

Isolation of lipase producing bacteria from olive extract to improve lipase production using a submerge fermentation technique

Samira Moradi, Seyed Hadi Razavi, Seyed Mohammad Mousavi

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Abstract

The tremendous mounting interest in lipase production is pertinent to the biotechnological applications that these enzymes offer. Lipase, belonging to the hydrolyze enzymes, is involved in copious biological functions including: hydrolysis insoluble triacylglycerol to mono and diacylglycerols and glycerol. Submerge fermentation technique was applied for the efficient production of this. Briefly, olive oil emulsified by Arabic gum (3% olive oil with 8% Arabic gum) and coriander seed extract (7%) were used as supplementation. Extract was isolated from the olive used to isolation lipase producing bacteria. Among the isolates, mesophilic OE3 strain was selected as the best lipase producing bacterium with high hydrolytic activities (663U/ml). From Rhodamin B, we applied agar plate technique to screen lipase production bacteria based on e formation of the fluorescence halo around the colony under radiation of UV. The isolate was short rod- and coccoid-like. The isolate was Gram-positive, aerobic, non-motile, non-endospore-forming, non-acid fast, oxidase-negative, and catalase-positive. The selected isolate was positive for H₂S and indole production and urea hydrolysis; however, they could not reduce nitrates to nitrogen. The final achievement was a lipase activity 2645 U/ml after 48 h incubation in 37°C.

Keywords: Lipase enzyme, Olive extract, Submerge fermentation, Hydrolytic activities

Introduction

Microbial lipases (EC 3.1.1.3) with the capacity to catalyze the long chain triacylglycerol have very low solubility in water to form free fatty diacylglycerol, monoacylglycerol, and glycerol. Lipases act at the lipid-water interface (Carrière, et al. 1997). Besides their natural function, lipases can catalyze a wide range of reactions such as hydrolysis, inter esterification, esterification, alcoholysis, acidolysis, and aminolysis in non-aqueous (Joseph et al. 2007; Ruchi al. 2008). Of all known enzymes, lipase is the second most common enzyme worldwide next to protease and amylase and plays an integral role in the industry of application. Many applications of this enzyme comprise the industry of additives, fine chemistry, detergents, wastewater treatment, leather, pharmaceutical, treatment of fatty effluents, synthesis of bio surfactants, and removal of resins in processing paper from cellulosic pulp, and biotransformation reactions in the synthesis of drugs and medical field (Houde et al. 2004). Lipases have been secreted by many species of animals, plants, and microorganisms. Nonetheless, the enzymes from microbial sources are currently gaining more attention due to their interesting characteristics including:

S, Moradi., S, H, Razavi*(✉), S, M, Mousavi
Bioprocess Engineering Laboratory (BPEL),
Department of Food Science, Engineering &
Technology, Faculty of Agricultural Engineering and
Technology, University of Tehran, P.O.
Biotechnology Group, Chemical Engineering Department,
Faculty of Engineering, Tarbiat Modares University
E-mail address: srazavi@ut.ac.ir;
Tel: +98 26 3224 8804; Fax: +98 26 3224 9453)

action under mild conditions, stability in organic solvents, high substrate specificity, regio and enantio selectivity. Lipase-producing microorganisms, namely bacteria, fungi and yeasts have been isolated from a number of various environments such as soil, waste water of different industry, oil cake and etc (Burkert et al.2004; Kempka et al. 2008). Many microorganisms with commercial potential, such as *Candida*, *Burkholderia*, *Acromobacter*, *Acinetobacter*, *Candida*, *Mucor*, *Rhizopus* and *Pseudomonas* were exploited for lipase production (Sharma et al. 2001). A variety of conditions including the concentration of carbon such as lipids (Lipids are generally essential inducers to lipase production and nitrogen sources, pH, aeration, metals ions and temperature of the culture medium are most important parameters to the production of lipolytic enzymes (Castro-Ochoa et al.2005; Dominguez et al. 2003; Gharibzahedi et al. 2013; Jaeger et al. 1999).

The main objective of the present study was to isolate a bacterial strain that efficiently expresses a desirable extracellular lipase. The novel extracellular lipases were isolated from olive extract (OE) at the presence of some cost-efficient carbon substrates such as milled seeds of Coriander extract. We analyzed and screened the best ratio of Arabic gum/olive oil in order to produce the maximum amount of lipase, thereby reducing the development time and the overall costs.

Materials and methods chemicals and raw materials

P-nitro phenol (p-NP) and p-nitro phenyl palmitate (p-NPP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Rhodamine B, nutrient broth (NB), peptone, yeast extract, iso-propanol, Triton X-100, Tris HCl, Arabic gum (AG) and agar were provided by Merck Chemical Co. (Darmstadt, Germany). Coriander seeds and olive oil (OO) were

purchased from a local market in Tehran (Iran). All other chemicals were of analytical grade.

