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Production of extracellular lipase by *Enterococcus faecium* E68 with olive oil waste as substrate

Producción de lipasa extracelular por *Enterococcus faecium* E68 en residuos de aceite de oliva como sustrato

Merih-Kivanc, Esra Acu*
Eskisehir Technical University, Science of Faculty, Department of Biology, Eskişehir, Turkey

ABSTRACT

Green technologies eliminate the damages caused by agro-technological wastes to the environment. Our study aimed to both prevent the environmental harm by olive oil waste, and produce lipase enzyme, which is an important biotechnological product. *E. faecium* E68 obtained from milk and dairy products was used for lipase enzyme production. *E. faecium* E68 was cultured in lipase production medium containing 10 % olive waste, pH 6.5, at 37 °C with 120 rpm agitation for 48 h. The effect of temperature, pH metal ion, surfactant, and NaCl was also determined. The molecular weight of the partially purified extracellular lipase enzyme was estimated to be around 19-20 kDa by SDS-PAGE. The optimum temperature was 45°C, while the enzyme exhibited appreciable thermostability retaining activity at 55°C for 48h. The optimum lipase activity was at pH 10. One mM Ca^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+} and K^{+} ions modulated the enzyme activity, but was inhibited by Hg^{2+}, SDS and Triton X-100. The enzyme is halophilic and 25 % NaCl salt increased the activity.

Keywords: *Enterococcus faecium*, Lipase activity, Olive oil waste, lipase.

RESUMEN

Con las tecnologías verdes se eliminan los daños que ocasionan los desechos agrotecnológicos al medio ambiente. En nuestro estudio, el objetivo era prevenir el daño de los residuos de aceite de oliva al medio ambiente y producir la enzima lipasa, que es un producto biotecnológico importante. *E. faecium* E68 obtenido de leche y productos lácteos se utilizó en la producción de la enzima lipasa. *E. faecium* E68 se desarrolló en medio de producción de lipasa con un 10 % de orujo de aceituna, pH 6,5, a 37 °C con agitación a 120 rpm durante 48 h. También se determinó el efecto de la temperatura, el pH del ion metálico, el surfactante y el NaCl. El peso molecular de la enzima lipasa extracelular parcialmente purificada se estimó en alrededor de 19-20 kDa mediante SDS-PAGE. La temperatura óptima fue de 45 °C, mientras que la enzima exhibió una termoestabilidad apreciable reteniendo la actividad a 55°C durante 48 h. La actividad óptima de la lipasa fue a pH10. Los iones Ca^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+} y K^{+} (1 mM) modularon la actividad de la enzima, pero fueron inhibidos por Hg^{2+}, SDS y Triton X-100. La enzima es halófila y la sal de NaCl al 25 % aumentó la actividad.

Palabras clave: *Enterococcus faecium*, actividad de lipasa, residuos de aceite de oliva, lipasa.

INTRODUCTION

By-products generated in the agricultural industry cause much environmental pollution and adverse health effects (Hamrouni *et al*., 2020; Leite *et al*., 2021). Waste resulting from olive oil extraction creates severe environmental problems with its highly polluting properties (Mantzavinos and Kalogerakis, 2005; Sarika *et al*., 2005). Olive waste contains biodegradable compounds and phytotoxic phenolic compounds. Phytotoxic wastes correspond to about 80 % of olive oil production. These phenols tend to change into condensed high molecular weight polymers, which are difficult to degrade on storage (Ayed *et al*., 2005). For these reasons, olive oil wastes may lead to acute odor problems and, more importantly, serious risks for water and soil quality (Mantzavinos and Kalogerakis, 2005; Sarika *et al*., 2005). Today, with the increasing awareness of environmental protection, the use of biomass has gained importance (Hamrouni *et al*., 2020). Olive oil waste contains simple and complex sugars, lipids, residual oil, proteins, and mineral elements, besides phytotoxic wastes. These compounds can be directly recovered by chemical extraction followed by purification (Fki *et al*., 2005; Papadimitriou *et al*., 2005). Olive oil waste can be used as the basic compound for fermentative production processes (Fenice *et al*., 2003; Angenent *et al*., 2004). Various agro-industrial wastes have been used for biotechnological purposes, especially for enzyme production (Mahanta *et al*., 2008). Lipases are one of those enzymes, which are commercially important since they catalyze the hydrolysis of long chain fatty acids to glycerol and fatty acids.

