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Isolation and diagnosis of high lipolytic activity in *Enterococcus faecium* and application of its lipase product in beef meat burger

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ABSTRACT

Microbial lipases are a crucial group of enzymes used in many biotechnological industries, such as flavor enhancement technology in foods, especially meat products. Lipases are produced alone or in combination with esterase by a variety of microorganisms. In the present study, Bacterial colonies isolated from various food samples and our isolate *Enterococcus faecium,* which was previously identified and registered in the gene bank under the accession number PV299116, with 99.89% identification, were screened for lipolytic activity, by producing a bright orange halo around it on tributyrin-MRS agar and a clear zone diameter on Tributyrin agar. Of the ten colonies observed for lipolytic activity, colony number 6, *Enterococcus faecium*, achieved the highest lipase activity, with a maximum lipolytic zone of 23mm and 46U/ml lipolytic activity. Lipase was produced from *E. faecium* in MSM medium, then purified by acetone precipitation and gel chromatography. The application for adding extracted lipase to beef meat burger showed significant improvement in the meat sensory properties (appearance, colour, flavor, and overall acceptance) with values of 7.7, 7.1, 7.2, and 7.3, respectively for the longest storage time (4 weeks) at $p \leq 0.001$.

1- Introduction

During fat breakdown, triglycerides are hydrolyzed to free fatty acids and glycerol, with intermediates such as mono- and diglycerides. These then emulsify the other additional food ingredients, thus developing the texture in the final products. Microbial lipases contribute to various types of food at specific times and in higher concentrations in the process of releasing fatty acids, which play a role in the final flavor, which is important for the production of dietary supplements and new foods . Those who participate in the final flavor are of concern in the manufacture of dietary supplements and new foods [1, 2].

LAB (lactic acid bacteria) are largely responsible for the spoilage and the safety of the product, and they also contribute to the texture, flavor, and shelf life of the food product by breaking down fats. Lipase (Triacylglycerol acyl hydrolases EC 3.1.1.3) belongs to the serine hydrolase category, Lipase performs the process of hydrolysis, the Trans esterification of other esters in addition to the synthesis of esters and displays selective properties for isotopes. Lipases are responsible for digesting fats in pancreatic secretions, their breakdown, and absorption of fat-soluble vitamins. In addition, lipases play a crucial role in the mechanism of some cholesterol-lowering medications. The lipase produced by (LAB) plays a key role in breaking down the fats present in ground beef [3,4,5]. These enzymes work to break down fats into fatty acids and glycerol, which affects the flavor, juiciness, and texture of the meat. Additionally, lipases help improve the digestion process and enhance the fat-soluble absorption of vitamins such as A, D, E, and K [6]. The LAB strains were selected by the degree of fat breakdown. This property is of great importance for the fermentation of vegetables, applications in baked goods, and flavor development in fermented dairy products [7]. It accelerates the breakdown of free fatty acids, glycerol at the oil-water interface, and triglycerides. It also speeds up the hydrolysis and synthesis of other esters, as well as exhibiting remarkable selective properties. Lipases are used in industrial processing, pharmaceutical, and food industries, as these organisms are considered safe for use. Enterococcus bacteria are LAB that are ubiquitous, found in the environment, Food, and primarily reside in the digestive tract of animals and humans due to their resistant nature. They offer several technologically important benefits in food fermentation. *Enterococcus* also contributes to improvements in the aroma, texture, and flavor of fermented products. The protein activity has been recorded many times for LAB, including *Lactococcus*, *Lactobacillus*, *Enterococcus*, and *Pediococcus*. The bacterial species *E. faecium* is known for its ability to produce several different enzymes, including lipase, which is involved in the breakdown of fats, such as those found in beef, and affects the texture, flavor, and quality of the meat [2, 8]. Lipases can be acidic or alkaline in nature, and active lipases (pH 1.5- 2), like the gastric lipase that comes from mammalian sources. Alkaline lipases are considered to have great potential in cooking as food spices, cleaning agents, leather processing, cosmetics, and pharmaceutical industries [5]. The present study aims to obtain bacterial isolates characterized by high lipolytic activity by isolating them from various food sources. The goal is to extract and purify the enzyme and assess its efficacy on beef burger disks.

