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# ISOLATION, IDENTIFICATION AND DETERMINATION OF ENZYMATIC ACTIVITY OF LOCALLY ESTERASE PRODUCING BACTERIA ISOLATED FROM IMPORTED EXPIRED DAIRIES

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**ABSTRACT : Microbial esterase has received much more attention in industry applications mainly owing to availability of wide range enzymatic activities. In the current study, a total of thirty samples of dairies, including, various types of imported expired dairy products, cheese, yoghurt, butter and cream were tested for screening of microbial esterase producers. Tween 80 agar medium was used for investigation of esterase producing bacteria. The culture filtrate (extracellular enzyme) was used for detected esterase activity spectrophotometrically using *p*-nitrophenylacetate as substrate. Morphological and molecular features for the extracellular esterase producing bacteria were identified. In the present study, two strains "S2 and S5" were identified as *Lactococcus lactis* strain LLKS2 with Genbank acc. MK977610; *Lactococcus lactis* strain LLKS5 with Genbankacc. MK977611, respectively. Through matching the detected DNA sequences of local samples withthe regained DNA sequences, were shown high esterase activity according to experimental conditions, with enzymatic activity about (5.39-7.76 U/ml) and specific activity about (0.641-0.982 mg/ml) for the two isolates.**

***Key words :*** Esterase activity, genetic identification, esterase extraction, Tween 80, *p*-nitrophenylacetate.

## INTRODUCTION

Esterase (E.C. 3.1.1.X) belong to a various set of hydrolases, which catalyzing the cleavage and construction of ester bonds. Esterases are broadly produced by microorganisms, animals and plants (Sharma *et al*, 2016). An esterase preferably catalyzes the carboxyl ester linkages hydrolysis designed of short chain fatty acids, it also catalyze the synthesis of ester, which involved in traesterification, interesterification and trans-esterification in water-free or water-restricted medium (Sayali *et al*, 2013; Vaquero *et al*, 2015). Esterases are liberate acetyl groups from partially acetylated substrates (Kahya *et al*, 2017), these are enzymes dependent on serine, because it comprised a typical catalytic triad of Ser-His-Asp, with a typical sequence of Gly-X-Ser-X-Gly (where X symbolize an amino acid residue) around the serine active site. Serine residue within a catalytic triad of Ser-His-Asp playsan important and vital role inesterases enzymatic activity (Bornscheuer, 2002). Esterolytic enzymes are highly diversified in a broad array of industrial applications, such as pharmaceutical processing, Cancer gene therapy, cosmetics, Detoxification reactions and environmental Monitoring, Endobiotic compounds processing, organic chemical processing, Plastic depolymerization, synthesis of biosurfactants, detergent formulations, food industry, dairy industry, the agrochemical industry, paper manufacture, nutrition (Sharma *et al*, 2001; Jaeger and Eggert, 2002; Sood *et al*, 2016). Because of wonderful regioselectivity, chemoselectivity and no cofactor required, these features make esterases use as group of biocatalysts in organic chemistry as well to its role in a various biotechnological applications (Jaeger and Reetz, 2000; Chen *et al*, 2015).

Carboxylesterases (CEs) are members of multipurpose lipolytic enzymes have been extracted from different origins involving bacteria, Archaebacteria, algae, fungi, plants, animals and human beings (Fett *et al*, 2000; Wang *et al*, 2016; Sood *et al*, 2016; Kwon *et al*, 2019). Owing to the ease culturing and manipulating genetically the bacterial cells, the bacterial strains are represent superior origins for producing esterases than the higher organisms (Klenk *et al*, 1997). CEs are characterizing to tolerate the strict circumstances of industrial biotransformation like comprehensive pH range, high temperature and exposure to denaturing agents. The CEs were obtained from bacterial speciesare ecofriendly and

have natural advantage to attain biocatalysis at temperature beyond ambient (Sood *et al*, 2016).

