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Optimization of extracellular lipase production by *Debaryomyces hansenii* isolates from dry-salted olives using response surface methodology

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ABSTRACT

The lipase production by *Debaryomyces hansenii* strains isolated from dry-salted olives cv. Thassos was investigated. Glucose, olive oil and pH were essential to obtain a high lipase yield. Optimization of the medium components which enhance lipase production by the strain *D. hansenii* YLL29 was achieved with the aid of response surface methodology. The composition of the optimized medium to enhance lipase production by *D. hansenii* is as follows (g/L): yeast extract 5.0, peptone 10, K_2HPO_4 4.0, $MgSO_4 \cdot 7H_2O$ 1.0, glucose 13.1, olive oil 19, Tween 80 3.8, and pH 6.4. Practical validation of the optimum medium gave lipase activity 7.44 U/mL, which was 2.28-fold higher than the unoptimized conditions. Under the optimized conditions the twenty *D. hansenii* isolates showed increased lipase activity fluctuating between 6.00 and 7.44 U/mL. The results corroborated the validity and the effectiveness of the model, as the statistical approaches proved to be suitable in predicting the optimum production medium composition for maximum extracellular lipase yield. The high lipolytic activity of *D. hansenii* YLL29 (7.44 U/mL) indicates the possible commercial importance of this isolate.

Keywords: Extracellular lipase production; *Debaryomyces hansenii*; Batch culture; Dry-salted olives; Experimental design

1. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are defined as glycerol ester hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol, and act at the interface between oil and water (Treichel et al., 2010). As surface-active enzymes, their activity is highly influenced by the interfacial area (Talukder et al., 2006). In non-aqueous environments lipases can reverse the reaction to synthesize triacylglyceride from glycerol and free fatty acid. Therefore, lipases can catalyze a wide range of reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis, and aminolysis (Joseph et al., 2008). Lipases are an important group of enzymes with biotechnologically relevant applications in food, dairy, detergent and pharmaceutical industries. In the food industry commercial lipases are utilized for flavour improvement of dairy products and processing of meat, vegetables, fruit juices, etc. (Singh and Mukhopadhyay, 2012). As they are indispensable for the bioconversion of lipids in nature, there is an increased interest for established technical applications of lipases as well as for entirely new areas of application.

Although the carbon metabolism of microorganisms is primarily based on carbohydrates, the presence of lipases enables many microorganisms to utilize lipidic carbon sources. As lipids cannot passively cross cell membranes, they have to be degraded into free fatty acids outside the cell before free fatty acids are absorbed by the cell. This process requires that microbial lipases are excreted into the medium where lipids are hydrolyzed (Najjar et al., 2011). Many lipase-secreting microorganisms including bacteria, fungi and yeasts have been isolated from lipid-rich environments. However, lipase production from yeasts remained a much neglected area in comparison to bacteria or fungi (Ali et al., 2010).

Debaryomyces hansenii is a non-pathogenic, osmotolerant and lipid-accumulating oleaginous yeast. Oleaginous yeasts

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accumulate lipids and contribute to lipid metabolism. Thus the capacity of *D. hansenii* to synthesize, accumulate and store lipids is advantageous for the biotechnological processes (Breuer and Harms, 2006). Moreover, *D. hansenii* is a halophilic yeast, as it grows optimally at 3–5% (w/v) salt, and is able to grow in concentrations of sodium chloride up to 2.5 M (Breuer and Harms, 2006; Prista et al., 2005). The lipase production by *D. hansenii* has been barely explored (Takaç and Şengel, 2010). The incidense of *D. hansenii* in salty environments and the peculiar behaviour of this yeast contributed that the consortium Génolevures selected *D. hansenii* to sequence and annotate its genome, available at http://cbi.labri.fr/Genolevures/. The increased lipase production by *D. hansenii* during the fermentation process is essential for industrial applications. However no published results are reported concerning the improvement of the fermentation process taking into account the variation of the medium components and their interactions.

Dry-salted olives are a special type of naturally black olives called "naturally black dry-salted olives Thassos style" as they are traditionally cultivated on the island of Thassos in Greece. The olives are harvested fully mature and completely black in colour, placed in concrete tanks in layers with coarse sodium chloride in a proportion of up to 40% and gradually lose water and oleuropein (the phenolic compound which causes the bitter taste of olive). In 30–60 days, olives become debittered, wrinkled and eatable. The microflora of the product is comprised of yeasts (Panagou et al., 2002).

