

*Full Length Research Paper*

# Lipase and esterase activities of lactic acid bacteria isolated from different biotopes

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The lipolytic and esterase activities of fifteen strains of lactic acid bacteria isolated from different biotopes of Algeria and Mauritania were tested on MRS medium supplemented with lipidic substrates. Five of them showed maximum activity in the presence of tributyrin; the activity is therefore a tributyrin esterase. These strains were identified by MALDI-TOF in *Enterococcus faecium* and *Enterococcus durans*. The study of growth kinetics as a function of time shows a start of fatty acid production during the exponential phase to reach its maximum in the stationary phase. A better esterase activity is observed at between pH 6 and 9 and at an optimal temperature of 30 to 40°C for the five strains. The influence of metal and additive ions on the esterase activity varies between bacteria but generally, total inhibition was observed in all strains tested in the presence of SDS, NaN<sub>3</sub>, CuCl<sub>2</sub>, EDTA, AgNO<sub>3</sub> and HgCl<sub>2</sub>.

**Key words:** Lipolytic activity, esterase activity, tributyrin, tributyrin esterase, MALDI-TOF, *Enterococcus*, growth kinetics, metal ions.

## INTRODUCTION

Lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) are carboxyl esterases that hydrolyze the carboxylic ester linkages of triacylglycerols to release diglycerides, monoglycerides, free fatty acids and glycerol. The lipolytic and/or esterase activity of lactic acid bacteria contributes to the production of new foods or food supplements (García-Cano et al., 2019). These bacteria with probiotic potential can also produce conjugated fatty acids by hydrolyzing triacylglycerol thanks to their lipase activity (Kuhl et al., 2016). It is generally recognized that lactic acid bacteria play an important role in quality, flavor, and maturation in cured meat production (Dinçer and Kivanç, 2018).

Carboxyl esterases act only in ester-water interface and are of considerable physiological and industrial importance (Martinelle et al., 1995). The water activity in the reaction medium controls the balance of the reactions (Borrelli and Trono, 2015). In case of low water activity, lipases catalyze other reactions (esterification, interesterification, acidolysis, alcoholysis, and aminolysis reactions) (Joseph et al., 2008; Bajaj et al., 2010).

Generally, esterases hydrolyze only short chain fatty acid triglycerides while lipases are active on water insoluble substrates and hydrolyze long chains to fatty acids (Kilcawley et al., 1998).

Microbial lipases are widespread in bacteria, especially

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**Table 1.** Bacterial strains used in this study.

Origin	Code of strain	Species
Camel's milk (Illizi, Algeria)	BH21	<i>Lactobacillus plantarum</i>
Camel's milk (Nouakchott, Mauritania)	CAM18	<i>Enterococcus</i> sp.
Camel's milk (Timimoun, Algeria)	CAT13; CAT18	<i>Enterococcus</i> sp.
Camel's milk (Béchar, Algeria)	CHBK320	<i>Leuconostoc mesenteroides</i> ssp <i>dextranicum</i>
Camel's milk (Tindouf, Algeria)	CHTD27	<i>Lactobacillus brevis</i>
Cow's Milk (Oran El-Kerma, Algeria)	LKV11	<i>Enterococcus</i> sp.
Olive brine (Sig, Algeria)	OV5	<i>Lactococcus lactis</i> ssp <i>diacetylactis</i>
Fresh beef meat (Mostaganem, Algeria)	V6-2 ; V17 ; V18 V9	<i>Lactococcus lactis</i> ssp <i>lactis</i> <i>Lactococcus lactis</i> ssp <i>cremoris</i>
Fresh sheep meat (Relizane, Algeria)	VO19	<i>Enterococcus</i> sp.

in Gram+ (Fickers et al., 2008). Among them are lactic acid bacteria, which are considered slightly lipolytic in comparison with other bacterial species (Brennan et al., 2002). This activity does not influence bacterial growth. In fact, these enzymes do not show any nutritional role (Fernández et al., 2000; Nardi et al., 2002). However, their presence in cheeses, and at higher concentrations and at precise periods, leads to the release of fatty acids responsible for the final taste (Das et al., 2005). *Lactobacillus plantarum* CCFM12 shows good esterase activity responsible for a considerable improvement in the production of ethyl esters and which leads to the fruity taste of camembert cheese (Hong et al., 2018).

