooled in an ice bath before reading the absorbance at 540 nm with a spectrophotometer (Biochrom Libra S11).

### Biomass

The biomass concentration was determined by measuring the absorbance at 600 nm using a spectrophotometer (Biochrom Libra S11) and a biomass standard curve.

### Lipase Activity

Lipase activity was determined using p-nitrophenyl laurate (pNFL) as substrate. The pNFL solution was prepared by dissolving 9 mg of pNFL in 0.5 mL of dimethyl sulfoxide (DMSO). Next, 50 mL of potassium phosphate buffer (PBS) solution (50 mM, pH 7.0) was added, as described by Brígida et al. [22]. A 380 μL aliquot of the resulting solution was mixed with 50 μL of the enzyme crude extract, and it was incubated at 40 °C for 10 min. After this time, the reaction was stopped by adding 800 μL of acetone [41] and the reaction was followed by measuring the absorbance at 410 nm using a spectrophotometer (Biochrom Libra S22). One

**Table 2** Costs in dollar cents (¢) of main components used in the formulation of the culture medium for lipase production. Different formulations were studied, namely synthetic medium (experiment 1), medium 1 (experiment 2), medium 2 (experiment 3), medium 3 (experiment 4), medium 4 (experiment 5), and optimized medium 4 (experiment 6)

| Component | Purchase cost (¢/kg) | Concentrations for each experiment | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| Glucose (g/L)[a] | 70 | 20 | – | 20 | 20 | – | – |
| Yeast extract (g/L) [a] | 170 | 4 | 4 | – | 4 | – | – |
| Oleic acid (% *v*/*v*)[b] | 119 | 1 | 1 | 1 | – | – | – |
| Peptone (g/L) [a] | 610 | 3 | 3 | 3 | 3 | 3 | 3 |
| $MgSO_4.7H_2O$ (g/L)[a] | 30 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| $KH_2PO_4$ (g/L)[a] | 120 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Molasses (g/L)[b] | 9.8 | – | 50.5 | – | – | 50.5 | 25.2 |
| CSL (g/L)[b] | 0.5 | – | – | 8.6 | – | 8.6 | 8.6 |
| OMW (% *v*/*v*)[c] | 0.0 | – | – | – | 1 | 1 | 1 |

[a] Cardoso et al. [34]

[b] https://www.alibaba.com

[c] No commercial value

unit of enzyme activity is defined as the amount of enzyme that released 1.0 μmol of p-nitrophenol per minute under the assay conditions.

## Results and Discussion

### Screening of Agro-Industrial Residues for Lipase Production by *D. rugosa* NRRL Y-95

In this work, the combined use of agro-industrial residues as feedstock for lipase production was evaluated through a classical one-factor-at-a-time method. First, the lipase production by *D. rugosa* NRRL Y-95 was evaluated using a synthetic medium, for comparison reasons. Next, the effect of replacing medium constituents (one at a time) by agro-industrial residues on lipase production was assessed, and results are summarized in Table 3.

Compared with the synthetic medium, the results obtained using the alternative media, particularly medium 2–4, were promising. These preliminary experiments showed that molasses, CSL, and OMW can be effectively used as carbon source, nitrogen source, and inducer, respectively, since the lipase activity was improved when *D. rugosa* NRRL Y-95 was cultivated using the alternative nutrients. Indeed, this was very interesting results, strengthening the potential of such residues for lipase production, by replacing the conventional carbon and nitrogen sources by more economic nutrients. Furthermore, culture medium 4, which combines the four residues, was the one that led to the greatest amount of lipase produced at 24 h of cultivation, which is almost 4-fold comparing with the amounts obtained using the synthetic medium. The other culture media were also satisfactory for lipase production; for instance, culture medium 2 allowed an extracellular lipase activity of 424.0 ± 20 U/L, which is almost 3-fold compared with the synthetic medium. In summary, from this preliminary evaluation, the concentration of residues in medium 4 was selected to be further investigated through a fractional factorial experimental design.

