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THE SENSE OF DISTRIBUTION OF STAPHYLOCOCCUS AUREUS, ITS CHRACTRISTICS ON NUTRIENT AGAR, MANNITOL SALT AGAR, STEPH-110 MEDIUM AND BLOOD AGAR AND THEIR HEMOLYTIC PROPERTIES

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Abstract:
Microorganisms, which are largely found in our environment, play an important role in stabilizing ecosystems such as primary energy and basic cycle. Microorganisms are everywhere like sea salts, high air pressure, very high and low temperatures. They are abundant in highly contaminated areas due to resistance. These organisms can be classified by ordinary carbon and energy sources converted into amino acids, starches, nucleotides, vitamins and different oils. Catalysts are mechanically extracted from microbes to perform different metabolic processes that are present in the world, and their addition is mainly due to these organisms. Lipase catalyzing the breakdown of fats produced by many microorganisms, including Gram-positive Staphylococcus aureus (Staph.aureus). Twenty soil (n = 20) samples contaminated with oil were collected from 2 oil production industries in Rawalpindi and Faisalabad city (10 samples from each factory). All oil samples were taken in the sterile sample collection tubes with a pipette and transferred to the microbiology laboratory, in the Institute of Microbiology, Faculty of Veterinary Sciences, University of Agriculture, Faisalabad.

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INTRODUCTION:

Micro-organisms that are numerously present in our environment carried out important function to stabilize ecosystems like primary energy and elemental cycling. Considering the total cell biomass, cell numbers and species diversity, micro-organisms form the largest part of pool containing living organisms e.g. bacteria, archaea, eukarya include cell numbers as 6 $x10^{30}$, 1.3 $x10^{28}$, 3.1 x 10^{29}, respectively and cell biomass as 6 x 10^{18} kg, 1.3 x 10^{16} kg, 3.1 x 10^{17} kg respectively. Because of this diversity, micro-organisms form the largest gene pools which have importance in research applications and industries. (Beloqui, *et al.* 2008).
Micro-organisms are present at every place as in sea salts, at high air pressure, at very high and low temperature. They are also present in abundance in highly polluted areas due to their resistance. These organisms can be assorted out by ordinary carbon and energy sources which change into amino acids, starches, nucleotides, vitamins, and different fats. Catalysts are mechanically extracted from microbes to perform different metabolic process few present in the earth, and its addition is mainly due to these organisms. According to capacities of microbes to adjust themselves in verities of conditions and produce particular compounds, they have central focused in bio-production lines. (Sanchez 2005).

Lipase that catalyzes the breakdown of fats produces by many microorganisms including gram-positive Staphylococcus aureus (Staph.aureus). The word Staphylococcus drives from Greek word "staphyle" means clusters or bunches of grapes. This Staphylococcus divides itself in such a way to form grapes like clusters. S. Aureusis, the most dangerous form of staphylococci discovered in the 19th century. This S. Aureusis contains strains characterize by a golden-yellow pigment staphyloxanthin. This Staphylococcus has also critical in producing the coagulase protein which converts fibrinogen to fibrin use in coating the bacteria to protect the bacteria from an attack of host cells.
Claude Bernard, who was studied role of pancreas, firstly reported the Lipase and its activity in fat digestion in 1856 (Hassan *et al.,* 2006; Peterson and Drablos, 1994). This enzyme is glycoprotein in nature and breakdown the largest lipids to monomers with low molecular weight products, having range from 20,000 to 60,000 (Veerapagu *et al.,* 2013; He and Tan, 2006). Microbial lipase has more commercial significance than other sources (Hsu *et al.,* 2002). This widely used enzyme has more than 20% role in biotransformation (Gitlesen *et al.,* 1997). The characteristics that made it more industrial significant are its selectivity, stability, and broad substrate specificity (Griebeler *et al.,* 2011). Production of this enzyme has increased because of these characteristics in last year (Elibol and Ozer, 2000). This enzyme is also used in the treatment of waste water to save the marine life e.g. treat the residual water of the industry of leather (Subramanian*et al.,* 2012). Many otherworks of lipases are observed in pharmaceuticals, in detergents, in the product of cosmetics, food flavouring, fats processing, fat removal from skin and hides, clothing, industries of paper and diagnostic marker for triglyceride test. One of application is in soap industry (He and Tan, 2006; Snellman and Colwell, 2004).