Response surface methodology (RSM) is a useful statistical technique for the investigation and optimization of complex processes. It is a collection of mathematical and statistical techniques, and is widely used in different biotechnological processes to study the effects of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. Central composite design (CCD) is a widely used response surface design when the experimental region is defined by the upper and lower limits of each factor and not extended beyond them (Neter et al., 1996). A combination of factors generating a certain optimal response can be identified. Also, significant interactions between variables can be identified and quantified by this technique. The production process of lipase by *Rhizopus delemar* was optimized by RSM (Açikel et al., 2010).

The present work was aimed at optimization of medium components which enhance lipase production by *D. hansenii* isolates with the aid of RSM. A CCD was employed to optimize the carbon and lipidic carbon sources as well as pH value of the production medium, which have significant influence on lipase production and the results were analyzed by RSM.

2. Materials and methods

2.1. Isolation and screening of lipase producing yeasts

Yeast strains with lipolytic activity were isolated from dry-salted olives of Thassos variety. Samples of 20 g olives obtained after removing the pit were homogenized for 60 s with 180 mL of sterile saline (0.85 g/L NaCl) containing Tween 80 (1 mL/L) using a Stomacher Lab-Blender 400 (Seward Medical, London, UK). Appropriate dilutions of the sample homogenates were inoculated on YMPG (yeast–malt–peptone–glucose) agar medium (Difco, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulphate (Oxoid) as selective agents for yeasts and incubated up to 5 days at 27 °C. A total of 97 colonies showing variations in appearance on YMPG agar (concerning surface, texture, shape, margin and diameter) were selected and purified on the above medium. The pure cultures were observed under a phase contrast microscope (Nikon Eclipse 50i, Japan) to distinguish cell morphology. For maintenance and storage of pure cultures YMPG agar was used, incubated at 27 °C and stored at 4 °C.

The 97 isolates were studied for lipase activity using rhodamine olive-oil agar method (Kouker and Jaeger, 1987) as described by Rodríguez-Gómez et al. (2010). Each isolate was cultivated in 5 mL of YMPG broth for 24 h at 30 °C. After centrifugation at 12,000 × *g* for 10 min, the cell pellets were washed twice in sterile 50 mM phosphate buffer (pH 7) and re-suspended in 2 mL of the same sterile buffer. Amounts of 10 μL of the whole cell were placed on rhodamine olive-oil agar. After 48 h of incubation at 30 °C, colonies were irradiated with UV light at 350 nm. The occurrence of orange fluorescent halos around the colonies indicated lipase production.

2.2. Characterization and identification of yeast strains

The 20 isolates showing lipase production were identified to species level according to the methodology and characteristics given by Kotzekidou (1997), Psani and Kotzekidou (2006) and Suzuki et al. (2011).

2.3. Production of lipase by D. hansenii YLL29 in shake flask culture

Lipase production was studied in 250 mL Erlenmeyer flasks containing 50 mL of the basal medium with the following composition (g/L): yeast extract 5.0, K_2HPO_4 4.0, $MgSO_4 \cdot 7H_2O$ 1.0, peptone 10. The production medium is composed of the basal medium supplemented with different concentrations of glucose and olive oil whereas the pH of the medium was adjusted to different values (i.e. 5.5, 6.5, and 7.5). According to the concentration of olive oil, Tween 80 was added to the production medium in a concentration of 20% (w/w) of olive oil. The medium was sterilized at 121 °C for 15 min. After cooling, the production medium contained in each flask was sonicated on a Vibra Cell Ultrasonic processor (Sonics & Materials, Inc., Newtown, CT, USA) that was equipped with a micro tip for 30 s to create an emulsion. The flasks were inoculated with 10% of freshly prepared culture of *D. hansenii* YLL29 grown in YMPG broth at 30 °C for 24 h. The flasks were incubated at 30 °C for 72 h in a rotary shaker incubator (Lab Line Orbit-Environ Shaker, Lab-Line Instr., Melrose Park, IL, USA) at 150 rpm. Data are the mean of three independent experiments. The data were analyzed by ANOVA and Tukey's test at $\alpha = 0.05$ using SPSS statistical software (SPSS Statistics 17.0, Chicago, IL, USA).

2.4. Experimental design and statistical analysis

Lipase activity can be calculated as a function of the levels of the three independent variables (glucose, olive oil, pH) with a significant influence on the response variables. Each parameter had three levels: the maximum value corresponds to +1, the minimum one to −1, and the centre point to 0, as shown in Table 1. A second-order polynomial equation, which

Table 1 – Codes and actual levels of independent variables for design of experiment.

