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Original scientific paper

Lipase production by *Yarrowia lipolytica* using olive oil processing wastes as substrates

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Abstract: In this study, the solid and liquid wastes from the olive oil processing industry were evaluated as substrates for *Yarrowia lipolytica* growth with the aim of lipase production. Olive mill wastewater and olive oil cake seemed to provide the necessary nutrients and physical support for yeast growth and enzyme production. The highest lipolytic activity of 850 IU dm^{-3} was achieved after 4 days of submerged cultivation in supplemented olive mill wastewater. In addition, olive oil cake appeared to be a convenient substrate for lipase production under a solid-state fermentation mode. Lipase production was further improved by media supplementation and/or change in the physical settings of the experiment. However, the most significant improvement of lipase production under solid-state fermentation was achieved by an alkaline treatment of the substrate (more than 10-fold), when the amount of produced lipase reached up to ≈40 IU g^{-1} of substrate.

Keywords: olive oil cake; olive mill wastewater; *Yarrowia lipolytica*; solid-state fermentation; lipase production.

INTRODUCTION

Olive oil is considered one of the best edible oil in terms of tastiness, stability and lipid profile. It is very rich in oleic acid and contains omega-6 and omega-3 essential fatty acids in a favorable ratio, making it one of the healthiest cooking oils. In addition, olive oil contains large amounts of plant-derived anti-

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oxidants (such as hydroxytyrosol, oleocanthal and oleuropein), phyto-sterols and vitamins.[1] The growing awareness of the importance of a proper diet rich in essential fatty acids and antioxidants on human health, as well as its distinct taste, has resulted in a worldwide increase in olive oil consumption.

Almost all of the produced olive oil (estimated at over 2.5 million tonnes per year) originates from the Mediterranean region.[2] Libya, as a Mediterranean country, has millions of olive trees, which are native to the region. According to the Food and Agriculture Organization of the United Nations, Libya produced 15000 tonnes of olive oil in 2009.

The olive oil production industry generates large amounts of waste, of which olive mill wastewater (OMW) and crude olive oil cake (solid waste – OOC) have the highest organic load and therefore present the largest pollutants of the oil processing industry. The quality and quantity of the constituents of these wastes are dependent of many factors, such as type and maturity of the olives, climatic conditions and region of origin, cultivation methods, and the technology used for oil extraction.[3] Traditional pressing and the three-phase system are relatively obsolete technologies for oil extraction that are being replaced by a new centrifugation two-phase system labeled as ecological due to the reduced generation of OMW. The processing of 1 t of fresh olives by the most frequently employed three-phase system yields about 210 kg of olive oil, 550 kg of crude olive cake, and 1–1.6 m^3 of OMW.[3] The phytotoxic and antimicrobial nature of these wastes are credited to their high phenolic and residual lipid contents.

Although several methods for olive oil processing waste have been proposed, *i.e.*, anaerobic digestion, ultrafiltration, precipitation/flocculation and electrocoagulation, olive oil processing waste is mostly improperly managed.[3,4] In most cases, olive mill wastewater is spread on ground or collected in vaporization ponds, causing pollution of the underlying soil. Solid residues (OOC) are sometimes used as fuel due to their high-energy content. Several pyrolysis methods were developed for the production of fuel from this waste.[5,6] Due to its content and unfavorable effects on plants and animals, its use as a component of fertilizers or animal feed is limited.[7,8] Thus, very little of the large quantity of olive oil processing wastes is being valorized.

However, as with the wastes of other agricultural processing industries that are difficult to treat and valorize, these substances can be used as starting materials for biotechnological applications, in particular for the synthesis of high value metabolites, such as single cell proteins, microbial lipids, organic acids, bio-surfactants and enzymes.[9] The use of these substrates for fermentation processes aimed at enzyme production decreases the final cost of the enzyme, making industrial enzymatic processes cost-competitive with chemical ones.[10]

Yarrowia lipolytica is strictly an aerobic yeast widely used in industrial applications due to its ability to produce a wide spectrum of products, such as

organic acids and/or extracellular enzymes, in adequate amounts. Several processes in which this strain is employed were classified as generally recognized as safe (GRAS) by the Food and Drug Administration (USA).[11] The yeast is also widely used in bioremediation processes due to its specific metabolic pathways and the ability to alter its cell surface, thereby enabling the efficient degradation of hydrophobic substrates, such as *n*-alkanes, fatty acids, fats, and oils.[12] Of a total of 300 yeast isolates obtained from samples of agro-industrial wastes, two strains, identified as *Y. lipolytica* species, have been proven to be the most capable to produce valuable products, such as lipase and citric acid, when grown on different waste substances.[9]

