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Olive Oil as Inductor of Microbial Lipase

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1. Introduction

Increasing interest in lipases has been observed at the end of the last century, due to their potential application, in (bio)degradation as well as in (bio)synthesis of glycerides. The advantages of the enzymatic hydrolysis over the chemical process consist of less energy requirements and higher quality of the obtained products. Beside this, lipases are also efficient in various reactions such as esterification, transesterification and aminolysis in organic solvents. Examples in the literature are numerous. Lipases are used in different fields such as resolution of racemic mixtures, synthesis of new surfactants and pharmaceuticals, bioconversion of oils and fats and detergency applications.

Lipase activity has been found in different moulds, yeasts and bacteria. Numerous papers have been published on selection of lipase producers and on fermentation process. This kind of information is important in order to identify optimal operation conditions for enzyme production. Previous studies on the physiology of lipase production showed that the mechanisms regulating biosynthesis vary widely in different microorganisms. Obtained results showed that lipase production seems to be constitutive and independent of the addition of lipid substrates to the culture medium. However, their presence can enhance the level of produced lipase activity. On the other hand, it is well known that, in other microorganisms, lipid substrates are necessary for lipase production. These enzymes are generally produced in the presence of a lipid such as oil or triacylglycerols or any other inductor, such as fatty acids. Lipidic carbon sources seem to be essential for obtaining a high lipase yield. The review is focused on the olive oil as lipase inductor.

1.1 Lipases

Lipases, (triacylglycerol acylhydrolases; EC 3.1.1.3.) are one of the most important classes of hydrolytic enzymes that catalyse both hydrolysis and synthesis of esters. Hydrolysis of a triacylglycerols by lipases can yield di- and monoacylglycerols, glycerol and free fatty acids. Lipases are valuable biocatalysts with diverse applications. Although lipases share only 5% of the industrial enzyme market, they have gained focus as biotechnologically valuable enzymes. They play vital roles in food, detergent and pharmaceutical industries.

Commercial microbial lipases are produced from bacteria, fungi and actinomycetes (Babu & Rao, 2007). Their industrial importance arises from the fact that they act on a variety of substrates promoting a broad range of biocatalytic reactions. Lipases from different sources

show different substrate specificities and they are widely used in industrial applications for biosynthesis (Jaeger & Eggert, 2002).

Most of the lipases, which are used in laboratory investigations and/or in industrial production, are substrate tolerant enzymes, which accept a large variety of natural and synthetic substrates for biotransformation. Microbial lipases are mostly inducible extracellular enzymes, synthesized within the cell and exported to its external surface or environment. Lipases are ubiquitous enzymes which are widely distributed in plants, microbes and higher animals. Microbial sources are superiour to plants and animals for enzyme production and this can be attributed to easy cultivation and genetic manipulation (Hasan et al., 2006). Each microorganism requires a different carbon source to produce lipase at its maximum level.

Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition besides physicochemical factors such as temperature, pH, and dissolved oxygen. The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or triacylglycerol or any other inductor, such as fatty acids, hydrolysable esters, Tweens, bile salts, and glycerol. Lipidic carbon sources seem to be essential for obtaining a high lipase yield. However, nitrogen sources and essential micronutrients should also be carefully considered for growth and production optimization. These nutritional requirements for microbial growth are fulfilled by several alternative media as those based on defined compounds like sugars, oils, and complex components such as peptone, yeast extract, malt extract media, and also agroindustrial residues containing all the components necessary for microorganism development. A mix of these two kinds of media can also be used for the purpose of lipase production. The main studies available in the literature since 2000 covering these subjects are presented below, divided by the kind of microorganisms used (Fernandes et al., 2007; Li et al., 2004; Tan et al., 2003).

