Morphological And Biological Characterisation Of Lysinibacillus Fusiformis L52 Isolated From Dairy Industry Wastes

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Abstract: Present study deals with isolation of lipolytic bacteria from dairy wastes collected from Vaishnavi dairy industry, Wyra, Khammam district, Telangana. The samples of different wastes like paneer, curd, milk and total effluent were collected and screened for the isolation of lipolytic microbes. Screening medium was prepared with olive oil emulsion (1% v/v) and later with Tributyrin (1% v/v) to the lipolytic microbes from dairy waste water. Lipolytic bacteria showing maximum zone of clearance around the colonies were found majorly in total effluent wastes, was focussed for the present study. Isolated bacteria were identified up to the genus level with the help of morphological characterisation by Bergey's manual, biochemical characterisation (Aneja 2003) and molecular characterisation by 16s rRNA sequencing and was identified as Lysinibacillus fusiformis L52 strain.

Keywords: Biochemical characterisation, Lipase, Tributyrin, Bergey’s manual, 16s rRNA sequencing

1 INTRODUCTION:
Microbes in the dairy waste are responsible for the organic and inorganic luxury uptake occurring in the treatment plant (G.W. Fuhs & M.Chen;1975). Lipase producing microbes are generally widespread in nature (Veerapagu M et al., 2013). Lipases are ubiquitous enzymes, found in animals, plants, fungi and bacteria. Lipolytic enzymes (Triacylglycerol hydrolases EC 3.1.1.3) are capable of hydrolyzing triglycerides, diglycerides and monoglycerides into fatty acids and glycerol (Yasuo et al., 2002). Microbial lipases have already established their vast potential regarding their production of lipase. Hence the present study mainly focuses on the selective isolation of lipolytic Lysinibacillus spp. from dairy waste water.

2 MATERIALS AND METHODS:

2.1 Collection of dairy waste water samples:
Samples from dairy industry wastes were collected in a sterile container and stored at 4°C until the analysis was carried out according to the standard methods of APHA [American Public Health Association] and Trivedy and Goel (1984). All experiments in the study were carried out to isolate lipolytic bacteria in triplicates and the mean values of the results were displayed. In the present study, lipolytic bacteria is isolated from dairy waste samples.

2.2 Preparation of screening media:
Preparation of screening media: Preparing medium was prepared by using Peptone (0.5% w/v), KH2PO4 (0.15% w/v), Na2HPO4 (0.1% w/v), MgSO4 (0.05% w/v), (NH4)2SO4 (0.5% w/v), NaCl (0.5% w/v), Olive oil emulsion (1% v/v) was prepared by mixing with 1 volume of the olive oil with 4 volumes of 5% Poly Vinyl Alcohol (PVA). This emulsified solution was kept in orbital shaker at 250 rpm for overnight at 37°C. Then the prepared oil emulsion was autoclaved at 121°C for 20 min at 15 lbs pressure and the pH was adjusted to 7.0. Then 5 ml of each sample was added to 50 ml of enrichment media in 150 ml flasks. The flasks were incubated on a shaker at 200 rpm at 37°C for 72 hrs.

2.2.2 Preparation of screening media:
Screening media is prepared by using Peptone (0.5% w/v), Yeast extract (0.3% w/v), Agar (2% w/v) and Olive oil emulsion 1%. The pH was adjusted to 7.0 and autoclaved at 121°C/20 min/15 lbs. The suspensions enriched with lipolytic organisms were diluted with sterilized water and plated on the screening media and incubated at 37°C for 3 days. Lipase producing colonies were indicated by the appearance of clearance zones. Colonies with large clearance zones were selected for further isolation by sub culturing on the same medium several times until the morphology of colonies was uniform for 3 days.

2.3 Preparation of confirmatory media:
Further to confirm the lipolytic isolates, the tributyrin agar medium was used as confirmatory media. This medium consists of components like Tributyrin (1% v/v), Yeast extract (0.3% w/v), Special peptone (0.5% w/v) & Agar (1.5% w/v).

2.3 Screening of lipolytic isolates:
2.3.1 Primary Screening of Lipase producing bacteria: 5ml of each sample collected from dairy industry wastes were suspended in 100 ml of enrichment media and agitated in orbital shaker incubator at 37°C at 200rpm. The enriched suspensions were used to make the dilutions from $10^{-1}$ to $10^{-10}$ by serial dilution method.