A variety of wastes have been studied as substrates for the growth of different strains of *Y. lipolytica* as a means of waste disposal and/or upgradation by additionally generating value-added products.[13] However, only few reports were focused on the production of lipase by the yeast when grown on olive oil processing wastes. For example, De Felice *et al*. investigated lipase production as well as OMW degradation in batch cultures of *Y. lipolytica* W29 (ATCC 20255), showing that the yeast was capable of metabolizing the waste and, under optimum conditions, a lipase activity of 770 U dm^{-3} was obtained.[14] Gonçalves *et al*. also cultivated *Y. lipolytica* W29 on OMW for lipase production under batch and fed-batch culture conditions, finding that the enzyme yields were higher in the former.[15,16] Although a few other reports considering lipase production by *Y. lipolytica* using OMW as a substrate have been published,[17–19] to the best of our knowledge, no attempts have hitherto been made to use the olive oil cake as a substrate for the production of lipase by this yeast using solid state fermentation (SSF).

The present study was aimed to valorize olive oil processing wastes (OMW and OOC) as substrate mediums for the cultivation of the yeast *Y. lipolytica* in order to facilitate the production of lipases. Some of the factors affecting the growth and the production of extracelluar lipase by the yeast strain were studied. To the best of our knowledge, this is the first report on a more complete assessment of lipase production by the yeast using different olive oil processing wastes.

EXPERIMENTAL

Organism

The microorganism used in this study, *Yarrowia lipolytica* NRRL Y-1095, was donated by the Agricultural Research Centre (USA) to the Microbiological Laboratory of the Faculty of Technology and Metallurgy, Belgrade, Serbia. The employed yeast strain was maintained on malt agar slants at 4 °C. A one-day-old culture grown in malt broth was used as the inoculum (approximately 5×10^7 cells cm^{-3}).

Characterization of the substrates

The OMW samples were collected from various traditional olive oil mills in Lasaba and Gharian, Libya, and used as fermentation medium for the submerged yeast cultivation. The

substrate samples were characterized before fermentation for total solids, chemical oxygen demand (*COD*), pH, crude protein, phenols, reducing sugars and total lipids. The total phenols were determined photometrically at 765 nm using the Folin–Ciocalteu method and are expressed as gallic acid equivalents.[20] The crude protein content was determined by the Kjeldahl method and multiplying the nitrogen content by 6.25.[21] The lipid content was determined gravimetrically after lyophilization of the sample and subsequent extraction of the samples with *n*-hexane at 60 °C for 20 h using a Soxhlet extraction apparatus.[21] The DNS (3,5-dinitrosalicylic acid) method was used for carbohydrate detection.[22] The *COD* was assessed using the standard method of titration.[23]

Samples of OOC were also obtained from various traditional olive oil mills (Libya) and used as a natural substrate for the SSF. They were packed in vacuum-sealed packages and stored at 4 °C until use. The moisture content of the cake was determined gravimetrically as described in detail elsewhere.[21] The ash and crude fiber contents were determined as previously described.[21] The total nitrogen of the substrate was determined following the standard Kjeldahl method while the oil content of the OOC was determined gravimetrically after extraction of the samples with *n*-hexane using a Soxhlet extraction apparatus.[21]

Submerged fermentation using olive mill wastewater as the substrate

The submerged fermentation was performed by transferring 100 cm^3 of undiluted samples of OMW to Erlenmeyer flasks and sterilizing them at 121 °C (at 1.2 bar pressure) for 30 min prior to inoculation with 1 % (v/v) of the yeast culture in malt broth. The fermentation was performed in thermostat shaker at 30 °C at 150 rpm. Samples were withdrawn at 24 h intervals and tested for lipase activity.

Optimization of the substrate

Composition of the OMW was optimized by the addition of ammonium sulfate (0.6 % w/v), yeast extract (0.1 % w/v), maltose (0.5 % w/v), olive oil (0.3 % w/v) and peptone I (0.1 % w/v).