1.2 Lipase catalytic properties

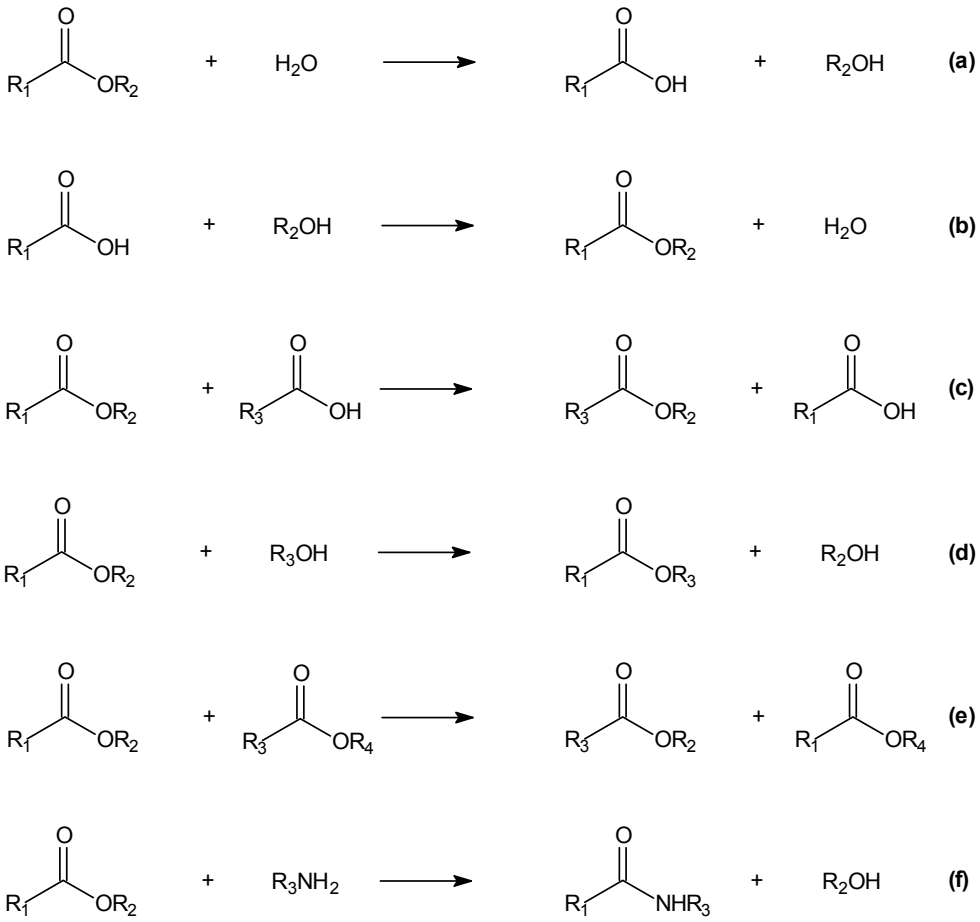
Lipase hydrolysis of water-insoluble substrates results from adsorption of the enzyme to the substrate-water interface, which can induce a conformational change in the enzyme structure, causing reaction rates to be influenced by both this adsorptive interaction as well as interaction with substrates. When lipases are active in organic solvents in which substrates are soluble, reactions follow normal enzyme kinetic models (Martinelle & Hult, 1995).

Reactions in which lipases may be involved, both in nature and in laboratory or industrial application, are: (a) enzyme-catalyzed hydrolysis, (b) enzyme-catalyzed esterification, (c) enzyme-catalyzed transesterification by acidolysis, (d) enzyme-catalyzed transesterification by alcoholysis, (e) enzyme-catalyzed interesterification and (f) enzyme-catalyzed aminolysis (Scheme 1).

1.3 Plant oils

The major components of fats and vegetable oils (98%) are triacylglycerols, which consist of glycerol molecules esterified with three long-chain fatty acids. The remainder of the oil,

although only a small part in proportion to triacylglycerols, includes a very large number of minor compounds, including the phenolics and the sterols. These compounds give olive oil its unique flavour and contribute greatly to the nutritional benefits.



Scheme 1. Processes catalyzed by lipases: (a) enzyme-catalyzed hydrolysis, (b) enzyme-catalyzed esterification, (c) enzyme-catalyzed transesterification by acidolysis, (d) enzyme-catalyzed transesterification by alcoholysis, (e) enzyme-catalyzed interesterification and (f) enzyme-catalyzed aminolysis.

