A STUDY OF THERMOSTABLE AND UV RESISTANT LIPASE PRODUCING BACILLUS SPECIES

Vishnu Priya Kalapatapu and Dr. I. Bhaskar Reddy

ABSTRACT

The objective of present study is to identify, screen and isolate a lipase producing bacterium which is stable at different temperatures and which is UV resistant. Screening and isolation of lipase producing strains of bacteria was carried out from eleven different soil samples collected from various places in Andhra Pradesh and Hyderabad. The isolates were positive on tribuytrin agar media and thus were selected as lipase producing strain. The strain was identified and characterised by the microscopic and biochemical tests as Bacillus.sps, a lipase producing organism. The optimisation of various cultural conditions was carried out by which the lipase production was enhanced with the optimal parameters being incubation period of 48 hours (5.9U/ml), palm oil as carbon source (5.9/ml), peptone as nitrogen source (6.2U/ml), initial pH of 7.0 and incubation temperature of 36ºc (6.7U/ml).The optimum agitation speed of 160 rpm produced lipase having (7.9 U/ml) activity. Finally, the enzyme lipase was purified by ammonium sulphate fractionation, dialysis and column chromatography. The ammonium sulphate precipitation and dialysis showed an increased specific activity of 1.71 U/ml and 6.17 U/ml when compared to crude enzyme which showed specific activity of 0.45 U/mg. Further purification was carried out by ion-exchange chromatography using DEAE column. The purified enzyme showed higher specific activity (15.24 U/mg) with a purification fold of 33. The molecular mass of purified lipase was estimated to be approximately 40.14 kDa by SDS-PAGE. Six cultures have shown tolerance to various organic solvents (acetone, benzene, toluene, ethyl benzene, 1-butanol, n-hexane and n-heptane) with hydrolytic zones when flooded on tribuytrin agar. It is evident from the results that the mutants LUV-8, LUV-9 and LUV-10 showed higher lipase activity than the parent strain-26, with the mutant LUV-9 showing the maximum activity of 9.5 U/ml after uv-irradiation. The sequence was deposited in NCBI and was identified to be a novel strain with an accession number which can be beneficial for industries.

Key words:  Bacillus sps, lipase, chromatography, dialysis.
1. Introduction

Lipases catalyse the hydrolysis of triglycerols releasing fatty acids, an alternate source of energy other than carbohydrates in all organisms universally [1]. These are mostly helpful in food and drug industry. Lipases blood serum can be used as a diagnostic tool for detecting conditions such as acute pancreatitis and pancreatic injury. A relatively smaller number of bacterial lipases have been well studied if compared to plant and fungal lipases. [2],[3] Most of the bacterial lipases reported so far are constitutive and are nonspecific in their substrate specificity, and a few bacterial lipases are thermostable. Among bacteria, Achromobacter sp, Alcaligenes sp, Arthrobacter sp, Pseudomonas sp., Staphylococcus sp., and chromobacterium sp have been exploited for the production of lipases [4]. Microbial enzymes have a great number of uses in food, pharmaceutical, textile, paper, leather and other industries[5],[6].

2. Material and methods

2.1 Microorganisms

Eleven different soil samples were taken for isolation of lipase producing organisms under laboratory condition. Sources are: bakery, automobile industry, groundnut field, sunflower field, Hussain Sagar effluent, oil sediment, diary industry oil cake, marine sediment, coconut oil mill, and vegetable crop soil. The labelled samples were spread on to the isolated media and were incubated at 37 degrees centigrade for 48 hours after serial dilution of 10^-1 to 10^-5 times.

2.2 Isolation and screening of lipase producing strains

The isolated bacteria were identified based on cellular morphology, growth condition, gram staining, endospore staining, capsule staining and biochemical tests (Sneath and Halt; 1986).

*Tribu-trin media:*

The media used for optimum production of lipase consisted of Ammonium sulphate 0.5, K2HPO4 -0.5%, MGSO4.7H2O-0.3%, yeast extract-0.03%, CaCO- 0.05%, olive oil -1% at ph 7.

2.3 Optimisation of various cultural conditions for enhanced production of lipase:

Selection of a suitable carbon source is done. Selection of a suitable nitrogen source, effect of ph, effect of incubation temperature, effect of incubation period, effect of agitation speed
(rpm) were all taken into account. In all the studies, experiments were carried out in triplicate and the average values are presented. The optimised parameters in each step were employed in subsequent experiments. The newly isolated bacterial species isolate L-3 that produces lipase was employed in the present study. The isolate was subcultured onto nutrient agar slants and incubated at 37 degree centigrade for 24 hours. These slants were subcultured at monthly intervals and stored at 4°C in the refrigerator.

2.4 Effect of incubation period on lipase activity:

L2 was cultured in Tributyrin broth containing yeast extract, NaCl, peptone and 1% (w/v) olive oil at 36°C in an inorbital shaker at agitation speed of 150rpm. The culture broth was harvested at 8 hour intervals by centrifugation at 10,000 g, 30 min, 4°C. The supernatant collected was used as crude enzyme solution and was assayed for enzyme activity.

2.5 Effect of different oils as carbon source on lipase activity:

Olive oil present in the growth media was replaced with different oils like palm oil, ghee, coconut oil, groundnut oil, sunflower oil and mustard oil at a final concentration of 1% (w/v).

2.6 Effect of different nitrogen sources on lipase activity:

Different nitrogen sources like yeast extract, soya bean meal, NaNO, tryptone and peptone were added to the broth at a final concentration of 1% (w/v).

2.7 Effect of agitation speed on lipase activity:

To determine the optimal agitation speed for peak enzyme activity, the L2 was cultured in an orbital shaking incubator at 36°C at varying agitation speed from 120-200 rpm.

2.8 Effect of temperature on lipase activity:

For selection of optimum temperature for the production of lipases, the temperatures varying from 21to 42°C were selected.

2.9 Effect of pH on lipase activity:

The optimum pH for enzyme production was selected by varying the pH of the tributyrin broth from 5 to 9.
Lipase assay by titration method

Mostly bacteria species secrete extracellular, inducible, alkalophilic lipase to hydrolyse fats and oils or lipids. Lipases act on lipids releasing fatty acids. These released fatty acids can be measured by titration with 50 mM NaOH solutions. Olive oil emulsion was prepared by mixing 2.5 ml of olive oil with 7.5ml of 1% gum Arabic solution. It was homogenised for two minutes.

Reaction mixture was prepared by adding 2.5 ml of olive oil emulsion, 2ml of 50mMTris-buffer (pH-8.0), 0.5ml of 110Mm CaCl₂ and 0.5ml of enzyme extract. This reaction mixture was incubated at 50°C for about 1 hour under orbital shaking at 160 rpm. The reaction was immediately stopped after the incubation period by addition of 2ml of Acetone: ethanol (1:1 v/v) mixture. Two-three drops of Phenolphthalein indicator was added to it. The released fatty acids were titrated with 50mM NaOH. Sodium hydroxide was standardised with 0.01N oxalic acid. One lipase activity unit was defined as the amount that released 1 µmol of fatty acid per minute.

- Calculation of lipase activity: Lipase activity (Units/ml) =N₂× (V₂-V₁) ×1000/T
  - N₂=Normality of NaOH (0.0)
  - V₂-V₁= Difference between the volume of alkali solution consumed for the test and the control
  - T= Incubation period of 60 minutes.

One unit of lipase activity was defined as amount of lipase capable of releasing one micromole of free fatty acid per ml per minute under the assay conditions and reported as U/ml.

2.10 Purification methods used:

- Ammonium sulphate fractionation.
- Dialysis
- Column chromatography
- SDS-PAGE
3.0 Results and discussion

3.1 Isolation and screening of lipase producing bacteria:

A total of 158 colonies were selected and isolated from the 11 samples. The lipase enzyme producing microbial colonies were identified by the clearing zones around the colonies. The selected isolates were transferred onto nutrient agar slants and incubated for 24 hours. The number of isolates from each sample and the zone of hydrolysis are analysed.

Out of 158 isolates, 28 were selected based on their macroscopic characters, eliminating those that appeared close to each other and zone of clearance greater than 1.0 cm. The results indicated that the isolate L-26 showed maximum lipolytic activity (A/B=2.8) followed by the isolates L-28 (A/B=2.7) and L-27(A/B=2.6).