2-Materials and Methods

2-1 Collection of Samples: Samples of milk, dairy, yogurt, cheese, cream, and pastirma were collected and brought to the laboratory. 1g was taken from each sample and serially diluted from 10^{-1}-10^{-5} with 9 ml distilled water [9].

2-1-1 Bacterial activation: The genetically identified isolate *Enterococcus faecium* PV299116 from our previous study was active on MRS broth medium at 37°C. [9].

2-2 Primary screening for isolation of lipolytic bacterial colonies

For screening of lipolytic bacteria, 0.1 ml from each dilution was spread separately onto MRS agar containing tributyrin as a substrate. After 48 h of incubation at 37°C, colonies with lipase activity were detected by exposing them to UV light at 350 nm [10].

2-2-1 Secondary screening for selection of high lipolytic activity colonies

Tributyrin agar (TBA) as designed by [11] was prepared by using casein peptone 2.5 g/L, meat peptone 2.5 g/L, yeast extract 3 g/L, and Agar-agar 10 g/L. It was adjusted to pH 7, then sterilized in an autoclave and allowed to cool to 60ºC. This was followed by the addition of 1 mL of neutral substrate tributyrin with agitation, then poured into plastic Petri dishes. Lipolytic activity was tested by agar well test method as described by [12]. 6 mm diameter wells were created in TBA medium by cork borer, then all wells were inoculated with 0.1ml of filter-sterilized supernatant of overnight MRS broth cultures of lipase-producing colonies separately. The plates were incubated at 30°C for 6 days. The TBA showed a clear zone around the wells, and then events measured (mm) of zones to get the chosen colony with the highest measure of lipase activity.

2-3 Identification of study bacterial isolate

The isolate bacteria was identified biochemically and genetically in a previous study [9], the method outlined by [13] was followed to PCR amplify the 16S rRNA gene of the selected bacterial isolate using the primer pair: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') [14, 15]. PCR reactions were also carried out in tubes containing a volume of 25 µL. For reaction mixture included PCR master mix (12.5 µl), template DNA (5 µl), forward primer (1 µl), reverse primer (1 µl) and Nuclease free water (5.5 µl). The amplification program included initial denaturation at 95°C for 10 min and 40 cycles of denaturation at 94°C for 15 sec., annealing at 60°C for 1 min, extension at 72°C for 15 sec, with a final extension at 72°C for 10 min. After detection of the PCR products by electrophoresis, they were exported to BIONEER Company, South Korea for determination of nitrogen bases sequencing and were then processed and revalidated before they were assayed in the BLAST program supplied by the National Center for Biotechnology Information Service (NCBI) (http://www.ncbi.nlm.nih.gov).

2-4 Lipase production and its activity determination

According to the procedures of [16, 17], the lipase production and activity measurement of the selected bacteria were conducted with the determination of the effect of pH, temperature, and incubation time on production. A single colony was transferred into 5 ml MSM medium, shaken at 37°C and 120 rpm until OD600 = 1.0. 1% was inoculated into a 150 ml conical flask containing 100 ml MSM medium, pH 7.4, and incubated at 37°C with 120 rpm shaking incubator for 48 h. The medium was collected at different incubation conditions and centrifuged at 12,000 rpm and 4°C for 20 minutes, and the crude lipase filtrate was collected. Lipase activity was determined by titration under the conditions of pH 9 and 55°C with olive oil emulsion as substrate. Protein content was quantitated using the BCA protein assay kit.

