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ISOLATION, GENETIC IDENTIFICATION AND DETECTION OF LIPOLYTIC BACTERIAL STRAINS FROM VARIOUS SOURCES OF MILK PRODUCTS

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ABSTRACT : The increase in industrial demand for lipase has resulted in the discovery of new sources of unusual properties of enzymes. Lipases are the most important enzymes in numerous areas of life, occupy a popular biocatalyst situation and have a wide variety of biotechnological applications. A total of twenty-five samples of milk products, Several kinds of expired imports of dairy products, yogurt, butter, cheese, and cream were tested for screening of microbial lipases producers in the present study. Rhodamine B-olive oil-agar and Tween 80 agar plate were used for the investigation of lipase-producing bacteria. Morphological and Molecular features for the extracellular esterase-producing bacteria were identified. In the present study, two strains "S3 and S4" were identified as *Lactococcus lactis* strain LLKS3 with Genbank acc. MK977608.1; *Lactococcus lactis* strain LLKS4 with Genbank acc. MK977609.1 respectively were shown high activity of lipase according to experimental circumstances.

Key words : Genetic identification, lipolytic activity, Tween 80 plate assay, rhodamine-B agar plate assay.

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INTRODUCTION

As biotechnologically valuable enzymes, lipases have been in a special situation and play essential roles in different industries (Panesar *et al*, 2016). In nature, lipases occur broadly and only microbial lipases are important. Due to the wide range of catalytic activities available, the high yields necessary, ease of genetic manipulation, daily supply due to the absence of seasonal variations and rapid growth of microorganisms on inexpensive media (Mahale *et al*, 2014).

Lipases are a group of enzymes that catalyze the hydrolysis of the carboxyl ester bonds of triacylglycerols (fats and oils) to monoglycerides, diglycerides, glycerol and free fatty acids, the position where the enzyme is dissolved is at the oil-water interface between the insoluble substrate phase and the aqueous phase. In addition, lipases also catalyze synthesis reactions include esterification, transesterification and others, so constituted as one of the most important groups of biocatalysts for biotechnological applications (Sharma and Kanwar, 2014; Borrelli and Trono, 2015; Alkabee *et al*, 2020; Sabir *et al*, 2021 and Alobaidi *et al*, 2021).

Dairies are mostly consumed nutrients and are considered an exceptional medium for the growth and reproduction of microorganisms (Parkash *et al*, 2007). The key spoilage is microorganisms, which are responsible for various health threats in the human food chain and are a major concern worldwide. and substantial economic sufferers (Pitt and Hocking, 2009; Kara Ali and Kacem Chaouche, 2019). The present work aims to screening, isolation and identification of lipolytic bacteria strains and estimate the lipolytic activity of the bacterial isolates recovered from different sources of dairies (Braun and Fehlhaber, 2020).

MATERIALS AND METHODS

Primary screening and Isolation of lipase producing bacteria

Twenty five samples of milk products, including, different types of bring-in expired dairy products as yogurt, butter, cheese and cream were collected. 1g of each sample was serially diluted individually with 10 mL sterile distilled water (Vanniyasingam *et al*, 2019). To select the lipase-producing isolates, Rhodamine B-agar plates (ROA) were used. The medium was equipped as stated by Akanbi *et al* (2010). One ml from 10^{-1} to 10^{-5} were spread separately onto ROA agar plates. The isolates were detected after 48h of incubation at 37°C by exposure to U.V. Light at 350 nm (Rabbani *et al*, 2013).

Secondary screening of Lipolytic bacterial strains

The isolates were culture overnight in a liquid medium, prepared according to Sagar *et al* (2013). According to Aruna and Khan (2014), 5% overnight culture media of all lipase producing isolates is inoculated from the broth media.

The lipolytic bacteria were typically detected and screened through the appearance of white zones by using a selective medium based on Tween80 hydrolysis, the medium containing 1% Tween 80 (polyoxyethylen - sorbitanmonooleate) as a substrate in three replicates (Kim *et al*, 2007). The ability to hydrolyse esters was tested on the medium prepare according to Duza and Mastan (2014). The sterile agar plates were punched aseptically with sterile cork borer to obtain 4mm diameter wells in the four halves of the plates with approximate separation distance of 15mm. These agar plates were loaded with the crude lipase was extracted according to (Zouaoui *et al*, 2012). For each lipase producing bacteria of 0.1ml in each well separately and incubated at 37°C in the incubator for 48 hrs. The developed clear zones around the wells were measured (mm)

Lipase assay

Titration method was used to estimate lipase activity based on Bhavani *et al* (2012).