Esterases of lactic acid bacteria preferentially degrade para-nitrophenyl or beta-naphthyl derivatives of C4 or C6 fatty acids, and a good activity was also observed in the presence of tributyrin. Esterasic activity decreases considerably with lengthening of the chain (Corrieu and Luquet, 2008).

Lipases and esterases enzymes of lactic acid bacteria are either extracellular or intracellular (Katz et al., 1997; Meyers et al., 1996), hence in the second case the need for cell lysis to promote their access to the substrate.

Several esterases of lactic acid bacteria such as *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, *Streptococcus thermophilus* and *Enterococcus faecium*, were previously characterized. These enzymes have an optimal activity in a temperature range of 30 to 45°C depending on the strains considered, as well as a neutral or slightly alkaline pH. Their activators are Zn<sup>2+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup> ions as well as NaCl. Phenylmethylsulfonyl fluoride (PMSF), para-chloromercuribenzoate (PCMB), and Hg<sup>2+</sup>, Cu<sup>2+</sup>, Ag<sup>2+</sup> ions inhibit esterase activity (Tsakalidou et al., 1994; Holland and Coolbear, 1996; Fernandez et al., 2000; Nardi et al., 2002; Chich et al., 1997; Fenster et al., 2000; Gobetti et al., 1997a; Castillo et al., 1999; Choi et al., 2004; Liu et al., 2001; Mobarak-Qamsari et al., 2011; Salwoom et al., 2019).

The objective of this research was to highlight the lipase and esterase activity of lactic acid bacteria cultivated in the presence of different lipid substrates. Studies of the kinetics of growth and production of esterases and/or lipases depending of various physico-chemical parameters (pH, temperature, surfactants, metal ions and other additives) will be useful in order to optimize the use of these enzymes.

## MATERIALS AND METHODS

### Bacterial strains

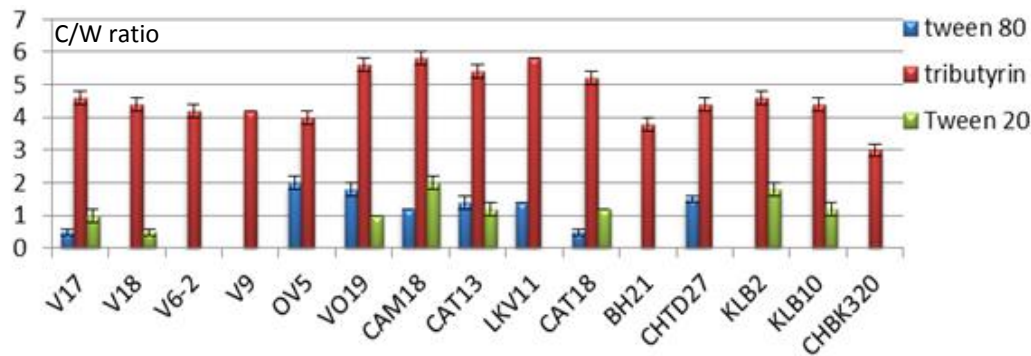
The 15 lactic acid bacterial strains in the genus *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Leuconostoc* have been isolated by serial dilution plating on MRS medium (De Man et al., 1960) from raw camel or cow milk, olive brine and fresh meat collected in Algeria and Mauritania (Table 1). The bacterial cultures were maintained at -20°C in MRS broth containing 20% glycerol (v/v). Working cultures were prepared by two consecutive transfers in MRS broth at 30°C for 18 h.