Solid-state fermentation (SSF) on olive oil cake

The substrate was dried at 105±5 °C for 1 h, and sieved to provide particles of size between 0.2 and 0.5 mm. The experiments were performed in 150 cm^3 Erlenmeyer flask with 5 g of well ground dry substrate supplemented with 0.15 g of yeast extract. Then, 1 cm^3 of distilled water was added and the contents of the flask were mixed and autoclaved at 121 °C for 20 min. Unless otherwise stated, SSF was realized by inoculating olive oil cake (initial moisture content adjusted to 50 %) with 500 μL of inoculum followed by incubation at 30 °C. The water added with the inoculums was also considered in the moisture content. Optimization studies were performed by varying the moisture content of the substrate and amount of inoculum. The effect of the addition of various carbon and nitrogen supplements was also studied for optimal lipase production.[21] Carbon (maltose, oleic acid and starch) and an inorganic nitrogen source (NH_4NO_3) were used at the 1 % level, while organic nitrogen sources (peptone and yeast extract) were used at the 3 % level to investigate their effect on lipase production.

Alkaline treatment of OOC

The alkaline treatment has been performed by mixing dried OOC with 3 % (w/v) NaOH and holding overnight at 20–22 °C. The pretreated substrate was then washed with distilled water until pH 7, dried, autoclaved at 121 °C for 20 min and used for fermentation. The pH of the substrate was not followed during fermentation due to the non-homogeneity of the fermen-

tation mixture. After 5 days of fermentation, the pH of a homogenized suspension of 1 g of fermented cake in 10 cm^3 of deionized water was measured using a pH meter.

Extraction of the enzymes

The crude enzymes were extracted by mixing a known quantity of fermented substrate with sterilized distilled water (1:5, w/w) following incubation in a KS 4000 orbital shaker (Ika-Werke, Germany) at 30 °C and 180 rpm for 30 min. A part of the liquid phase was used for the determination of yeast cell growth, while the rest was centrifuged at 12,000 rpm for 10 min. The supernatant was used for the determination of the lipase activity.

Lipase activity assay

Lipase activity was determined by hydrolysis of *p*-NPP (*p*-nitrophenyl palmitate) substrate by lipases according to a previously described method.[24] The amount of liberated *p*-nitrophenol was measured photometrically (410 nm) during the first 3 min of reaction. One unit of enzyme activity (IU) is defined as the amount of enzyme that formed 1 μmol of *p*-nitrophenol per minute (ε =1500 dm^3 mol^{-1} cm^{-1}) under the assay conditions.

Growth studies

Determination of yeast cell growth was performed by spreading suitably diluted cell suspensions on malt agar plates and counting the yeast cell colonies after incubation for 48 h at 30 °C.

RESULTS AND DISCUSSION

Characteristics of the substrates

Although composition of OMW can vary, this waste was a dark, acidic liquid (pH 4.8±0.6) with a characteristic odor and high organic load (*COD* 220±35 g dm^{-3}). The solid content was 127.5 g dm^{-3}. The OMW samples contained 11.9 g dm^{-3} of reducing sugars (expressed as the glucose equivalent) and 18.9 g dm^{-3} of lipids. The organic fraction of OMW also included phenols (6.9 g dm^{-3}) and proteins (195 mg dm^{-3}). Almost all phenolic matter of the olive fruit was reported to remain in the OMW (53 %) and OOC (45 %).[16] Since phenols were reported to act as enzymatic inhibitors, they could prevent spontaneous microbial processes, making these wastes serious pollution threats.[17] The mean moisture content of the olive oil cake was 51.4±1.5 mass%, while the composition of the cake on a dry-weight basis, except moisture, was 4.1±0.4 mass% proteins, 11.1±0.5 mass% lipids, 20.6±0.9 mass% carbohydrates, 60.1±1.3 mass% fibers and 4.1±0.4 mass% ash.[21]

Submerged fermentation using olive mill wastewater as the substrate

In this study, the yeast *Y. lipolytica* was tested for its ability to grow and produce lipase in such a medium. The OMW was inoculated with the strain for a preliminary study to estimate the suitability of olive oil wastewater as a substrate for growth and lipase production.