Isolation and screening of lipolytic bacteria

The sample of olive extract (OE) was used to isolate a bacterial strain with the highest lipolytic activity. To enrich the sample, 1.0 g was cultured into 250 mL-Erlenmeyer flasks containing 50 mL of broth (NB, 8 g/L (w/v); NaCl, 3 g/L (w/v) and emulsified olive oil (EOO), 25 g/L (w/v) at pH 6.5. The flasks were incubated at 37°C with a constant shaking rate of 160 rpm for 24 h. Appropriate dilutions of the sample were inoculated on a specific medium containing 10 g/L extract yeast, 3 g/L NaCl, 25 g/L EOO, 0.01 g/L Rhodamine B and 20 g/L agar at pH 7.0 by serially spreading. We incubated the samples in 37°C for screening mesophilic bacteria. The formation of orange fluorescent halos around colonies under ultraviolet light (350 nm) indicates lipase positive isolates (Jaeger et al. 1999). Bacterial colony with the largest halo was initially isolated by the repeated pure culture technique, and afterward, it was selected for further studies.

Selection of the best lipase-producing bacteria isolate

One loopful from orange halo colonies of fresh cultures was inoculated into a medium containing 10 g/L extract yeast, 3 g/L NaCl and 25 g/L EOO at pH 7.0, and then incubated for 24 h at 37°C under a shaking rate of 160 rpm. Then, the cultures allocated to monitor enzyme activity were centrifuged twice at 6000 rpm for 30 min (Hettich Centrifuge, D-78532 model, Tuttlingen, Germany), and they were filtrated through 0.2 µm filters to remove the cell mas and other solids. The obtained supernatant was used in a lipase activity (LA) assay.

Morphological and biochemical identification of selected isolate

The selected bacterium was identified by morphological and biochemical properties

according to Bergey's Manual of Determinative Bacteriology (Dominguez et al. 2003).

Effect of gum arabic, olive oil and coriander seed extract on lipase production

The batch experiments for investigating the effect of Gum Arabic, olive oil and extract Coriander seed lipase production were carried out in 500 mL-Erlenmeyer flasks based on the milled seeds of coriander. This extract was prepared following being washed with distilled water and next air-drying. The powders were sieved based on the particle diameter of 1 mm (Tyler Standard Sieve Series of 9). The powders in mesh textile were placed into beaker, after that they were subjected to heating on the heater in 60 °C for 1 h, the range of 3% to 10% concentration was used. A stable oil-in-water emulsion was prepared for usage in the culture medium. The coarse emulsion was produced by Ultra-Turrax (IKA T25 Digital, Germany) in 24,000 rpm for 2.5 min and, further emulsification was performed using a 20 kHz-ultrasonic homogenizer (UP200S, Hielscher Ultrasonics GmbH, Teltow, Germany) equipped to a water bath (45°C) at a total nominal output power of 45 W for 3 min (Winkler and Stuckmann 1979). Each sterilized flask was inoculated with the constant amount of the selected isolate and then incubated at 37°C for 24 h; the used ranges for ratio of GA to OO and concentration of coriander seed extract (CSE) were 1 to 2 and 3% to 10% (w/v), respectively.

Lipolytic activity determination

Lipase activity was also measured using p-nitrophenylpalmitate (pNP-palmitate) as substrate. Briefly, the activity was determined by addition of 0.1 mL of the crude enzyme to a solution of 0.9 containing 3 mg p-NPP dissolved in 1 mL isopropanol which was diluted in 9 mL of 50 mM Tris-HCl (pH 8.0) containing 40 mg of Triton X-100 and 10 mg of GA (Gharibzahedi et al. 2013). The hydrolysis reaction was carried out and measured over time up to 10 min at 410 nm. The reactions were performed under initial rate conditions. One

unit of lipase activity (U) is defined as the amount of enzyme which releases 1 μmol of p-nitrophenol per minute under the assay conditions. The calibration curve was prepared via using p-NP as the standard (Winkler and Stuckmann 1979).