### Bacterial identification

The bacteria were streaked on MRS agar plates and incubated at 30°C for 24 to 48 h. Single isolates were identified by Bruker Daltonic's MALDI-TOF Biotyper – CM according to the manufacturer instructions. The identification is carried out by mass spectrometry analysis coupled with a source of laser ionization assisted by a MALDI (Matrix Assisted Laser Desorption Ionization) and a TOF (Time of Flight) analyser. For the MALDI-TOF MS analysis, the strains were grown on MRS medium for 24 h. Each colony was smeared on the target and then covered with 1 µl of formic acid and 1 µl of the matrix. It is then identified by the MALDI-TOF.

### Detection of intracellular lipolytic and esterase activity

Bacterial preculture was grown on MRS broth at 30°C until it reaches OD600 ~1.2. Tubes of 10 ml of fresh MRS broth were inoculated with 0.1 ml of the bacterial preculture and incubated at 30°C for 18 h. The intracellular enzymes from the isolate were extracted by using glass bead stirring. After centrifugation at 8000



**Figure 1.** Lipolytic activity on artificial lipid substrates in lactic strains (C: clarification area, W: well diameter).

rpm, the supernatant is recovered. It constitutes the crude enzymatic extract.

The activity of enzymes was investigated according to the diffusion method in buffered agar at pH 7 (0.1 M phosphate buffer) containing various natural or artificial lipid substrates as follows:

1. 3% olive oil, almond oil, argan oil or oleic acid. The media are then opacified with  $\text{CaCO}_3$  in order to visualize a possible lipase activity.
2. 0.25% of tributyrin is added and the emulsion is homogenized by sonication. In case degradation of tributyrin an observed clarification reflects the presence of esterases (Medina et al., 2004).
3. 3% Tween 80, 0.01%  $\text{CaCl}_2$  and 0.5% NaCl. Under these conditions, the presence of lipases is manifested by opacity (Guiraud and Galzy, 1980).

Plates were incubated at 37°C for 72 h. The strains as well as the lipid substrate where the activity is maximal are selected for further work.

#### Kinetics of growth and release of fatty acids as a function of time

Tubes of 10 ml of fresh MRS broth were inoculated with 0.1 ml of the bacterial preculture as described above and incubated at 30°C. The kinetics of bacterial growth is determined by measuring OD600 nm every 4 h.

In parallel, 0.5 ml of enzymatic extract is mixed with 1 ml of pH 7 phosphate buffer and 1.5 ml of lipid substrate. After 60 min of incubation at 37°C with stirring, in order to promote lipid-lipase source contact, 1 ml of 95% ethanol is added to extract the free fatty acids. Titration with 0.05 M KOH solution in the presence of phenolphthalein was carried out to determine the concentration of free fatty acids. The control consists of the same reaction mixture but without enzymes (Ginalska et al., 2004). The results were expressed in  $\mu\text{mol}$  of fatty acid / ml of sample (Sokolovska et al., 1998).

#### pH and temperature determination for lipase activity

The lipase activity was measured in a pH range of 4 to 9. This pH range is obtained using 0.1 M buffer solutions of acetic acid-sodium acetate buffer (pH 3-5), or phosphate buffer sodium (pH 6-7), or Tris-HCl buffer (pH 8) or glycine-NaOH buffer (pH 9). The concentration of released fatty acids was carried out by titration in the same manner as previously described.

The optimal temperature for lipase activity was determined in the same way and in the presence of the optimal pH buffer obtained previously for each strain. To determine the optimal temperature for lipase activity, incubation was performed at 20, 30, 37, 40 and 45°C.