MATERIALS AND METHODS:

3.1 Collection of oil samples

About twenty (n=20) oil contaminated samples from oil contaminated wastes were collected from 2 oil manufacturing plants (10 samples from each plant) situated in the city of Rawalpindi & Faisalabad. An industry (Darja-e-awal ghee) was located in Faisalabad city while other industry (Zaiqa ghee mill) was located in Rawalpindi city. All the oil samples were taken in the collecting tube with the help of pipette and transferred to the Microbiology lab. Of Institute of Microbiology, Faculty of veterinary Sciences, University of Agriculture Faisalabad, with ice boxes maintaining the temperature @ 4ºC. It is only due to the production of microorganisms which produced protein in nature enzymes and these enzymes might not be denatured. All the collected samples were kept in the refrigerator for further processes.

3.2 Cultivation and Isolation of Bacteria

3.2.1 Sample Processing and Cultivation

All the collected samples were refined (cultured) in nutrient broth for enhancement of bacteria avoiding from contaminations.

Nutrient broth preparation

1- Suspended 3.0 g Yeast extract, 5.0 g NaCl and 5.0 g Peptone in 1000ml deionized water.
2- Gently Mixed to obtain homogenous mixture.
3- Homogenized solution was autoclaved for about 15 minutes @ 121°C.
4- Then transferred into the test tubes.
5- Inoculation was started with the transferring the materials in nutrient broth.

Nutrient agar is only the medium for the microorganism's growth and it is used only for the cultivation of less fastidious microorganism.

Nutrient Media Preparation

1- 28 g were dissolved in 1 liter of deionized water.
2- Gently Mixed to obtain homogenous mixture

3- Homogenized solution was autoclaved for about 15 minutes @121°C.
4- Cooled @ 45°C.
5- Then transferred into petri plates.
6- Streaking was completed with the help of disinfected inoculation loop.
7- Plates were incubated for about 24 hours @ 37°C.

In the next step, the media (Seph-110) for cultivation of lipase producing *Staphylococcus aureus* was used. It is the only medium for differentiation and isolation of different Staphylococci based pathogenic for mannitol fermentation, pigmentation and gelatin liquefaction.

Preparation of Steph-110 media:

1- Dissolve75 g NaCl, 2.5 g yeast extract, 15.0g agar, 10.0 g tryptone, 3.0g gelatin 2g lactose 5.0 g dipotassium hydrogen phosphate and 10g Mannitol was dissolved into 1.0 liter deionized (distilled) water.
2- Gently Mixed to obtain homogenous mixture
3- Homogenized solution was autoclaved for about 15 minutes @ 121°C.
4- Cooled @ 45°C.
5- Then transferred into petri plates.
6- Streaking was completed with the help of disinfected inoculation loop.
7- Plates were incubated for about 24 hours @ 37°C.

3.2.2 Isolation of *Stephylococcus aureus*

Goldish, glittering, raised colonies.
These colonies were recognized on the bases of hemolytic properties with biochemical confirmation.

3.2.3 Blood agar Cultivation

To observe the hemolytic properties, blood agar was streaked with isolates.

Blood agar Preparation

1- About 28 g powder of nutrient agar was suspended in 1000 ml of deionized water.
2- Properly heated the mixture until powder was fully dissolved.
3- Homogenized solution was autoclaved for about 15 minutes @ 121°C.
4- Cooled @ 45°C.
5- Then transferred into petri plates for solidification. 50 ml blood sample of sheep was added.
6- Petri plates were incubated in anaerobic jar with 5% CO_2 after the process of inoculation for 48 hours.
7- Then observed the results.

3.2.4 Cultivation on Mannitol Salt Agar

Mannitol salt agar is selective medium for *Staphylococcus aureus.* Mannitol is specific sugar that have indicator. *Staphylococcus aureus* by the process of fermentation of mannitol wasproduced goldish colour, glistening and raised colonies.

Preparation of Mannitol salt agar

1- 1 g Beef extract, 75 g NaCl, 0.025 g Phenol red. 10 g Mannitol and 25 gm agar into were dissolved in 1000 ml of deionized water.
2- Properly heated the solution until the power was fully dissolved
3- Homogenized solution was autoclaved for about 15 minutes @ 121°C.
4- Cooled @ 25°C.
5- Transferred it into petri plates for solidification.
8- Petri plates were incubated in anaerobic jar with 5% CO_2 after the process of inoculation for 48 hours.
9- Then observed the results.

3.3 Gram Staining

It was done to check the suspected isolated *Staphylococcus aureus* species.