Y. lipolytica demonstrated the ability to grow in unsupplemented OMW, yielding up to ≈160 IU dm^{-3} lipolytic activity on the fifth day of fermentation

(Fig. 1). Suitability of the OMW medium for growth of various *Y. lipolytica* strains was already demonstrated by many researchers.[12,25] Lopes *et al*. studied two *Y. lipolytica* strains, ATCC20460 and IMUFRJ 50 682, which produced up to 30 IU dm^{-3} lipolytic activity in unsupplemented OMW.[2] Additionally, Gonçalves *et al*. studied three *Y. lipolytica* strains in OMW from different stages of olive processing, achieving 317-1041 IU dm^{-3} lipolytic activity while simultaneously reducing the *COD* and phenolic content by up to 51 % and 35 %, respectively.[16] In addition, almost all of the 62 *Y. lipolytica* strains studied by Lanciotti *et al.* were able to grow and produce lipase in OMW.[19] Papanikolaou *et al.* examined the capability of this yeast to produce citric acid in an OMW-based medium. These fermentation processes resulted in significant reductions in the phenolic content and *COD*.[26]

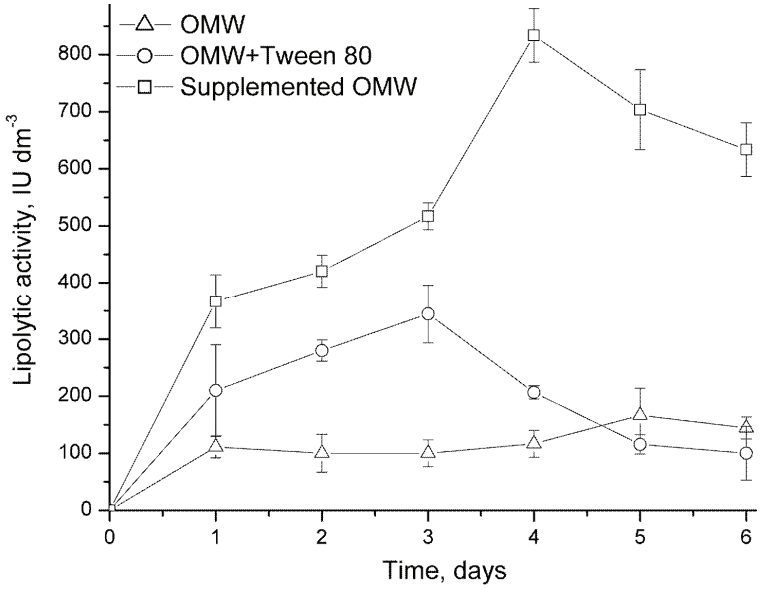

Fig. 1. Lipase production by *Y. lipolytica* in olive mill wastewater. The OMW was supplemented by the addition of ammonium sulfate (0.6 % w/v), yeast extract (0.1 % w/v), maltose (0.5 % w/v), olive oil (0.3 % w/v) and peptone I (0.1 % w/v).

Since the OMW contained a relatively large amount of residual fat, studies aimed at determining the influence of the addition of Tween 80 were undertaken with the intention to increase the oil dispersion in the medium. The addition of Tween 80 was shown to improve lipase secretion significantly (Fig. 1). Such a phenomenon of increased lipase production could be credited to changes in the permeability of cell membranes, facilitating the release of various metabolites, including enzymes, out of the cell. The literature review particularly confirmed this indicating that *Y. lipolytica* produced various lipases (extracellular Lip2p,

cell-bound lipases I and II, as well as intracellular lipolytic enzymes).[27] Surfactant supplementation seemed to lead to a 2-fold increase in lipase production, shifting the maximum of the enzyme production to the third day of fermentation. However, this effect of surfactant addition to the medium was not always registered and literature data showed significant variations in the influence of surfactant, even among the same species. Although the addition of surfactant appeared to stimulate biomass production of several *Y. lipolytica* strains, lipase production was inhibited in such media.[2,28] On the other hand, the addition of Tween 80 led to a 3-fold increase in lipase production by *Y. lipolytica* 681.[29]

Since a literature survey showed that OMW lacked some basic nutrients, the effect of substrate supplementation on lipase production was investigated. It seemed that the supplementation led to a 5-fold increase in lipase production in comparison to enzyme production in non-supplemented media. The level of produced enzyme reached up to ≈850 IU dm^{-3}, which is comparable to or higher than data found in literature related to OMW treatment with *Y. lipolytica* spp., as well as with other microorganisms.[2,16,30]

Solid-state fermentation (SSF) on olive oil cake

Although submerged fermentation is the most commonly used method for lipase production,[31] recently production by SSF has been gaining significant attention.[32–34] The advantages of SSF are low total capital investment and low production costs, relatively high productivity and better product characteristics. It was reported that for a production scale of 100 m^3 lipase concentrate per year, the total capital investment needed for the submerged fermentation technology was 78 % higher than that needed for SSF.[35] Despite all the advantages of SSF over submerged fermentation, the use of this technique for the lipase production is still far from being applied on the industrial scale.

