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# Extracellular Lipase Production and Estimation from Staphylococcus and Aspergillus Species Isolated from Oil Contaminated Soil

Pallavi Singh<sup>1\*</sup>, Diksha Srivastava<sup>2</sup>, Diksha Kumari<sup>2</sup>, Samita Tiwari<sup>2</sup>

Head of Department, Department of Biotechnology, IILM Academy of Higher Learning, Greater Noida, India<sup>1</sup>

B.Tech Students (4th year), Department of Biotechnology, IILM Academy of Higher Learning, Greater Noida India<sup>2</sup>

**ABSTRACT:** Importance of enzyme is ever growing, specifically microbial lipases as they hydrolyze fats into fatty acids and glycerol, and reverse the action in non-aqueous media. Lipases are of great significance in fat and oil processing, detergents, pulp and paper industry, oleochemical industry, cosmetics and perfumery. They are also used in biosensors. Lipases are produced by various microorganisms such as Bacillus, Staphylococcus, Pseudomonas of bacterial genera and Aspergillus, Penicillium and Candida of fungal genera. Isolation of lipolytic microbes was done from oil contaminated soil and Rotten samples of coconut and Onion. Both the microorganisms were screened on enrichment media followed by morphological tests and biochemical tests which included Tributyrin Agar Test, Catalase test, and Triple Sugar Iron test followed by Gram staining to characterize and identify the type of lipolytic microorganisms present. Isolation, Screening and Identification of lipolytic bacteria was followed by production of lipase in various kinds of mineral media having diverse types of oil sources like Olive oil, Coconut oil, Groundnut oil and Castor oil. Quantitative estimation of lipase produced under submerged culture conditions on these various sources under different pH and temperature conditions was carried using titration. Results obtained suggest that the highest lipase production is seen in ground nut oil at pH 7 with culture of Staphylococcus species and in case of media inoculated with Aspergillus species, maximum lipase production is observed in olive oil at pH 5. Current studies have significantly generated avenues for accentuating lipase production with microbial cultures of Staphylococcus and Aspergillus species with the incorporation of groundnut oil in production media.

**KEYWORDS:** Lipase, Staphylococcus, Aspergillus, Tributyrin agar, Titration, Groundnut oil, Enrichment culture, Oil-spilled soil.

### I. INTRODUCTION

Lipases are serine hydrolases that hydrolyze fats into fatty acids and glycerol at the lipid-water interface. They reverse the action in non-aqueous media (Tembhurkar V. R et al, 2012). Under experimental conditions, lipases can hydrolyze reverse reactions including synthesis of esters by esterification, transesterification and interesterification (Franken, Eggert, Jaeger & Pohl, 2011). The use of enzyme-mediated processes can be traced to ancient civilizations. Today, nearly 4000 enzymes are known, and of these, about 200 are in commercial use. The majority of the industrial enzymes are of microbial origin (Godfrey and West, 1996). The demand of enzyme in the world is satisfied by 12 major producers and 400 minor suppliers. Around 60% of the total world supply is produced in Europe (R. Sharma et al, 2001). Due to the ability of biotransformation, lipases find an increasing range of applications such as in the detergent, food, pharmaceuticals, organic synthesis, cosmetics, and paper and pulp industry (M.P. Prasad January 2015).

The common lipase producing microorganism are Bacillus, Pseudomonas, Burkholderia and Staphylococcus, Aspergillus, Candida, Penicillium, Rhizopus, Zygosaccharomyces, Pichia, Lachancea Kluyveromyces, Saccharomyces, Candida, and Torulaspora (Afshin. Farahbakhsh et al, 2013). The most important characteristics of the lipase are its ability to utilize all mono-, di-, and tri-glycerides as well as the free fatty acids in transesterification, low product

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inhibition, high activity/yield in non-aqueous media, low reaction time, resistance to altered temperature, pH, alcohol and reusability of immobilized enzyme (Kumar A et al,2012) Bacterial lipases have a neutral or alkaline optimum pH with the exception of lipase from *P. fluorescence* SIK W1 that had an acidic optimum pH 4.8 (Bradoo S., et al,1999). The optimum temperature ranges of bacterial lipases are 30-60°C (Lithauer D. et al, 2002). Lipases form an inherent part of the industries ranging from food, dairy, pharmaceuticals, agrochemical and detergents to oleo-chemicals, tea industries, cosmetics, leather and in several bioremediation processes (Patil K.J et al, 2011).