The structure of triacylglycerol molecule is depicted in Fig. 1. The seed triacylglycerols are usually characterized by predominance of C_{18}-unsaturated and polyunsaturated fatty acids, and this distinguishes them from animals fats, which are generally of a more saturated nature. The C_{18}-unsaturated fatty acids (oleic, linoleic, and linolenic) are particularly important and govern, to a large degree, the physical properties of the oil and hence its use and commercial value.

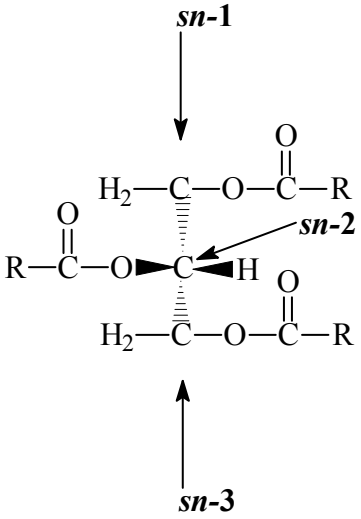


Fig. 1. The particular fatty acids in the plant triacylglycerols are not distributed randomly between the different *sn*-carbon atoms. It is a general rule that saturated species of fatty acids are confined to the positions *sn*-1 and *sn*-3 with some enrichment in the first position, and that the polyunsaturated C_{18} fatty acids are located mainly at position *sn*-2 (Gunstone & Ilyas-Qureshi, 1965; Gunstone et al., 1965)

2. Lipase biosynthesis

Lipase production requires carbon and nitrogen sources as required by any fermentation process. Most of the lipases production studies do not use simple sugars as carbon sources. They rather use lipid substrates as sole carbon sources (Zhang et al., 2009a; Zhang et al., 2009b; Hun et al., 2003). Lipase production is rarely constitutive and the quantity of the extracellular lipase produced is meagre (Lee et al., 2001). Hence inductors like vegetables oils (Kumar et al., 2005), Tween 20, Tween 80 (Li et al., 2004), hexadecane (Boekema et al., 2007), and synthetic like tributyrin and tripalmitin, are used. Generally, production of lipases increases when the relative percentage of C18:n fatty acid esters in the respective vegetable oil is increased; this indicates the importance of such substances in the synthesis and secretion of the enzyme (Lakshmi et al., 1999). Among vegetable oils, olive oil has also been referred as one of the best inductors of lipase production (Table 1; Sokolovska et al., 1998). To elucidate some aspects of the induction effect of lipid related substrates on lipase production, mixed carbon sources consisting of a soluble compound selected for its growth-promoting capacity and a fatty acid selected as inductor of the enzyme production were used (Dalmau et al., 2000). This strategy allows for the microorganism to use both substrates in a sequential or simultaneous way, depending on its metabolism. Biomass production can be higher in most cases, no increase in lipolytic activity can be observed. This suggested a possible competing effect of some soluble carbon sources or a close relation between extracellular lipase activity production and consumption of fatty acids.

Source of lipase	References
Aspergillus sp.	Cihangir & Sarikaya, 2004; Papanikolaou et al., 2011
Aspergillus niger	Pokorny et al., 1994
Aspergillus niger MYA 135	Colin et al., 2011
Bacillus sp.	Sugihara et al., 1991; Eltaweel et al., 2005
Bacillus subtilis NS 8	Olusean et al., 2011
Burkholderia cepacia LTEB11	Baron et al., 2011
Candida sp.	Annibale et al., 2006; Brozzoli et al., 2009
Candida cylindracea (ATCC 14830)	Salihu et al., 2011
Candida rugosa (DSM 2031)	Lakshmi et al., 1999
Geotrichum candidum 4013	Stránsky et al., 2007; Brabcová et al., 2010
Mycotorula sp.	Peters & Nelson, 1948
Penicillium sp.	Lima et al., 2003; Papanikolaou et al., 2011
Penicillium aurantiogriseum	Lima et al., 2003
Penicillium cyclopium	Chahinian et al., 2000
Pseudomonas aeruginosa KKA-5	Sharon et al., 1998
Rhizopus arrhizus	Elibol & Oyer, 2000
Rhizopus delemar	Acikel et al., 2011
Rhizopus oryzae	Hiol et al., 2000; Salleh et al., 1993; Nunes et al., 2011
Rhodotorula glutinis	Papaparaskevas et al., 1992
Serratia rubidaea	Immanuel et al., 2008
Yarrowia lipolytica	Dominguez et al., 2003; Pignčde et al., 2000; Najjar et al., 2011