Dairies are broadly consumed nutriments and considered an exceptional medium for the growth and reproduction of microorganisms (Parkash *et al*, 2007). Microorganisms are the main spoilage and constitute a serious problem worldwide, responsible for numerous healthy risks in human food chain and substantial economic sufferers (Braun and Fehlhaber, 2002; Pitt and Hocking, 2009; Kara ALi and KacemChaouche, 2018). In view of the vital role of Esterases in the industrial biotechnological processes, the present work aims to Screening, Isolation and Identification of esterase producing bacteria and to estimate the Esterolytic activity of the bacterial isolates recovered from imported expired dairies.

## MATERIALS AND METHODS

### Primary screening and Isolation of esterase producing bacteria

Thirty samples of dairies, including, various types of imported expired dairy products, cheese, yoghurt, butter, and cream from different origins were collected. 1g of each sample was serially diluted individually with 10 mL sterile distilled water (Vanniyasingam *et al*, 2019). Subsequently homogenization, one ml from $10^{-1}$ to $10^{-5}$ were spread separately onto agar plates containing 1% Tween 80 (polyoxyethylen - sorbitanmonooleate) as a substrate in three replicates (Kim *et al*, 2007). The ability to hydrolyse esters wastested on the medium prepare according to Duza and Mastan (2014). The plates were incubated for 24-48 h at 37°C for the growth of microorganisms. Microbial colonies, which characterized as esterase producing microorganisms were purified on the nutrient agar and subjected to identification and quantitate screening of esterase producing microorganisms (Parkash *et al*, 2007).

### Production of extracellular bacterial esterase

The esterase producing isolates were grown in the broth medium prepared according to Sharma *et al* (2016), supplement with Tributyrin, 10 mL/L.7.5% (v/v) of the overnight culture was transferred to 50 mL production medium kept under shaking (110 rpm) at 37°C for 24 h. The crude extracellular esterase was extracted by low speed centrifugation (5,500 g for 15 min at 4°C of production culture, the supernatant was collected and passed through 0.22ìm. filter to remove any remaining cells.

### Esterase assay

Esterase activity was assayed spectrophotometrically in the culture broth according to Winkler and Stuckman (1979) by measuring the micromoles of *p*-nitrophenol released from *p*-nitrophenylacetate. The quantity of *p*-nitrophenol (*p*-NP) released was measured at A410 by UV- visible spectrophotometer (Shimadzu) against a control.

### Esterase activity

One unit (U) of esterase activity was defined as quantity of enzyme desired to release one micromole of *p*-NP from the substrate *p*-nitrophenylacetate "*p*-NPA" per minute for one mL of the prepared enzyme under standard assay conditions. Protein content valuation was carried out by Bradford (1976).

### Identification of Esterase producing bacterial isolates

The morphological and genetic features of isolates were identified according to Bergey's manual of determinative bacteriology.

### DNA sequencing of PCR amplicons

The microbial isolate which shows esterase activity on Tween 80 plate assaywas considered as positive colonies for Esterase enzyme production, the isolates were identified according to molecular genetic study by using of *16SrRNA* gene sequencing.

The resolved PCR amplicons were sequenced from both termini of forward and reverse based to the sequencing company instruction manuals. The results of the PCR products sequencing of the isolate was analyzed as long as with the respective sequences in the reference database. The detected variations in each sequenced sample were numbered in PCR amplicons along with its consistent locus within the referring genome.

A specific comprehensive bacterial tree was constructed in this study according to the protocol described by Al-Shuhaib *et al* (2019). The observed bacterial variants were compared by reference sequences neighbor homologous by means of NCBI-BLASTn server (Zhang *et al*, 2000). Then, the results of BLAST of the observed variations were linked and aligned together using a Clustal Omega based tools. A full inclusive tree, including the observed variant was imagined according to (http://tree.bio.ed.ac.uk/software/figtree/) (Rambaut, 2014).