### Optimization of the Selected Alternative Culture Medium

After these initial trials, a fractional factorial experimental design was conducted to evaluate the effects of the different constituents' concentrations (medium 4) on the lipase production towards its maximization. As it can be seen in Table 4, enzyme production ranged from 383 to 641 U/L, depending on the medium composition. These experimental results were used to estimate the variable main effects and the interactions among them. Figure 1 shows a Pareto chart that illustrates the impact of CSL, OMW, and molasses on lipase production. All medium components were found to have a positive effect on the production of the lipase. However,

**Table 3** Enzymatic activity of lipase produced by *D. rugosa* NRLL Y-95 after 24 h of cultivation using different culture media (as defined in Table 1)

| Culture medium | Biomass concentration (g/L) | Lipase (U/L) |
|---|---|---|
| Synthetic | 3.50 ± 0.04 | 143.0 ± 18 |
| 1 | 3.60 ± 0.05 | 143.0 ± 20 |
| 2 | 4.88 ± 0.03 | 424.0 ± 20 |
| 3 | 4.83 ± 0.39 | 213.1 ± 10 |
| 4 | 4.39 ± 0.04 | 561.8 ± 30 |

**Table 4** Effect of the substrates in alternative culture medium number 4 in activity lipase. The fractional factorial design ($2^{3-1}$) with 3 factors (molasses, CSL, and OMW concentrations) and 3 repetitions at the central point was conducted in a total of 7 trials

| Assays | Molasses (g/L) | OMW (% *v*/*v*) | CSL (g/L) | Activity (U/L) |
|---|---|---|---|---|
| 1 | 5 | 0.5 | 6 | 587.0 ± 02.0 |
| 2 | 15 | 0.5 | 2 | 383.0 ± 18.0 |
| 3 | 5 | 1.5 | 2 | 418.0 ± 39.0 |
| 4 | 15 | 1.5 | 6 | 641.0 ± 09.0 |
| 5 (C) | 10 | 1 | 4 | 427.0 ± 26.0 |
| 6 (C) | 10 | 1 | 4 | 392.0 ± 11.0 |
| 7 (C) | 10 | 1 | 4 | 402.0 ± 02.0 |

only for CSL, this effect was statistically significant, at a significance level of 0.1, which means that the higher the CSL concentration, the higher the lipase activity.

Overall, the most important finding was that CSL exhibited a strong positive effect on the lipase activity. This agro-industrial residue is rich not only in carbohydrates and proteins, but also contains surface active molecules (Vecino et al. 2014). Therefore, CSL may have the same effect as Tween that is commonly used in the synthetic culture media reported for lipase production. Several reports suggest that Tween stimulates lipase production and aids its excretion, thus resulting in an increased lipase activity (Kim et al. 2010; Dalmau et al. 2000).

For the next steps, based on the results obtained with the fractional factorial experimental design, medium 4 was modified (medium 4*) to the following composition: molasses (5 g/L), CSL (6 g/L), OMW (0.5%), ammonium sulfate (0.5 g/L), and peptone (3 g/L). It is important to note that ammonium sulfate and peptone concentrations were kept constant.

## Influence of CSL Concentration on Lipase Production by *D. rugosa*

Based on the previous results, the impact of changing the CSL concentration on lipase production was further investigated. As the factorial design revealed that only CSL presents a significant effect on the lipase production, it was not possible to proceed with the culture medium optimization by response surface methodology. Since it is not possible to build a factorial experiment with a single variable, a univariate analysis was conducted, and results are shown in Fig. 2. However, the increase in the CSL concentration did not promote an increase on lipase production. On the contrary, the enzyme activity decreased when more than 6 g/L of CSL was used.

According to the literature, in batch cultivations, no differences have been observed in terms of biomass and lipase production when the nitrogen source is used in excess, considering the stoichiometry calculated from the elementary composition of the microorganism [19, 20]. The C/N ratio of modified medium 4 (medium 4*) is about 4.68, which is close to the amount necessary for biomass production (C/N 4.51). At carbon concentrations higher than the one at this ratio (4.51), the metabolic pathway of the yeast cannot be directed for the production of additional amounts of enzyme. Some authors mention the importance of nitrogen to protein metabolism [19], in order to assure lipase production. Furthermore, the cell morphology also is also affected when the limiting nutrient is exhausted in the culture broth. Culture conditions can lead to changes both in the shape and length of the cells, organized in chains. The same authors observed a change in the shape and length of cells, which have become predominantly rounded or ellipsoidal, when