Identification of bacterial isolates

According to Bergey's manual of determinative bacteriology, the morphological, biochemical and genetic characteristics of isolates were established. The bacterial isolates, which displays highest activity of lipase assay is considered as positive isolates for lipase production. According to molecular genetic testing, the isolates were identified using *16SrRNA* gene sequencing as previous study (Alkabee *et al*, 2020).

In the current study a particular comprehensive bacterial tree was created according to Al-Shuhaib *et al* (2019).The detected bacterial variants were contrasted by homologous neighbor comparison sequences using the NCBI-BLASTn server (Zhang *et al*, 2000; Rambaut, 2014).

RESULTS AND DISCUSSION

Screening and isolating of lipolytic bacterial strains

The lipase activity investigation for the selected isolates was seen in Table 1 and Figs. 1 and 2, respectively. It appears the colonies with lipolytic activity tend to exhibit orange fluorescence halos on Rhodamine-B agar plates after development and appear as white

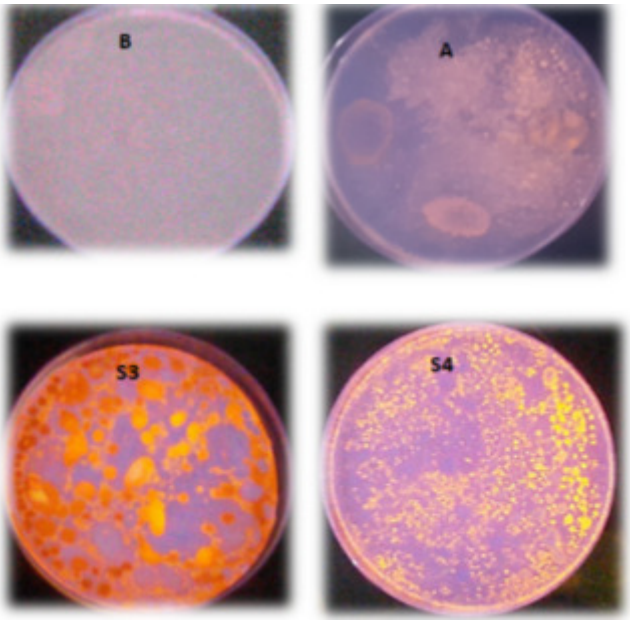

Fig. 1 : Lipolytic activity on ROA plate. A: Negative test, B: Control plate, [S3 and S4] positive test.

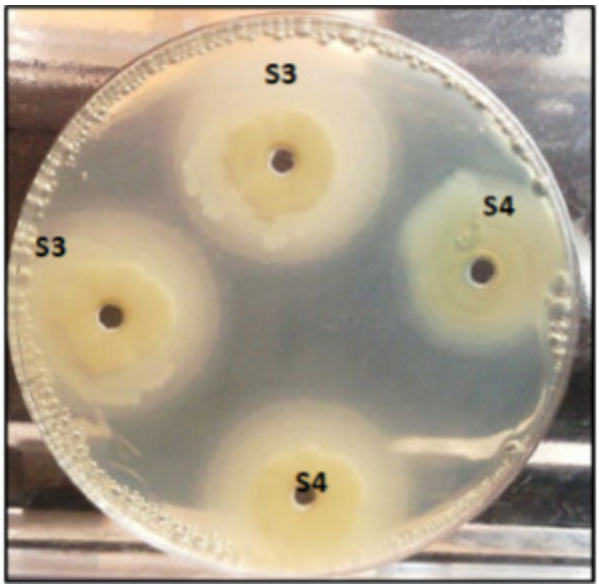

Fig. 2 : Tween 80 plate assay for the two isolates of *Lactobacillus lactis* (S3 and S4).