Fat and oil is one of the prime areas in food processing industries where lipases modify the fatty acid chain in glyceride (Gupta. R et al, 2003). Enzymes had played important role in enhancement of quality of food products, flavour development, modifying the structure of inter or transesterification to increase nutritional values (Ghosh.P.K et al, 1996). Using lipases in detergents had added value in their increasing use in washing machine, where it impart softness, resiliency to fabrics, antistaticness, dispersible in water and mild to eyes and skin (Weerasooriya M.K.B et al 2012). Fermentation is a biological technique for converting substrate into simpler compounds by using microorganisms under controlled environmental parameters (Masoumeh. Ghasemi et al, 2013). Lipase production is influenced by the type and concentration of carbon source, type of culture medium, pH, temperature etc. Because of the extracellular production of lipases by the microorganisms as a result of metabolism, lipase extraction becomes easier from the fermentation culture (Luciane Maria Colla et al, 2014). The objective of this study was to conclude the best carbon source and pH for lipase production. For that, we isolated the lipolytic microorganism, bacteria from oil-spilled soil sample in nutrient broth media and fungi from rotten coconut in the potato dextrose broth media. Screening of isolated microorganisms was done on enrichment mineral media. Further biochemical tests were performed to check the lipolytic activity in microorganisms isolated after screening. Several biochemical tests performed were Gram staining, triple sugar iron agar test, tributyrin agar test and catalase test.

Gram staining is a staining technique used to differentiate bacterial species into two large groups that are gram positive and gram negative. Bacteria are differentiated on the basis of chemical and physical properties of their cell walls by detecting peptidoglycan, present in the cell wall of gram positive bacteria. Gram positive bacteria possess a thick cell wall made of peptidoglycan (50-90% of cell envelope), whereas gram negative bacteria possess a thinner layer (10% of cell envelope). Gram staining is not used to classify archaea, as these microorganisms yield widely varying responses that do not follow their phylogenetic groups (Sridhar Rao P.N). The Triple Sugar Iron test is a microbiological test which is used to test the ability of microorganisms to produce hydrogen sulfide (M. Patel et al, 2016). TSI agar medium was developed based on Kligler's iron agar. Bacteria that ferment any of the sugar will produce by-products. These by-products are usually acids, which will change the colour of the red pH sensitive dye to yellow colour. Some bacteria utilize thiosulfate anion as a terminal electron acceptor, reducing it to sulfide. If this occurs, the newly formed hydrogen sulfide reacts with ferrous sulfate in the medium to form ferrous sulfide, which is visible as a black precipitate (G.Narasimha et al, 2010). Tributyrin agar is a differential medium that tests the ability of an organism to produce an exoenzyme, called lipase that hydrolyses tributyrin oil. Lipases break down lipids and tributyrin oil is a type of lipid called a triglyceride (Davender Kumar et al, 2012). Tributyrin oil forms an opaque suspension in the agar. When an organism produces lipase and breaks down the tributyrin, a clear halo surrounds the areas where the lipase producing organism has grown. Catalase is an enzyme, produced by microorganisms that live in oxygenated environments and neutralize toxic forms of oxygen metabolites, hydrogen peroxide. Catalase enzymes are not found in anaerobes. Catalase brings the breakdown of hydrogen peroxide into oxygen and water. Catalase positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor (Doaa El Hadeedy et al). After that carbon sources and pH were varied to assess the best carbon source and optimum pH for the production of lipases by measuring the lipase activity titrimetrically. Titration is a method of quantitative chemical analysis which is used to determine the unknown concentration of a known analyte (Annalisa Adele).

## II. RELATED WORKS

Tembhurkar V. R. et al had isolated staphylococcus species from oil contaminated soil which was used for lipase production and enhanced by UV mutagenesis. Optimum pH and temperature and effect of trace elements were

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observed. M. P. Prasad had observed the effect of different growth media and conditions on lipase production by staphylococcus aureus. Sabiha Naz had studied the estimation of lipase production capability of Some Fungal Species and their application in oil spillage degradation. Margarita Stoytcheva had studied on analytical methods for lipases activity determination. M.Veerapagu et al had isolated produced and optimized bacterial lipase for industrial production.