#### Effect of metal ions and additives on lipase activity

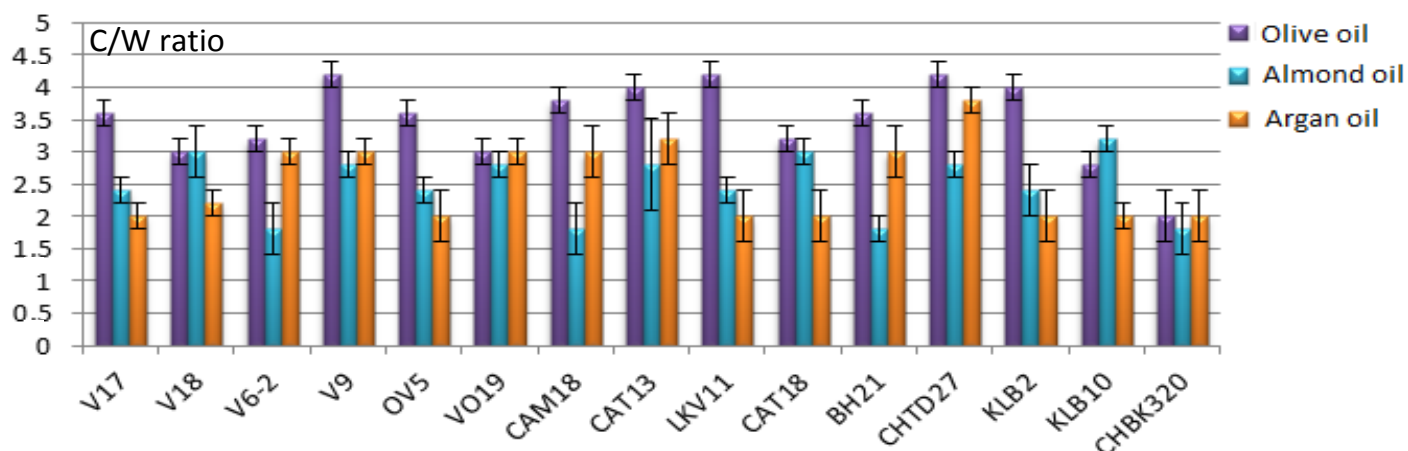
The enzymatic extract was incubated in the presence of 10 mM of various additives and metal ions for 1 h at a temperature of 37°C. The ions, additives and surfactants used are: Ascorbic acid, aspartic acid, folic acid, glutamic acid, nicotinic acid, serine, cysteine, riboflavin, EDTA, Triton X100, beta-mercaptoethanol, ammonium sulfate, barium sulfate, magnesium sulfate, lithium sulfate, sodium thiosulfate, SDS, copper chloride, silver nitrate, iron, calcium chloride, manganese chloride, mercury chloride, zinc chloride, urea, sodium azide, calcium carbonate, Tween 80, Tween 20, sodium molybdate and potassium permanganate. The reaction mixture was supplemented with lipid substrate and different buffers at optimal pH and then incubated for 60 min at optimal temperatures for each strain in order to obtain the released fatty acids.

## RESULTS AND DISCUSSION

#### Intracellular lipolytic and esterase activities

The results of lipolysis obtained on medium supplemented with different lipid substrates are shown in Figures 1 and 2. An example of lipase effect on tributyrin is shown on Figure 3. The lactic acid bacterial strains degrade the lipid substrates differently with a maximum of activity when adding 0.25% tributyrin. This occurs especially in strains VO19, CAM18, CAT13, CAT18 and LKV11 of the genus *Enterococcus* (Table 1). Ginalska et al. (2004) and Gobbetti et al. (1997b) reported that the lipolytic activity is often observed in enterococci and it shows higher activity than strains of most other genera of lactic acid bacteria.

Our results agree with the work of Cardenas et al., (2001) who showed that bacterial lipases tend to reveal better hydrolytic activity on tributyrin, which is a



**Figure 2.** Lipolytic activity on natural lipid substrates in lactic strains (C: clarification area, W: well diameter).