Variables	Symbols	Coded levels		
		−1	0	+1
Glucose (g/L)	X_1	1	10	19
Olive oil (g/L)	X_2	1	15	29
pH	X_3	5.5	6.5	7.5

includes all interaction terms, is used to calculate the predicted response:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where Y represents the predicted response, i.e. lipase activity (LA) released in the culture medium expressed in U/mL, β_0 is the intercept, β_i first order coefficient, β_{ii} second order coefficient, β_{ij} the coefficient of interaction effect and x_i, x_j the coded levels of variables X_i and X_j investigated in experiments. The variable X_i was coded as x_i according to the equation: $x_i = (X_i - X_0)/\Delta X_i$ where x_i is coded value of the variable X_i (dimensionless), X_0 is the value of X_i at the centre point level and the step change value ΔX_i = (high level − low level)/2. Coding is required since the factors are expressed in different units (Table 1).

CCD is a useful design to acquire data to fit the above polynomial. A 2^3 full factorial design with six replicates at the centre point resulting in 20 experiments was used to investigate the three selected variables (i.e. glucose, olive oil and pH) for determination of the production medium components for lipase production. The levels of the variables (Table 1) were chosen after a series of preliminary experiments. The experiments were designed by using the Statistica software package (StatSoft, Tulsa, OK). The experimental design is presented in Table 2. The experimental design was repeated in three replications.

The experimental data were subjected to multiple regression analysis using the STATISTICA Program (StatSoft, Inc., Tulsa, OK) to obtain the coefficients of the second-order polynomial. The *F* test was used to evaluate if the model was significant. The multiple coefficients of correlation *R* and the determination coefficient R^2 were calculated to evaluate the performance of the regression equation. By analyzing the graphical presentation of the equation the behaviour of the model in the experimental area was investigated. The optimum levels of the variables to obtain maximum response is denoted by the vector $X_s = -½\ B^{-1}b^*$, where the elements of the matrix B are the estimated coefficients at the second-order terms and the elements of the vector b^* are the estimated coefficients of the first-order terms (Neter et al., 1996). Statistical testing of the model was done in the form of analysis of variance (ANOVA), which is required to test the significance and adequacy of the model. In the Pareto plot of standardized effects, the important effects are visually identified. The bars correspond to the absolute magnitudes of the estimated effect coefficients. An effect that exceeds the vertical line ($p = 0.05$) may be considered significant.

2.5. *Assay of lipolytic activity and biomass production*

After 72 h of incubation, the content of each flask was analyzed. All data were assumed as the average values of three independent experiments.

The determination of lipase activity was based on the procedure described by Tommaso et al. (2011) with some modifications. Lipase assay was performed using olive oil as a substrate. Lipase activity was calculated by titrating the fatty acids liberated from olive oil with alkali. The assay mixture contained 2 mL of filter-sterilized olive oil through nitrocellulose filter with a porosity of 0.22 μm (Millipore, Bedford, MA, USA), 4.5 mL of 100 mM phosphate buffer pH 7, 0.5 mL of 100 mM $CaCl_2$ and 0.5 mL of culture supernatants (12,000 × *g*, 10 min). The mixture was incubated for 30 min at 30 °C with continuous stirring at 200 rpm. The enzyme reaction was stopped by adding 20 mL of ethanol. Fatty acids released during the incubation of the sample and a blank assay carried out adding the ethanol direct after the addition of the enzyme solution, were determined by titration with 50 mM NaOH in the presence of phenolphthalein indicator. One unit of lipase activity was defined as the activity which liberated 1 μmol of free fatty acid per min under the above conditions.

Ten millilitre samples from the fermentation broth were filtered through preweighed Whatman GF/C filter discs (diameter 47 mm). The harvested biomass was washed twice with distilled water and then with three aliquots (10 mL, each) of n-hexane to remove possible traces of residual oil; the filter was dried at 105 °C, for 24 h to constant weight, cooled in a desiccator and weighed.

2.6. *Lipase and biomass production by D. hansenii isolates under optimized conditions*

The 20 *D. hansenii* isolates from dry-salted olives of Thassos variety were grown in 250 mL Erlenmeyer flasks containing 50 mL optimized medium with the following composition (g/L): yeast extract 5.0, K_2HPO_4 4.0, $MgSO_4 \cdot 7H_2O$ 1.0, peptone 10, glucose 13.1, olive oil 19, Tween 80 3.8 and pH was adjusted to 6.4. The medium was sterilized at 121 °C for 15 min. After cooling, the content of each flask was sonicated, inoculated and incubated in a rotary shaker incubator as described above. Lipase activity and dry biomass were determined as described previously. All data were assumed as the average values of three independent experiments. The data were analyzed by one way analysis of variance (ANOVA) and pair-wise multiple comparison procedures (Tukey's test at $\alpha = 0.05$) using SPSS statistical software (SPSS Statistics 17.0, Chicago, IL, USA).