2-4-1 Purification of the produced lipase

Lipase was separated from crude supernatant made as per [18] using chemical precipitation and gel chromatography in two successive steps. The technique of acetone precipitation was used with the varying ratios of acetone (1:1, 2:1, 3:1, 4:1, and 5:1). The protein precipitates were then stored in the refrigerator at 4°C for the subsequent process.15 ml of protein precipitate was filled in dialysis bags and was stored in a 500 ml beaker with a 20 mM Tris HCl buffer, and stored at 4°C for 24-48 hours, and the buffer was being changed every 6 hours. The protein precipitated with acetone was purified using the filter column gel chromatography, where the column resin Sephadex G-150 was appropriately prepared according to the manufacturer's procedure.

2-5 Application of lipase in beef burger

According to the protocol given by [19,20], beef samples were collected from local markets in Basrah city and stored in sterile polyethylene bags and sterile plastic containers with ice until shipped to the laboratory, where the samples were surface washed with distilled water after stripping the outer lipid layer and bone. The meat was cut and chopped with an electric chopper and each 100g of it was packed in an air-emptied polyethylene bag, then bags were divided into two sections: a control section without lipase added and a treatment section with 20% lipase added, and all then closed tightly and kept in the refrigerator at 4°C for different periods (0, 2, 4 weeks), before incubated them at 37 ˚C for 72 hours to allow the effect of lipase on the meat to carry out the later examinations.

2-5-1 Sensory evaluation of the beef burger

The sensory evaluation of beef burger disks was conducted following their grilling in an electric oven at 200°C for 30 minutes by a panel of ten expert evaluators on a 10-grade evaluation sheet with five sensory parameters: appearance, color, taste, flavor, texture, and general acceptance of treated and control burger products after refrigerated storage for the periods (0, 2, 4) weeks to determine the impact of lipase addition in a burger.

2-6 Statistical analysis

With the assistance of statistical software SPSS (2019, Version 26). Complete randomized design (CRD) was used for data analysis. Means were compared using the Revised Least Significant Difference (RLSD) test in the program and in accordance with the following mathematical model, $Yij=\mu+ Ti+ eij$ where Yij is the value of the observation for each trait, μ is common mean, Ti Effect of bacterial colony eij represent the experimental error with mean=zero and a variance=ϙ2e [21].

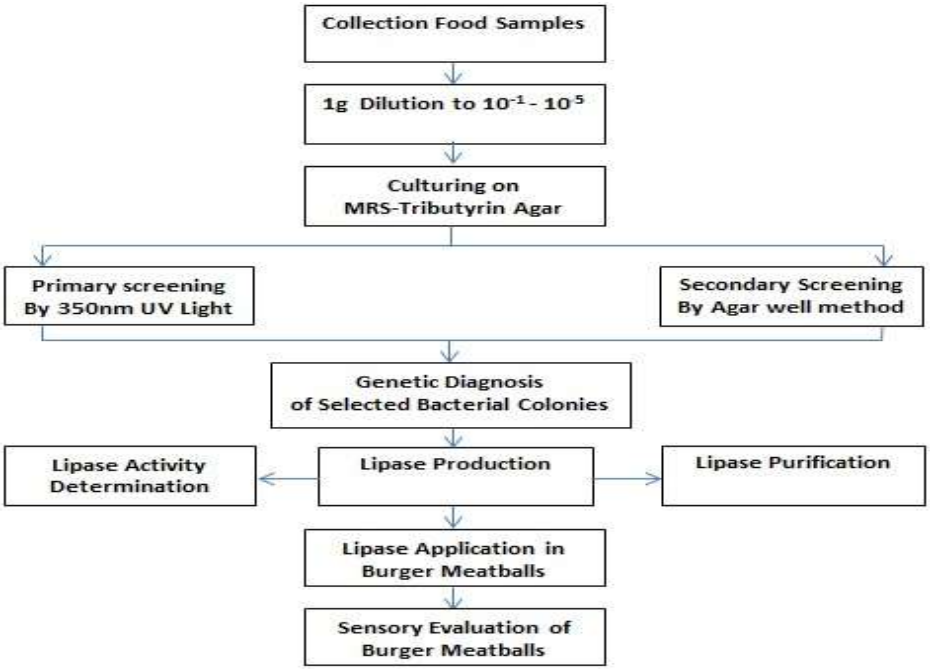

Fig.1: Outline of search work steps.