Results and discussion

Isolation of lipase producing bacteria

The aim of this investigation was to isolate potential lipolytic bacteria with novel properties. Rhodamin B agar plate was used for the isolation of lipase producing bacteria; in this culture medium, orange fluorescent halo around the colonies under ultraviolet light (350 nm) indicates lipase positivity. Additionally, olive oil as a substrate was added to optimize the production of lipase in liquid media. Based on the results, four lipase producing bacteria were selected (OE1, OE2, OE3, OE4). The OE3 bacterium exhibited a maximum lipase activity of 663 U/ml so that it was selected for further investigations (Fig. 1). Bacterium was isolated and identified by morphological and biochemical characteristics (table 1). The morphological characteristics of OE3 was short rod- and coccoid-like, the isolate was Gram-positive, aerobic, non-motile, non-endospore-forming, non-acid fast, oxidase-negative, and catalase-positive. The selected isolate was positive for H₂S and indole production and urea hydrolysis, yet they could not reduce nitrates to nitrogen. The biochemical tests of Voges-Proskauer and citrate utilization were negative and positive, respectively. The isolate appeared to be capable of producing acid in the presence of D-glucose, D-mannose, D-tagatose, L-rhamnose, lactose, maltose, sucrose, trehalose, gentiobiose, melezitose and melibiose. However, it was inept in utilizing inulin, inositol, D-adonitol, D-arabitol, L-arabinose, arbutin, cellobiose, raffinose and cellobiose.

Table 1. Identification of potential lipolytic bacterial isolate by morphological and biochemical characterization

Characteristic	result
Morphological Configuration, Gram's reaction and shape, Motility	coccoid-like, Gram, short rods non-motile
Growth on: Indole, citrate, H ₂ S production, urea hydrolysis, catalase	Positive
Voges-proskauer, oxidase, Nitrate reduction	Negative
Hugh Leifson's: (a) aerobic and (b) anaerobic	aerobic
Tolerance to NaCl (% , w/v)	0-5%
Temperature range (°C)	15-45
Acid production from: D-Adonitol, D-Arabitol, L-Arabinose, Arbutin, Cellobiose, Raffinose	Negative
Gentiobiose, D-Glucose, Inositol, Inulin, Lactose, D-Lyxose, Maltose, D-Mannose, Melezitose, Melibiose, L-Rhamnose, Sucrose, D-Tagatose, Trehalose, D-Xylose	positive

The isolate was able to grow at 15-45°C, 0-5% and 6.5-8 optimum range for growth temperature, salinity (tolerance level to NaCl) and pH, respectively. Rhodamine B-agar plate method was used to determine lipase producing bacteria. It is suggested that the formation of fluorescent halos under the UV rays in Rhodamine B plate agar can be due to Rhodamine B dimers complied with mono or diglycerides and fatty acid liberated by the

enzyme into the medium (Castro-Ochoa et al. 2005). High activity of produced lipase by the isolated from E03 can probably be attributed to the ideal growth conditions and substrate type present in olive (Gharibzahedi et al. 2013). Olive is consist of the best carbon sources for lipase bacteria. Indeed, it comprises of substantial amount of oleic acid which is essentially a specific inducer in the lipase producing bacteria.

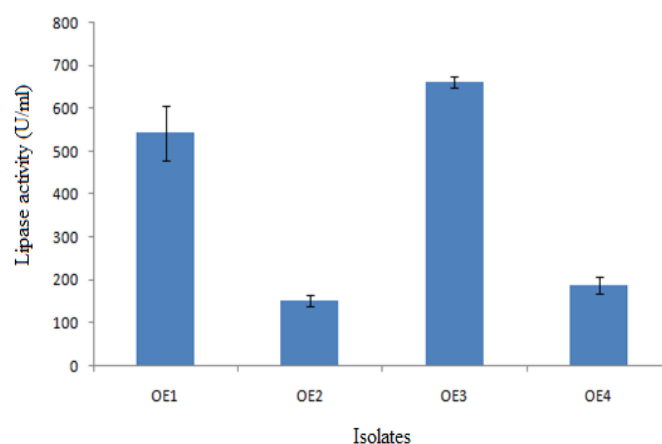


Fig 1. The lipase activities of the screened bacterial isolates

Effect of gum arabic, olive oil and coriander seed extract on lipase production

Since olive oil has a very low solubility in water and enzymes act in the interface of oil-water, the substrate utilization efficiency of lipase producing bacteria is limited. Therefore, appropriate emulsion of olive oil by Arabic gum

might be helpful to enhance cell growth, thereby increasing the lipase productivity. To confirm this, Arabic gum, a common surfactant and stabilizer used in drink industry, was added into the fermentation broth to emulsify olive oil (Winkler and Stuckmann. 1979).