## RESULTS AND DISCUSSION

### Screening and Isolation of Esterase producing bacteria

Tween 80 plate method was applied for screening of esterase activity. The results in the Fig. 1 showed the growing of bacterial isolates on Tween 80. The results

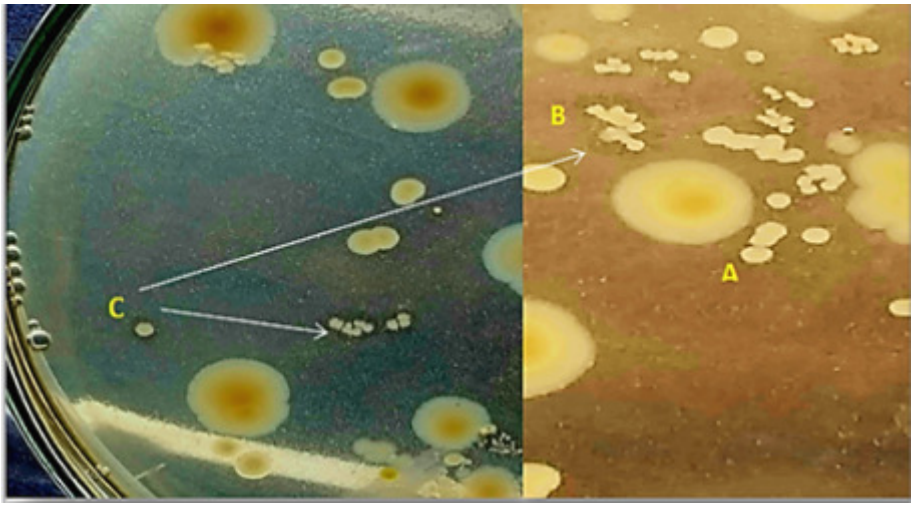


**Fig. 1 :** Esterase producing bacteria on Tween 80 agar plate: **A:** Negative control; **B:** Positive strains (Esterase produced); **C:** Clearness Zone (Halo zone).

appear that there were sixty five isolate (S1- S65) from the primary screening of esterase activity, only four strains (S2, S5, S6 and S7) were characterized by esterase activity isolated from cream. The appearance of a halo of clearness zone around the growth of bacterial colonieson the Tween 80 agar was indicated for esteraseactivity result from hydrolysis of Tween 80. The study of Alkhafaji *et al* (2018)was appeared in our study that Tween 80 plate had the ability to detect esterase activity (resulting a halo of clearness zone) after incubation the growth of bacterial colonies, whereas the study of Slifcan (2000) and Kumar *et al* (2012) they reported that the development of obscure region (turbid zone) around bacterial colonies indicated for lipases activity when they were used Tween 80 plate.

Standard microbial investigations are executed in order to determine whether the isolated strains has esterase activity and it serves for the close characterization or selection for bacterial strains, which have biotechnological applicability. Tween 80, Tween 20, Tributyrin and vegetable oil are represents as appropriate compounds can be added to culturing agar plates (Emanuilova *et al*, 1993; Gopinath *et al*, 2013; Pohanka, 2019; Singh *et al*, 2019). The existing of esterase producing isolateshad been identified in diverse habitats containing oils, which results from importance bacterial lipid metabolism or their involvement in bacterial pathogenesis (Kalyani and Saraswathy, 2014).

## Esterase activity

The esterase activity to the two selected isolates (S2 and S5) were analyzed using p-nitrophenyl ester (p-NP). Specifically with substrate p-nitrophenyl acetate (p-NPA). The results in the Figs 2 and 3 showed esterase activity and specific activity for crud esterase to the two