3.2 Secondary screening:

The results indicated that the isolate L-26 exhibited maximum lipase activity (4.4 U/ml) followed by the isolates L-27(4.2 U/ml) and L-28 (4.2 U/ml). Hence, further studies were focused on the isolate L-26. To perform further investigation, the selected L-26 strain was grown on Tributrin medium and incubated at 37ºc for 18 hours and stored until use at 4ºc in refrigeration.

3.3 Identification and characterisation of the isolate L-26 isolate

Colony morphology: Colonies are large, opaque, irregular, producing green colour pigment. Based on the results of morphological studies and biochemical tests conducted, the isolated organism L-3 was identified as Bacillus species.

3.4 Microbial characterization:

Phenotypic characterisation:

The isolate selected creamy white smooth widely spreading large size rod shaped opaques on nutrient agar media. The bacterium is a rod shaped spore producing gram negative bacterium. Based on the results of morphological studies and biochemical tests conducted (Table-3) and following the Bergey’s manual (Sneath, P.H.A.1986), the isolate L-26 was identified as likely to be belonging to genus Bacillus sps.
Results of morphological and bio-chemical tests

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony morphology</td>
<td>Creamy, white smooth, widely spreading, large size</td>
</tr>
<tr>
<td>Gram staining</td>
<td>Negative</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Rods</td>
</tr>
<tr>
<td>Spore formation</td>
<td>Positive</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>Positive</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>Positive</td>
</tr>
<tr>
<td>Acid production</td>
<td>Positive</td>
</tr>
<tr>
<td>Gas production</td>
<td>Negative</td>
</tr>
<tr>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>Hydrogensulphide production</td>
<td>Negative</td>
</tr>
<tr>
<td>Urease</td>
<td>Positive</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>Positive</td>
</tr>
<tr>
<td>Oxidase</td>
<td>Negative</td>
</tr>
<tr>
<td>Denitrification</td>
<td>Positive</td>
</tr>
<tr>
<td>Methyl red</td>
<td>Positive</td>
</tr>
<tr>
<td>Voges proskauer</td>
<td>Positive</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>Positive</td>
</tr>
<tr>
<td>Indole test</td>
<td>Negative</td>
</tr>
<tr>
<td>Growth at 7% NaCl</td>
<td>Positive</td>
</tr>
<tr>
<td>Utilisation of glucose</td>
<td>Positive</td>
</tr>
<tr>
<td>Utilisation of sucrose</td>
<td>Positive</td>
</tr>
<tr>
<td>Utilisation of lactose</td>
<td>Negative</td>
</tr>
<tr>
<td>Utilisation of citric acid</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Molecular characterisation:

The novel strain identity was confirmed by sequencing of 16S rRNA gene. The sequence comparison was done using multiple sequence alignment with the aid of BLAST programme and utilisation of Blast technique (Gene KJ603220 (Bacillus sp. MK1 16S ribosomal RNA gene, partial sequence) and the sequence showed a similarity of 100% with the Bacillus sp and 89% similarity with bacillus subtilis and Phylogenetic tree and BLAST analysis data is as below:

The GenBank accession numbers for BankIt1682396 seq1 KJ603220 (Bacillus sp. MK1, 16S rRNA partial sequence)

>gi|641451095|gb|KJ603220.1| Bacillus sp. MK1 16S ribosomal RNA gene, partial sequence
AGCCTTTTGGCTCCCTTGATGTTAGCGGCAGACGGGTCGATTAACACGTGGGTAACCT
GCCTGTAAGACTGGGATAAACTC
CGGGAAACCCTTAAATACGCTGACGGTTAGGTTAGTTGAAACGCAAGTGTACACATGAAAAGTGCGTTTT
CGGCTACACCTTTA
GATGGAAAATCCGGCCTTCCTATCTTATAATTTTGAAGGTTACGGCTC
ACAAAGGCAAGCGTAACCCACCTGAAGAGGTGAT
CCCGAACACTGGAATAAAACCGCCCACCTCCT

Bacillus sp. MK1 16S ribosomal RNA gene, partial sequence  Sequence ID: gb[KJ603220.1]

Length: 273    Number of Matches: 1

Related information

Range 1: 1 to 273 GenBank Graphics  Next Match Previous Match

Multiple sequence alignment using BLAST programme

Alignment statistics for match #1

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>505 bits(273) 2e-139</td>
<td>273/273(100%)</td>
<td>0/273(0%)</td>
<td>Plus/Plus</td>
<td></td>
</tr>
</tbody>
</table>