3. Results and discussion

3.1. *Isolation, identification and selection of lipase producing yeasts*

Dry-salted olives of Thassos variety have been selected as a biotope for the isolation of lipase producing yeasts because the product fulfils the following requirements: the strains have a long tradition as food-grade yeasts because they are part of the microflora of a traditional food product and the enzyme produced may be generally recognized as safe (GRAS) which is particularly attractive for lipase used in the food, dairy, pharmaceutical and cosmetic industries.

A total of 97 yeast colonies were isolated from olive samples, including representatives of all colony types seen. Of the 97 strains evaluated, 20 yeast strains showed increased lipase activity on rhodamine olive-oil agar. The above strains were tested for 32 morphological and physiological characters and identified as *D. hansenii* (Kotzekidou, 1997; Psani and Kotzekidou, 2006; Suzuki et al., 2011). The biochemical and

Table 2 – Experimental design of lipase production by *Debaryomyces hansenii* YLL29 grown on basal medium supplemented with glucose and olive oil at adjusted pH values in shake flask culture at 30 °C for 72 h.

Run	Coded level			Lipase activity (U/mL)	
	Glucose	Olive oil[a]	pH	Experimental[b]	Predicted
1	−1	−1	−1	$3.25^{j} \pm 0.1$	3.27
2	−1	−1	1	$5.67^{i} \pm 0.13$	5.85
3	−1	1	−1	$8.53^{cd} \pm 0.23$	9.06
4	−1	1	1	$8.00^{de} \pm 0.21$	8.05
5	1	−1	−1	$6.90^{gh} \pm 0.12$	6.95
6	1	−1	1	$10.12^{a} \pm 0.19$	9.69
7	1	1	−1	$8.33^{d} \pm 0.14$	8.25
8	1	1	1	$7.33^{fg} \pm 0.12$	7.41
9	−1	0	0	$6.67^{h} \pm 0.33$	5.96
10	1	0	0	$7.00^{gh} \pm 0.3$	7.48
11	0	−1	0	$5.33^{i} \pm 0.13$	5.51
12	0	1	0	$7.67^{ef} \pm 0.27$	7.26
13	0	0	−1	$8.90^{bc} \pm 0.17$	8.47
14	0	0	1	$9.15^{b} \pm 0.22$	9.34
15	0	0	0	$7.33^{fg} \pm 0.13$	7.35
16	0	0	0	$7.33^{fg} \pm 0.12$	7.35
17	0	0	0	$7.00^{gh} \pm 0.12$	7.35
18	0	0	0	$7.27^{fg} \pm 0.12$	7.35
19	0	0	0	$7.27^{fg} \pm 0.11$	7.35
20	0	0	0	$7.33^{fg} \pm 0.13$	7.35

[a] Tween 80 was added in each run in a concentration of 20% (w/w) of olive oil.
[b] Data are shown as mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's test ($p < 0.05$).

technological characteristics of the 20 isolates with increased lipase activity characterized as *D. hansenii* are presented in Table 3. An important characteristic of the 20 isolated strains is the capacity to adapt to stringent environmental conditions, i.e. low temperature (the strains were able to grow at 4 °C), and high concentration of salt. The main characteristics of *D. hansenii* are that the microorganism grows faster in the presence of up to 1 M NaCl due to increase in the plasma membrane fluidity as a consequence of the increase of fatty acid unsaturation at higher NaCl concentrations compared to lower concentration of salt (Turk et al., 2007).

The yeast strain forming the most intense orange fluorescent halo around the colonies on rhodamine olive-oil agar (i.e. *D. hansenii* YLL29) was selected for optimization experiments of lipase production. Preliminary results showed that growth conditions such as availability of carbon and nitrogen sources, the presence of surfactants, incubation temperature, pH, level of inoculum and aeration influence the extracellular lipase production.

3.2. *Lipase production in shake flask culture*

The lipase production was undertaken in shake flask cultures as the enhanced aeration rate favours the production of extracellular enzymes by aerobic organisms. Preliminary experiments showed that aeration is essential for lipase production and the highest activity is obtained in shake flask cultures compared with static cultures.