**Figure 3.** Tributyrin esterase activity of lactic strains.

triglyceride composed of short chain fatty acid, namely butyric acid. Tributyrin can be easily broken down by esterases acting on short lipid chains (<10C). Likewise, the lipases of lactic acid bacteria have an optimal efficacy with tributyrin and lower with natural lipids (Talon and Montel, 1994; De Roissart and Luquet, 1994; Guiraud and Galzy, 1980), which has been observed in the presence of natural substrate, in particular olive oil. The lipase activity was less effective with olive oil where in comparison with tributyrin; on the other hand, the presence of olive oil leads to an increase in lipase activity compared to other oils supplemented. This is due to the presence of oleic acid in large quantities in olive oil (78%) because lipases are of the inductive type with a preference for monounsaturated long chain fatty acids. These results are consistent with several studies on microbial lipases showing a high production of lipase in

the presence of olive oil among several oils tested (Feitosa et al., 2010; Sooch and Kauldhar, 2013; Iqbal and Rehman, 2015; Quian and Chun-Yun, 2009; Nwachukwu et al., 2017; Vishnupriya et al., 2010; Esakkiraj et al., 2010; Dandavate et al., 2009). An important lipolytic activity is also observed with 2% olive oil in other bacterial species, like *Pseudomonas aeruginosa* KM110 and *Bacillus* sp ZR-5 (Mobarak-Qamsari et al., 2011; Soleymani et al., 2017).

In the presence of Tween 80 and Tween 20, the lactic strains are weakly lipolytic (Figure 1), so the degradation of these two substrates differs depending on whether the bacteria hydrolyze the Tween 20 containing the lower chain lauric acid esters and the Tween 80 composed of oleic acid degraded respectively by esterases and lipases (Kumar et al., 2012). Therefore, the five strains of *Enterococcus* mentioned above therefore show better

**Table 2.** Identification of lactic strains by MALDI-TOF.

Analyte name	Organism (best match)	Score value
CAM18	<i>E. faecium</i>	2,306
CAT13	<i>E. faecium</i>	2,344
CAT18	<i>E. faecium</i>	2,333
LKV11	<i>E. durans</i>	2,151
VO19	<i>E. faecium</i>	2,177

degradation in the presence of tributyrin, the activity then turns out to be a tributyrin esterase.

These strains are retained for further work and are identified by MALDI-TOF; the results obtained are shown in the Table 2.

### Kinetics of growth and release of fatty acids

The kinetics are carried out every 4 h by measuring the bacterial growth at 600 nm and assaying of freed fatty acids obtained by the action of intracellular tributyrin esterase on the tributyrin supplemented therefore 1  $\mu$ mol of fatty acid released / ml / min corresponds to an enzyme unit (EU). The results obtained are shown on Figure 4a to e.

The results showed the presence of tributyrin-esterase in the intracellular content of the five strains tested from the exponential phase and with maximum production during the stationary phase. On the other hand, the enzymatic activity varies from one strain to another. In addition, according to the phases, a significant production is then observed in the CAT13 and VO19 strains during the exponential phase, which is maintained until the stationary phase. The production of lipases is therefore associated with cell growth, this agrees with studies showing that bacterial lipases are produced during the growth phase or late in the same phase (Papon and Talon, 1988; Makhzoum et al., 1995; Gupta et al., 2004). Other works on intracellular extracts of *Lactobacillus* species had shown that ester activity was present from the start of the exponential phase and then increased to reach a maximum value at the start of the stationary phase (El Soda et al., 1986; Khalid et al., 1990). Serio et al. (2010) also noted an ester activity on strains of *Enterococcus* in stationary phase; similar results are observed in our study where strains CAT18, LKV11 and CAM18 activity is moderate in the exponential phase with maximum production during the stationary phase.