Table 1. Sources of lipases induced by olive oil

The induction process can be accomplished by adding edible oils such as butter fat, olive, canola and fish oils to the fermentation medium. It is well known that certain lipids in the culture medium can influence the production and activity of lipases from microorganisms. Generally, the activity of intra and extracellular lipases increases with increasing lipid concentrations, although excessive levels in the growth medium may be cytotoxic. The mechanisms regulating lipase biosynthesis vary widely in different microorganisms.

2.1 *Aspergillus niger*

Lipases from *Aspergillus niger* were induced by solid-state fermentation using, as substrate, agroindustrial residue supplemented with by-products from corn oil refining process or olive oil. Based on the values of lipase activity obtained after 48 hour fermentation by-products from corn oil refining were tested as inductors in the preparation of fermentation

medium. The best results were achieved with soapstock and stearin, reaching values of 62.7 and 37.7 U/gds, respectively, which are higher than the value for olive oil (34.1 U/gds). The use of fatty acids residue inhibited lipase production. This kind of inhibition has already been reported by other authors (Corzo & Revah, 1999; Li et al., 2004). The inhibition effect was not observed for low fatty acid concentrations using palmitic and oleic acid during lipase production by *Candida rugosa* (Dalmau et al., 2000) and *Rhyzopus arrhizus* (Li et al., 2006), respectively.

2.2 *Candida rugosa*

The synthesis and secretion of lipases in *C. rugosa* have been studied with carbon sources that are known to affect the production of lipase in two opposite ways: glucose (repressor) and oleic acid (inductor; Ferrer et al., 2001). In these studies, lipase production was monitored both by enzyme activity and by immunodetection with specific antibodies. These studies showed that, according to their regulation, lipase-encoding genes might be grouped in two classes, one of which is constitutively expressed and the other is induced by fatty acids. The synthesis of inducible enzymes is inhibited at the level of transcription by the addition of glucose, and, conversely, oleic acid appears to hinder the synthesis of the constitutive lipase (Lotti et al., 1998).

The studies clearly show that different inductors may change the expression profile of individual lipase genes. A differential transcriptional control of *lip* genes had been previously suggested from several studies on the relationship between culture conditions of *C. rugosa* and the lipase/esterase profiles secreted by this organism (Gordillo et al., 1995; Lotti et al., 1998; Linko & Wu, 1996). *Lip* isoenzymes have differences in their catalytic properties (Rua et al., 1993; Diczfalusy et al., 1997; Tang et al., 2000).

Del Río et al. (Del Río et al., 1990) demonstrated the diauxic growth of *C. rugosa* on olive oil. Two stages could be observed in the consumption of the olive oil: the first one was related to the glycerol depletion without lipase production, and the second one was associated with the fatty acids consumption when the enzyme appeared in the medium. According to this observation, the initial presence of a small quantity of lipase would be sufficient to hydrolyze the triacylglycerol to glycerol and fatty acids. Therefore, production of high levels of lipase would be associated with the consumption of fatty acids. Similar results have been obtained by Sokolovska et al. (Sokolovska et al., 1998), who used olive oil and oleic acid for lipase production. It has been observed that the uptake of oleic acid by *C. rugosa* is favored by the presence of extracellular lipases (Montesinos et al., 1996). Based on the observations and hypothesis just described, Serra et al. (Serra et al., 1992) calibrated and validated a model for lipase production on olive oil and free fatty acids in batch fermentation. Sokolovska et al. (Sokolovska et al., 1998) did not observe significant differences in lipase production using these substrates. Montesinos et al. (Montesinos et al., 1996) developed a simple structured mathematical model for lipase production by *C. rugosa* in batch fermentation. Lipase production is induced by extracellular oleic acid present in the medium. The acid is transported into the cell, where it is consumed, transformed, and stored. Lipase is then excreted to the medium, where it is distributed between the available oil-water interface and the aqueous phase. Cell growth is modulated by the intracellular substráte concentration. Model parameters were determined in a calibration step, and then

the whole model was experimentally validated with good results. This model was later modified to be applied from batch to fed-batch and continuous lipase production (Montesinos et al., 1997). Finally, it was exploited in simulations and for the design of new operational conditions as discussed next.