## III. MATERIAL AND METHOD

**i]Collection of sample-** Oil-spilled soil sample was collected aseptically from petrol pump of Pari chowk Greater Noida and oil mill of Kanpur. Rotten coconut was collected from the local shop in Greater Noida.

### ii]Isolation of lipolytic microorganisms

**From oil spilled soil sample-**The collected oil-spilled soil sample were serially diluted to 10 folds. 5ml of 10th fold serially diluted soil sample was inoculated in the nutrient broth media and incubated in orbital incubator shaker at 37°C for overnight at 100 rpm. After that culture plates were made for oil spilled soil sample by streak plate method, incubated at 37°C overnight. Further single colony was picked up from the culture plates of oil spilled soil sample and streaked on nutrient agar media.

**From rotten coconut sample-**1gm of rotten coconut sample was inoculated in potato dextrose broth (PDB) media and incubated at 30°C for 7 days at 100 rpm. Culture plates were made for rotten coconut sample by spread plate method for 5 days at 30°C and 100rpm. Further single colony was picked up from the culture plates of rotten coconut sample obtained and streaked on potato dextrose agar (PDA)media.

### iii] Screening of lipolytic microorganisms

#### A]Enrichment culture test

**For oil contaminated soil sample-**Enrichment mineral media (100ml) was prepared containing ammonium dihydrogen orthophosphate, potassium chloride, magnesium sulphate, yeast extract, olive oil and bromophenol blue. Supplemented the media with 0.5% olive oil in first two flasks, 1% olive oil in another two, 1.5% olive oil in next two and 2% olive oil in last two flask and autoclaved at 121°C, 15 psi for 20 minutes. Picked up the single colony from nutrient agar plates with a sterile loop and added to the flasks. These flasks were incubated in orbital incubator shaker at 37°C for 5 days at 100 rpm and observed.

**For rotten coconut sample-**Enrichment mineral media was prepared containing ammonium dihydrogen orthophosphate, potassium chloride, magnesium sulphate, yeast extract, olive oil and bromophenol blue. Supplemented the media with 1% olive oil in first flask, 2% olive oil in second flask, 3% olive oil in third flask and 4% olive oil in fourth flask and autoclaved the media at 121°C, 15 psi for 20 minutes. Picked up the single colony from potato dextrose agar plates with a sterile loop and added to the flasks. These flasks were incubated in orbital incubator shaker at 30°C for 5 days at 100 rpm and observed.

**B]Morphological analysis-**Colonies were picked from the culture plates of oil contaminated soil sample and rotten coconut sample and smeared on two cleaned glass slides and observed under the compound microscope.

#### C]Biochemical tests-

**TSI agar test-**TSI agar media (150ml) was prepared using beef extract, yeast extract, peptone, protease peptone, lactose, glucose, sucrose, ferrous sulphate, sodium chloride, phenol red, agar, sodium thiosulphate and then autoclaved at 121°C, 15psi, for 20 minutes. Then the media was cooled and TSI slants were made in culture tube at a slanted angle for soil sample and for rotten coconut sample. Inoculation was done on the slants by the culture obtained from enrichment culture having maximum colour change. These slants were incubated in BOD incubator at 37°C & 30°C for oil-spilled soil sample and rotten coconut sample overnight, respectively.

**Tributyryl agar test-**Tributyryl agar media (150ml) was made using tributyrin oil, yeast extract, agar and then autoclaved at 121°C, 15psi, for 20 minutes. Culture plates were made by dividing the media equally in the culture rotten coconut obtained from enrichment culture having maximum colour change. The culture plates were placed in BOD incubator at 37°C & 30°C for oil-spilled soil sample and rotten coconut sample overnight, respectively.

**Gram's staining (for bacteria)-**In a clean glass slide smear the loop full of culture and heat fixed it. A crystal violet solution was added drop wise, after 1 min it was washed under running water. Then Gram's iodine solution was added

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drop wise, after 1 min it was washed under running water. Then acetone-alcohol decolouriser was added in drop wise manner, after 30 seconds it was washed under running water. Then the glass slide was flooded with safranin and washed under running water. Observed the glass slide under compound microscope.

**Catalase test-**A small amount of culture was transferred onto a clean and dry slide by using a sterile loop. A drop of hydrogen peroxide was added on the slide and observed.

### iv]Lipase production-

**Lipase production in submerged culture by varying carbon source (for both samples)**-Prepared the mineral media (100ml) containing different carbon sources as olive oil, coconut oil, groundnut oil and castor oil, peptone, yeast extract, glucose, sodium chloride, and magnesium sulphate. Autoclaved the media at 121°C for 15 minutes. After cooling, inoculated the media with one loop full of culture from the plate of oil spilled soil sample and another with rotten coconut sample. Incubated the flasks in an orbital incubator shaker at 37°C & 30°C for oil-spilled soil sample and rotten coconut sample respectively for 8 days at 150rpm. Results were observed.

**Lipase production in submerged culture by varying pH from 5-9 (for both samples)**-Prepared the mineral media (100ml) containing carbon source as olive oil, peptone, yeast extract, glucose, sodium chloride, and magnesium sulphate. Maintaining the pH of the flasks as 5, 6, 7, 8 and 9 for 2% olive oil concentration for soil sample and 3% olive oil concentration for rotten coconut sample and autoclaved the media. Add a loop full of bacteria from the plates obtained on enrichment culture having maximum colour change (2% olive oil concentration for soil sample and 3% olive oil concentration for rotten coconut sample). Incubated the flasks in an orbital incubator shaker at 37°C & 30°C for oil-spilled soil sample and rotten coconut sample respectively for 8 days at 150rpm. Results were observed.

**v] Estimation of lipase activity titrimetrically**-Samples were collected from the culture obtained from oil spilled soil sample and rotten coconut sample in two flasks. Centrifuged them for 15 minutes at 5,000rpm for extracellular lipase activity. Substrate media was made with olive oil, calcium chloride and phosphate buffer. 1ml of culture was added to the supernatant media. Placed the samples in orbital incubator shaker at 150rpm for 1 hour, 30°C. 20ml of acetone: alcohol (1:1) was added to the mixture so as to stop the reaction. Titrated the liberated fatty acids with 0.1N NaOH and phenolphthalein as an indicator. Lipase activity was then calculated.

## IV. RESULT AND DISCUSSION

### i]Isolation of microorganisms from oil spilled soil and rotten coconut



Fig.1 showing culture of Staphylococcus isolated from oil contaminated soil by streak plate method on nutrient agar media.

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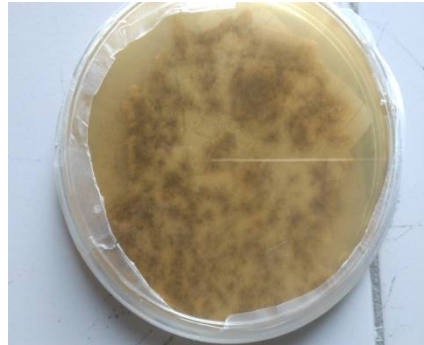


Fig.2 showing culture of Aspergillus isolated from rotten coconut by spread plate method on potato dextrose agar media.

### ii) Screening for lipolytic microorganisms

**Enrichment culture test**-Continued the enrichment until the colour of the medium changes from dark brown to yellow, indicating acidification as a result of lipolysis. Further, the medium showing highest colour change were cultured in plate by streak plate method.



Fig.3 (a) Before incubation



Fig.3(b) After incubation

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Fig.3 showing the positive result of screening of extracellular lipase producing microorganism using enrichment mineral media as the colour changes to yellow/ greenish yellow from culture grown in nutrient agar. Thus, indicating acidification as a result of lipolysis.

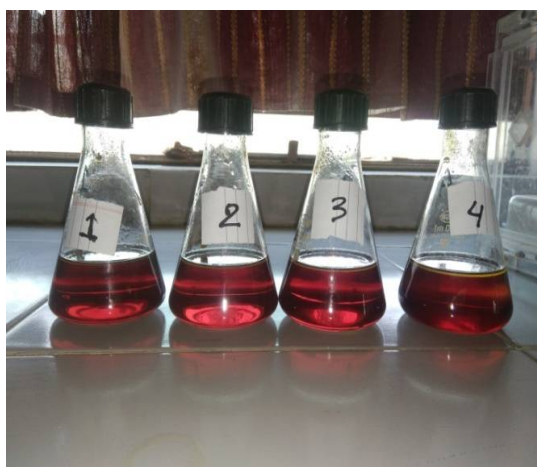


Fig. 4(a) before incubation



Fig.4 (b) after incubation

Fig.4 showing the positive result of screening of extracellular lipase producing microorganism using enrichment mineral media as the colour changes to yellow/ reddish yellow from culture grown in PDB agar plate. Thus, indicating acidification as a result of lipolysis.

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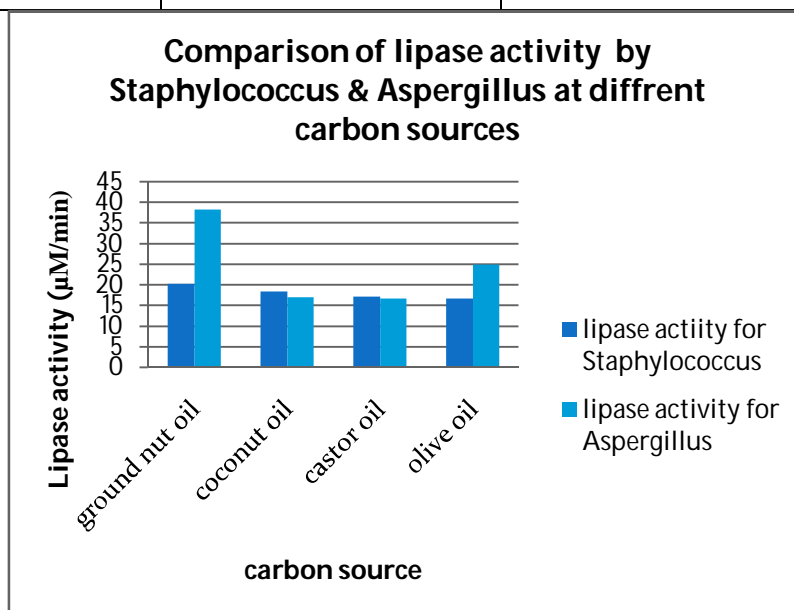
### Bio-chemical characteristics:

Biochemical tests	For oil contaminated soil sample	For rotten coconut sample
Tributylin agar test	+	+
Gram staining	+	-(only for bacteria)
Morphology	Cocci in clusters	Columnar, uniseriate conidial head
Triple sugar iron agar test	+	+
Catalase test	+	+

iii] **Lipase production & its estimation**-Lipase was produced by varying carbon sources such as ground nut oil, castor oil, coconut oil, olive oil for both the samples which shows ground nut oil is the best source among all the four sources chosen. Lipase was produced on olive oil at different pH varying from 5 to 9 for both the samples. After incubation for 7 days, lipase activity was maximum for *Staphylococcus* at pH 7 and for *Aspergillus* at pH 5.

### iv] Lipase production on different carbon source

Carbon source	EU of lipase ( $\mu\text{M}/\text{min}$ ) For <i>Staphylococcus</i>	EU of lipase ( $\mu\text{M}/\text{min}$ ) For <i>Aspergillus</i>
Groundnut oil	20.3	38.3
Coconut oil	18.5	17.2
Castor oil	17.3	16.7
Olive oil	16.7	25



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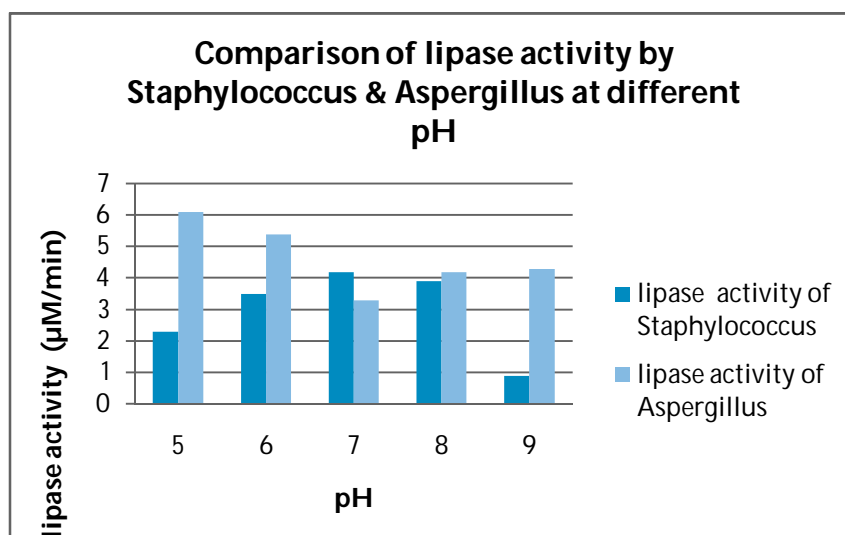
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### v]Lipase production at different pH

pH variations at 30°C		
pH	EU of lipase ( $\mu\text{M}/\text{min}$ ) For <i>Staphylococcus</i>	EU of lipase ( $\mu\text{M}/\text{min}$ ) For <i>Aspergillus</i>
5	2.3	3.35
6	3.5	5.46
7	4.2	7.7
8	3.9	4.24
9	0.9	4.32



### IV. CONCLUSION

The lipolytic microorganisms isolated and screened was identified by analysing the colour change indicating acidification as a result of lipolysis in enrichment media, the maximum colour change in the flask was plated on nutrient agar media for oil-spilled soil sample and for rotten coconut on potato dextrose agar (PDA) media and incubated followed by morphology analysis. The morphology for oil-spilled soil sample was cocci in clusters and for rotten coconut; the morphology was columnar, uniseriate conidial heads. Then various bio-chemical tests such as gram staining, triple sugar iron agar test, tributyrin agar test, catalase test were performed. The purple colour at the end of gram staining confirmed the presence of gram's positive bacteria. Further, the tributyrin agar test confirmed the presence of lipolytic microorganism which was cross checked by TSI test proved that sugar fermenting microorganisms were there in the sample. On performing catalase test, we got to know that the microorganisms were catalase positive. These tests confirmed the presence of *Staphylococcus* & *Aspergillus*.

Lipase was produced on mineral media using four different carbon sources namely ground nut oil, coconut oil, castor oil, and olive oil and varying the pH from 5 to 9, by *Staphylococcus* and *Aspergillus*. To estimate the lipase activity, titration of the sample with 0.1N NaOH gave different enzyme units for different carbon sources at different pH. The maximum lipase activity was observed in ground nut oil for *Aspergillus* at pH 5 and for *Staphylococcus* at pH 7. Hence, groundnut oil is the best carbon source for lipase production amongst olive oil, castor oil and coconut oil.



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## BIOGRAPHY



Dr.Pallavi Singh

Currently working as head in Department in Biotechnology at IILM Academy of Higher Learning, Greater Noida, U.P, India. She has enriched research experience in domain of Bioinformatics and Microbiology along with more than a decade, teaching experience at B.Tech and M.tech level. She has guided several projects at B.Tech and M.tech level.

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Ms. Diksha Srivastava

Currently she is an undergraduate student of B.Tech Biotechnology 4<sup>TH</sup> Year, at IILM Academy of Higher Learning, Greater Noida, U.P, India. She has done her summer internship from NICPO, Noida in division of molecular genetics and biochemistry.



Ms. Diksha Kumari

Currently she is an undergraduate student of B.Tech Biotechnology 4<sup>TH</sup> Year, at IILM Academy of Higher Learning, Greater Noida, U.P, India. She has done her summer internship from SHRM biotechnologies Pvt. Ltd., Kolkata in Molecular Biology and ESIC government hospital, New Delhi in microbiology.



Ms. Samita Tiwari

She is an Undergraduate student of B.Tech Biotechnology 4<sup>TH</sup> Year, at IILM Academy of Higher Learning, Greater Noida, U.P, India. She has done her summer internship from NICPO, Noida in division of molecular genetics and biochemistry. NICPO, Noida in division of molecular genetics and biochemistry