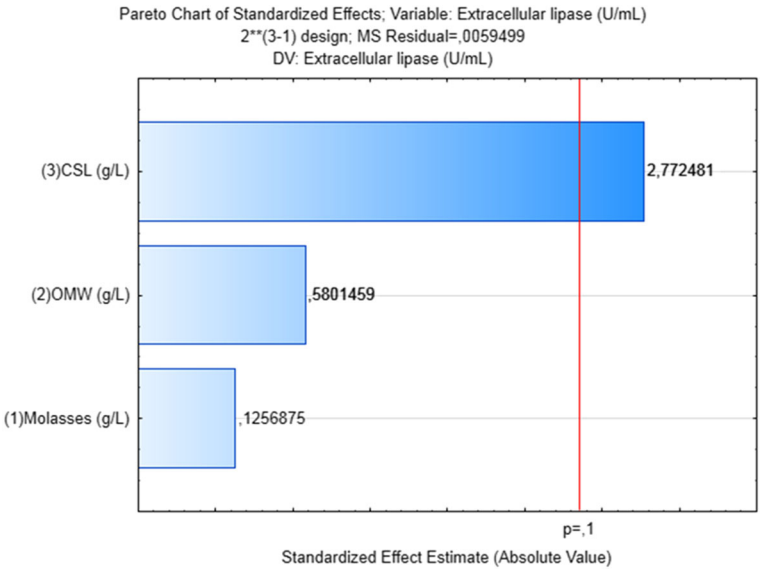


**Fig. 1** Effect of the three medium components, CSL (corn steep liquor), OMW (oil mill waste), and molasses, on the lipase activity obtained after 24 h in submerged culture

nitrogen is limiting [19, 20]. In addition, lipase production was interrupted when this rounded or ellipsoidal morphology was present [20].

## Lipase Production in Shake Flasks and in a Bench-Scale Bioreactor

After defining the concentration of medium 4 constituents, lipase production as a function of time was investigated both in shake flasks and in a bench-scale bioreactor. Figure 3 shows the results of biomass concentration and lipase activity, respectively.

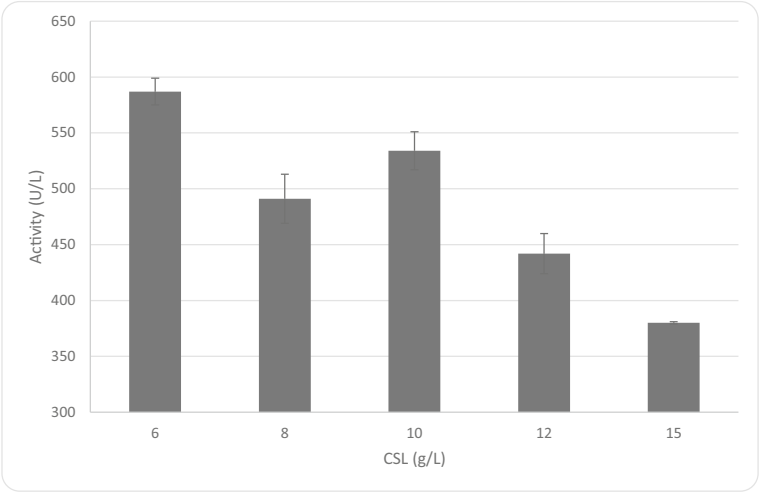


**Fig. 2** Lipase production by *D. rugosa* NRRL Y-95 as a function of the concentration of CSL in flasks at 30 °C and 170 rpm for 48 h. All experiments were conducted in triplicates