2.3.1. a) Serial dilution: 10 test tubes were taken with 9 ml of freshly prepared enrichment media in each tube and all the tubes with media were autoclaved at 121°C /15 lbs/20 min. After cooling 1ml of enriched suspension was diluted serially to get the dilutions of $10^{-1}$ to $10^{-10}$ dilutions and incubated for 72 hrs at 37°C in orbital shaker for 3 days. 100μl from each diluted samples was used as inoculum in olive oil emulsion agar base medium for primary screening of lipase producing bacteria by spread plate method on screening media. The experiment was done in triplicates and incubated at 37°C for 72 hrs. The bacterial colonies which were suspected with lipase production (i.e., with clear zone formation) were counted and the better colonies were isolated and screened further.

2.3.2 Confirmation of lipolytic bacteria: Lipolytic bacterial colonies were screened by qualitative plate assay method in the presence of tributyrin agar medium for 72 hrs. Zone of clearance was observed due to hydrolysis of tributyrin, due to extracellular lipase production by lipolytic bacteria.

3. LIPASE ACTIVITY DETERMINATION: The better clearance zone formed colonies were selected for further studies. Qualitative test was done by agar well diffusion method hydrolysing the substrate present in the medium and quantitatively by using titrimetric method.

3.1 Qualitative assay for lipase activity

3.1.1 Agar well diffusion method:

Tributyrin agar is prepared and allowed to solidify in the petriplates. As the agar medium is loaded with the Tributyrin, where the enzyme released by bacteria interacts with substrate by forming clearance zone around the wells after 24 hrs of incubation at 37°C.

3.1.2 Quantitative assay of lipase activity: Extracellular lipase assay was done with crude enzyme which is collected by centrifuging the bacterial culture for 10min. 1ml of the crude enzyme is mixed with 4ml of olive oil emulsion and 5 ml of 0.5M phosphate buffer with pH 7. Then this mixture was incubated for 15min at 37°C. After incubation, reaction was terminated by the addition of 2ml of acetone: ethanol mixture (1: 1). Then 2 drops of phenolphthalein indicator (pp) was added and titrated against 0.1 N NaOH until the color changes to pale pink. Lipase activity can be calculated by the following equation (1). One lipase unit (U) is defined as the amount of lipids required per minute to liberate 1 mol of fatty acids. In the current study lipase activity is measured in the presence of olive oil as the substrate. Lipase activity (U / ml) = $\frac{(A(B) x N \times (NaOH)\times 1000)}{VE}$ -----(1)

Description:

A = Volume (ml) of NaOH for titration of the sample, B = Volume (ml) NaOH for blank titration,1000 = conversion to mmol to mol, VE = total volume of the mixture of crude extract.

4. CHARACTERIZATION OF THE BACTERIAL ISOLATES:
The enriched lipolytic bacteria was observed for their morphological, biochemical and molecular characteristics.

4.1 Morphological characters:

Morphological characterisation of the isolated bacteria includes the size, shape, colour, margin, elevation, opacity and consistency, Gram nature, Shape and Arrangement of cells. Further biochemical characterisation and 16srNA analysis was done and identification of bacteria according to Bergey's Manual of Determinative Bacteriology.

4.2 Biochemical characters:

Biochemical characterisation was done by performing the tests like hydrolysis of Gelatin, Catalase, hydrolysis of Casein, Citrate Utilization, Hydrolysis of Tween 20 & Tween 80, Indole production, H₂S production, Nitrate reduction, Hydrolysis of Sterch, Voces-Proskauer, Hydrolysis of Urea and Oxidase (Aneja 2003).

4.3 Molecular characterisation by 16s rRNA sequencing:

4.3.1 DNA Preparation:

Genomic DNA from the isolated bacterial culture was done by using DNA extraction kit method.

Procedure: 1.5ml of overnight bacterial culture was pelleted out by centrifugation at 8000 rpm for 2 min. Then the supernatant was discarded and pellet was resuspended in 180μl of lysis buffer with addition of 20 μl of RNase solution and incubated at room temperature for 2 min. 20 μl of Proteinase K solution was added, mixed well and incubated further for 30 min at 55°C. Then 200 μl of lysis buffer was added, vortexed thoroughly (15 sec) and incubated at 55°C for 10 min. 200 μl of ethanol (96-100%) was added to this solution and again mixed thoroughly by vortexing for 15 sec and was loaded on Hi-elute column and centrifuged at 6500Xg for 1 min. After that 500 μl of prewash solution was added to the Hi-elute mini prep spin column and centrifuged at 6500Xg for 1 min (Discard the flow through liquid and reuse the same collection tube with the column). 500 μl of wash solution was added to the column and centrifuged for 3 min at maximum speed (14000 rpm) to dry the column (The column must be free of ethanol before eluting the DNA) The column was centrifuged for the additional 1min at maximum speed if residual ethanol is seen. Then 200 μl of elution buffer was added directly to the column without spilling to the sides and then incubated for 1 min at room temperature and centrifuged at 10000 rpm for 1 min to elute the DNA bound to the column. DNA was extracted to amplify 16s rRNA gene as template DNA.