The suitability of OOC as a substrate for lipase production in the SSF cultivation mode was verified by inoculating 5 g of previously moistened OOC (moisture content 50 mass%) with 500 µL of a one-day-old culture in malt broth. Growth and lipase production curves are given in Fig. 2.

The studied yeast strain seemed to effectively grow and produce lipase in such a medium, achieving the maximum yield of produced lipase (3.8 IU g^{-1} of substrate) on the second day of fermentation and decreasing thereafter. Generally, these results corresponded to the literature data suggesting similar pattern of lipase production by *Y. lipolytica* in which lipase production occurred during the intensive cell growth.[27,36] The decrease in the lipolytic activity in the later phases of growth was usually accredited to nitrogen and carbon utilization and pH decrease as a result of the production of organic acids, such as citric acid.[28,29] However, this growth to lipase production ratio was not a common pattern for other microorganisms, particularly in the SSF mode of culture. For instance,

Candida utilis growth in the same medium reached maximum lipase activity on the 4th day of fermentation, which was followed by cessation of yeast growth.[21] The abrupt decrease in viable cell counts on the 5th day could be due to a decrease in pH and sugar consumption.

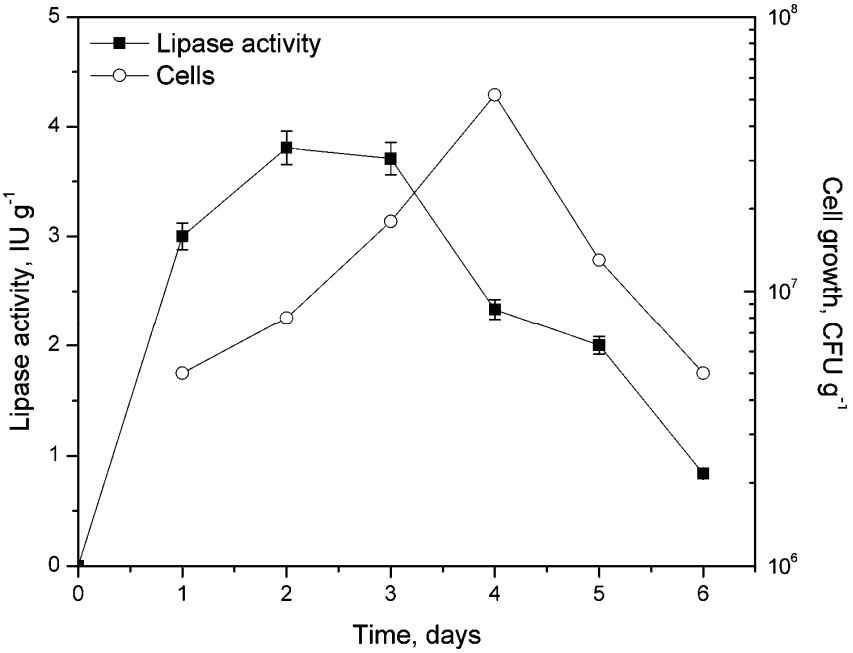

Fig. 2. The kinetics of growth and lipase production by *Y. lipolytica* in unsupplemented olive oil cake under solid-state fermentation conditions (30 °C, moisture content: 50 %). CFU g^{-1} – colony forming units per g.

Effect of moisture content and inoculum size

Since the capability of *Y. lipolytica* growth and lipase production were confirmed, studies were undertaken to optimize lipase production in this medium. In view of the fact that the moisture content and inoculum size could be critical factors in solid-state fermentations, the effect of both different initial moisture levels and inoculum size on lipase production and cell growth were evaluated. The optimal moisture content for lipase production appeared to be at 55 % initial moisture content of substrate, as shown in Fig. 3, similar to previous results considering lipase production by *C. utilis* on the same medium, where the optimal moisture content was found to be 55–60 %.[21] According to a literature survey on SSF lipase production on various food and agricultural wastes, it was suggested that an excessive moisture content has a negative effect on the physical properties of the substrate, causing agglutination of substrate particles that led to retarded oxygen transfer and over-intrusion of the substrate particles. However, sub-optimal

moisture levels adversely affected microbial growth due to insufficient particle swelling, which had a negative impact on the enzymatic activity.[21] The optimum substrate moisture content corresponded well to most of the literature data. For instance, Imandi *et al*. found the maximum lipase activity was achieved when *Y. lipolytica* was grown in seed oil cake at 60 % initial moisture.[34] In general, the differences in moisture requirements in SSF depend not only on the microorganism employed, but also greatly on the type of substrate, especially in terms of the water-holding capacity of the substrate.