The addition of glucose in the production medium was essential to achieve a high biomass production which is a prerequisite for lipase production; in order to achieve maximum lipase production the microorganism must grow and produce or release lipase under the prevailing growth conditions. Lee et al. (2007) found glucose as the best carbon source for lipase production. Whereas, increased lipase activity was achieved by *Candida cylindracea* Y-17506 in an optimized liquid medium where glucose had been used as the sole carbon source (Muralidhar et al., 2001). Lipase production is induced in the presence of olive oil from which oleic acid is released (Dalmau et al., 2000). Thus, it is evident that the glucose and olive oil are essential components for the growth and secretion of lipase. As surfactant Tween 80 (polyoxyethylenesorbitan monooleate) was added in the production medium in a proportion of 20% (w/w) of olive oil as preliminary experiments showed that this was the optimum proportion because higher concentration of Tween 80 was proved to inhibit lipase activity. Tween 80 is a non-ionic polyoxyethylene detergent, the hydrophobic part of which usually consists of an alkyl chain, and the hydrophilic part is made of uncharged ethylene oxide units (Helisto and Korpela, 1998). Tween 80 stimulates lipase biosynthesis and secretion as it increases cell permeability and facilitates the export of lipase through the cell membrane (Dalmau et al., 2000). Ultrasonication has been applied to the production medium in order to effectively disperse the substrate. In the ultrasonicated system the interfacial area is enhanced via formation of an emulsion of aqueous microdroplets in a biphasic system (Talukder et al., 2006). The enzyme activity highly depends on the property of "interfacial-activation," which can be described as the enhancement of lipase activity when it is exposed to oil–water interface.

3.3. *Optimization of lipase production*

The goal of the investigation was the optimization of extracellular lipase production by *D. hansenii* strains isolated from a product rich in olive oil and increased salt concentration. Different carbon and nitrogen sources as well as incubation temperature and pH of the production medium were evaluated. The simple one-at-a-time optimization strategy was applied for screening process variables that make the greatest impact on the process. After preliminary studies glucose, olive

Table 3 – Biochemical and technological characteristics of 20 strains of *Debaryomyces hansenii* isolated from dry-salted olives of Thassos variety (% of strains positive for each character).

Characteristics	*D. hansenii*
Urea hydrolysis	0
Glucose fermentation	40
Growth with 50% glucose	100
Growth with 60% glucose	100
Growth with 10% NaCl	100
Assimilation of	
Glucose	100
D-Galactose	100
L-Rhamnose	85
Sucrose	100
Trehalose	100
α-Methyl-D-glucoside	100
Cellobiose	100
2-Keto-gluconate	100
Salicin	100
Arbutin	100
Melibiose	80
Raffinose	100
Glycerol	100
Xylitol	100
D-Mannitol	100
D-Lyxose	80
D-Turanose	85
Gentibiose	100
D-Arabitol	100
N-acetyl-D-glucosamine	90
Nitrate	0
Growth at:	
4 °C	100
25 °C	100
37 °C	15
Morphology	
Ascospores round	100
Pellicle formed	40
Ballistoconidia	20

oil and pH were selected as components of the production medium for the optimization process.

RSM was adopted to optimize the medium in order to obtain an increased lipase activity. Statistical design of experiments offers efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments. CCD was used as a very useful tool to determine the optimal level of the medium constituents and pH as well as their interaction. Minimum and maximum levels of each parameter (glucose concentration, olive oil concentration and pH) were determined after preliminary experiments. In the basal medium the minimum or maximum concentration of glucose and olive oil were added, the pH of the medium adjusted to values 4.5, 5.0 and 7.5 and the lipase activity was determined after incubation at 30 °C for 72 h. At minimum glucose and olive oil concentrations when the pH value of the production medium was 4.5 or 5.0 the lipase activity determined was negligible or very low, so the pH range tested was 5.5–7.5. The concentrations of glucose and olive oil and the pH values investigated for their effect on lipase production by *D. hansenii* YLL29 are presented in Table 1. The lipase activity determined after incubation at 30 °C for 72 h is presented in Table 2. As shown in Table 2, the highest and the lowest lipase activity was detected in the media 6 (LA 10.12 U/mL) and 1 (LA 3.25 U/mL), respectively. Data were fitted by the following second order polynomial equation, which accounts for the

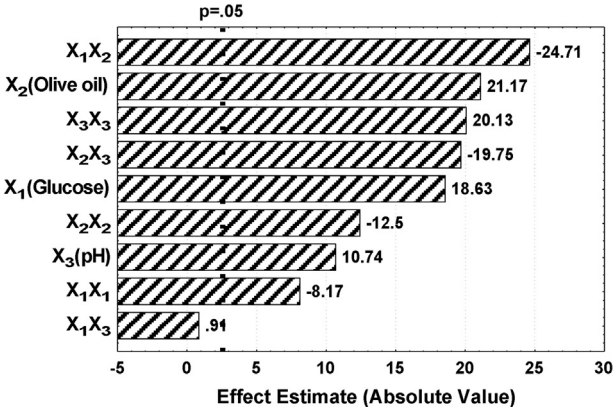

Fig. 1 – Standarized effects of glucose (X_1), olive oil (X_2) and pH (X_3) added in the basal medium on lipase production by *Debaryomyces hansenii* YLL29 in shake flask culture.

lipase produced by *D. hansenii* YLL29.

$$\text{LA (U/mL)} = 59.39 + 0.34X_1 + 0.72X_2 - 18.9X_3 - 0.01X_1^2 - 0.01X_2^2 + 1.56X_3^2 - 0.01X_1X_2 + 0.01X_1X_3 - 0.06X_2X_3 \quad (1)$$

where LA is the lipase produced per mL of the production medium as a function of the concentration of glucose and olive oil (in g/L) and the pH value.