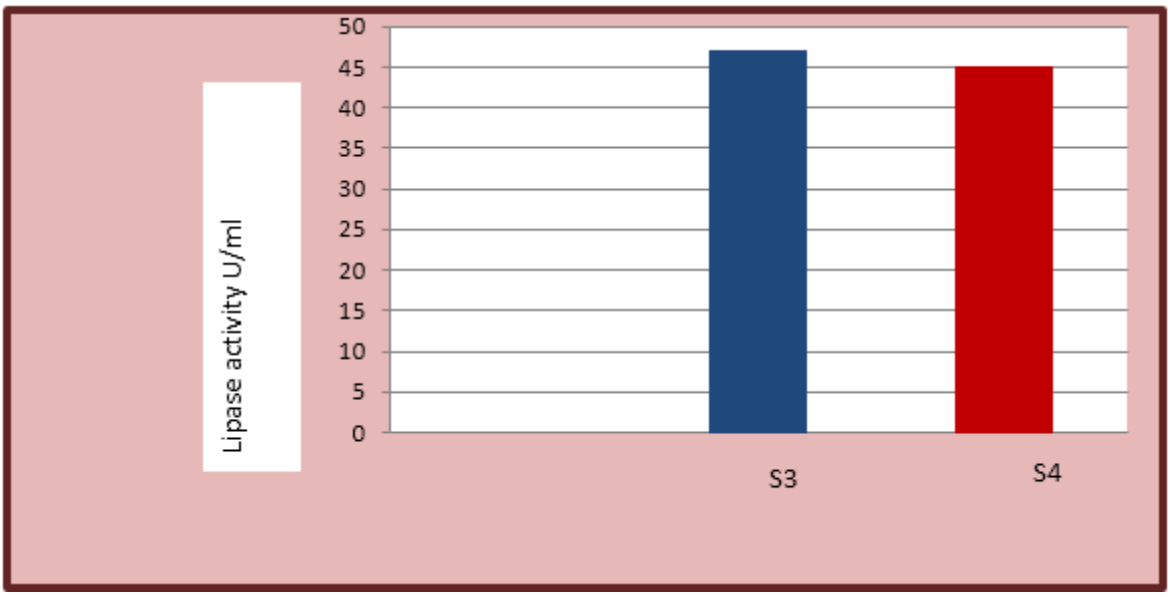

Fig. 3 : Lipase activity for two isolates S3 and S4 by titration method.

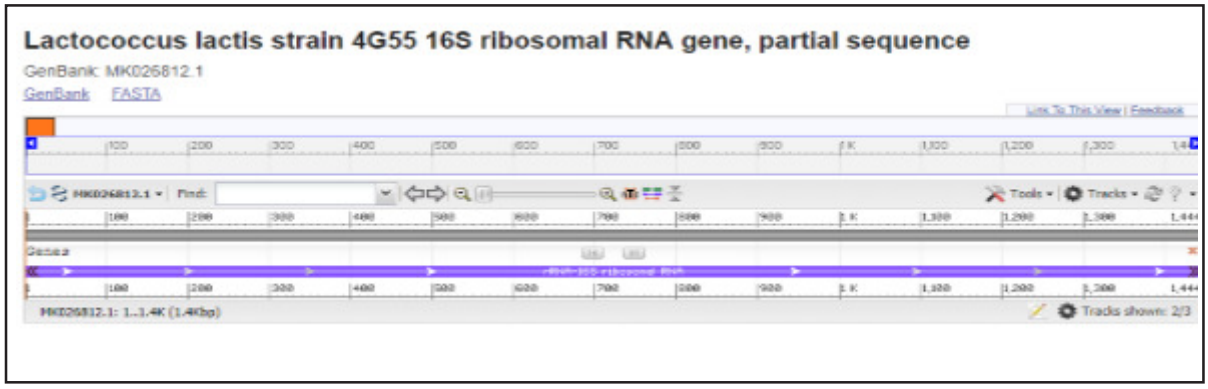

Fig. 4 : The exact position of the 1444 bp amplicon obtained, which partially filled a portion of the *16S rRNA* locus of *Lactobacillus lactis* (GenBank acc no. MK026812.1) genomic DNA sequences. The green arrow refers to this amplicon's beginning point, while the cyan arrow refers to its endpoint.

zones on the Tween 80 assay after 48 hours of incubation, the findings revealed that during the present analysis, two bacterial isolates of S3 and S4 from fifteen isolates were distinguished by high activity of lipase, the findings of the present research agree with study of Kukreja and Bera (2005), Akanbi *et al* (2010), Boonmahome and Mongkolthanaruk (2013), Alkabbi and AlHamdani (2017). The results appear the colonies of lipase producers surrounded with Halos of white zones on the Tween 80 assay with diameters (22-20) mm for the isolates St3 and St4, respectively. Two of the selected bacterial strains display noticeable precipitates on the colonies that may be an indicator of lipolytic activity due to the release and precipitation of Tween80 fatty acids as calcium salts, the clear apparition of the zone of hydrolysis around colony were considered as essential parameters for selection of lipase producer strains, the evaluation of the lipase efficiency based on the clear zones around colonies were