Most commercial lipases are produced by microorganisms (Babu and Rao, 2007; Treichel *et al*., 2010; Bharathi and Rajalakshmi, 2019; Adetunji and Olaniran, 2021). Especially since the lipase enzyme produced from lactic acid bacteria is considered safe, it is preferred in the food industry (Meyers *et al*., 1996; Liu *et al*., 2001; Lopes *et al*., 2002; Couto and Sanroman, 2006; Ramakrishnan *et al*., 2013; 2015; 2016; Sukohidayat *et al*., 2018; Dellali *et al*., 2020; Acu *et al*., 2021).

In our study, it was aimed to produce, partially purify and characterize the lipase enzyme from *E. faecium* E68 strain in olive oil waste.

*Autor para correspondencia: Merih Kivanc
Correo electrónico: mkivanc@eskisehir.edu.tr
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MATERIAL AND METHODS

Bacteria

In the study, *Enterococcus faecium* obtained from Eskişehir Technical University microbiology unit was used. *E. faecium* E68 was inoculated in M17 broth and incubated at 37 °C for 24 h under 10 % CO_2 conditions. Growing cultures from M17 broth were inoculated on M17 agar and incubated at 37 °C for 24 h under 10 % CO_2 conditions. The morphological features of the colonies formed after incubation were examined. Then, the purity of the cultures was checked by microscopic examination by Gram staining.

Olive oil waste

Olive oil waste with dry matter content of 0.82 g, nitrogen content of 1.593 % and oil content of 0.036 % was obtained from olive oil production facilities and used in the studies.

Lipase production

E. faecium E68 was inoculated onto M17 agar and incubated at 37 °C for 48 h. It was then inoculated as a single colony into M17 broth and incubated at 37 °C for 24 h. Culture was set to an Optical Density (OD) of 1 at 600 nm in the spectrophotometer. Then, 1 % of the culture was inoculated into the lipase determination medium.

The study was carried out in 2 parts. In the first part, without adding olive oil waste to the medium, and in the second part, by adding 10 % olive oil waste to the medium.

For enzyme production, 500 mL of lipase assay medium was prepared, added with 5 % peptone as nitrogen source, 3 % glucose as carbon source and other components (0.1g/L $CaSO_4$, 0.5g/L KH_2PO_4, 0.1g/L $MgSO_4$ x $7H_2O$, 1 % tributrine) and the pH adjusted to 6.5-7. It was incubated for 48 h in a 37°C shaking oven (120 rpm) under 10 % CO_2 conditions. After incubation, it was centrifuged at 9,798 x g for 30 min at 4 °C. The cooled acetone was mixed with the obtained filtrate at a 1:5 ratio, kept at 4 °C for 24 h, and centrifuged at 9,798xg, 4 °C for 15 min.

In the application where olive oil waste is used, the same processes were applied by adding 10 % olive waste to the lipase medium (0.1 g/L $CaSO_4$, 0.5 g/L KH_2PO_4, 0.1 g/L $MgSO_4$ x $7H_2O$, 1 % tributrine).

Then, 3 mL of the enzyme was placed into the dialysis tube (Sigma PURX12015), which in turn was placed in Tris HCl buffer. Tris HCl buffer was changed every 24 h. After 48 h, partially purified enzyme was obtained. Partially purified enzymes were used in the experiments. Experiments were carried out in two replicas each.