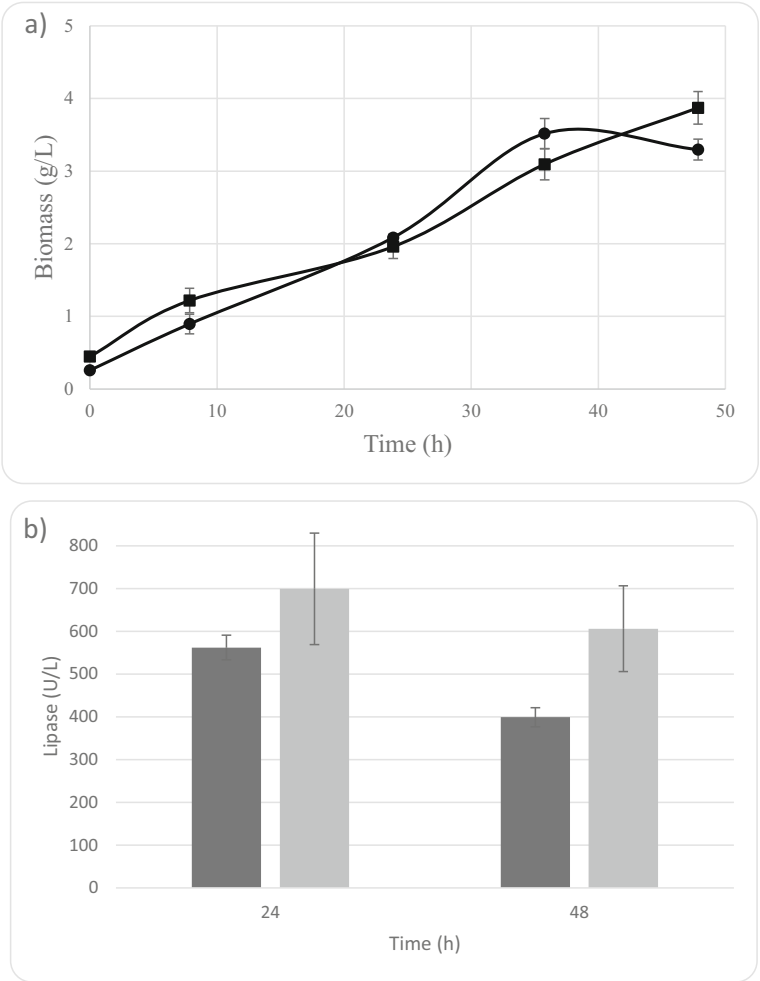


**Fig. 3** Cultivation of *D. rugosa* NRRL Y-95 at 30 °C: **a** cell growth in flasks (circles) and using a stirred tank reactor (squares) and **b** lipase production in flasks (dark gray) and using a stirred tank reactor (light gray)

In general, the experiments conducted in shaken flasks led to lower biomass concentration and lipase activities than the ones obtained in bioreactor. Regarding the best cultivation time, a similar trend was found, i.e., a maximum lipase activity was achieved at 24 h of cultivation. This occurs most probably because at 48 h of cultivation, some nutrients are exhausted and therefore are no longer available for enzyme production. Although the final biomass concentration was similar in flasks and bioreactor, the behavior was different. In the experiments conducted in flasks, biomass reached stationary phase around 36 h. On the other hand, in the bioreactor, biomass constantly increases until 48 h, although, as mentioned, a decline in the lipase production was observed.

These differences observed in the lipase production in flasks and bioreactor have been widely reported [21, 42]. For instance, Bussamara et al. [42] reported a lipolytic activity of 386 U/L and 1232 U/L obtained in in flasks and bioreactor, respectively. The lipase activity values herein reported are lower, not only because the microorganism is different but also

because no oxygen supply was used. Many studies report significant differences in the lipase production when air is supplied [10, 43, 44], which may be an alternative for further investigation aiming higher lipolytic activity.

## Culture Medium Costs

Table 5 shows the comparison of lipase production and costs of the different culture media investigated in this work. As previously mentioned, the medium composition strongly influenced the lipase production, especially when yeast extract was replaced by CSL (experiments 3, 5, and 6), leading to a higher lipase activity. Regarding the culture medium cost (MC), a decrease was observed, as expected, when the alternative raw materials were used to replace glucose, yeast extract, or olive oil. The effect of the inducer was the most pronounced among the nutrients evaluated. Media containing OMW (experiments 4, 5, and 6) presented lower costs when compared with the ones containing oleic acid (experiments 1, 2, and 3), thus reducing more than 20% the culture medium cost. The combined use of residues (alternative raw materials), in the best conditions (experiment 6), provided the lowest MC, which was approximately 43% of the cost of the synthetic medium (experiment 1). When lipase production was taken into account, by analyzing UPC, again the experiment 6 stands out with the lowest production cost. UPC using synthetic medium is 9.2-fold higher than using modified medium 4*. In addition to the lower cost of the medium, higher soluble protein production using the alternative media contributed to mitigate enzyme costs.

Figure 4 shows the cost distribution across the different components for the synthetic medium (experiment 1) and the modified medium 4 (experiment 6). The carbon source plays an important role in the total culture medium cost, representing 28% in the synthetic medium and 12% in the complex medium. Nevertheless, organic nitrogen sources were the major contributors to the culture medium costs, representing 50% and 85% in the synthetic medium (peptone and yeast extract) and modified medium 4 (peptone), respectively. Similarly, other researchers have reported that nitrogen sources are more relevant than carbon sources in the culture media costs [39]. Moreover, those researchers also indicate that these compounds are potential targets to reduce the enzyme production costs. In the current work, considering all the medium components, peptone exhibited the greatest impact on the medium cost (e.g., 85% of medium 4). Therefore, additional efforts should be made to replace this nutrient, for instance by using other protein hydrolysates (such as hydrolyzed casein from whey), as its prices may be crucial to the overall culture medium cost.