Table 1 : Lipolytic activity on ROA and Tween 80 plates assay for the two isolates of *Lactobacillus lactis.*

Isolate symbol	ROA assay	Tween 80 plate assay(mm)
S1	+	3
S2	+	3.5
S3	+	22
S4	+	20
S5	+	4
S6	+	3
S7	+	2
S8	+	3.3
S9	+	4
S10	+	3
S11	+	2
S12	+	5
S13	+	7
S14	+	4
S15	+	4

Fig. 5 : Alignment of the DNA sequences of the local *16S rRNA* sample with their consistent 1444 bp amplicon reference sequences within the genomic DNA of *Lactobacillus lactis*. The observed substitution mutations were highlighted according to their position in the PCR products. The symbol "ref" refers to the NCBI referring sequence, "S3 and S4" refer to the samples no. 3 and 4, respectively.

showed that all of them could produce lipase (Lee *et al*, 2015).

Determination of lipase activity

Fig. 3 appears maximum crude lipase extract production for the isolates S3 and S4 during 72hrs of incubation. Lipase activity for the two isolates S3 and S4 are 47U/ml and 45U/ml, respectively.

Identification of Esterase producing bacterial isolates

The results in the present study revealed that the two isolates (S3 and S4) from 15 isolates that show high lipase activity on Rhodamine B- olive oil-agar plates and Tween 80 plate assay were identified as gram-positive, and non-motile and cocci shape bacteria.

In the current research, the two samples were interested in amplifying 1444 bp genetic sequences in the genome of *Lactobacillus lactis*. Two strains "S2 and S5" were identified as *Lactococcus lactis* strain LLKS3 with Genbank acc. MK977608.1; *Lactococcus lactis* strain LLKS4 with Genbank acc. MK977609.1, respectively. The sequencing reactions revealed that for these PCR amplicons (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE TYPE = BlastSearch) the exact identification after performing NCBI blastn. For supposed ribosomal 1444 bp amplicons, the NCBI BLASTn engine showed almost 100 percent sequence similarity between the sequenced samples and the predicted reference target sequences. Through comparing the observed DNA sequences of these native samples with the recovered DNA sequences (GenBank acc. MK026812.1) (Fig. 4).