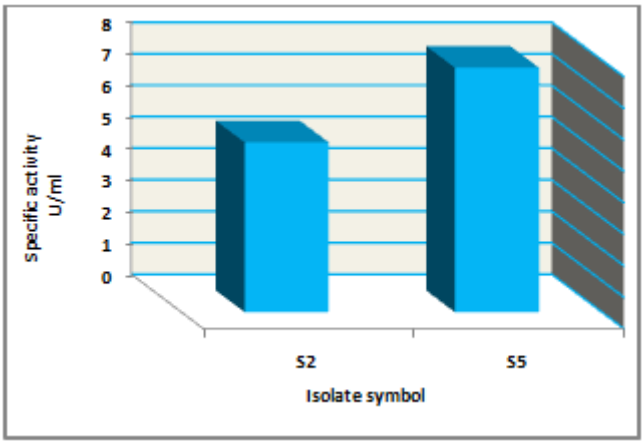


**Fig. 2 :** Esterase activity for crud enzyme of the two isolates of *Lactobacillus lactis*.

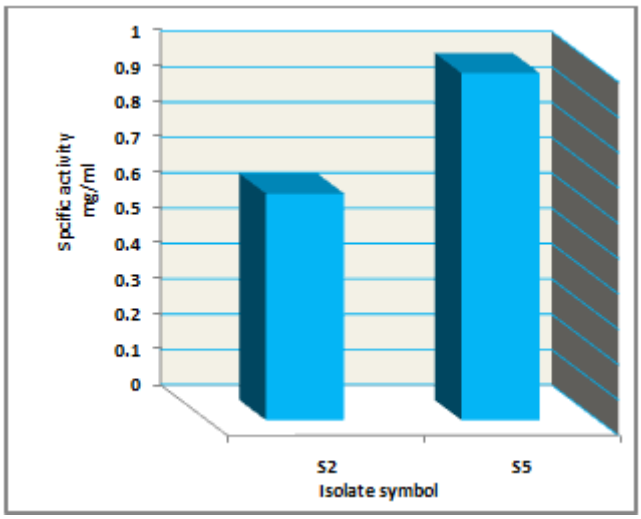


**Fig. 3 :** Esterase specific activity of crud enzyme of the two isolates of *Lactococcus lactis*.

strains of *Lactococcus lactis*. The results appear that the two isolate in the current study has ability to hydrolysis p-nitrophenyl acetate (p-NPA). Also the results of