**Table 5** Lipase activity (U/L) in the reactor medium ($P_P$), culture medium cost (MC), and unit production cost of lipase (UPC) for each culture medium used for enzyme production by *D. rugosa* NRRL Y-95. MC was expressed in dollar cents per liter (¢/L) and UPC in dollar cents per unit of enzyme produced (¢/U). Media composition is described in Table 2

| Experiment | Culture medium | $P_p$ (U/L) | MC (¢/L) | UPC (¢/U) |
|---|---|---|---|---|
| 1 | Synthetic | 143 | 5.04 | $3.53 \times 10^{-2}$ |
| 2 | Medium 1 | 143 | 4.14 | $2.90 \times 10^{-2}$ |
| 3 | Medium 2 | 424 | 4.37 | $1.03 \times 10^{-2}$ |
| 4 | Medium 3 | 213 | 3.98 | $1.87 \times 10^{-2}$ |
| 5 | Medium 4 | 561 | 2.40 | $0.43 \times 10^{-2}$ |
| 6 | Medium 4* | 562 | 2.15 | $0.38 \times 10^{-2}$ |

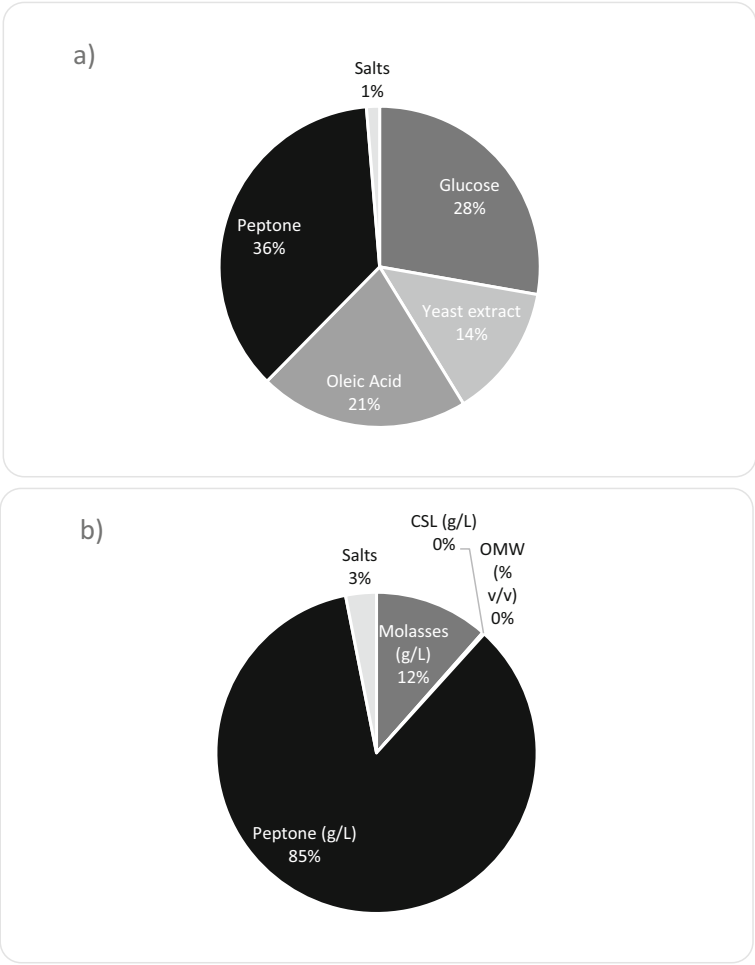


**Fig. 4** Cost distribution across the different components of the culture medium: **a** synthetic Medium and **b** modified medium 4

## Effect of pH and Temperature on Lipase Activity

Figure 5 shows the effect of pH on the lipase activity at a constant temperature of 40 °C using pNFL as substrate. The highest activity value was observed at pH 7.0, decreasing rapidly at higher pH values and exhibiting very low activity (< 30%). At pH 6, the enzyme maintained 60% of its maximum activity, but at more acidic pH values, similarly to what was observed at basic pH values, the residual activity was very low (< 20%). Similar results were observed by other authors working with *D. rugosa* lipase [45] and lipases from other microorganisms [25, 42, 46]. In fact, it is well known that changes in the pH of the enzyme microenvironment may play an important role in the movement of the lipase lid and, consequently, in its enzymatic activity [47].