The goodness of fit of the regression equation was evaluated by the coefficient of correlation (R) and the determination coefficient (R^2). The above second-order model is adequate predictor of the experimental results as the value of R (0.98) indicates a good agreement between the experimental and predicted values. The value of determination R^2 (0.96) indicates that the response model can explain 96% of the total variations. The value of adjusted determination coefficient ($R^2_{Adj} = 0.92$) was high enough to indicate the significance of the model. According to the corresponding analysis of variance (ANOVA) of the regression model the F-test for the regression was significant at a level of 5% ($p < 0.05$).

The standardized effects of the independent variables and their interactions on the dependent variable were investigated by preparing the Pareto chart (Fig. 1). The positive coefficients for the model components (X_1, X_2, X_3 and X_3^2) showed a favourable or synergistic effect on lipase production by *D. hansenii* YLL29, while the negative coefficients for the model components (X_1^2, X_2^2, X_1X_2 and X_2X_3) indicated an unfavourable effect on lipase production. The interaction of glucose and olive oil was the most significant effect, followed by the linear effect of olive oil. In addition to the above observations, as showed in Fig. 1 the quadratic effect of pH value had a significant positive influence on lipase activity meaning that a little variation in pH will alter the lipase production to a considerable extent. Lipase production by *Pichia lynferdii* Y-7723 increased proportionally with pH up to optimum value (pH 7.0) and decreased sharply thereafter (Kim et al., 2010). Linear effects of glucose and olive oil are highly significant for lipase production by *D. hansenii* YLL29 (Fig. 1) and explain that they can act as limiting nutrients and subtle variation in their concentrations will alter the lipase production. The fact that the bar for the interaction of glucose and pH (X_1X_3) remained inside the reference line, and the smaller coefficient for this term compared to the other terms in Eq. (1), indicated that this term contributed the least in the prediction of lipase production by *D. hansenii* YLL29.

The contour and three-dimensional plots of interactions among the above variables showed an increase in lipase production as the concentration of olive oil increased up to 19 g/L (Fig. 2a) and the concentration of glucose increased up to optimum level, i.e. 13.1 g/L (Fig. 2b) by pH increasing up to the maximum level. In Fig. 2c presenting the interaction of olive oil and glucose the maximum predicted lipase yield is indicated by the surface confined in the smallest ellipse in the contour diagram. The hyperbolic contour plots in Fig. 2a and b (saddle or minimax contours) indicate that the centre is neither a maximum nor a minimum point. Two-dimensional contour plots are the graphical representations of the regression equation and help in the identification of the interactions between test variables. The shapes of the contour plots provide a measure of the significance of the mutual interactions between the variables. Each contour curve represents an infinite number of combinations of the two test variables (i.e. olive oil and glucose with pH maintained at the optimum value). The elliptical contour plot in Fig. 2c indicates a significant interaction between glucose and olive oil concentration. Figs. 1 and 2 show that increasing concentrations of glucose and olive oil had positive influence on lipase production until the optimum value; whereas increased concentrations of the above constituents had significantly negative influence on lipase production.

The predicted optimum levels of the tested variables, namely glucose, olive oil and pH were obtained by applying regression analysis of Eq. (1). The optimal levels for *D. hansenii* YLL29 were as follows: glucose 13.1 g/L, olive oil 19 g/L, pH 6.4 with the corresponding lipase activity 7.59 U/mL. Verification of the predicted value was conducted by using optimal conditions in inoculation experiments. Three repeated experiments were performed under the predicted optimum conditions. The mean value of lipase activity was 7.44 U/mL, which is in excellent agreement with the predicted value (7.59 U/mL). Optimization resulted in 2.28-fold increase in lipase production compared with the unoptimized conditions (Table 2, run 1: 3.27 U/mL).

In order to visually compare predicted and observed data, predicted values plotted against observed values as shown in Fig. 3. The results corroborated the validity of the model as also indicated by the calculated bias factor ($B_f = 1.00$) which is a measure of the overall agreement between predicted and observed values (Ross, 1996). The model can be considered quite reliable for predicting the lipase production from *D. hansenii* YLL29.