Preparation of Slide Smear:

1- Put the drop of water with the help of loop on the glass slide.
2- A very little culture from petri plate was moved to the glass slide.
3- Culture was accurately mixed into drop of water.
4- Dried the culture.
5- Slide was heated on a flame for 3-4 seconds by moving 2 to 3 times in a circular motion.

Gram Staining:

1- Inundated the slide with Crystal violet. Slide was slightly washed with water after one minute.
2- With gram Iodine inundated the slide. Slide was slightly washed with water after one minute.
3- Slide was washed with acid alcohol. Slide was slightly washed with water after thirty seconds.
4- With Safranin again inundated the slide with counter stain Safranin. Slide was slightly washed with water after sixty seconds.
5- Dried the slide
6- Observed by using oil immersion under microscope.

3.4 Microscopy

Stephlococci appeared in round and grape like cluster as gram positive under the microscope. These *Stephlococci* were further characterized on the basis of biochemical tests.

3.5 Screening of *Staphylococcus aureus* for Lipase Production

After determined Staphylococcus *aureus*, it was refined on Tributyrin agar. Which is specific media for the determination of microorganisms help in producing lipase. Tributyrin is naturally present in the butter. It is an ester composed of butyric acid and glycerol. Amongst other things; it is used as a constituent for making fat. It is found in butter and defined as a liquid fat with a bitter taste. It is also used in microbiological laboratories to recognize the Tributyrin is an unchanging and quickly absorbed prodrug of butyric acid which improves antiproliferative properties of dihydroxycholecalciferol in human colon cancer cells.

Preparation of Tributyrin agar

1- In 990 ml of deionized water 23 g was suspended.
2- 10ml Tributyrin agar was added.
3- Properly heated the solution until the powder was fully dissolved
4- Homogenized solution was autoclaved for about 15 minutes @ 121°C.
5- Shake the individual plate and flask to maintain uniformity.
6- Autoclaved Tributyrin agar was poured into petri plates for solidification.
7- Stored in dark place.

3.6: Biochemical Tests

The isolates were exposed for biochemical endorsement by Catalase and coagulase test.

3.6.1: Catalase Test

$2H_2O_2 \longrightarrow 2H_2O + O_2$ (gas bubbles)

Procedure

To determine the catalase enzyme by *Staphylococcus aureus* presence hydrogen peroxide was used. The cultures were established @ 35°C for 48 hours. Catalase test was achieved by affecting the growth from the plate of blood agar with a needle or a wooden stick on glass slide. A drop with 3% H_2O_2 was used for the cluster on the slide and noticed for bubbling that specifies +ve effect (Murray *et al.*, 2003).

3.6.2 Coagulase Test

Coagulase was produced by some bacteria. The capability to form coagulase is expected to be linked to the virulence of staphylococci and the test applied to differentiate between coagulase +ve and coagulase -ve staphylococci.

Procedure

The test was done by Hanging one colony from the assumed pure culture with disinfected loop in 0.5 ml of plasma from man, rabbit or horse on a glass slide, then it was incubated for four hours @ 37ºC. Plasma has a clumping factor and responsible for coagulation.

3.7 Statistical analysis:

The data thus obtained was subjected for statistical analysis thorough analysis of variance (ANOVA) technique (Montgomery, 2008).

RESULTS AND DISSCUSION:

During proposed study total 20 (n= 20) samples were collected from Zaiqa Ghee mil and Darja-e-awwal Ghee mil that situated in Rawalpindi and Faisalabad. 10 samples were collected from each oil mill. 10, 10 samples were taken from Zaiqa Ghee mil and Darja-e-awal Ghee mil, respectively.

4.1 Distribution of *Stephylococcus aureus*

A total of 4 isolates were found positive for *Stephylococcus aureus* out of 20 samples during proposed study. The overall prevalence of *Stephylococcus aureus* was 20% (Fig 4.1). it means that 2 isolates out of 10 were found in Zaiqa ghee mill and 2 out of 10 from Darja-e-awwal.