4.3.2 PCR Amplification for 16s rRNA gene:
The DNA isolated was further amplified for 16s rRNA gene by Polymerase chain reaction with Random primers (with forward primer 5'-AGAGTTTGATCTTGAGCT-3’ and Reverse Primer 5'-AAGGAGGTGATCCAGCC-3’). The denaturation was carried out at 94°C for 3 min 30 sec, annealing for 40 sec at a temperature of 58.2°C and further extension for 1 min at...
68°C of temperature. Then PCR was carried out for 35 cycles and final extension was carried out for 5 min at 68°C. Then the amplified DNA was sequenced and analysed by DNA analysis software like chroma or sequencer. The phylogenetic analysis was performed and phylogenetic tree was constructed by using bioinformatics tools.

III RESULT AND DISCUSSION:

3.1 Isolation of lipase-producing bacteria:
The method most commonly used to screen the lipolytic bacteria is based on the formation of clear halos around colonies grown on olive oil emulsified agar plates as stated previously (Jaeger et al. 1994; Bora and Kalita 2007; Ertugrul et al. 2007). Oils and fats are insoluble in water; hence a fine and stable oil emulsion is important to the subsequent medium preparation for an enzyme activity assay. The samples namely milk, curd, paneer, total effluent were collected from dairy industry.

3.2 Primary screening of lipolytic microbes

3.2 a) Samples directly spread over screening media:
All the five samples were directly placed on the screening media with olive oil where no clear zone was observed and results were shown in the Fig 1.

Fig 1

| Table 1: Zone formation with different Industrial samples |
|-----------------|-----------------|-----------------|-----------------|
| S.NO | Sample | Substrate | Zone |
| 1 | Milk wastes | Peanut oil | Negative |
| 2 | Paneer wastes | Peanut oil | Negative |
| 3 | Total wastes | Peanut oil | Negative |
| 4 | Curd wastes | Peanut oil | Negative |

The samples directly spread on the media with peanut oil without emulsification, where no lipolytic zones were observed. However the bacterial growth was observed for all industrial samples except paneer sample.

3.2 b) Enrichment of sample with olive oil emulsion:
To obtain lipolytic bacteria, in industrial waste samples were further enriched with olive oil emulsion and plated on screening media containing olive oil emulsion. The results clearly showed the appearance of clearance zone around the bacterial colonies in total effluents as shown in the Fig 2. Hence only total effluent sample is selected for further studies.

Table 2: Zone formation with total effluent waste by enriching in olive oil emulsion

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Sample</th>
<th>Substrate</th>
<th>Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total wastes</td>
<td>Olive oil emulsion in Enrichment media &amp; screening media</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Only total effluent sample from dairy industry has shown more number of lipolytic zones with olive oil emulsion in enrichment media and hence total effluent sample is selected to isolate lipolytic bacteria by using serial dilution method.

3.3 Serial dilution of the total effluent waste sample:
Total effluent sample enriched with olive oil emulsion was used for serial dilution, where we observed formation of clear zone with \(10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}\) dilutions and the number of colonies were represented in Fig 3. In which \(10^{-3}\) dilution has shown maximum number of colonies and \(10^{-10}\) has shown the on screening media. Only one colony was observed in \(10^{-10}\) dilution.

Fig 3

| Table 2: Zone formation with total effluent waste by enriching in olive oil emulsion |
|-----------------|-----------------|-----------------|-----------------|
| S.NO | Sample | Substrate | Zone |
| 1    | Total wastes | Olive oil emulsion in Enrichment media & screening media | Positive |

3.4 Confirmation on tributyrin agar plate for lipolytic bacteria:
The lipolytic bacteria from single colony was collected from \(10^{10}\) and further confirmed further for clearance zone on Tributyrin Agar as shown in Fig 4. The results clearly indicate the formation of clearance zone around the bacterial colony after 72 hrs for morphological characterisation. The morphological characteristics of the colonies were observed under microscope.
The bacterial colony showing maximum clearance zone due to release of extracellular lipase was checked for lipolytic activity by titrimetric method and the activity was found to be 6 IU/ml.