For the 20 strains of *D. hansenii* isolated from dry-salted olives of Thassos variety endowed with high lipase production in rhodamine olive-oil agar, the optimized medium was used for lipase and biomass production in shake flask cultures for 72 h at 30 °C. The results obtained are reported in Table 4. There were statistically significant differences between the *D. hansenii* isolates concerning lipase activity and biomass production. Although the strains YLL3, YLL17, YLL19, YLL21, YLL29, YLL30, YLL31, YLL33, YLH91 did not differ significantly on lipase production, there were statistically significant differences ($p < 0.05$) between the above strains on biomass production (Table 4). The strain *D. hansenii* YLL29 showed both the highest lipase production and also the highest biomass compared to the other strains indicating that lipase production is growth associated. The lowest lipase activity of the investigated strains was 6.00 U/mL and the highest 7.44 U/mL. Low lipolytic activity was obtained by a *D. hansenii* strain isolated from waste of a dairy industry (Prista et al., 2005). The highest lipase activity detected using *Candida cylindracea* NRRL

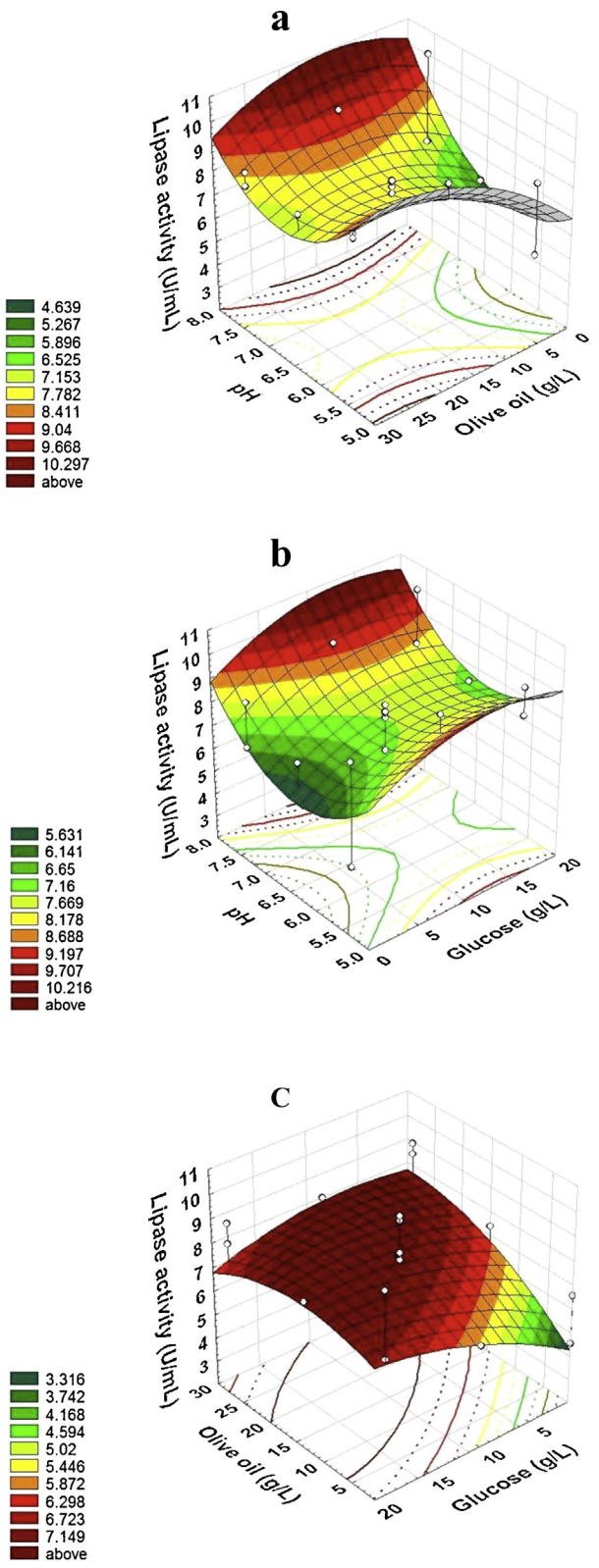

Fig. 2 – Response surface plot and corresponding contour plot for lipase production by *Debaryomyces hansenii* YLL29 on production medium supplemented with different concentrations of glucose and olive oil and adjusting pH to different values in shake flask culture: (a) glucose at the optimum concentration of 13.1 g/L; (b) olive oil at the optimum concentration of 19 g/L; (c) pH at the optimum value 6.4.