3-Results and Discussion

3-1 Selecting and isolating of lipolytic bacterial colonies

Ten colonies with lipolytic activity were isolated and selected from the dietary samples of the study, where these colonies showed a bright orange halo after growing on the tributyrin-MRS agar plates and exposed to 350 nm UV light (Fig.2). Also, the colony number 6 was obtained, which had the highest lipolytic activity (zone =23 mm) in agar well assay on TBA plates (P= 0.001, LSD=2.78) unlike the other remaining colonies, which had halo areas of less than 20 mm (Fig. 3).

3-2 Determination of lipase activity

Colony 6 *E. faecium* PV299116 was picked up and the lipolytic efficiency of its crude extract was estimated, during 72 hr. of incubation, by 46U/ml (P=0.001, LSD=4.16) compared to other colonies (Fig. 4), and then it was genetically diagnosed.

All these results in our study concur with the previous studies [22,23,24,25,26,27], which showed bright orange halos surround bacterial colonies of lipase-producing strains and exert zones of activity with large diameters by evaluating the agar well method. The selected bacterial strains showed visible deposits on their colonies that may be indicative of the fatty activity resulting from the release of fatty acids and their deposition as calcium salts, as reported in a study [28]. The appearance of a hydrolysis zone around the colonies was also considered a key sign for the selection of the lipase-producing bacterial strains.

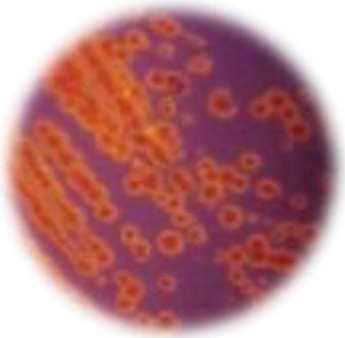

Fig.2: Selected lipolytic bacteria on tributyrin-MRS plate

3-3 Genetic identification of selected bacterial colony

[9] Ali, A. Z., reported that the diagnosis outcome of the selected high lipolytic colony (C6) was gram-positive, non-motile, and coccoid in shape. After the isolation of bacterial DNA, 1222 bp of the genome was amplified through the polymerase chain reaction (PCR) technique to submit it for genetic sequencing before the precise identification carried out in NCBI blast (https://blast. ncbi.nlm.nih.gov/ Blast. cgi? PAGE TYPE= Blast Search). The NCBI BLASTn engine revealed about 99.89% sequence similarity of the 1222 bp sequenced sample amplicons to the predicted reference target sequences of Enterococcus faecium (GenBank acc. no. PV299116). (Table 1) through comparison of the newfound DNA sequences of these original samples with the retrieved DNA sequences. Their sequence information was given after the positioning of the 1222 bp sequence, according to the position of both forward and reverse primers to the 1222 bp amplicon primers (Table 2). This observation is in agreement with a study by [29], who achieved the diagnosis of six Enterococcus species, including *E. faecium*, isolated from foods by sequence analysis of the 16S rRNA genome and establishing the PCR method.