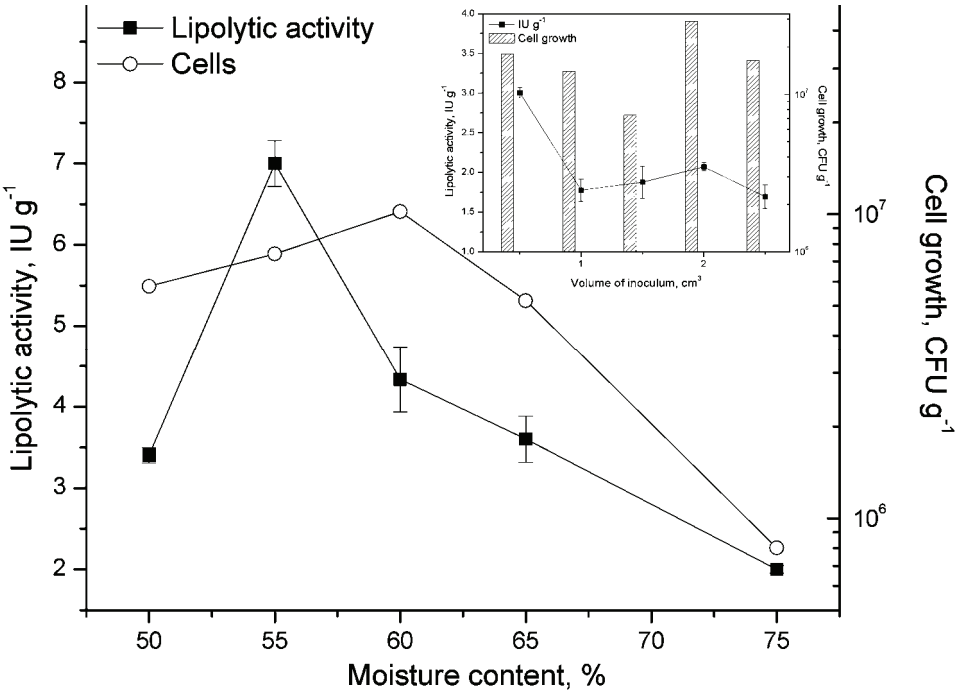

Fig. 3. Effect of moisture content and inoculum size on lipase production and growth of *Y. lipolytica* on olive oil cake (30 °C, 3rd day of fermentation).

The highest yield of produced lipase was achieved in OOC inoculated with 0.5 cm^3 of yeast culture in malt broth (approximately 5×10^7 cells cm^{-3}). It was interesting that higher cell concentrations did not result in higher lipase activity under the same culture conditions. The inhibition of the biosynthesis of lipase at higher cell concentrations might be related to inferior oxygen transfer into the culture media. It is known that low oxygen transfer negatively affects the metabolism of several microorganisms.[37] At higher cell concentrations, the oxygen transfer appeared to be lower, altering the yeast metabolism and, consequently, the production of lipase. The other possible explanation might be related to sugar consumption and pH decrease because of the production of organic acids, result-

ing in lipase deactivation. In addition, the decrease of lipase production could be due to the action of proteases.

Although microbial growth seemed to be influenced strongly by the level of inoculum employed, a significant impact of this factor on lipase production was not detected. Contrary to the present results, other researchers using the same range of inoculum volumes found that this factor had a major impact on lipase production by *Y. lipolytica* on seed oil cake in SSF, with the maximum activity being obtained when 2 cm^3 of inoculum was used.[34] However, the inoculum concentrations were not specified.

Effect of medium supplementation

To increase lipase production by the yeast and considering that the choice of the carbon source could be of crucial importance for the reduction of catabolite repression and induction of lipase biosynthesis, the basal medium (OOC) was supplemented with various carbon sources. For comparison, the yeast strain was also grown with OOC as the sole carbon source. Additionally, the medium was supplemented with several nitrogen sources. The achieved lipolytic activities in the control and enriched mediums are presented in Fig. 4.