Annibale et al. (Annibale et al., 2006) and Brozzoli et al. (Brozzoli et al., 2009) confirmed that lipase production by *Candida* sp. was found to be completely repressed by the presence of simple sugars and induced by using natural oils.

2.3 *Pseudomonas* sp

An extracellular lipase was isolated and purified from the culture broth of *Pseudomonas aeruginosa* SRT 9 (Borkar et al., 2009). Production medium was prepared containing olive oil (1% w/v) as inductor. Marked stability and activity of induced lipase in organic solvents suggest that this lipase is highly suitable as a biotechnological tool with a variety of applications including organo synthetic reactions and preparation of enantiomerically pure pharmaceuticals. A strain of *Pseudomonas mendocina* producing extracellular lipase was isolated from soil (Dahiya et al., 2010). The bacterium accumulates lipase in culture fluid when grown aerobically at 30 °C for 24 h in a medium composed of olive oil (1%) as substrate. This lipase was capable of hydrolyzing a variety of lipidic substrates and is mainly active towards synthetic triglycerides and fatty acid esters that possess a butyryl group. The medium for lipase production from *Pseudomonas fluorescens* P21 had glucose as carbon source (Cadirci & Yasa, 2010). When glucose was replaced by various lipids, olive oil was the effective lipid for lipase production (3.5 U/l). When glucose in the medium was replaced with olive oil, the lipase yield was increased by 48.9% between 12 and 18 h.

2.4 *Mucor hiemalis*

The influence on lipase induction in *Mucor hiemalis* of different types of triacylglycerols containing mainly oleic acid (olive oil), erucic acid (mustard oil), or saturated fatty acids of 8 to 16 carbons (coconut oil) was studied (Akhtar et al., 1980). The fungus produced a significant amount of lipase in the presence of glucose, but the lipase activity increased markedly when olive oil was added to the medium at the beginning of the fermentation. Among the various sources of triacylglycerols used as the carbon source, olive oil was found to be most effective in inducing the lipase. The lipase of *M. hiemalis* can be considered to be both constitutive and inducible.

2.5 *Penicillium restrictum*

While supplementation with olive oil gave the best lipase results, the highest values of glucoamylase and protease activities (de Azeredo et al., 1999) were achieved with starch enrichment. This indicates that the type of carbon source used as supplementation plays a determinant role in the kind of major enzymes that will be produced by *P. restrictum*. Enriching the babassu cake with different carbon sources favours the synthesis of different enzymes: olive oil supplementation results in high lipase activities, while starch supplementation results in high glucoamylase activities. Therefore, according to the application desired, the basal medium may be differentially enriched to give high yields of the desired enzyme.

2.6 *Rhizopus homothallicus*

Different mixtures of triacylglycerols (Rodriguez et al., 2006): olive, sunflower, corn, peanut, walnut and grape seed oils, were used as energy and carbon sources in addition to lactose, and with urea as nitrogen source. It should be emphasized that the presence of the carbohydrate account for the early growth of the strain *Rhizopus homothallicus* and later growth occurs due to the added oil (Pokorny et al., 1994). This fungal strain was able to produce similar high lipase activities with all studied oils. In the fermentation system, lipase synthesis was not prevented at 4% of triglycerides. To complete these studies, the above medium using olive oil and urea was chosen to evaluate the effect of different carbohydrates on lipase production: glucose, fructose, glycerol, xylose, sucrose and lactose. There were little or no differences with these substrates. This fact suggests that carbohydrate type does not influence lipase production, probably because the carbohydrate concentration is low (5 g/ l) compared to the oil amount (40 g/l) added to culture media and because they are probably utilized before the oil and consequently, before lipase production (Cordova et al., 1998).