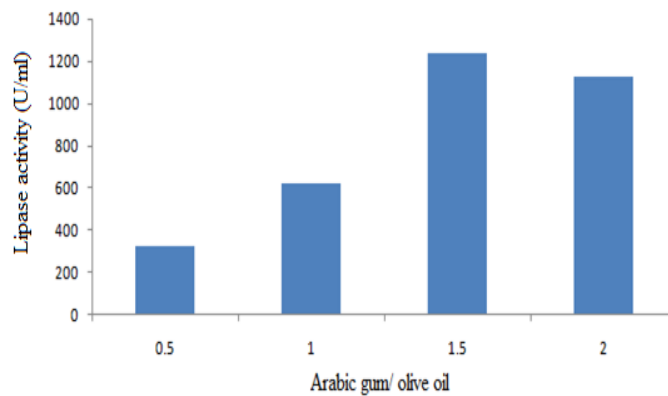


Fig 2. Lipase activity of the *OE3* isolated from olive extract as a function of different ratio of arabic gum/olive oil

As indicated in Fig. 2, when olive oil was emulsified by ratio of 1.5;1 Gum Arabic/oil, lipase production increased. The increase in lipase production by using emulsified olive oil (3%) as an inducer could influence oleic acid concentration, which became higher due to emulsification. Increase of enzyme activity in the presence of OO inducer in the culture

medium could be attributed to its high content of long, unsaturated fatty acyl chains, such as oleic acid (Holt et al. 1994; Hou, and Johnston 1992; Joseph et al. 2012; Silva et al. 2005). In earlier studies, olive oil was demonstrated to be a preferential inducer for maximum lipase production from the lipase producing bacterium (Chen 1999).

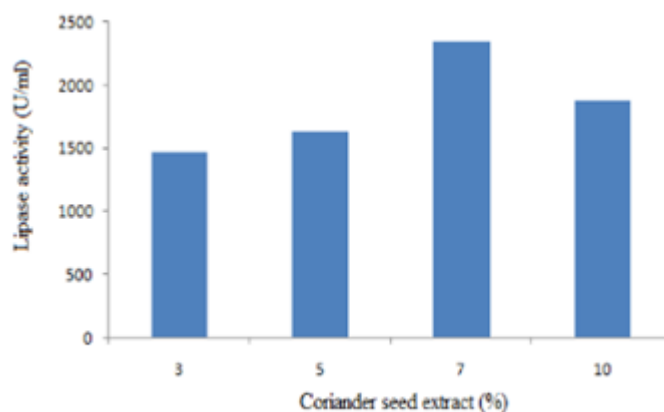


Fig 3. Lipase activity of the *OE3* isolated from olive extract as a function of different coriander seed extract concentration

Fadiloglu and Erkmen (2002) reached to some promising results by supplementation of *Candida rogusa* culture medium with olive oil. On the other hand, the presence of an excessive amount of olive oil could lead to decrease in lipase production. Based on the result Fig3, the coriander seed extract with 7% concentration was the best substrate for OE3 isolate. The content of protein, lipid and dry material in the coriander seed extract were 9.12, 6.4, and 1.2%, respectively which verify it as an appropriate substrate for lipase production. According to the previous reports, high content of nitrogen SC is one of the most effective contributory factors in enhancing the production of lipases by microorganisms (Dheeman et al. 2011). The substrate (CS) supplementation with other compounds has been attempted initially for analyzing the microorganism nutritional needs in the hope of maximize lipase production. Some of these supplements were also characterized with respect to nitrogen content and oil (Fadiloğlu, and Erkmen 2002).

In fact, microorganisms provide high yields of lipase when organic nitrogen sources are available. Growth curve and lipase activities of isolate in optimized condition are shown in Fig 4. It is evident from the results that there was no enzyme activity and lipase production at 0 h. It increased gradually from 20 h and after 27 h, the cell biomass and lipase activity after 48 h reached their highest values. In addition, Lipase production was observed at 48 h 2645 U/ml. A thermophilic microorganism SBS-4S was isolated from a hot spring located in Gilgit, northern areas of Pakistan. It was an aerobic, gram-positive, rod-shaped, thermophilic bacterium that grew on various sugars, carboxylic acids and hydrocarbons at temperatures between 45°C and 75°C (Tayyab et al. 2011). A lipase *Burkholderia sp. C20* strain was also isolated from the food waste products. It is of hydrolytic activities when used in olive oil as a substrate (Liu et al. 2012). In

addition to that, thirty-two lipase producing bacterial were isolated so far from different soil samples (Abdel-Fattah et al. 2012).

Conclusion

Of all the olive extracts used to screen microbial lipase production capacity, the OE3 isolate was selected as the best in that regard. The first step involved determination of optimal medium compositions so as to optimize lipase production by using various ratio of Arabic gum to olive. In the second step, we applied different coriander seed extract concentrations as a carbon and nitrogen source, which led to an increase in lipase production by 3.98 fold. Lipase production by fermentation will be an advantage for the largescale application. From the economic perspective, its cost is a major restriction in operating an enzyme catalyzed reaction.

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