The effect of temperature on the lipase activity was investigated at temperatures ranging from 30 to 70 °C, as shown in Fig. 6, at a constant pH of 7.0. The maximum activity was obtained at 40 °C. This optimum temperature value is in good agreement with other studies

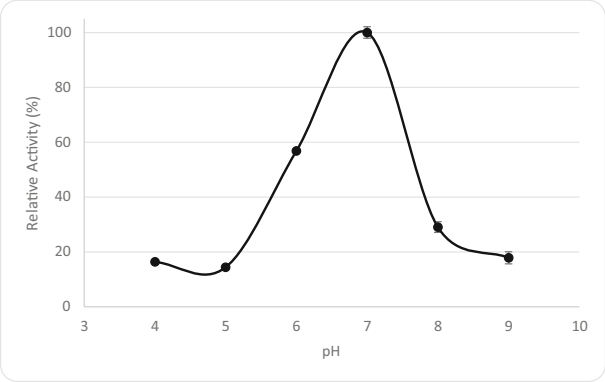


**Fig. 5** Effect of the pH on lipolytic activity of CRL at 40 °C and 50 mM sodium-acetate buffer (pH 4.0 and 5.0), 50 mM sodium-phosphate buffer (pH 6.0–8.0), or 50 mM sodium-bicarbonate buffer (pH 9.0). Results correspond to the average of triplicate experiments ± standard deviation. Relative activity is expressed as a ratio of the maximum activity

reported in the literature [25, 42, 46]. However, it is important to mention that *D. rugosa* lipase (CRL) retained more than 80% of its initial activity when incubated at 50 and 60 °C. A further increase in temperature led to a loss of activity, and the enzyme retained around 50% of its initial activity at 70 °C. Considering that the enzyme was incubated without the addition of the substrate, these results show that the enzyme may be used at higher temperatures. Another key point is that CRL can also be used in applications with low boiling-point compounds or labile substances, since it is also active at low temperatures (30 °C).

It is important to mention that temperature affects not only enzyme activity but also stability, which is the enzyme capacity in retaining activity. Loss of stability may be caused by protein denaturation (unfolding and/or structural changes), and temperature is one of the variables that accelerates unfolding. Therefore, the optimum temperature is as compromise between increasing activity while maintaining stability which is not easy to determine. Although stability depends on the duration of the assay, while the activity is essentially time-independent, it is not always possible to conduct true initial rate tests. In some cases, inactivation is so fast that initial reaction rates remain constant for a very short period of time,

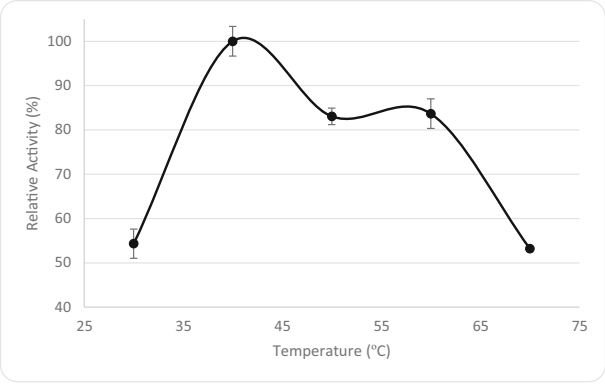


**Fig. 6** Effect of the temperature on CRL activity at pH 7.0 (50 mM phosphate buffer). Results correspond to the average of triplicate experiments ± standard deviation. Relative activity is expressed as a ratio of the maximum activity

making it very difficult to quantify them [48]. Hence, it would be interesting to further assess the enzyme stability to different temperatures.

## Conclusion

This work opens new perspectives in the valorization of agro-industrial residues, namely molasses, CSL, and OMW, in enzyme production by submerged cultures. Lipase was produced by *D. rugosa* NRLL Y-95 in a medium containing 5 g/L of molasses, 6 g/L of CSL, 0.5% of OMW, 0.5 g/L of ammonium sulfate, and 3 g/L of peptone. This medium favored the production of lipase both in shake flasks and in a bench bioreactor, thus highlighting its interest for industrial enzyme production. However, the nitrogen source prices seem to be determinant, and further efforts to replace peptone must be conducted. Last but not least, the lipase produced can be used in a wide range of temperature, which is an interesting property for biocatalytic reactions.

**Funding** The study is funded by CAPES, CNPq, and FUNCAP (from Brazil) for the financial support that made this work possible. In addition, the study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UIDB/04469/2020 unit, the BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020-Programa Operacional Regional do Norte, and the Project LIGNOZYMES (POCI-01-0145-FEDER-029773).

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