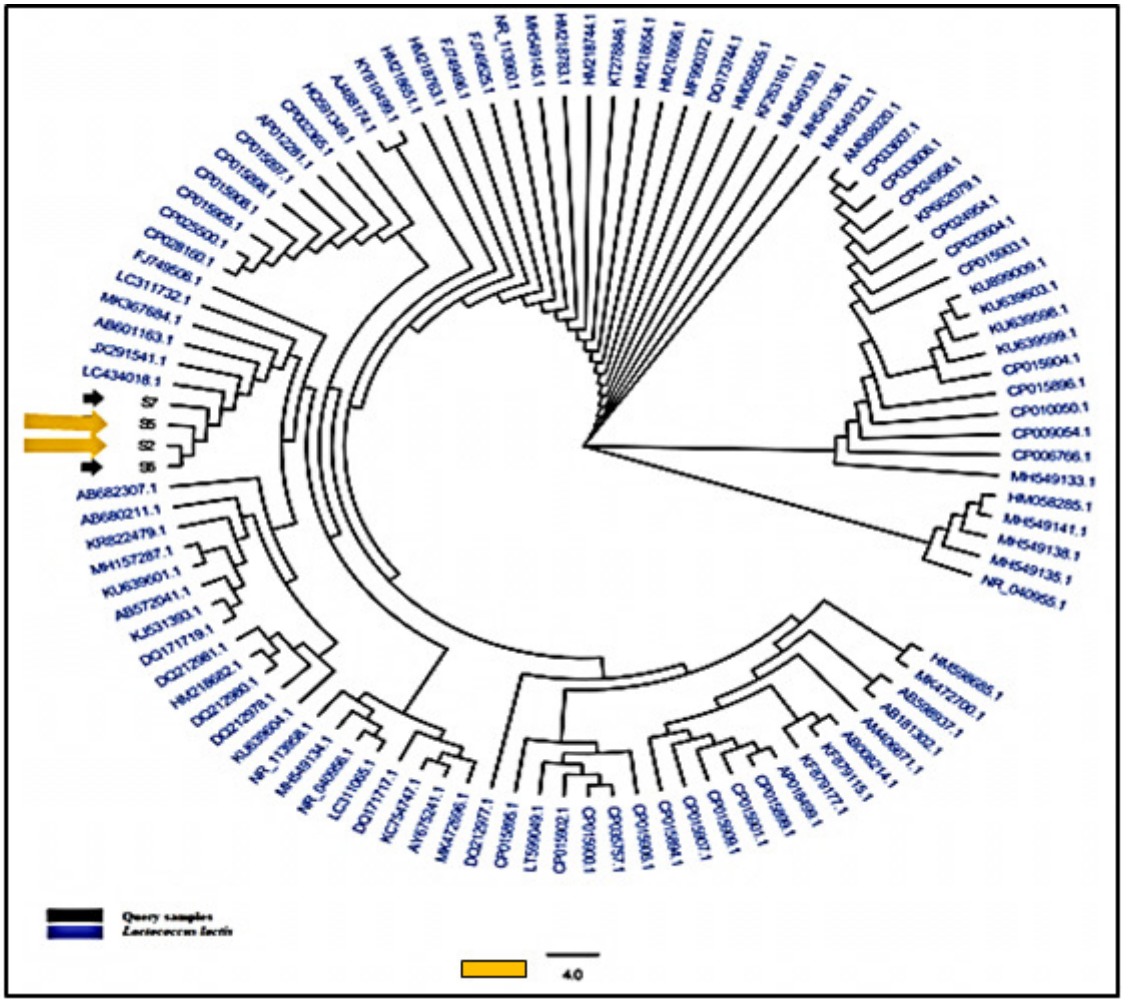


**Fig. 4 :** The comprehensive phylogenetic tree of the 16S rRNAgenetic fragments for *Lactococcus lactis* local isolates. The yellow color and arrows refers to the sequenced query samples, while the cyan color refers to other referring NCBI *Lactococcus lactis*.

esterase activity of all isolates showed variation in production of esterase points towards their ability to turns the growth and metabolic activities with some time lag. The study of Alkhafaji *et al* (2018) was reported that the purified esterase appear maximum esterase activity towards p-NPA followed by P-NPBZ, while the study of Singh *et al* (2019) reported that hyper- thermo alkaline esterase was gottenfrom strain *Rhodococcus* sp LKE-021.

## Identification of Esterase producing bacterial isolates

The results in the current study showed that the two isolates (S2 and S5), which show esterase activity on Tween 80 plate assay were identified as gram-positive and non-motile and cocci shape bacteria. Within this locus, the present study were included the two samples that had shown to amplify 1508 bp genetic sequences in *Lactococcus lactis* genome. Two strains "S2 and S5" were identified as *Lactococcus lactis* strain LLKS2 with Genbank acc. MK977610; *Lactococcus lactis* strain LLKS5 with Genbank acc. MK977611, respectively. The sequencing reactions indicated that the exact identity after performing NCBI blastn for these PCR amplicons (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Concerning the supposed ribosomal 1508 bp amplicons, NCBI BLASTn engine shown about 99% sequences similarities between the sequenced samples and the intended reference target sequences. Through matching the detected DNA sequences of local samples with the regained DNA sequences (GenBank acc. LC434018.1), the approximately locations and other details of the regained PCR fragments were identified (Fig. 4).

The exact position of the retrieved 1508 bp amplicon that partially covered a portion of 16S rRNA locus of *Lactococcus lactis* genomic DNA sequences (GenBankacc no. LC434018.1).

After positioning the 1508 bp amplicons' sequences, in terms of the positioning of both forward and reverse primers of the 1508 bp amplified amplicon (Table 1).

**Table 1 :** The position and length of the 1508 bp PCR amplicons used to amplify a portion of 16S rRNA locus of *Lactococcus lactis* genomic DNA sequences (GenBankacc no. LC434018.1).