### Determination of optimum pH and temperature

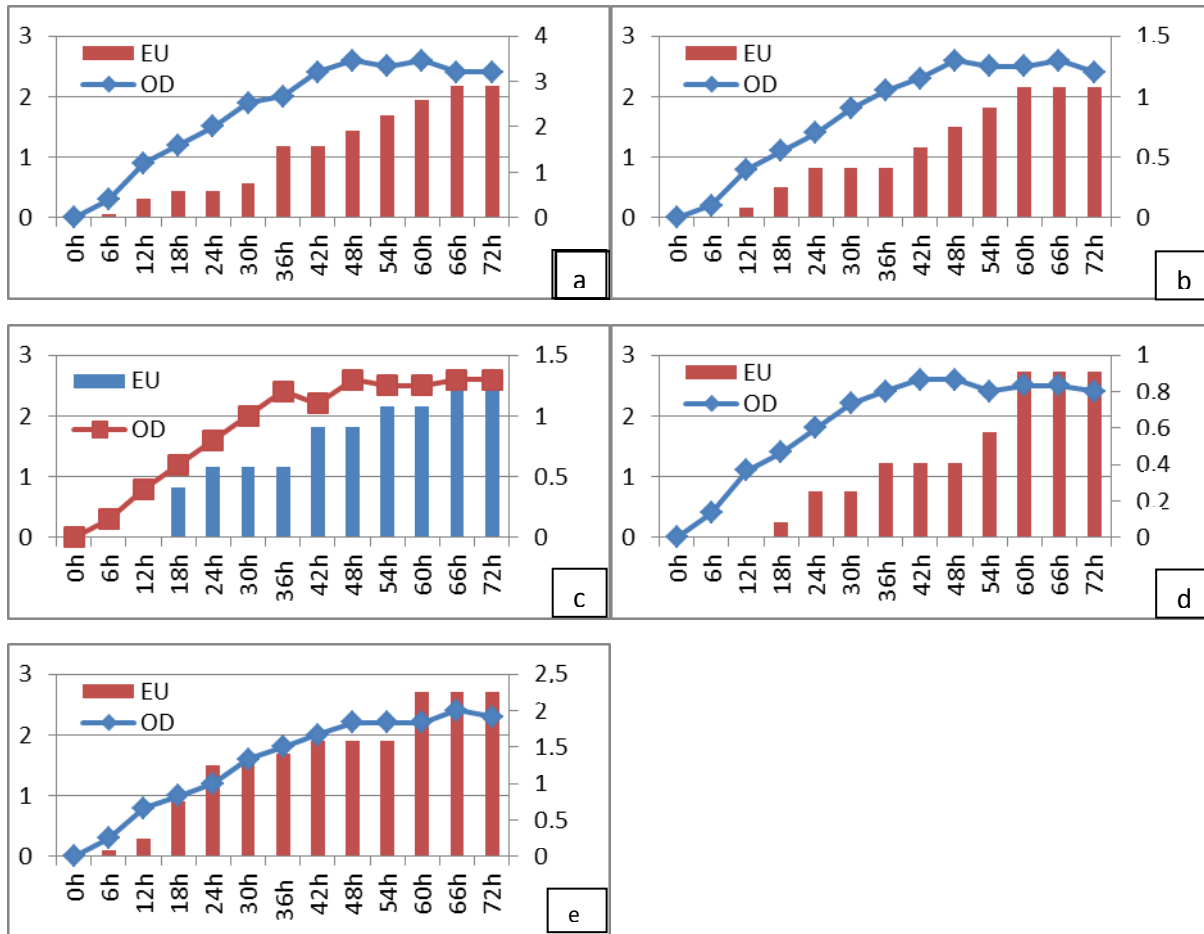
Bacterial lipases are generally neutral (Dharmsthiti and Kuhasuntisuk, 1998; Dharmsthiti and Luchai, 1999; Lee

et al., 1999), or slightly alkaline (Kanwar and Goswami, 2002; Schmidt-Dannert et al., 1994; Sidhu et al., 1998a, b; Sunna et al., 2002). These results are observed in five strains tested with an optimum pH between pH 6 and 9 (Figure 5b), which is consistent with the work of Esteban-Torres et al. (2014b) who reported a noticeable lipolytic activity at pH 7. Tributyrin esterase of *Lactobacillus plantarum* strain 2739 had an optimum pH of 7 (Gobbetti et al., 1996, 1997a), while *Lactobacillus plantarum* MF32 lipase shows maximum activity at a more alkaline pH (pH 9.3) (Andersen et al., 1995).