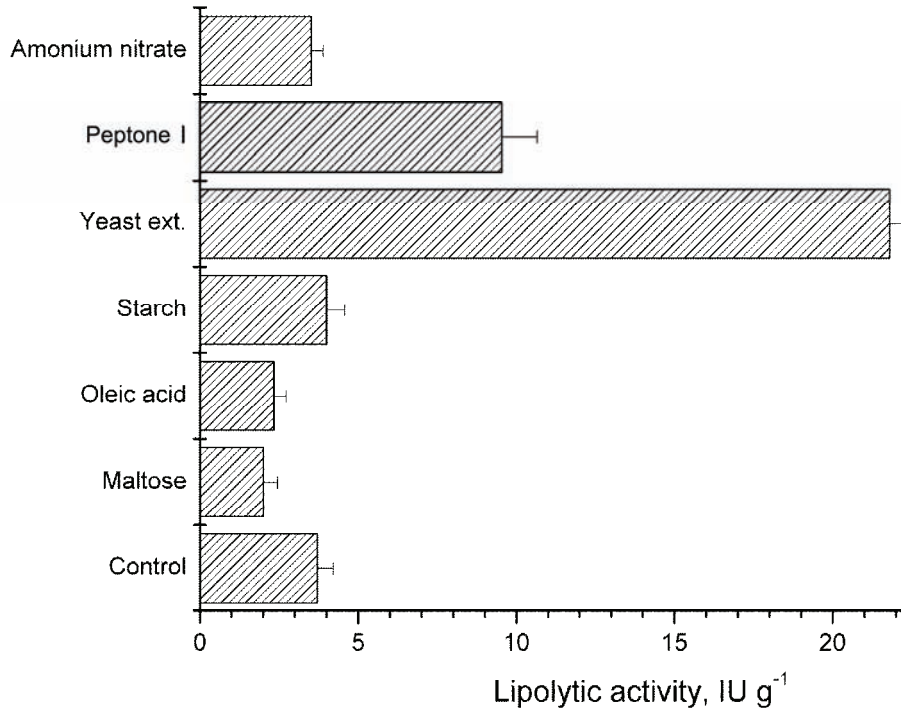

Fig. 4. Effect of supplementation of olive oil cake on lipase production by *Y. lipolytica* (30 °C, 3rd day of fermentation).

As can be seen in Fig. 4, additional oil and maltose supplementation inhibited the production of lipase. Among investigated carbon sources, starch appeared to enhance lipase production. However, the effect of starch addition was negligible as the increase in produced amounts of enzyme was only to 10 % with the respect to the control. These results differed significantly from those obtained with *C. utilis* on the same medium. A previous study on lipase production with this yeast strain showed a significant influence on lipase production on OOC supplemented with maltose, while starch inhibited enzyme secretion.[21]

On the other hand, lipase production seemed to be strongly influenced by the addition of nitrogen sources. The addition of organic nitrogen sources to the basal medium was found to be effective in enhancing the production of lipase by *Y. lipolytica*, while addition of inorganic nitrogen did not affect the enzyme production. Yeast extract addition caused a 6-fold increase in the secreted lipase unlike in a previous study with *C. utilis*, which showed that nitrogen addition had a negligible effect on lipase production. Moreover, all the employed nitrogen sources, except the yeast extract, inhibited lipase production by *C. utilis* on the same medium.[21]

In accord with the present results, a study of *Y. lipolytica* growth on seed oil cake showed a major positive impact of medium supplementation with nitrogen sources such as urea, peptone or yeast extract on the yield of the produced lipase, while NH_4NO_3 also had a negligible effect on enzyme production.[34]

Effect of alkaline treatment of the substrate

Since alkaline treatment of the OOC caused significantly better utilization of the substrate in a previous study on lipase production by *C. utilis* on the same media,[21] the same preparation method was employed to verify the possibility of improving the consumption of the substrate by *Y. lipolytica* and subsequently enhancing lipase production. The treatment was conducted based on the assumption that it could cause swelling and disruption of the structure of the substrate cell wall, facilitating thereby the access of degradative enzymes.[38] Namely, studies showed that alkaline treatment of olive oil pomace led to a significant reduction in the contents of cellulose, hemicellulose and lignin.[39] In addition, literature data showed that alkaline pretreatment of other substrates, such as bagasse, coir pith and rice husks, had tremendous effects on the physical properties of the substrates, leading to significant improvement of cellulase production by several microorganisms under SSF conditions.[39–41] The effects of this treatment on both lipase production and cell growth are shown in Fig. 5.