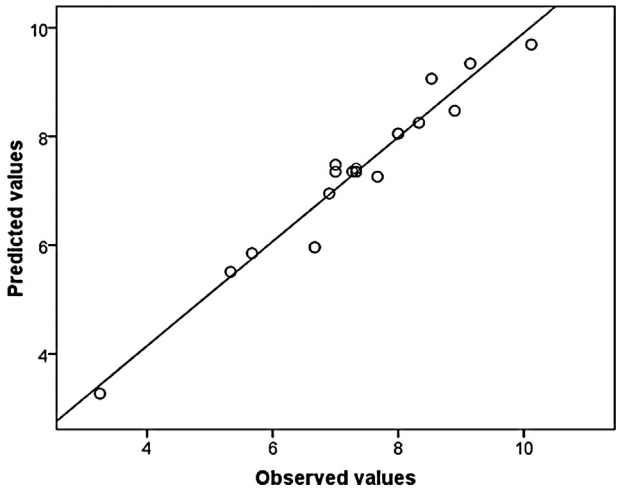

Fig. 3 – Observed versus predicted values of lipase activity determined according to the experimental design for *Debaryomyces hansenii* YLL29.

Table 4 – Lipase and biomass production by *Debaryomyces hansenii* strains isolated from dry-salted olives Thassos variety grown in optimized production medium in shake flask culture at 30 °C for 72 h.

D. hansenii strain	Lipase activity (U/mL)[a]	Dry biomass (g/L)[a]
YLL3	7.33 ^{a} ± 0.19	3.50 ^{def} ± 0.23
YLL8	6.22 ^{d} ± 0.15	2.71 ^{gh} ± 0.29
YLL10	6.55 ^{bcd} ± 0.18	3.05 ^{efgh} ± 0.18
YLL17	7.00 ^{ab} ± 0.31	3.25 ^{defg} ± 0.12
YLL19	7.22 ^{a} ± 0.16	3.36 ^{defg} ± 0.15
YLL21	7.33 ^{a} ± 0.3	3.92 ^{cd} ± 0.14
YLL29	7.44 ^{a} ± 0.28	5.26 ^{a} ± 0.27
YLL30	6.77 ^{abc} ± 0.19	4.42 ^{bc} ± 0.36
YLL31	7.11 ^{ab} ± 0.16	3.15 ^{efgh} ± 0.21
YLL33	7.23 ^{a} ± 0.2	3.54 ^{de} ± 0.19
YLL36	6.11 ^{d} ± 0.19	1.83 ^{i} ± 0.16
YLL39	6.22 ^{d} ± 0.15	2.5 ^{hi} ± 0.18
YLL40	6.55 ^{bcd} ± 0.18	3.46 ^{def} ± 0.17
YLL55	6.22 ^{d} ± 0.29	3.02 ^{efgh} ± 0.29
YLL59	6.22 ^{d} ± 0.1	3.15 ^{efgh} ± 0.17
YLL63	6.11 ^{d} ± 0.16	2.69 ^{gh} ± 0.23
YLL69	6.00 ^{d} ± 0.23	2.85 ^{fgh} ± 0.14
YLH86	6.33 ^{cd} ± 0.31	4.68 ^{ab} ± 0.32
YLH87	6.22 ^{d} ± 0.17	3.11 ^{efgh} ± 0.12
YLH91	7.14 ^{ab} ± 0.26	5.02 ^{ab} ± 0.24

[a] Data are shown as mean ± standard deviation of three replications. Means within the same column with different superscript letters are different by Tukey's test ($p < 0.05$).

Y-17506 growing on olive-mill wastewater supplemented with NH_4Cl (2.4 g/L) and olive oil (3.0 g/L) was 9.23 U/mL (D'Annibale et al., 2006). In a medium incorporating cheese whey, a brewery co-product, yeast extract, malt extract, Tween 80 and olive oil a lipase activity of 20.2 U/mL was achieved by *Candida rugosa* (Tommaso et al., 2011). As lipases are designed by nature for the hydrolysis of the ester bonds in triglycerols in the presence of excess water their industrial uses are enormous as they contribute to save energy and minimize thermal degradation during hydrolysis.

4. Conclusion

Central composite design and response surface analysis were useful to determine the optimum levels of the process variables that significantly influence the lipase production by *D. hansenii* isolated from dry-salted olives of Thassos variety. Maximum lipase production was a function of close interaction between the concentration of glucose and olive oil and initial pH of the medium. The results corroborated the validity and the effectiveness of the model, as the statistical approaches proved to be suitable in predicting the optimum production medium composition for maximum lipase production. The 20 *D. hansenii* isolates showed increased lipase activity growing in the optimized medium. The strain *D. hansenii* YLL29 showed both the highest lipase and biomass production indicating that it is a suitable strain for scale-up studies to achieve commercial exploitation of the batch process.

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