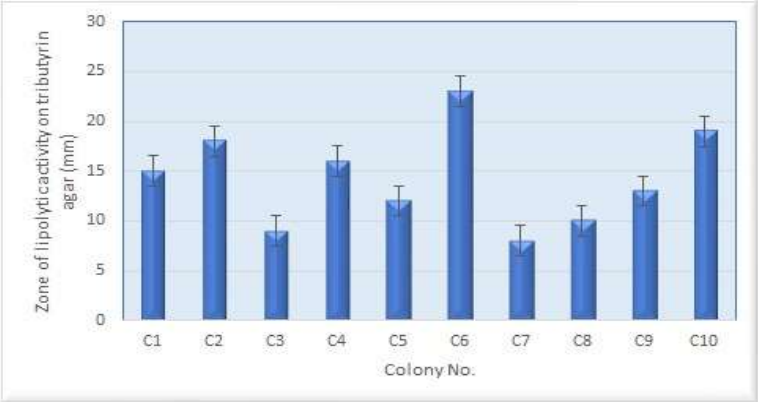

Fig. 3: preliminary screening of lipolytic activity on TBA agar plates for selected colonies
*(zone < 15mm) = weak activity; (zone 15–20mm) = medium activity ; (zone >20 mm)= strong activity.
LSD= 2.78; P value= 0.001

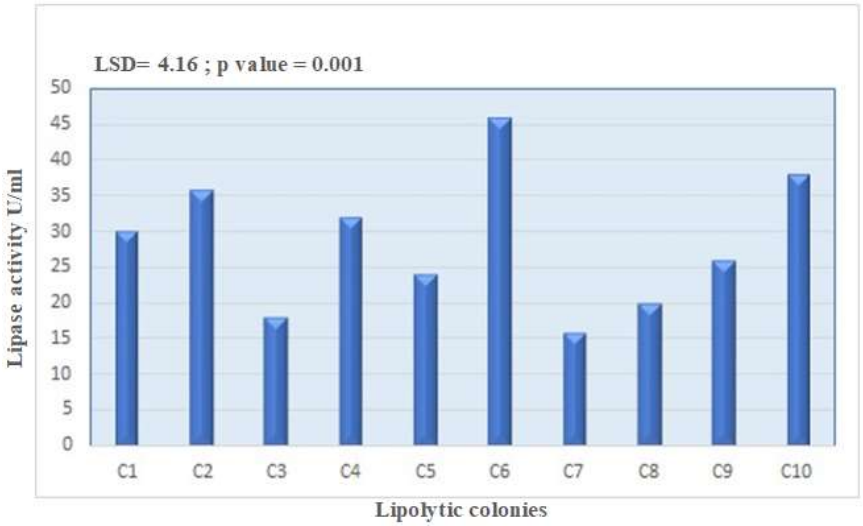

Fig. 4: Lipase activity for ten selected isolates by the titration method

Table 1: The precise location of the 1222 bp amplicon obtained, which partly covered a portion of the 16SrRNA locus of genomic DNA sequences of *Enterococcus faecium* (GenBank acc. no. PV299116) [9]

Sample ID	Percentage of identity	Sequence size	Accession number	Scientific name Reference copy (NCBI)Data
Z2	99.89%	1222 bp	PV299116	*Enterococcus faecium* (OQ940305)

Table 2: The length and location of the 1222 bp PCR amplicons used to amplify the 16S rRNA locus part of the genomic DNA sequences of Enterococcus faecium (GenBank acc. no. PV299116) [9].

Amplicon	Referring locus sequences (5′ - 3′)	Length
16S rRNA sequences	GGGAAAGGGGGGGGCCTATACATGCAGTCGTACGCTTCTTTTTCCACCGG AGCTTGCTCCACCGGAAAAGAAGAGTGGCGAACGGGTGAGTAACACGTGG GTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACC GTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTG TCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGC TCACCAAGGCCACGATGCATAGCCCACCTGAGAGGGTGATCGGCCACATT GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCT TCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGG TTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAA CTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTG CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGG GCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG CTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGG AGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAAC ACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTTAGGCTCGAA AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC GATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAAC	1222 bp

GCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG
CAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGA
TAGAGCTTCCCCTTCGGGGGCAAGTGACAGGGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAGTCCCGCACGAGCGCACCCTTATTGT
TAGTGCCATCATTCACTTGGGCCTCTTCCACAATGCCGGGACAACCGAGA
AAGGGGGGGAGGACGCCCATCATCCTGCCCCTTTTAACTGGGGTACCACC
GCTTCATGGGAGGAAAACCACT

