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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.

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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

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