Assay of lipase activity

Para-nitrophenyl palmitate (p-NPP) method, which is a spectrophotometric method, was used for lipase activity determination (Arora, 2013). This method determined lipase activity by measuring p-NPP at a wavelength of 405 nm in a spectrophotometer. One unit (U) of lipase activity is the amount of enzyme that releases 1 µmol p-nitrophenol per unit time (min).

Determination of the molecular weight of lipase

The protein amount of the lipase enzyme was determined by the Bradford method (Bradford, 1976) in a spectrophotometer at 590 nm using Coomassie Brilliant Blue G-250 dye. The molecular weight of the enzyme was determined by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to the method of Laemmli (1970).

Determination of Factors Affecting Partially Purified Extracellular Enzyme Activity

Effect of temperature and pH on enzyme activity

To determine the effect of temperature on partially purified extracellular enzyme activity, the enzyme was incubated for 1 h at 5 °C, 20 °C, 30 °C, 37 °C, 40 °C, 45 °C, 55 °C and 65 °C in 50 mM sodium phosphate buffer (pH 7).

To determine temperature stability, lipase enzyme was placed in 50 mM sodium phosphate buffer (pH 7) and incubated at 5 °C, 20 °C, 30 °C, 45 °C and 55 °C for 5 min, 1 h, 4 h, 24 h and 48 h. After cooling, the remaining enzyme activity was measured (Esteban-Torres *et al*., 2015).

The effect of pH on lipase activity was determined by keeping the enzyme in buffers prepared between pH 3-11. Acetic acid-sodium acetate buffer was used for pH 3-5, sodium phosphate buffer for pH 6, Tris-HCl buffer for pH 7-8, and glycine NaOH buffer for pH 9 (Esteban-Torres *et al*., 2015).

In order to determine the enzyme pH resistance, 200 µL of buffers at different pHs were placed in the microtubes. On top of it, 200 µL of enzyme were added and incubated at 45 °C for 2 and 3.5 h. The remaining enzyme activity was determined (Esteban-Torres *et al*., 2015).

Effect of some surfactants and cations on enzyme activity

To determine the effect of some cations, surfactants, and solutions on the enzyme activity, $MnCl_2$, $CuCl_2$, $MgCl_2$, KCl, $NiCl_2$, $CaCl_2$, $HgCl_2$, and $ZnCl_2$ of 1 mM were added, and the enzyme activity determined at 405 nm in a spectrophotometer.

The effect of urea, EDTA, SDS, tween 20, tween 80, and triton X-100 on enzyme activity was determined by adding 1 µL to the medium (Esteban-Torres *et al.,* 2015, Ramakrishnan, *et al*., 2016).

Effect of salt on enzyme activity

To determine the effect of sodium chloride (NaCl), it was added to the buffer at concentrations of 0 %, 1 %, 5 %, 10 %, 15 %, 20 %, 25 % (w/v) and the enzyme activity determined in spectrophotometer at 405 nm (Esteban Torres *et al*., 2015).

RESULT AND DISCUSSION

In recent years, producing useful substances from waste materials has been of great importance. Thus, products with economic importance can also be obtained while preventing environmental pollution. In the study, it was determined that olive waste could be used in the nutrient medium and, in this way, a product of high economic importance can be obtained.

The extracellular lipase enzyme obtained without using olive oil waste and using 10 % olive oil was partially purified and used in the tests. The wet and dry weights and protein contents of the obtained lipase enzymes are given in Table 1.

Table 1. Dry weight and protein amounts of extracellular lipase enzymes from *E. faecium* E68.

Tabla 1. Peso seco y concentración proteica de las enzimas lipasa extracelulares de *E. faecium* E68

	Protein (BSA mg/mL)	Dry weight (mg/mL)	Wet weight (mg/mL)
Medium	0.315	0.396	1.078
Olive oil waste	0.691	0.360	1.133

The protein amounts in the extracellular enzymes were found to be 0.315 mg/mL for the lipase enzyme produced in the medium, while it was 0.691 mg/mL for the partially purified enzyme produced in olive oil waste.