2.7 *Rhizopus oryzae*

In the study (Hama et al., 2006) utilizing *Rhizopus oryzae* cells as whole-cel biocatalysts, various substrate-relate compounds such as olive oil, oleic acid, oleyl alkohol, methyl carpate and Tween 80 were tested. It was found that the addition of olive oil on lipase production and localization in suspension cells were therefore investigated (Ban et al., 2001). Olive oil increased intracellular lipase production. However that extracellular hydrolysis activity was much higher in the absence of olive oil. Because the *Rhizopus oryzae* cells used in the study were able to produce lipase constitutively regardless of whether substrate-related compounds were present, it seems likely that these compounds are effective in retaining lipase within the cells.

Nitrogen and carbon sources influencing the growth and production of lipase by *Rhizopus oryzae* were studied by Fadiloglu & Erkmen (1999). High yields of enzyme activity were obtained when protease peptone was the nitrogen source in media with olive oil and without olive oil. Carbon sources increased lipase activity in the media without olive oil, but decreased it slightly in the presence of oil. Lipase activity was significantly higher in the media with olive ail than that without olive ail. Biomass concentration was also higher in the presence of oil (Fadiloglu & Erkmen 1999). Rapid induction of enzymes able to break down foodstuffs appearing in the environment of the micro-organism is clearly of great ecological advantage. This induction process effects a change in the phenotype allowing further production of energy required for metabolism and/or growth (Wiseman, 1975).

2.8 *Geotrichum candidum*

The fungus *Geotrichum candidum* 4013 produces two types of lipases (extracellular and cell-bound; Stránsky et al., 2007). Both enzymes were induced by addition of olive oil. The differences in the abilities of these two enzymes to hydrolyze *p*-nitrophenyl esters were observed. Yan and Yan (2008) tested a combination of different experimental designs to optimize the production conditions of cell-bound lipase from *Geotrichum* sp. A single factorial design showed that the most suitable carbon source was a mixture of olive oil and citric acid and the most suitable nitrogen source was a mixture of corn steep liquor and

NH_4NO_3. Burkert et al. (2004) studied the effects of carbon source (soybean oil, olive oil, and glucose) and nitrogen source concentrations (corn steep liquor and NH_4NO_3) on lipase production by *Geotrichum* sp. using the methodology of response surface reaching a lipase activity of 20 U mL^{-1}.

3. Conclusion

Inducible enzyme systems in micro-organisms display many features of microbiological and biochemical interest. There is no doubt that in the microbial systems investigated, the major induced enzyme (usually a hydrolase) was fonned *de novo.* Enzyme formation occurs from amino acids, rather than from inactive peptide or protein precursor existing prior to the addition of the inductor to the culture (Wiseman, 1975).

Most published experimental data have shown that lipid carbon sources (especially natural oils) stimulate lipase production. Among vegetable oils, olive oil has been referred as one of the best inductors of lipase production. The review showed that olive oil plays significant role in lipase production. It could be concluded that the higher content of unsaturated free fatty acids contained in oil, the higher intracellular and extracellular lipase activity could be obtained with the oil as inductor for cells cultivation.

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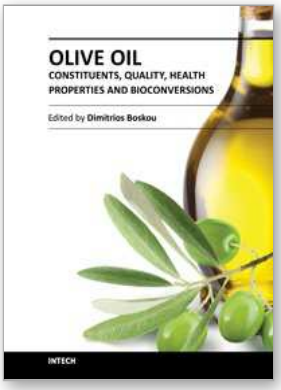

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The health-promoting effects attributed to olive oil, and the development of the olive oil industry have intensified the quest for new information, stimulating wide areas of research. This book is a source of recently accumulated information. It covers a broad range of topics from chemistry, technology, and quality assessment, to bioavailability and function of important molecules, recovery of bioactive compounds, preparation of olive oil-based functional products, and identification of novel pharmacological targets for the prevention and treatment of certain diseases.

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