3-4 Effect of incubation conditions on lipase production

The activity levels show lipase activity variability with incubation time, temperature and pH variability in the production medium. The highest enzyme activity (46U/ml) was recorded at 48 hours incubation, 37°C temperature and a pH value of 7 (Fig. 5,6,7). This what promotes the level of production compared to other values. When comparing these results with those of [30], pH was in agreement but varied with the incubation period and temperature, which were 24 hr. and 30°C, respectively. [31] Reported the best enzyme production at 40°C and pH 9, but the results were entirely in agreement with another study by [15] about the production conditions of lipase from *Bacillus subtilis*.

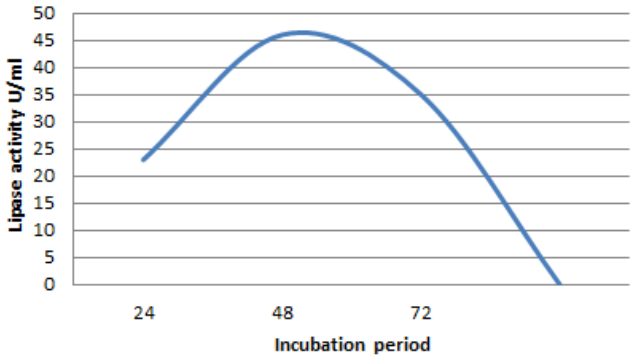

Fig.5: Lipase production at different incubation periods

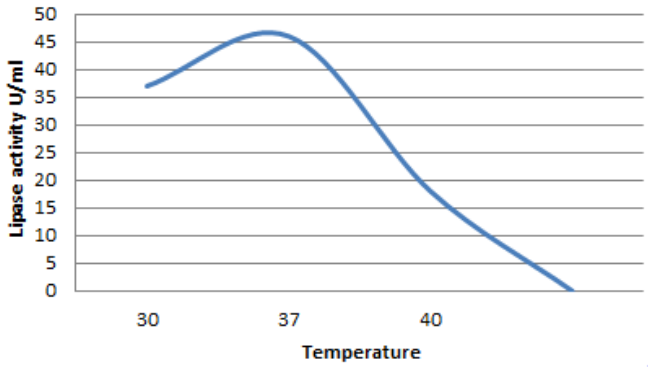

Fig. 6: Effect of different temperatures on lipase production

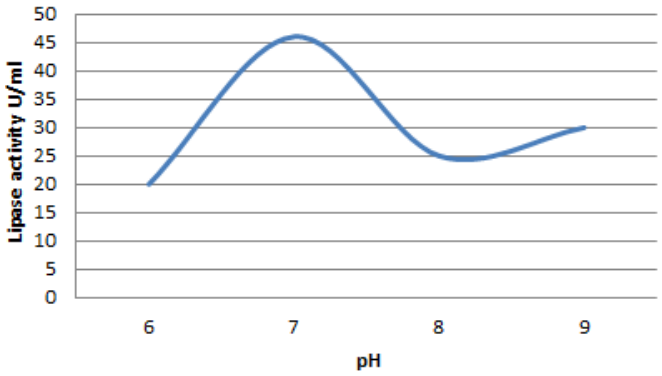

Fig.7 : Effect of medium pH on lipase production

3-5 Sensory evaluation of beef burgers

Table (3) shows the sensory evaluation of meat at different storage periods in the presence of the 20% enzyme and without adding it, where significant differences were observed, because it was significantly better than p = 0.001 when the enzyme was present for all sensory qualities. The scores awarded by evaluators for the sensory qualities of cooked beef burger treated with 20% lipase compared to the control samples during cryopreservation periods, especially for flavor, appearance, and general acceptance. While the scores were close during the preservation period (0 days), the difference widened between them, especially during the preservation period (4 weeks), possibly because of enhancing the storage shelf life of the treated samples, which was also reflected positively in the experimental samples' higher average scores of evaluations compared to control samples. The findings were in accordance with those of an equivalent study [19].