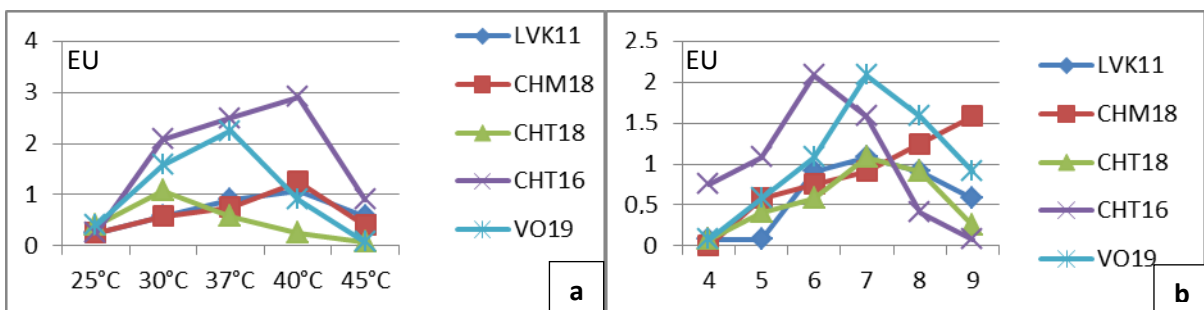
The bacteria studied in this work have an optimal temperature ranging from 30 to 40°C (Figure 5a), some work has shown that bacterial lipases have an optimal temperature of 30 to 60°C (Lesuisse et al., 1993; Wang et al., 1995; Dharmsthiti et al., 1998; Litthauer et al., 2002). This has been observed in lipases of *Lactobacillus plantarum* with an optimal temperature of 35°C (Andersen et al., 1995; Gobbetti et al., 1996; 1997a; Lopes et al., 2002), and in *Enterococcus faecium* where maximum activity occurs at 40°C (Ramakrishnan et al., 2016).

### Effect of metal ions and additives on lipase activity

Several studies show the effect of metal and additive ions on lipase and esterase activity, despite their concentration and the mechanism of induction may vary from one species to another (Saxena et al., 1994). Supplemented metal ions and additives act differently on the esterase activity of the strains tested as shown in Figure 6. The activity is completely inhibited by SDS, sodium azide, EDTA, copper chloride, silver nitrate and mercury chloride in the five bacteria studied. Some studies showed a considerable decrease in activity in the presence of EDTA, which can influence the interfacial zone between substrate and lipase (Sztajer et al., 1992). The activity of lipase Lp\_3562 is strongly inhibited by  $Hg^{2+}$ ,  $Cu^{2+}$  and SDS (Esteban-Torres et al., 2014a). A significant inhibition is observed in Lp\_1760 in the presence of  $Hg^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$  and SDS (Esteban-Torres et al., 2014b). Significant tributyrin-esterase activity is detected when adding Tween 20 and barium sulfate in VO19 and cysteine in CAM18. It was reported that Tween 20, 40, 60 and Triton X-100 could activate lipases in



**Figure 4.** Growth kinetics and fatty acid production as a function of time. a: CAM18; b: LKV11; c: CAT13; d: CAT18; e: VO19. EU: Enzyme unit ; OD: Optical density.