As a result of *E. faecium* E68 SDS-PAGE analysis, the molecular weight of lipase enzymes was determined at around 19-20 kDa (Figure 1).

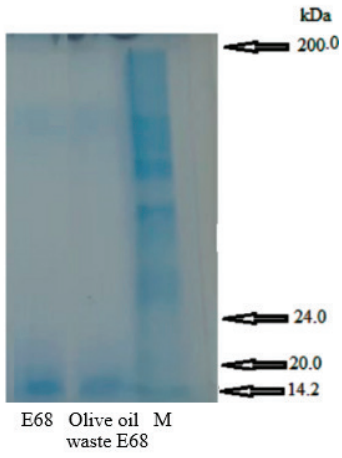

Figure 1. SDS-PAGE analysis of lipase enzymes from partially purified *E. faecium* E68. M. marker.

Figura 1. Análisis SDS-PAGE de enzimas lipasa de *E. faecium* E68 parcialmente purificado. Marcador M.

Lipase enzyme production was performed with *E. faecium* E68 in lipase medium containing 10 % olive oil waste (pH 6.5) after 48 h of incubation at 120 rpm at 37 °C.

Higher enzyme activity was obtained in the extracellular enzyme produced by *E. faecium* E68 in the fattening medium where olive waste was used. Lipid sources, such as natural oils have been shown to stimulate lipase production. Olive oil is one of the best inducers of lipase production (Zarevúcka, 2012). The presence of a certain amount of olive oil in olive oil waste stimulated lipase production. The activity of intracellular and extracellular lipases increases with increasing lipid concentration (Zarevúcka, 2012). The reason for the higher activity of the lipase enzyme produced using olive oil waste may be related to the increased lipid concentration.

The effects of temperature, pH, cations, and surfactants on the enzyme activity were determined.

The partially purified extracellular enzyme showed high lipolytic activity between 5 °C and 65 °C. The highest activity of the enzyme produced in the lipase medium was between 5 - 20 °C (Figure 2). Partially purified extracellular enzyme activity obtained with *E. faecium* E68 in olive waste was highest at 45 °C (Figure 2). Temperatures above 45 °C caused a decrease in enzyme activity (Figure 2). It has been reported that the partially purified extracellular enzyme obtained from *E. durans* E114 shows maximum activity at 30 - 45 °C (Acu *et al*., 2021). Maximum activity in the *E. faecium* lipase enzyme was observed at 40 °C (Ramakrishnan *et al*., 2016). Dellali *et al*. (2020) reported that the optimum lipase activity of *E. faecium* strains was 30-40 °C.

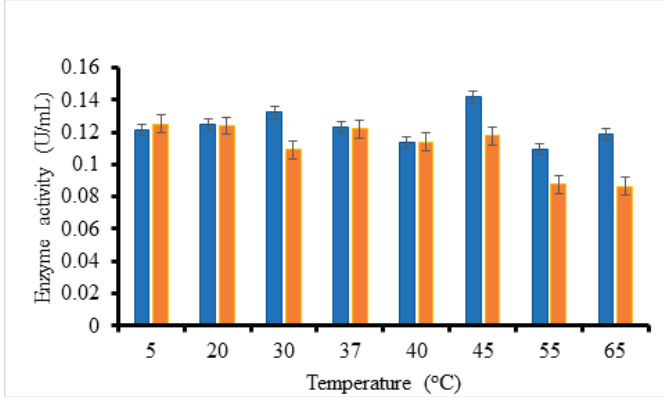

Figure 2. The effect of temperature on the *E. faecium* E68 extracellular lipase enzyme activity. Olive oil waste, medium.

Figura 2. Efecto de la temperatura sobre la actividad enzimática de la lipasa extracelular de *E. faecium* E68. Residuos de aceite de oliva, medio.