3.5. Characterisation of isolated bacteria:

3.5.1 Morphological Characterisation:

The isolated bacteria showing positive lipid hydrolysis activity was further evaluated for their morphological characters like size, form, elevation, margins, color, texture, and opacity. Observing under microscope showed that these isolated lipolytic bacteria were rod shaped, convex by elevation with an entire margin, identified as opaque with respect to opacity and also found to be aerobic in nature.

3.5.2 Biochemical Characterisation:

Biochemical characterisation of isolated bacteria was found to be Gram Positive, Indole Negative, Methyl red Negative, Voges-Proskauer(VP) Positive, Catalase Positive, Starch hydrolysis Negative, Hydrogen sulphide production Negative, Nitrate reduction Negative, Citrate utilisation positive, Urea hydrolysis Positive, Tween 20 & 80 hydrolysis Negative. Being catalase positive, it was confirmed as aerobes. Positive VP test denote that they can ferment the glucose and convert to acetoin and 2,3-butanediol. Positive Citrate utilisation positive indicates the sodium citrate in the media as carbon source. Delayed Urea hydrolysis indicates that organism is able to hydrolyse the urea present in the medium only after 48 hrs.

3.5.3 Molecular characterisation:

The extracted DNA, from the isolated bacteria was used as template for 16s rRNA gene sequence analysis by using PCR amplification. The random forward and reverse primers picked for 16s rRNA gene which was further amplified by PCR amplification. The sequence was analysed by chroma or sequencer software. The DNA sequence was analysed by nucleotide blast in which the top 6 sequences were selected among 100 sequences showing maximum sequence similarities and the graphical summary is mentioned in the table 4 and depicted in Fig 5. The result clearly indicates that the isolated micro organism belongs to Lysinibacillus fusiformis L52.

Results in the table 4 depicts the maximum similarity showing score 313 and the percentage of similarity was identified to be 83.50%. Hence the isolated microorganism was identified as Lysinibacillus fusiformis L52

3.5.3 (a) Graphical summary:

In Fig 5, graphical summary depicts that the top 6 hits in the blast has shown similarities with the isolated microorganism greater than 200 nucleotide sequences and the results are shown.

| Table 3: Zone formation with total effluent wastes by using tributyrin |
|---|---|---|---|
| S.NO | Sample | Substrate | Zone |
| 1 | Total wastes | Tributyrin | Positive and clear |

| Table 4: Top 6 sequences showing maximum similarities with isolated microbe |
|---|---|---|---|---|---|
| Description | Max Score | Total Score | Query Cover | E value | Accession |
| Lysinibacillus fusiformis strain NBRC 15717(T) 16S ribosomal RNA gene, partial sequence | 313 | 7e-88 | 31 | 83.50% | KY328837.1 |
| Lysinibacillus fusiformis strain NBRC 15717(T) 16S ribosomal RNA gene, partial sequence | 296 | 7e-83 | 29 | 82.08% | MK424286.1 |
| Lysinibacillus fusiformis strain NBRC 15717 16S ribosomal RNA, partial sequence | 296 | 7e-83 | 29 | 82.08% | NR_112569.1 |
| Lysinibacillus fusiformis strain DSM 2898 16S ribosomal RNA, partial sequence | 296 | 7e-83 | 29 | 82.08% | NR_112628.1 |
| Lysinibacillus fusiformis strain NBRC 15717(T) 16S ribosomal RNA gene, partial sequence | 292 | 9e-82 | 29 | 82.15% | MH321607.1 |

Fig 4: Confirmation of lipase producing colonies on tributyrin agar

![Confirmation of lipase producing colonies on tributyrin agar](image)

![Graphical summary depicting the maximum similarity showing score 313 and the percentage of similarity being 83.50%](image)
3.5.3(b) Phylogenetic tree analysis:
Further the phylogenetic tree was constructed for the isolated microorganism based on morphological, biochemical and molecular characterisation was identified as Lysinibacillus fusiformis sps as shown in Fig 6:

![Fig 6](image)

Fig 6: Represents the phylogenetic tree analysis of isolated Lysinibacillus fusiformis L52 strain

5 ACKNOWLEDGMENT
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6 CONCLUSION:
Among all the samples collected from dairy industry wastes only the total effluent was used for isolation of lipolytic bacteria which has shown highest lipolytic activity with clear zones due to hydrolysis of olive oil and tributyrin substrates. Based on morphological, biochemical and molecular characterisation the isolated microorganism was found to be Lysinibacillus fusiformis L52 strain which is gram positive, rod shaped, convex in elevation with an entire margin, identified as opaque. This organism has also shown maximum growth at 72 hrs of incubation in tributyrin broth.

7 REFERENCES: