Results
4. Results

4.1 ISOLATION OF STRAINS AND IDENTIFICATION OF LIPASE PRODUCING MICROORGANISMS FROM CONTAMINATED SOIL SAMPLE

4.1. (a) Collection of sample (Soil samples and de-oiled seed cakes)

Based on the morphological characteristics, total 31 isolates were isolated from soil samples for the production of lipase using de-oiled seed cake from Gulbarga India. The places from India such as Biodiesel Information and Demonstration Centre (BIDC), Gulbarga University Gulbarga, Venkatesh Hero-motors service Centre, Lahoti motors service centre, Hyundai motors service centre, M.S. Ghanti Oil Merchant and Different Oil mills. The soil samples were brought to the laboratory and tested for its capability of lipase production by utilizing seed cake as a substrate.

It is reported that lipase utilizing microorganisms were found in oil deposited soil. (Dobson R et al., 2012) In this work, the seed cake for the production of lipase has taken from Biodiesel Information and Demonstration Centre (BIDC), Gulbarga University, Gulbarga, Karnataka.

4.1. (b) Enrichment of soil microorganisms

Enrichment of soil samples was carried out so that the microorganisms present in the soil sample get adapted towards the medium (The medium used for bacterial enrichment process was containing the following components (%, w/v): trybutyrin-1.0(v/v), yeast extract-0.3, peptone-0.5 and pH-7.5) under which they are subjected to grown and this process is carried out in following steps.
About 1.0 g of soil sample was inoculated to a 250 ml flask with 50ml of enrichment medium and then incubated aerobically at 37°C on a rotatory shaker at 150rpm. After one day incubation, 0.1ml of culture was transferred to another fresh flask containing the same enrichment medium and then incubated for overnight. Four rounds of such enrichment operation were done. Thus, the bacteria that could use seed cake as substrate for lipase production and grow quickly under aerobic condition would be enriched.

Fig-5: Collected soil samples from different regions of Gulbarga.

Fig-6: Collected seed cakes from Biodiesel information and demonstration centre, GUK, Kalaburagi
4.1. (c) Screening of isolates for Lipase production

The 31 isolates were screened for production of lipase by growing them on tributyrin plates. Among 31 isolates two isolates Kar_21 and AB 1 were found to produce larger zone of hydrolysis and Kar_21 was selected for production screening.
4.2 SCREENING OF ISOLATES FOR LIPASE PRODUCTION

4.2.1 Screening of microbes:

4.2.1(a) Primary Screening:

Screening of the isolates using Tributyrin (Qualitative assay): Lipolytic organisms were screened by qualitative plate assay, using tributyrin agar base and incubated at 37°C for 48hrs. Potential isolates were selected by measuring the diameter of zone of hydrolysis exhibited by the isolates.

4.2.1(b) Secondary Screening:

The isolates showing potentiality in primary screening were further screened for the ability to utilize the de-oiled seed cake as substrate. This was done in two different steps, firstly the isolates were cultured containing 1% tributyrin as substrate, and in second step the isolates were cultured containing 1gm of *pongamia pinnata, simarouba, jatropha, neem, mauha* seed cake as substrate, and incubated for 18hrs at 37°C in orbital shaker at 160rpm.

The 31 isolates were screened for production of lipase by growing them on tributyrin plates. Among 31 isolates two isolates KAR-21 and AB-1 were found to produce larger zone of hydrolysis and KAR-21 was selected for production screening.

4.3 BIOCHEMICAL AND PHYSIOLOGICAL STUDIES

4.3.1 Biochemical Characterization:

Biochemical characterization was done to know the response of microorganism towards different substrate. All the biochemical reactions that occur both inside and outside of the cells are precisely controlled by some governing factors, the enzymes. The enzymes are either exoenzymes or endoenzymes.
Exoenzymes which are few in numbers are released from the cell and act on the substrate. In the laboratory, presence of exoenzymes is assayed by looking for a change in the substrate outside a microbial cell.

In order to find out enzymatic activities of isolates various kind of biochemical tests were performed.

4.3.1(a) Indole production test

Tryptophan is an essential amino acid, which is oxidized by some bacteria by the enzymes tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The indole test is carried out by inoculating a bacterium into tryptophan broth, the indole produced during the reaction is detected by adding Kovac’s reagent (dimethyl amino benzaldehyde) which produces a cherry red reagent layer.

![Fig-9: Indole test](image)

The isolates KAR21 and AB1 showed no colourless so it’s negative reaction.
4.3.1(b) Methyl red and Voges-proskauer tests

The Methyl red and Voges-proskauer tests are used to differentiate between two major types of facultative anaerobic enteric bacteria that produce large amounts of acids and those which produce neutral product acetoin as an end product. Both these tests are performed continuously because they are physiologically connected and they performed same medium in MR-VP broth. In these tests, if an organism produces large amount of organic acids from glucose, then the medium remains red (a positive test) after the addition of methyl red which is a pH indicator (i.e. pH remaining below 4.4). In other organisms methyl red will turn into yellow (a negative test) due to the elevation of pH above 6.0 because of the enzymatic conversion of the organic acid to non acidic product.

4.3.1(b)(i) MR test

Negative reaction was shown by both KAR21 and AB1 isolates, which is indicated by no change in colour of the medium when methyl red was added.

4.3.1(b)(ii) Voges-Proskauer test

The crimson- to ruby pink colour was developed, which was the indication of the positive VP test. The positive test was shown by AB1 and no change in colour shows the negative VP test which was shown by isolate KAR21.
4.3.1(c) Catalase test

In the presence of oxygen, microorganisms that produce hydrogen peroxide which is lethal to the cell. The catalase enzyme which is found in some the microorganisms gets breaks down into hydrogen peroxide to water and oxygen and helps in their survival. The catalase test is performed by the addition of H2O2 to trypticase soya broth. If the microbe’s releases free oxygen gas bubbles then it is a positive catalase test.

The gas bubbles were shown by both KAR21 and AB1. Hence the isolates were seems to be positive for catalase test.
Fig-11: Catalase test

Table-13: Results of Biochemical Characterization

<table>
<thead>
<tr>
<th>Biochemical Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KAR 21</td>
</tr>
<tr>
<td>Indole Production Test</td>
<td>Negative</td>
</tr>
<tr>
<td>Methyl Red Test</td>
<td>Negative</td>
</tr>
<tr>
<td>Voges Proskauer Test</td>
<td>Negative</td>
</tr>
<tr>
<td>Catalase Test</td>
<td>Positive</td>
</tr>
</tbody>
</table>

4.3.1(d) Fermentation of sugars

Fermentative degradation of different carbohydrates such as glucose (a monosaccharide), fructose (disaccharide), cellulose (polysaccharide) by microbes, under anaerobic conditions is carried out into a fermentation tube. A fermentation tube is a culture tube which contains a Durham tube for the detection of gas production, as an end product of metabolism. The fermentation broth which contains ingredients consisting of nutrient broth, a specific carbohydrate (glucose, lactose,
maltose sucrose or mannitol) and a pH indicator (phenol red), which is red at a neutral pH (7) and turns yellow at or below pH of 6.8. The production of gas in the Durham tube and change in colour of broth (from red to yellow) due to the production of acid are tabulated below:

Table-14: Results of Fermentation of sugars

<table>
<thead>
<tr>
<th>Carbohydrate Fermentation</th>
<th>Tests</th>
<th>KAR21</th>
<th>AB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars</td>
<td>Acid Production</td>
<td>Gas Production</td>
<td>Acid Production</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

4.4 STRAINS IDENTIFICATION WITH 16S rDNA

4.4(a) Characteristics of lipase producing bacteria

The biochemical tests revealed that among 31 isolates, two isolates KAR21 and AB1 were found to be more potent for the production. Thus these isolates had similar biochemical properties. The 16S rRNA sequencing of the isolates and its bioinformatics analysis revealed that, the KAR21 isolate belonged to *Micrococcus* genus, *M. luteus* species and the strain was designated as KAR21. The sequence submitted to genbank database can be retrieved through the accession number NR_114673.1, whereas, the AB1 isolate belonged to *Bacillus* genus, *subtilis* species and the strain was designated as AB1. The sequence submitted to genbank database can be retrieved through the accession no: NR_113265.1.
4.4(b) Amplification of 16S rDNA and Nucleotide sequence analysis

Amplification of 16s rDNA has showed the distinct fragment of approximately 1770 bp and 1799 bp in length of isolated two strains and the sequence results were obtained from Bhatt biotech pvt ltd. The sequences of the *Micrococcus luteus* KAR 21 and *Bacillus subtilis* AB stain with a sequence of 1777 bp and 1799 bp respectively has been deposited into the NCBI Genbank data library and assigned the accession number NR_114673.1 and NR_113265.1.

BLAST results showed that *Micrococcus luteus* has highest homology with micrococcus sp.ss1321 with a significant value of 99% homology, from the phylogenetic tree construction and the G+C contents of 16s DNA sequences, the strain is also similar with *Marine bacterium* HD-816s ribosomal RNA gene, *Micrococcus endophytic* stain YIM 56238, uncultured bacterium clone TS3G-25 sequences respectively. The analysis of the strain *Bacillus subtilis* AB strain showed highest homology with *Brevibacterium* Sp strain WHA-2, *Bacillus tequilensis* strain WHA-11 16s, *Bacillus subtilis subsp. spizizenii* strain DJY-7 16s ribosomal gene.
**Micrococcus luteus** KAR21

```
GATCGTGCGCTCGATGAATCCCAGCTTGCTAGGGATGAGATTATGTGGCG
AACGGGTTAGTAACACGTGAGTAACCTGCCCCTTGACTCTGGGATAAGCC
TGGGAAACTGGGCTTAATACCGGATAGGAACGTCCACCCGATGGTGGGT
GTTGGAAAGATTTATCGGTCATGGATGAGACTCGCAGCCTATACAGGCTTG
GGTGAGGTAATGGCTGCAAAAGGCAAGGAACACCGGCTTAACCTTTCAAGGA
GGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACCGGAGG
CAGCAGTGGGAATATTTGCAATGGGCGAAAGCCTGATGCAGCGACGC
CGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACTAGGAA
GAAAGCAGAATGACGCTACCTGCAAGAGGAAGACACCGGCTAACTACGTGC
CAGCAGCCGGGTAATACGTAGGGTGACGCACATTCCGGAATTATTTG
GCGTAAAGAGCTCGTACGGGCTTGTGCTGCTGCTGCTGAAAGTCCCGG
GCTTAACCCCGGATCTGCAGGTTACGGGCTAAGCAGACTAGAGTGACGTAGG
GGAGACTGGAATTCTCTGGTGATACGGGAGATGAGTGAGATATCAGGAGG
AACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAG
CGAAAGCATGGGGAGCAACAGATTAGATACCTGGTGTAGTCCCATGCCG
TAAACGTGGGCCACTAGTGTGGGGACCATCTCCAGGCTTCCGCGCCGCA
GCTAAGCGATTAGTGCCCGGCTGCTGGGAGTACGGGCAGACTAGAGTGCAGTAGG
GGAGACTGGAATTCTCTGGTGATACGGGAGATGAGTGAGATATCAGGAGG
AACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAG
CGAAAGCATGGGGAGCAACAGATTAGATACCTGGTGTAGTCCCATGCCG
TAAACGTGGGCCACTAGTGTGGGGACCATCTCCAGGCTTCCGCGCCGCA
GCTAAGCGATTAGTGCCCGGCTGCTGGGAGTACGGGCAGACTAGAGTGCAGTAGG
GGAGACTGGAATTCTCTGGTGATACGGGAGATGAGTGAGATATCAGGAGG
AACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAG
CGAAAGCATGGGGAGCAACAGATTAGATACCTGGTGTAGTCCCATGCCG
TAAACGTGGGCCACTAGTGTGGGGACCATCTCCAGGCTTCCGCGCCGCA
GCTAAGCGATTAGTGCCCGGCTGCTGGGAGTACGGGCAGACTAGAGTGCAGTAGG
GGAGACTGGAATTCTCTGGTGATACGGGAGATGAGTGAGATATCAGGAGG
```
GGGGAGACTGGAATTCTGGTAGCGGTGGAATGCGCAGATATCAGGA
GGAACACCGATGGCGAAGGCAGGATTAGATACCTGGGTAGTCCATGC
CGTAAACGTTGGGACTAGGTGTGGGGACCATTCCACGGTTTCCGCACCG
CAGCTAACCAGCATTAAGTGCCTCGCTGGGAGTACGGCCGAAGGCTAA
AACTCAAGGAATTTGACCGGGGCACCAACGGCGAGCATGCAGGA
TTAATTCGATGCAAACGGAAGAACCTTACCAAGGCTTTGACATGTTCCTGA
TCGCCGTTAGAGATACGGTTTCCCCTTTGGGGCCGGAATCAGGTGGTGA
TGGTTGTCGTCACTCGTGTCTGAGATGTTGATGTTAAATGGTCGCACAC
GCGCAACCCTCAGTTCCCATGTGAGACGCACGAATGATGGGGACTCATGGG
AGACTGCCGGGGTCAACTCGAGGAAGGTGGGACGCACGTCAAATCATC
ATGCCCTTATGTCTTGGGCTTCAGCATGCTACAATGGCC
GGTTGCGATACTGAGGTGGAGCTAACCCCATGAAATTCCGGGAAACCGGGGCTAATACCGGATGCTTGTT
TGAACCGCATGGTTCAAACATAAAGGTGGCTTCGGCTACCACTTACAG
ATGGACCCGCGGCGCGCATGAGGGTGATCGGACACTGGGACACCTGCTCCCTAAGCAGATGCGTAGCCGACC
TGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA

**Bacillus subtilis AB1 (1799bp)**

GGCAGTGTCGCTGCTATACTGCACTGAGCGGACAGATGGGAGCTTGCT
CCCTGATGTTACGGCGCGACGGGTTGAGTAACACGGTGAGTACCTGCTG
TAAGACTGGGATAACTCCGCGGAAACCGGGGCTAATACCGGATGCTTTT
TGAACCGCATGGTTAAACATTTAAGGTGGGCTCGCTACCACCTTACAG
ATGGACCCGCGGCGCGATTAGTGTGGTGAGGTAATGGGCTCACCAGG
CAACGATGCGTACGCGACCTGAGGAGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGAAATCTTCCGCAA
4.5 OPTIMIZATION CONDITIONS FOR LIPASE PRODUCTION

4.5.1 Optimization of pH and temperature

All microorganisms possess transport mechanism to keep the normal physiological H+ ion concentration within the cell and channels to promote nutrient absorption. The microbial growth is depending on the proper functioning of these transport systems and channels, which in turn are regulated by medium pH. The optimum pH for growth of Micrococcus sp. and lipase production was determined by adjusting the medium pH in range of 5 to 10 and the results are shown in graph-1.

The partially purified lipase was studied for the optimum pH by assaying the enzyme in different pH. The results (graph-1) show that the lipase was alkaline with optimum pH of 8.0. To find the optimum temperature the enzyme was assayed at different temperatures, which show that (graph-2) enzyme was active at 35°C.
Graph 1: Represents the determination of optimum pH

Graph 2: Represents the determination of optimum temperature

4.5.2 Kinetic Studies

The effect of p-nitrophenyl laurate concentration on the hydrolysis rate was measured at different substrate concentrations. The lipase showed a variable specificity towards various concentrations of triacylglycerols. The spectrophotometrically lipase assay applied for each substrate concentrations with
respect to time. According to absorbance values, enzyme activity calculations were done for each concentration. By combining the data, Lineweaver-Burk plot which was 1/enzyme activity (V) versus 1/substrate concentration(S), was drawn (graph-3).

The Lineweaver–Burk plots which were linear and indicated that hydrolysis of many triglyceride esters by lipase followed Michaelis–Menten kinetics. That graph gave two kinetic parameters, Km and Vmax, which show the substrate affinity of enzyme. The smaller Km value indicates the higher enzyme affinity to substrates. According to some of the report, the Km value of most of the industrial enzymes varies at the range of $10^0$ to $10^5$ M which acts on biotechnologically highly important substrates.

The effect of different substrate concentrations on enzyme activity was studied to find the Km and Vmax of the lipase. The figure below shows that the enzyme had a Vmax of 8.0 and Km of 5.26.

Graph-3: Represents the Kinetic studies.
4.5.3 Effect of metal ions and inhibitors

The spectrophotometric enzyme activities of lipase were studied in the presence of metal salts. Relative enzyme activities were calculated from the absorbance values of sample with respect to the time. In this study, the control sample which does not contain any of the additives was used and it is assumed 100% while calculating relative enzyme activities. Other trials were calculated according to control sample.

Various additives were used to find their effect on the enzyme activity. The results are presented in Table-15.

**Table-15: Relative enzyme activity values in the presence of metal ions.**

<table>
<thead>
<tr>
<th>Additives</th>
<th>Activity (U/ml)</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.6</td>
<td>0</td>
</tr>
<tr>
<td>PMSF</td>
<td>42.6</td>
<td>143.91</td>
</tr>
<tr>
<td>EDTA</td>
<td>36.4</td>
<td>122.97</td>
</tr>
<tr>
<td>1-10,Phenanthroline</td>
<td>21.6</td>
<td>72.97</td>
</tr>
<tr>
<td>NaCl</td>
<td>38.1</td>
<td>128.71</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>36.3</td>
<td>122.63</td>
</tr>
<tr>
<td>SDS</td>
<td>24.7</td>
<td>83.44</td>
</tr>
</tbody>
</table>

The inhibitions in presence of 1-10 phenanthroline indicate that the enzyme is a metallo enzyme and inductions in presence of PMSF indicate the possible absence of serine in the active site of the enzyme. The enzyme is found to be tolerant to salt, detergent and bleaching agent.
4.6 Large Scale Production of Lipase and Its Purification

4.6(a) Bulk Production:

The enzyme was produced by inoculating the culture in 500 ml of culture media. The supernatant obtained was used for purification.

4.6(b) Enzyme assay

Bulk product of lipase extracted by centrifuge and assayed. The activity was found to be 1.17 U/ml.

Graph-4: Representing the effect of different substrate concentrations on enzyme activity having a Vmax of 8.0 and Km of 5.26.

4.6(c) Estimation of total protein

The Bradford total protein assay is the spectroscopic analytical techniques which are utilized to determine the aggregate protein concentration of responsible sample. In this technique, Coomassie brilliant blue G-250 dye which binds to proteins and changes their colour from green to blue. That change in colour is observed at 595nm in UV-visible spectrophotometer. As the concentration of protein content is increase, the colour is getting darker and darker. Coomassie brilliant blue
G-250 binds to histidine, lysine, and arginine residues in protein samples. Bradford assay applied samples before loading on SDS-PAGE gel and after every purification steps in order to calculate lost and gain of protein amount.

A series of Bovine serum albumin (BSA) Standard in various concentrations were prepared. According to this BSA standard, standard calibration curve was drawn with reaction to their absorbance values. Total protein content was calculated from standard calibration curve equation.

The total protein was estimated by Bradford’s method with the help of BSA standard curve (graph-5). The protein was 4.51 mg/ml and specific activity was 14.86 U/mg proteins.

![Std. BSA Curve](image)

**Graph-5: Represent the std. BSA Curve**

### 4.6.1 Large-scale production

500 ml of nutrient broth was prepared 1% tributyrin was added to the broth. The culture was inoculated to the broth and kept for incubation at 37°C for 24 h. After a day incubation the broth was centrifuged at 10,000 rpm for 3 min. the enzyme content supernatant was collected.
4.6.1(i) AGROWASTE SCREENING:

Different agro-wastes like sugarcane waste, sugarcane baggasse, Pigeon Pea waste, Rice bran, sawdust, Dal husk, Pongamia seed cake, Jatropha seed cake, Simarouba seed cake, Azadirachta seed cake and Mauha seed cake (after detoxification) were used as complex nutrient source for the isolates to grow and produce lipase. The produced enzyme was assayed and Simarouba seed cake was found the most suitable for production of lipase by *Micrococcus luteus* KAR21.

Table-16: Effect of different agro-waste for production of lipase by *Micrococcus luteus* KAR21.

<table>
<thead>
<tr>
<th>Agro-waste</th>
<th>Lipase activity U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugarcane waste</td>
<td>53</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>39</td>
</tr>
<tr>
<td>Pigeon pea waste</td>
<td>80</td>
</tr>
<tr>
<td>Rice bran</td>
<td>82</td>
</tr>
<tr>
<td>Saw dust</td>
<td>113</td>
</tr>
<tr>
<td>Dal Husk</td>
<td>78</td>
</tr>
<tr>
<td>Pongamia seed cake</td>
<td>259</td>
</tr>
<tr>
<td>Jatropha seed cake</td>
<td>272</td>
</tr>
<tr>
<td>Simarouba seed cake</td>
<td>482</td>
</tr>
<tr>
<td>Neem seed cake</td>
<td>173</td>
</tr>
<tr>
<td>Mauha seed cake</td>
<td>243</td>
</tr>
</tbody>
</table>
Graph-6: Represents the effects of different agro-waste for the production of lipase.

4.6.1(ii) Media Optimisation

Mineral media such as MSS1 (K$_2$HPO$_4$-6.0, NaCl-6.0, MgSO$_4$.7H$_2$O-0.4, CaCl$_2$-0.1 and Na$_2$CO$_3$-10.0; pH 10.0), MSS2 (K$_2$HPO$_4$-4.0, NaCl-4.0, MgSO$_4$.7H$_2$O-0.2, CaCl$_2$-0.1 and Na$_2$CO$_3$-8.0; pH 10.0), MSS3 (K$_2$HPO$_4$-2.0, NaCl-1.0, MgSO$_4$.7H$_2$O-0.1, CaCl$_2$-0.05 and Na$_2$CO$_3$-5.0; pH 10.0), MSS4 (K$_2$HPO$_4$-2.0, NaCl-1.0, MgSO$_4$.7H$_2$O-0.05, CaCl$_2$-0.03 and Na$_2$CO$_3$-5.0; pH 10.0), MSS5 (K$_2$HPO$_4$-1.0, NaCl-0.5, MgSO$_4$.7H$_2$O-0.05, CaCl$_2$-0.02 and Na$_2$CO$_3$-5.0; pH 10.0), were added as additive mineral nutrients and production of lipase was monitored. The data suggested that MSS5 was better among other media’s used for the studies.
Table-17: Effect of different Media’s for the production of lipase by *Micrococcus luteus* KAR21.

<table>
<thead>
<tr>
<th>Media</th>
<th>Lipase activity U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSS1</td>
<td>199</td>
</tr>
<tr>
<td>MSS2</td>
<td>212</td>
</tr>
<tr>
<td>MSS3</td>
<td>273</td>
</tr>
<tr>
<td>MSS4</td>
<td>296</td>
</tr>
<tr>
<td>MSS5</td>
<td>342</td>
</tr>
</tbody>
</table>

Graph-7: Represents the effect of different mineral salts for lipase production.

4.6.1(ii)(a) Effect of Additives

Different carbon and nitrogen sources, additives were added into the media to study their effect on production of lipase by using *Micrococcus luteus* KAR21. The data suggested that addition of tributyrin has induced the production of lipase better.
than other additives. The lipase production was observed to be maximum with tributyrin by production of 482 U/ml.

Table-18: Effect of addition of additives for production on lipase

<table>
<thead>
<tr>
<th>Additives</th>
<th>Lipase activity U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>312</td>
</tr>
<tr>
<td>Peptone</td>
<td>378</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>357</td>
</tr>
<tr>
<td>Sucrose</td>
<td>290</td>
</tr>
<tr>
<td>Tributyrin</td>
<td>482</td>
</tr>
<tr>
<td>Tween 80</td>
<td>411</td>
</tr>
</tbody>
</table>

Graph-8: Represents the effect of additives on lipase production by *Micrococcus luteus* KAR-21
4.6.1(ii)(b) Effect of Temperature

Incubation temperature is considered as one of the most essential growth requirements for any microbe and associated metabolite production during bioprocess. It is well known that suitable growth temperature in the presence of nutrients provides the organism a favourable environment to grow, propagate and allow the accumulation of metabolites. Generally, temperature influences the metabolic activity of every cell. The influence of incubation temperature on lipase production was studied in the range of 37° to 55°C by Micrococcus luteus KAR21. Quantitative analysis of lipase revealed that Micrococcus luteus its accumulation had increased with an increase in temperature up to 37°C and decreased on increasing the temperature beyond 37°C.

The effect of temperature on growth of isolate and production of lipase was monitored and 37°C was found suitable as culture turbidity was more and activity of lipase was also recorded maximum.

Table-20: Effect of temperature on lipase production by KAR21 was monitored at 37°C and activity of lipase was also recorded maximum.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Lipase activity U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>437</td>
</tr>
<tr>
<td>45</td>
<td>373</td>
</tr>
<tr>
<td>55</td>
<td>78</td>
</tr>
</tbody>
</table>
Graph-9: Represents the effect of temperature on lipase production.

4.6.1(ii)(c) Effect of Incubation time

The optimum production time was screened by incubating the flasks for different time. The maximum production was found in 24 h which declined at 48 h and a sharp decrease was found at 72h.

Table-21: Effect of incubation time for production of lipase at 24h.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Lipase activity U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>449</td>
</tr>
<tr>
<td>48</td>
<td>321</td>
</tr>
<tr>
<td>72</td>
<td>98</td>
</tr>
</tbody>
</table>
Graph-10: Represents the effect of incubation time taken for the production of lipase by *Micrococcus luteus* KAR21.

4.6.1(ii)(d) Effect of Moisture

To determine the water content of soil sample by oven drying method/ sand bath method/ rapid moisture meter method.

5 g of seed cake was moistened with different volumes of media so that the effect of moisture on production of lipase could be monitored. Initial level of moisture was not effective, later the increased moisture levels have induced the production of enzyme, though a decline was noted at 1:2 levels.

**Table-22: Effect of different seed cakes moisture content for lipase production.**

<table>
<thead>
<tr>
<th>Moisture level</th>
<th>Lipase activity U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>01:0.5</td>
<td>142</td>
</tr>
<tr>
<td>01:01</td>
<td>466</td>
</tr>
<tr>
<td>01:02</td>
<td>433</td>
</tr>
<tr>
<td>01:04</td>
<td>364</td>
</tr>
</tbody>
</table>
Graph-11: Represents the effect of moisture content studies for the production of lipase.

4.6.1(ii)(e) Effect of Inoculums

The critical volume of inoculums required to initiate culture growth, due to the diffusive loss of cell materials into the medium. The subsequent culture growth cycle is dependent on the inoculums size, which is determined by the volume of medium and size of the culture vessel.

Different volumes of inoculums were added into the production mixture to monitor the effect on production of lipase.

Table-23: Effect of different volumes of inoculums suitable for production.

<table>
<thead>
<tr>
<th>Inoculums volume (ml)</th>
<th>Lipase activity U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>296</td>
</tr>
<tr>
<td>0.5</td>
<td>388</td>
</tr>
<tr>
<td>0.75</td>
<td>467</td>
</tr>
<tr>
<td>1</td>
<td>488</td>
</tr>
</tbody>
</table>
4.6.2 Purification of lipase enzyme

Lipases have been extensively purified and characterized in terms of their activity and stability profiles relative to pH, temperature, and effects of metal ions and chelating agents. In many cases, lipases have been purified to homogeneity and crystallized. Purification methods used have generally depended on nonspecific techniques such as precipitation, hydrophobic interaction chromatography, gel filtration, and ion exchange chromatography.

The enzyme was purified to homogeneity by ammonium sulphate precipitation and dialysis, sephadex column, ion-exchange chromatography and SDS-PAGE.

Lipase was purified to by a combination of DEAE-Sephadex G-75 ion exchange chromatography.

- The lipase activity assayed in the dialyzed extract was found to be 1.18 U/ml.
4.6.2 Column purification

4.6.2(a) Gel filtration column

Gel filtration chromatography is a separation technique based on size. It is also called molecular exclusion or gel permeation chromatography. In gel filtration chromatography, the stationary phase consists of porous beads with a well-defined range of pore sizes. The stationary phase for gel filtration is said to have a fractionation range, meaning that molecules within that molecular weight range can be separated. Proteins which are small in size are enough to fit inside all the pores in the beads and are said to be included. These small proteins have access to the mobile phase inside the beads as well as the mobile phase between beads and elute last in a gel filtration separation.
The dialyzed protein was located on a sephadex G-75 column for separation; the fractions were collected in 25 fractions of 2ml each.

The sephadex fractions were screened for lipase activity by performing the lipase assay method. The enzyme was eluted into all 25 fractions. The active fraction of highest O.D (i.e. 5-15) were selected for further study and stored in the deep freezer.

Graph-13: Elution profile of lipase from gel-filtration column
4.6.2(b) Ion-exchange column purification

Totally 4ml of the active fractions collected from gel filtration column purification, was loaded on to DEAE cellulose columns. 25 fractions were eluted of 2ml each (Fig.3).

The eluted DEAE fractions were screened for lipase activity by performing the lipase assay method the enzyme was eluted in to all the 25 fractions. The active fraction of highest O.D (i.e. 14-19) were selected for further study and stored in the deep freezer.
Graph-14: Elution profile of lipase from ion-exchange column

Fig-15: Showing ion exchange chromatography
Table-24: Purification of lipase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (Units/ml)</th>
<th>Protein Concentration (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Fold purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>1.17</td>
<td>4.51</td>
<td>14.86</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Dialysis</td>
<td>1.8</td>
<td>7.31</td>
<td>9.16</td>
<td>162.14</td>
<td>61.66</td>
</tr>
<tr>
<td>Ion-exchange</td>
<td>69.8</td>
<td>3.45</td>
<td>19.40</td>
<td>76.57</td>
<td>130.56</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>666.38</td>
<td>1.83</td>
<td>36.54</td>
<td>40.66</td>
<td>245.87</td>
</tr>
</tbody>
</table>

4.6.2(c) SDS-PAGE analysis

The separation of macromolecules in an electric field is called electrophoresis. An extremely regular technique for separating proteins by electrophoresis utilizes an irregular polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The technique is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Protein separation by SDS-PAGE can be utilized to appraise relative molecular mass, to determine the relative abundance of significant proteins in a specimen, and to decide the distribution of proteins among portions. The purity of protein tests can be evaluated and the progress of a fractionation or purification procedure can be followed. Different staining strategies can be utilized to identify rare proteins and to learn something about their biochemical properties. Specific procedures, such as Western blotting, two-dimensional electrophoresis, and peptide
mapping can be utilized to identify extremely scarce gene products, to know similarities among them, and to distinguish and separate iso-enzymes of proteins.

4.6.2(d) Molecular mass versus molecular weight

The PAGE analysis indicated three protein bands in the ion-exchange active fractions. The proteins were of molecular weight ~25kd (Fig.16).

![PAGE analysis of Lipase](image)

**Fig-16: PAGE analysis of Lipase**

4.7 Cloning of lipase gene by *Micrococcus luteus* KAR-21

Cloning of the lipase gene obtained from *Micrococcus luteus* KAR21. For cloning the gene encoding lipase, gene bank of *Micrococcus luteus* KAR21 was constructed by cloning the partially digested genomic DNA in Escherichia coli using vector pUC19 and screening the gene bank for the lipase gene(s) by direct expression. DNA was eluted with 50-60µl of elution buffer or nuclease free water. A single isolated bacterial colony was picked from the plate and inoculated in to 5ml of LB broth containing ampicillin and incubated overnight at 37°C. Overnight culture was taken in 1.5ml polypropylene tube and cells were pellet down by centrifugation at 10,000rpm for 10mins. Supernatant was transferred into new tube and then the pellet was washed with 70% ethanol and air dried to remove traces with ethanol.
Pellet was dissolved in 30µl of TE buffer containing RNase and then the mixture was incubated at room temperature for 20 minutes.

4.7.1 PCR amplification of the Lipase Gene

The PCR products were electrophoresed on 0.7 % agarose gel. Based on the Micrococcus luteus KAR21 strain lipase gene sequence the amplified PCR product showed approximately 371bp as shown in the fig.18. The optimum PCR reaction condition for the detection of the lipase gene in Micrococcus luteus KAR 21 strain included an annealing temperature of 55º C and MgCl2 concentration of 2.mM, so the present study, the sample were amplified under these optimized conditions and the electrophoresed. The PCR product was further purified for the cloning purpose.
4.7.2 Cloning of Lipase Gene

In the present study, the amplified gene product is purified and cloned in cloning vector PTZ57R/T vector, the cloning first of all the product is ligated using 1:3 inserts vector ratio and further transformed in DH5α. The recombinance as showed as blue and white colonies on the plate show in the fig-19.
The screening of the cloned colonies further processed using TBA plate assay. Among cultures of the transformed colonies screened for lipase activity. The random selected colonies 2, 4, 6, 8 showed clearance zones and the zone showed was prominent.

These cultures were thus taken as positive for the lipase activity. The quantification of enzyme activity of the clones were showed 0.272, 0.26, 0.248, 0.252 U/ml respectively.

Fig-19: Plate showing the white colonies which represents the cloned genes.