Partially purified enzyme activity produced in olive waste decreased at 20 °C and remained stable at 55 °C, although it was below the optimum activity. The lipase enzyme produced in the lipase medium maintained its activity at 20 °C (Figure 3). The temperature tolerance of the extracellular enzyme remained quite stable after an incubation period of 24-48 h at 30-45 °C. Ramakrishnan *et al*. (2016) reported that the *E. faecium* lipase enzyme activity is stable between 30-70 °C. Researchers have reported that the enzyme activity is stable at 80-100 °C, and that enzyme activity does not remain after 100°C. Esteban Torres *et al*. (2015) observed that the maximum activity of the *L. plantarum* esterase enzyme is at 40 °C. They reported that the enzyme showed only 40 % of its activity at 5 °C, and, after 10 h of incubation at 55 °C and 65 °C, 40 % of the activity remained. Francisco *et al*. (2019) reported that the decrease in enzyme activity with temperature is associated with the change in its three-dimensional structure. It has been found that the alpha helix decreases at temperatures above 50 °C. At temperatures above 70 °C, the beta sheet increases while maintaining a low alpha helix. Opening the protein results in permanent inactivation and denaturation (Ismail *et al*., 2021).

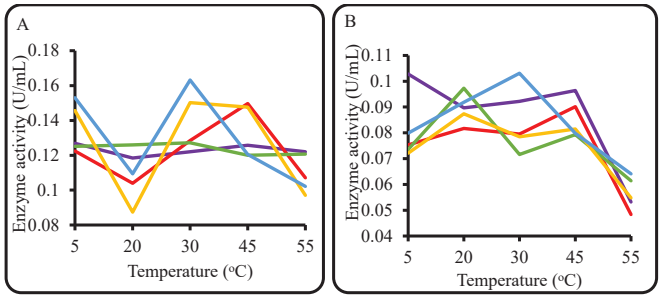

Figure 3. Temperature resistance of *E. faecium* E68 extracellular lipase enzyme according to different residence times at different temperatures. A; olive oil, B; Medium. 5 min, 1 h, 4 h, 24 h, 48 h.
Figura 3. Estabilidad a la temperatura de la enzima lipasa extracelular de *E. faecium* E68. A; aceite de oliva, B; Medio. 5 min, 1 h, 4 h, 24 h, 48 h.

The highest activity of the lipase enzyme, produced in olive waste and partially purified, was obtained at pH 10 (Figure 4). Partially purified enzymes were alkaline in nature. The optimum pH was found to be 10. While the lowest activity is obtained at pH 6, the enzyme has higher activity at pH 3. Acidic pH activity has been observed for a lipase from *E. durans* 27 isolated from fish processing waste. Lipase from ED-27 showed optimal activity at pH 4.6 and at temperature 30 °C (Ramakrishnan *et al*., 2015). A highly alkaline extracellular lipase that exhibits maximum hydrolytic activity at pH 10.8 has been reported from *E. faecium* MTCC5695 (Ramakrishnan *et al*., 2016). Dellali *et al*. (2020) reported that the optimum activity of the enzyme produced by *E. faecium* strains is between pH 6 and 9.

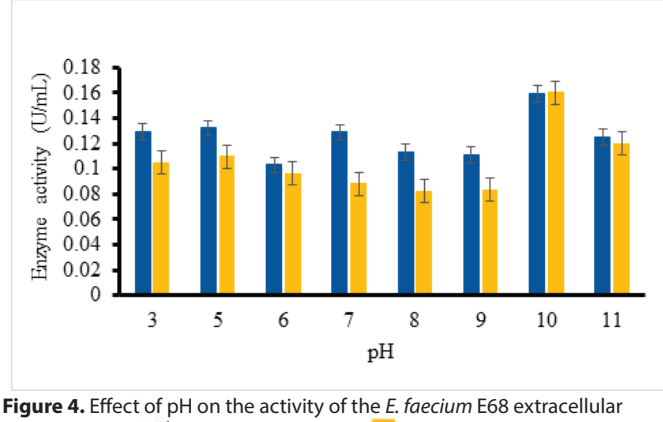

Figure 4. Effect of pH on the activity of the *E. faecium* E68 extracellular lipase enzyme. Olive oil waste (U/mL), Medium (U/mL)
Figura 4. Efecto del pH sobre la actividad de la enzima lipasa extracelular de *E. faecium* E68. Residuos de aceite de oliva (U/mL), Medio (U/mL)