Query 1  AGCCTTTTGCTCTCTTGATGTTAGCGGCAGACCGGTGTCGATTAACACGTTGGAACCTGC
Query 61  AGCCTTTTGCTCTCTTGATGTTAGCGGCAGACCGGTGTCGATTAACACGTTGGAACCTGC

Sbjct 1  AGCCTTTTGCTCTCTTGATGTTAGCGGCAGACCGGTGTCGATTAACACGTTGGAACCTGC

Query 121  AAGGTTCACACATAAAAAGTGGCTTTTCGCTACCATTTAAAGATGGACCGCCGCGTCCCTTA

Sbjct 121  AAGGTTCACACATAAAAAGTGGCTTTTCGCTACCATTTAAAGATGGACCGCCGCGTCCCTTA

Query 181  TCTAATTTTTGAGGTTCAGGCTCAAAGGCAACGTAACCCACCTGAAGAGGTGAT

Sbjct 121  AAGGTTCACACATAAAAAGTGGCTTTTCGCTACCATTTAAAGATGGACCGCCGCGTCCCTTA
The phylogenetic tree was constructed and represented in Figure 1.

3.5 Optimisation of various cultural conditions for enhanced production of lipase

Effect of incubation period on lipase activity: The L26 isolate was inoculated in Tributyrin broth and was harvested at 24 hours interval. Maximum enzyme activity was observed at 72 hours during the early stationary phase. The activity of the enzyme gradually decreased after 72 hours (figure 1). It took more time as it was harvested at 8 hrs and it was observed that the enzyme activity was high at 48 hours and gradually decreased as their sample was from oil contaminated soil [17].
Effect of different carbon sources

In this case, a range of lipids were screened for their ability to support lipase production. Peak enzyme activity 5.9U/ml was obtained when palm oil was used as substrate as observed in the previous papers [18].

(Table-5, Figures 18).

**Table-5 Effect** of different carbon sources

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>Lipase activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive oil</td>
<td>5.1</td>
</tr>
<tr>
<td>Mustard oil</td>
<td>4.6</td>
</tr>
<tr>
<td>Ghee</td>
<td>3.9</td>
</tr>
<tr>
<td>Palm oil</td>
<td>5.9</td>
</tr>
</tbody>
</table>
Effect of nitrogen sources

For selection of optimum nitrogen source, different nitrogen sources were used for the media. Peak enzyme activity was obtained when peptone oil was used as nitrogen [18].
Effect of different temperatures

Lipase activity was observed at broad range of temperature. The maximum activity was observed at 36°C temperature as found in the literature till date.

Effect of different PH

Lipase activity was observed at broad range of pH. Maximum activity was observed at pH, 7. The enzyme isolated is stable at both the acidic and alkaline pHs which is flexible and is of good industrial importance.
Effect of agitation speed

Lipase activity was observed at broad range of agitation. Maximum activity was observed at agitation speed of 160 rpm for about 24 hours when compared to previous papers as that was a plant source.

3.6 Organic solvent tolerance by lipase producers:

Tolerance of lipase producing bacteria to various organic solvents

Organic solvent tolerance of the selected cultures was investigated by their ability to grow on tributyrin agar plates flooded with different organic solvents such as benzene (log $P$ 2.0), toluene (log $P$ 2.5), ethyl benzene (log $P$ 3.1), n-hexane (log $P$ 3.5) and n-heptane (log $P$ 4.0) during incubation. Among 28 lipase producing cultures, 12 cultures exhibited growth as well as lipolytic activity in presence of all the organic solvents tested and retained above 80% of their initial activity which is measured and compared with D-d values. Bacterial culture AI91 retained 99% of its initial activity on plates flooded with n-hexane by comparing maximum zone of hydrolysis with that of controls (absence of n-hexane) which are shown in table 2.3.
Table 2.3: Lipolytic zones of OST bacterial cultures in presence of n-Hexane on Tributyrin Agar medium