**Figure 5.** Study of optimal parameters (a: Optimal temperature, b: Optimal pH).

*Cryptococcus* sp. (Thirunavukarasu et al., 2008). Tween 20 also increases the production of *Bacillus altitudinis* AP-MSU esterases (Palanichamy et al., 2012), but inhibition of lipase activity is detected in the presence of surfactants (Tween-20, Tween-80 and Triton X-100) tested on Celite-immobilized commercial lipase (Lipolase

100 L) (Sharma et al., 2016). It was also noted that lipases produced by *Pseudomonas aeruginosa* HFE733 are activated by beta-mercaptoethanol and cysteine (Jun et al., 2018). Other ions and additives tested had little or no effect on enzyme activity with a slight decrease or sometimes activation of tributyrin esterases. In some



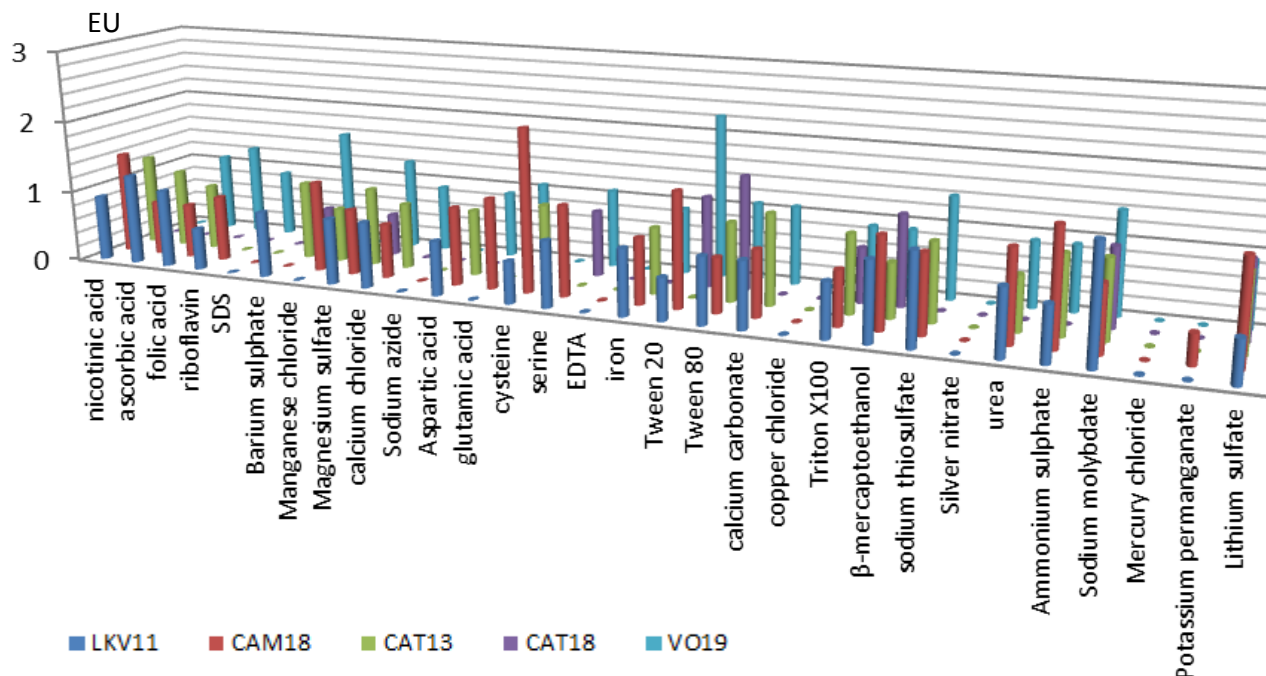


Figure 6. Effect of additives on the lipolytic activity of the five enterococcal strains.

cases the same ion or additive acts differently in the five strains tested by activating or inhibiting enzyme activity as shown in Figure 6.

## Conclusion

Five enterococcal strains show a maximum esterase activity in medium supplemented of tributyrin. The production of intracellular tributyrin esterases can be related to bacterial growth or sometimes maximal during the stationary phase, requiring a neutral or slightly alkaline pH and an optimal temperature between 30 and 40°C. The tributyrin esterases act differently when metal ions and additives are added, while SDS, sodium azide, EDTA, copper chloride, silver nitrate and mercury chloride were found to inhibit enzyme activity.

## CONFLICT OF INTERESTS

The authors declared no conflict of interests.

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