The results also showed a very strong odor after the incubation period of the lipase-treated samples, which could be attributed to the release of simple fatty acids due to lipase activity, which affected the appearance of the flesh (Fig. 8). This property of the enzyme can be used in the medical field to remove excess fat content in patients, and this conclusion is consistent with a study of [31].

Flavor: All samples were evaluated when fresh and after 2 and 4 weeks of storage. The results obtained indicated that the manufacture of functional burger samples by adding 20% lipase has acquired high degrees of flavor in fresh status ad throughout storage periods due to enzyme activity compared to control samples with lower grades. **Texture:** The manufacture of the burger with the addition of 20% Lipase enhanced the texture of the product, which obtained higher evaluation scores, while the samples produced without it obtained lower scores. **Appearance and color:** The addition of lipase to the burger made the product darker and fermented than the unadded samples. These findings were similar to those reported by [19], who demonstrated that the use of lipases in the production of meat burger products gives better sensory qualities than without. **Overall Result:** The obtained results proved that the addition of lipase in the manufacture of functional meat burger improved the overall result of the product as a sensory properties, Such as flavor, texture, appearance, and color of the product. As well as increasing its storage duration.

Table 3: Sensory evaluation of treated and non-treated meatballs

	Storage time (week)	Meat without lipase	Meat with 20% lipase	Mean Storage	LSD
Appearance	0	7.7	7.7	7.7	Treatment**=0.15
	2	7.6	7. 9	7.6	Storage**= 0.0.21
	4	6.3	7.7	7	treatment*Storage^{ns}=2.3
mean treatment	-	7.2	7.8		
Colour	0	7.5	7.8	7.65	Treatment**=0.08
	2	7.4	7.6	7.5	Storage**= 0.03
	4	6.3	7.1	6.7	treatment*Storage**=0.12
mean treatment	-	7.1	7.5		
Taste	0	7.7	7.8	7.75	Treatment**=0.07
	2	7.6	7. 9	7.6	Storage**= 0.02
	4	6.5	6.9	6.7	treatment*Storage^{ns}=2.49
mean treatment	-	7.3	7.5		
Flavor	0	7.8	7. 9	7.8	Treatment**=0.11
	2	7.5	7.7	7.6	Storage**= 0.09
	4	6.4	7.2	6.8	treatment*Storage**=0.05
mean treatment	-	7.2	7.6		
Texture	0	7.5	7.7	7.6	Treatment**=0.04
	2	7.4	7.8	7.6	Storage**= 0.02
	4	6.5	6.8	6.65	treatment*Storage**=0.06
mean treatment	-	7.1	7.4		
Overall Acceptance	0	7.6	7.9	7.75	Treatment**=0.12
	2	7.5	7.9	7.7	Storage**= 0.02
	4	6.4	7.3	6.85	treatment*Storage**=0.05
mean treatment	-	7.2	7.7		

** Significant p≤ 0.001, * Non significant

A B

Fig. 8: Effect of lipase activity on the beef burgers: A. before treatment, B. after treatment.

4- Conclusion

The study concluded that *E. faecium*, isolated from different food sources, had high lipolytic activity due to its production of lipase, which gave maximum lipolysis activity of 46U/ml and was effective in breaking down beef fat after a 72h.

Incubation period. The best conditions for producing lipase from these bacteria are 48 hours, 37°C temperature, and pH 7. Our findings also showed that the addition of lipase in the production of organic meat burger improves the sensory properties of the product and also increases its shelf life and storage, which supports and develops the animal meat industry. Consequently, we suggest that these bacterial strains serve as a promising microbial reservoir for lipases, presenting valuable potential for application within the biotechnological sector.

5- Acknowledgment

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