The pH stability of the extracellular enzymes obtained from *E. faecium* E68 was determined by incubating them at different pH values for 2 h and 3.5 h at 45 °C. The lipase produced in olive oil waste by *E. faecium* E68 remained significantly stable after 2 h and 3.5 h at pH 3 (respectively % 77,36 and % 70,79). However, the lipase activity produced in the lipase production medium was lower at pH 3. Similar acidic pH activity has been observed for a lipase from *E. durans* NCIM5427 from fish waste isolated from slaughterhouse waste (Ramakrishnan *et al*., 2015). For the enzyme produced in the lipase production medium, the activity loss was higher after 2 h and 3.5 h at pH 3. The highest loss of activity was observed at pH 5. Enzymes remained stable at alkaline pH for 2 h and 3.5 h of standing (Figure 5).

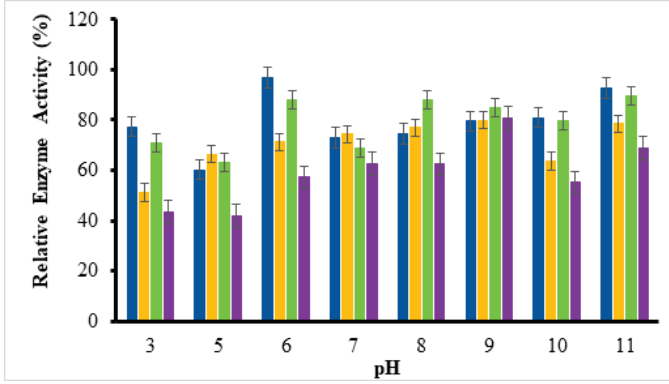

Figure 5. pH resistance of *E. faecium* E68 extracellular lipase enzyme according to different residence times (2 and 3.5 h) at different pH. Olive waste 2 h, Olive waste 3,5h; Medium 2 h, Medium 3,5 h.
Figura 5. Resistencia al pH de la enzima lipasa extracelular de *E. faecium* E68 según diferentes tiempos de residencia (2 y 3,5 h) a diferentes pH. Residuos de aceituna 2 h, Residuos de aceituna 3,5 h; Medio 2 h, Medio 3.5 h.

Lipolytic isoenzymes from a thermophilic *Bacillus sp*. have also been observed. It showed optimum activity at pH 8.5 and was reported to be very stable at pH 6.0 - 8.0 (Nawani and Kaur, 2007).

The effects of some ions and additives on the enzyme activity are given in Table 2. One mM Ca^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+} and K^{+} ions significantly increased the lipase activities produced in olive oil wastes. Contrary to our findings, Ramakrishnan *et al*. (2015) reported that it significantly reduced Ca^{2+} and Mg^{2+} lipase activity. Mercury (Hg^{2+}) led to a strong decrease in lipase activity. In the lipase enzyme produced in the lipase environment, metals other than Mg decreased the enzyme activity (Table 2). EDTA, which can affect the interface region between substrate and lipase, increased enzyme activity, however, some studies have reported that it reduces activity (Sztajer *et al*., 1992). The activity of lipase Lp_3562 was strongly inhibited by Hg^{2+}, Cu^{2+} and SDS (Esteban-Torres *et al*., 2014a). Urea, Hg^{+2}, Mn^{+2}, Cu^{+2}, Ni^{+2}, Zn^{+2} and SDS, inhibited the activity of the enzyme produced in the lipase medium. Similarly, Dellali *et al*. (2020) reported that although the effect of metal and additive ions on esterase activity varies from bacteria to bacteria, they inhibit SDS, NaN_3, $CuCl_2$, EDTA, $AgNO_3$ and $HgCl_2$ enzyme activity.