The biomass concentration gradually increased with fermentation period when the strain grew in alkali-treated OOC. A different pattern of cell growth was obtained for untreated OOC when the biomass concentration reached a maximum after 4 days and then decreased. This result is possibly related to the differences in the pH profile during fermentation in treated and untreated sub-

strates, although the pH was not controlled during the fermentation because of the non-homogeneity of the fermentation systems. Specifically, the measured pH after fermentation was 6.3 and 4.5 for treated and untreated substrates, respectively.

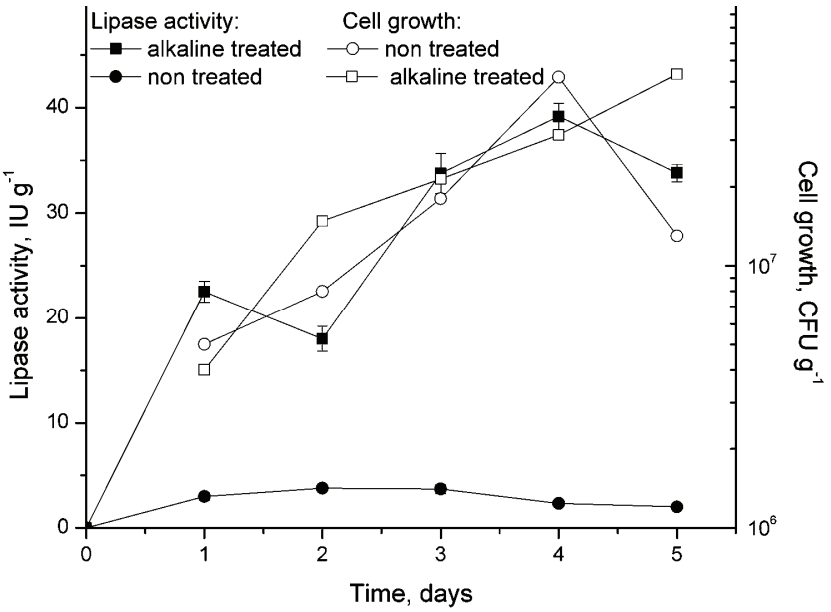

Fig. 5. Kinetics of *Y. lipolytica* growth and lipase production on alkaline-treated and non-treated OOC.

The treatment resulted in a more than a 10-fold increase in the yield of produced lipases, although it seemed that the level of produced biomass was unaffected. The amount of produced lipase reached up to ≈40 IU g^{-1} of substrate, exceeding numerous reported values considering lipase production in the SSF mode with different strains and substrates.[30]

CONCLUSIONS

This study showed that the *Y. lipolytica* NRRL Y-1095 strain could be successfully utilized for treatment and valorization of olive oil processing waste. OMW and OOC seemed to provide the necessary nutrients and physical support for yeast growth and enzyme production but this production could be further optimized by media supplementation and/or change in the physical settings of the experiment. Additionally, alkaline treatment of OOC appeared to improve remarkably lipase production by *Y. lipolytica* in SSF production. The amount of lipase in this initial study is promising and it could be of interest to attempt the production of other industrial enzymes from different microbes.

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ИЗВОД

ПРОИЗВОДЊА ЛИПАЗЕ ИЗ *Yarrowia lipolytica* КОРИШЋЕЊЕМ ОТПАДНИХ СИРОВИНА ИНДУСТРИЈЕ ПРЕРАДЕ МАСЛИНА КАО СУПСТРАТА

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У овом раду, течне и чврсте отпадне сировине које заостају приликом прераде маслина испитане су као потенцијални супстрати за раст квасца *Yarrowia lipolytica* са циљем производње липаза. Отпадна вода из млина, као и погача која заостаје након цеђења уља из маслина, показали су се као добри извори нутријената за раст овог квасца и производњу ензима. У оптимизованој течној подлози, принос липаза достиже и до 850 IU dm^{-3}. Поред тога, погача која заостаје након цеђења уља из маслина се показала као погодан чврсти супстрат за гајење производног микроорганизма. Продукција липаза на овом медијуму је додатно оптимизована суплементацијом различитим изворима азота и угљеника, као и променом осталих параметара ферментације. Утврђено је да се најзначајније побољшање продукције липазе остварује алкалним предтретманом супстрата (више од 10 пута).

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