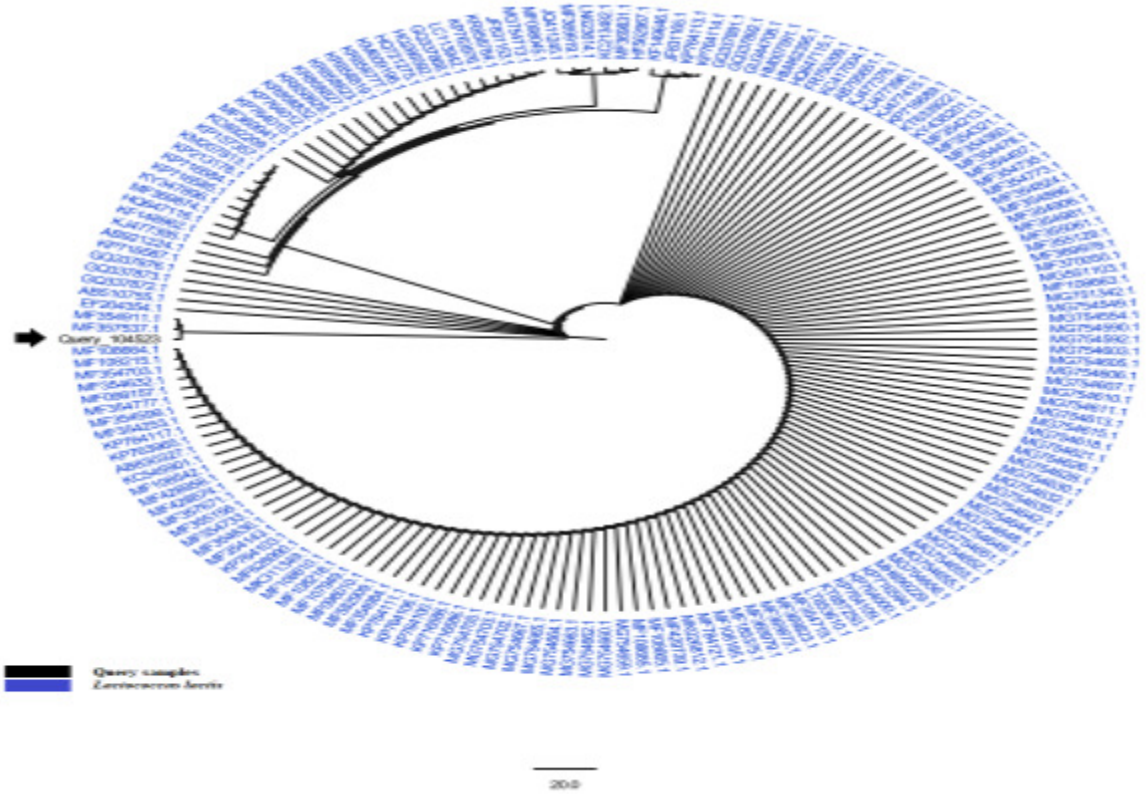

Fig. 6 : The comprehensive phylogenetic tree of the local isolates of the 16S rRNA genetic fragments for *Lactococcus lactis*. The black color indicates local samples for the sequenced query, while the cyan color refers to other indicates NCBI *Lactococcus lactis* deposited referring species. All the numbers listed referred to Genbank acc. no. of each species cited. At the bottom of the tree, the number '20.0' corresponds to the degree of scale range of the classified species of the full tree.

Table 2 : The length and position of the 1444 bp PCR amplicons used in the genomic DNA sequences of *Lactobacillus lactis* to amplify the 16S rRNA locus section (GenBank acc no. MK026812.1).

Amplicon	Referring locus sequences (5' - 3')	Length
16S rRNA sequences	TGCAGTTGAGCGCTGAGGTTGGTACTTGTACCGACTGGATGAGCAGCGAACGGGTG AGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAACGAATGCTAATACCG CATAAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAAG ATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAG CCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC GGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGC CGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACGTTGGTGA GAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTACGTG CCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGA.....ATACTCGTGCTA TTCCTAGAGATAGGAAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTC AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAG TTGGGCACTCTAACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCA TGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACAGTGAT GTTTAGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGC TAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC CGTCACACCACGGGAGTTGGGAGTACCCGAAGTAGGTTGCCTAACCGCAAGGAGGGCGCTTCCTAAGGTAAGA CCGATGACTGGGGTGAAGTC	1444 bp

The specifics of its sequences were illustrated after placing the 1444 bp amplicon sequences, in terms of the placing of both reverse and forward primers of the 1444 bp amplified amplicon primers (Table 2).

The alignment results of the 1444 bp samples revealed the lack of any observable polymorphism in this position distributed in the examined sample in contrast with the reference DNA sequences (Fig. 5).

A comprehensive phylogenetic tree was constructed, in which 171 sequences were the total number of aligned nucleic acid sequences in this comprehensive tree, independent of the local bacterial variants examined. The total number of the included species within this tree was only one type, including *Lactococcus lactis*. Thus, the

present constructed comprehensive tree showed the presence of only one bacterial species allover scanned sequences (Fig. 6) (Alhilfi *et el*, 2016). It was found that both studied variants located in one position within this comprehensive tree. This location was positioned in the vicinity to GenBank acc. no. MF357537.1 and MF354911.1, which represent two strains of *Lactococcus lactis*, including CAU5068 and CAU:1587, respectively. The currently constructed 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 particular, gives the commonly used *16S rRNA* specific primers a high detection capacity to differentiate between the organisms currently studied. In addition, another layer of clarification regarding the verified identity of S3 to S4 isolates was applied to the tree provided (Alhilfi *et el*, 2019; Al-Tameemi *et el*, 2020).

CONCLUSION

In the present study, Two strains "S3 and S4" were identified as *Lactococcus lactis* strain LLKS3 with Genbank acc. MK977608.1; *Lactococcus lactis* strain LLKS4 with Genbank acc. MK977609.1 respectively were shown high activity of lipase according to experimental circumstances.

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