<table>
<thead>
<tr>
<th>T&amp;OST bacterial cultures</th>
<th>Lipolytic zone (D-d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM2</td>
<td>13mm</td>
</tr>
<tr>
<td>CM9</td>
<td>28mm</td>
</tr>
<tr>
<td>CM5</td>
<td>11mm</td>
</tr>
<tr>
<td>DF79</td>
<td>13mm</td>
</tr>
<tr>
<td>DF75</td>
<td>16mm</td>
</tr>
<tr>
<td>DF83</td>
<td>12mm</td>
</tr>
<tr>
<td>BK34</td>
<td>17mm</td>
</tr>
<tr>
<td>BK47</td>
<td>15mm</td>
</tr>
<tr>
<td>GF55</td>
<td>23mm</td>
</tr>
<tr>
<td>GF70</td>
<td>18mm</td>
</tr>
<tr>
<td>AI91</td>
<td>29mm</td>
</tr>
<tr>
<td>AI94</td>
<td>23mm</td>
</tr>
</tbody>
</table>

Here, log $P$ is the logarithm of the partition coefficient of the solvent between n-octanol and water and is used as an index of the solvent polarity. Organic solvents with a log $P$ between 1.5 and 4.0 are extremely toxic to microorganisms and other living cells because they undergo partition preferentially in the cytoplasmic membrane, disorganizing its structure and impairing vital functions. Solvent toxicity depends not only on the inherent toxicity of the compound but also on the intrinsic tolerance of the bacterial species and strains. The solvent tolerance in certain strains has been attributed to the change in the composition of the membrane fatty acids and/or presence of active efflux pumps which maintain minimal sub-toxic concentration of toxic organic solvents within cells (Ramos et al., 2002). Several authors had reported tolerance of bacteria to various organic solvents (Torres et al., 2011), including Benzene tolerant *Rhodococcus* species (Paje et al., 1997) and *Bacillus* species (Moriya and Horikoshi, 1993), butane tolerant *Bacillus* species (Li and Poole., 1999), etc.

3.7 Effect of UV radiation on the survival of L-26 isolate

**UV irradiation of L-26 isolate and selection of mutants**

The isolate L-26 was subjected to UV treatment for six different time intervals. The number of survivals from each exposure is given in Table-10. At 20 and 25 minutes exposure, more than 99% deaths were recorded, while 30 minutes exposure resulted in 100% death of the bacteria. The UV survival curve was plotted (Fig -23) and the plates having less than 1% survival rate (20 and 25 min) were selected for the isolation of mutants.
A total of 15 mutants were selected and tested for lipase production along with the control (Fig-24, 25 (a-d). The results are presented in Table-11. It is evident from the results that the mutants LUV-8, LUV-9 and LUV-10 showed higher lipase activity than the parent strain-26, with the mutant LUV-9 showing the maximum activity of 9.5 U/ml.

### 3.8 Purification of lipase:

**Results:**

Enzyme purification is done by ammonium sulphate fractionation, dialysis and column chromatography. The ammonium sulphate precipitation and dialysis showed an increased specific activity of 1.71 u/ml and 6.17 u/ml when compared to crude enzyme which showed specific activity of 0.45 u/mg.

Further purification was carried out by ion-exchange chromatography using DEAE column. The purified enzyme showed higher specific activity (15.24 u/mg) with a purification fold of 33.8. The molwt of purified lipase was estimated to be 40.14 kDa.

(Fig.1) picture taken- im gel doc

A–Marker
B-Ammoniumsulphate
C-Dialysis
D-Column 6 tube
3.9 Conclusion

Isolate L-26 is identified to be Bacillus sps KJ603220 (Bacillus sp. MK1) novel strain from automobile shops near Medak. This is the first report to identify a novel thermostable, organic solvent tolerant, UV resistant lipase producing Bacillus from automobile soil sample of Medak district. It is evident from the results that the parameters found to be optimal for lipase production are incubation period of 72 hours (5.9 u/ml), incubation temperature at 36°C, (6.7 u/ml), pH 7.0, (7.1u/ml), carbon source as palm oil (5.9/ml), nitrogen source being peptone (6.2 u/ml), agitation speed at 160 rpm, (7.9 u/ml). The optimisation of medium and physical conditions increased the enzyme production from 4.4 u/ml to 7.9 u/ml, UV mutant LUV-9 showing the maximum activity of 9.5 u/ml, more than the parent strain with 7.5 u/ml. So the strain improvement by UV irradiation for high lipase production can be used for commercial purpose on a large scale.

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