| Amplicon | Referring locus sequences (52 - 32 ) | Length |
|---|---|---|
| 16S rRNA sequences | AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGT<br>GCCTAATACATGCAAGTTGAGCGCTGAAGGTTGGTAC<br>TTGTACCAACTGGATGAGCAGCGAAACG<br>GGTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATTTGG<br>AAACGAATGCTAATACCGCATAAAAACTTTAAACACAA<br>GTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAAGA<br>TGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGG<br>CTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGT<br>GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT<br>ACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGAC<br>GAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAG<br>GTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACG<br>TTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCA<br>GAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATA<br>CGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGC<br>GTAAAGCGAGCGCAGGTGGTTTATTAAGTCT<br>GGTGTAAAAGGCAGTGGCTCAACCATT<br>GTATGCATTGGAAACTGGTAGACTTGAGTGCAG<br>GAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAAT<br>GCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCT<br>GGCCTGTAACTGACACTGAGGCTCGAAAGCGTGGGGA<br>GCAAACAGGATTAGATACCCTGGTAGTCCACGCCG<br>TAAACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCT<br>CTGTATCGCAGCTAACGCAATAAGCACTCCG<br>CCTGGGGAGTACGACCGCAAGGTTGAAACT<br>CAAAGGAATTGACGGGGGCCCGCACAA<br>GCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA<br>AGAACCTTACCAGGTCTTGACATACTCGTGCTA<br>TTCCTAGAGATAGGAAGTTCCTTCGGGACACGG<br>GATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC<br>GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA<br>ACCCCTATTGTTAGTTGCCATCATTAAGTTGGG<br>CACTCTAACGAGACTGCCGGTGATAAACCGGAG<br>GAAGGTGGGGATGACGTCAAATCATCATGCC<br>CCTTATGACCTGGGCTACACACGTGCTAC<br>AATGGATGGTACAACGAGTCGCGAGACAGTGAT<br>GTTTAGCTAATCTCTTAAAACCATTCTCAGTTCGGA<br>TTGTAGGCTGCAACTCGCCTACATGAAGTCGGAAT<br>CGCTAGTAATCGCGGATCAGCACGCCGCGGTG<br>AATACGTTCCCGGGCCTTGTACACACCG<br>CCCGTCACACCACGGGAGTTGGGAGTACCCGAAGTA<br>GGTTGCCTAACCGCAAGGAGGGCGCTTCCTAAGGTAAG<br>ACCGATGACTGGGGTGAAGTCGTAACAAGGTAACC | 1508 bp |

The alignment results of the 1508 bp samples revealed the absence of any detected polymorphism in this position distributed in the investigated sample in comparison with the referring reference DNA sequences.

A comprehensive phylogenetic tree was constructed, in which the whole number of the aligned sequences of nucleic acid, irrespective of the investigated local bacterial variations was 100 sequences. The total number of the included species within this tree was only one type, namely *Lactococcus lactis*. Thus, in the current study the comprehensive tree which constructed indicated the existence of only one bacterial specie allover scanned sequences and all studied variants located in one position within this comprehensive tree (Fig. 4).

This location was positioned in the vicinity to GenBank acc. no. LC434018.1, which represent the African MB52 strain of *Lactococcus lactis*. However, extremely high similarity was found with other *Lactococcus lactis* strains, including JX291541.1 (Fang *et al*, 2016), AB601163.1, MK367684.1 (Nami *et al*, 2014), phylogenetic analysis provided an extremely powerful ability of the 16S rRNA fragment to achieve high detection specificity amongst the analyzed samples. Furthermore, this tree has given a highly discriminative power to dissociate all the analyzed

samples into one bacterial species. This 16S rRNA-based tree has provided a strong indication of the identity of these local studied isolates. This, in turns, gives a further tool of the power of the currently utilized "16S rRNA" specific primers to precisely identify the currently analyzed strains. Eventually, the presented tree has added another layer of confirmation about the confirmed identity of the two isolates S2 and S5.

## CONCLUSION

The extensive employment of microbial esterase consequence in the investigation of novel origins of Carboxylesterases "CEs", by way of suitable purposeful values for Biotechnological applications. The current study indicated that it is possible to isolate microorganisms from expired dairies, which characterized high esterase activity towards short chain p-nitrophenyl ester (p-NP). Microbial esterase may have a substantial role in the provide diverse industrial applications for human prosperity. Therefore, the current project recommends studying the genetic characteristics and enzymatic activity of esterase production for other stains isolated during the current study.

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