While Triton X-100 and Tween 80 of surfactants increased enzyme activity, Tween 20 and SDS decreased enzyme activity.

This enzyme has high salt resistance and shows halophilic properties, an important feature in the preparation of foods. Even an increase in enzyme activity was observed (Figure 6). The salt resistance of this enzyme is high, and it showed halophilic properties. This feature is important for the preparation of foods.

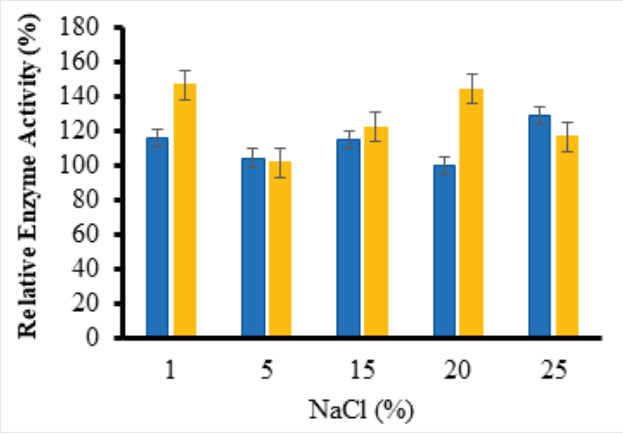

Figure 6. Effect of sodium chloride on the lipolytic activity of *E. faecium* E 68.
Olive oil waste; medium.
Figura 6. Efecto del cloruro de sodio sobre la actividad lipolítica de *E. faecium* E 68.
Residuos de aceite de oliva; medio.

Table 2. Effect of additives on the lipolytic activity of *E. faecium* E 68.
Tabla 2. Efecto de aditivos sobre la actividad lipolítica de *E. faecium* E 68.

Additives	Relative activity (%)	
	Medium	Olive oil waste
Control	100	100
$HgCl_2$	41.7	48.9
$CaCl_2$	104.7	79.3
$MnCl_2$	112.8	88.2
$CuCl_2$	133.9	85.8
$NiCl_2$	110.0	86.1
KCl	130.8	90.9
$ZnCl_2$	105.2	89.2
$MgCl_2$	150.9	123.3
EDTA	101.4	108.4
Urea	128.7	92.8
SDS	87.3	91.2
TritonX-100	81.5	120.5
Tween20	104.6	93.5
Tween80	105.4	128.5

The lipase enzyme produced in olive waste is promising in the food industry due to its resistance to 55 °C for 48 h, not losing its activity at low temperatures, and its halophilic properties. The use of these lipases is important as they can provide some advantages in food production.

The activity of lipase enzyme produced in olive oil waste was higher. The reason for this may be the presence of a small amount of olive oil residue in it. Olive oil has a significant effect for increasing lipase activity. It has been reported that the most suitable inducer in lipase production is olive oil. This has been associated with high levels of unsaturated grade free fatty acids, particularly oleic acid, in oil (Amenaghawon *et al*., 2022). This has been shown to facilitate cell growth and consequently increase both intracellular and extracellular lipase activity (Suci *et al*., 2018). A similar observation was reported by Brozzoli ve ark. (2009) and Rajendran and Thangavelu (2012).

CONCLUSION

The results revealed that natural substrate such as olive oil waste has good inducing properties for lipase synthesis. Therefore, it may be beneficial to use olive oil waste as a cost-effective source for lipase production. The relative stability of *E. faecium* E68 lipase at high temperatures may make it usable for biotechnological processes, as enzymes that can withstand high temperatures longer, attract the attention of industries. It is important that the lipase enzyme produced by *E. faecium* E68 has high activity at 45 °C and pH 10, as well as showing activity in acidic conditions such as pH 3. It is promising in the food industry with its resistance to 55 °C for 48 h, its effectiveness at low temperatures and its halophilic feature. Olive oil can be an important substrate for waste lipase production. Thus, environmental pollution can be prevented, and a biotechnological product is also obtained.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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