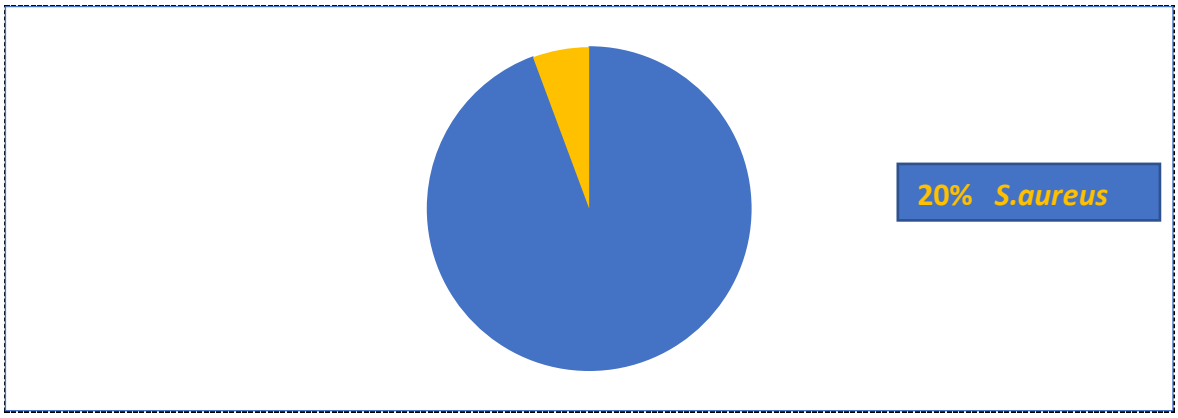

Fig 4.1 Distribution of *Stephylococcus aureus* in Faisalabad and Rawalpindi District.

4.2 Growth characteristics of *Stephylococcus aureus* on Nutrient broth

Fig 4.2 Growth of *S. aureus* into Nutrient broth

4.3 Growth characteristics of *Stephylococcus aureus* on Nutrient agar

After the cultivation of wastes effluents samples, *Stephylococcus* was obtained by culturing on Nutrient agar. Culture characteristics of isolates are described below.

Large, yellowish white, glistening colonies of *S. aureus* were obtained on Nutrient agar (Fig4.3)

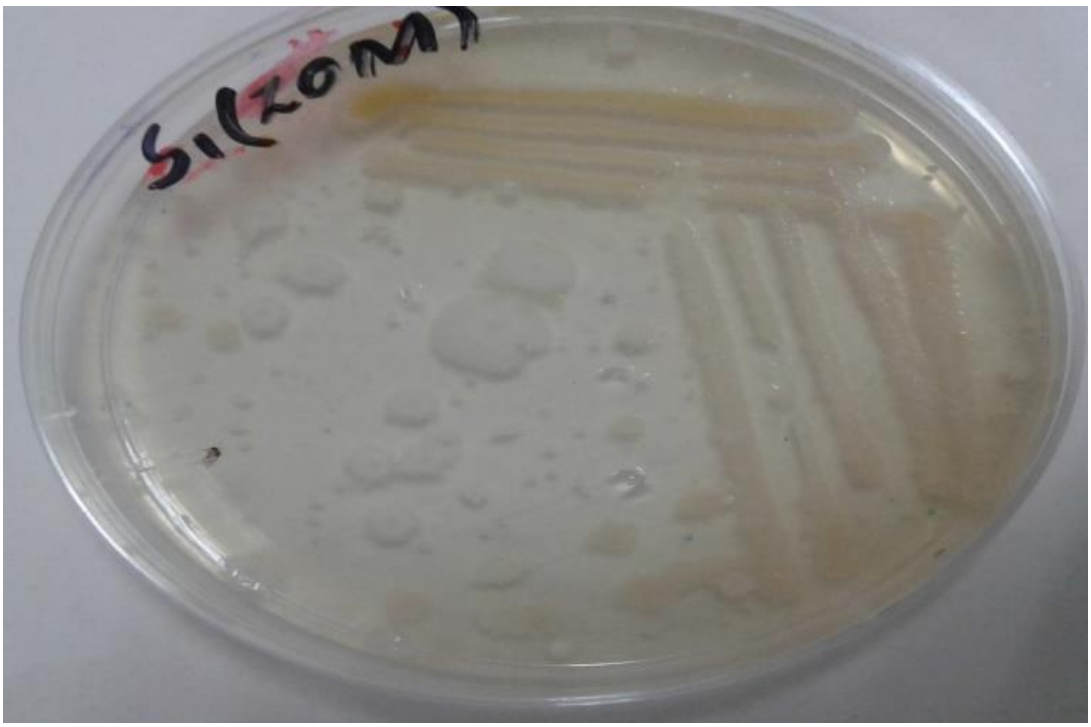

Fig 4.3 Colony morphology of *S. aureus*.

4.4 Growth characteristics of isolated *Stephylococcus aureus* on Mannitol Salt agar
Small, glistening, raised, golden colored colonies were obtained on Mannitol salt agar (Fig 4.4).

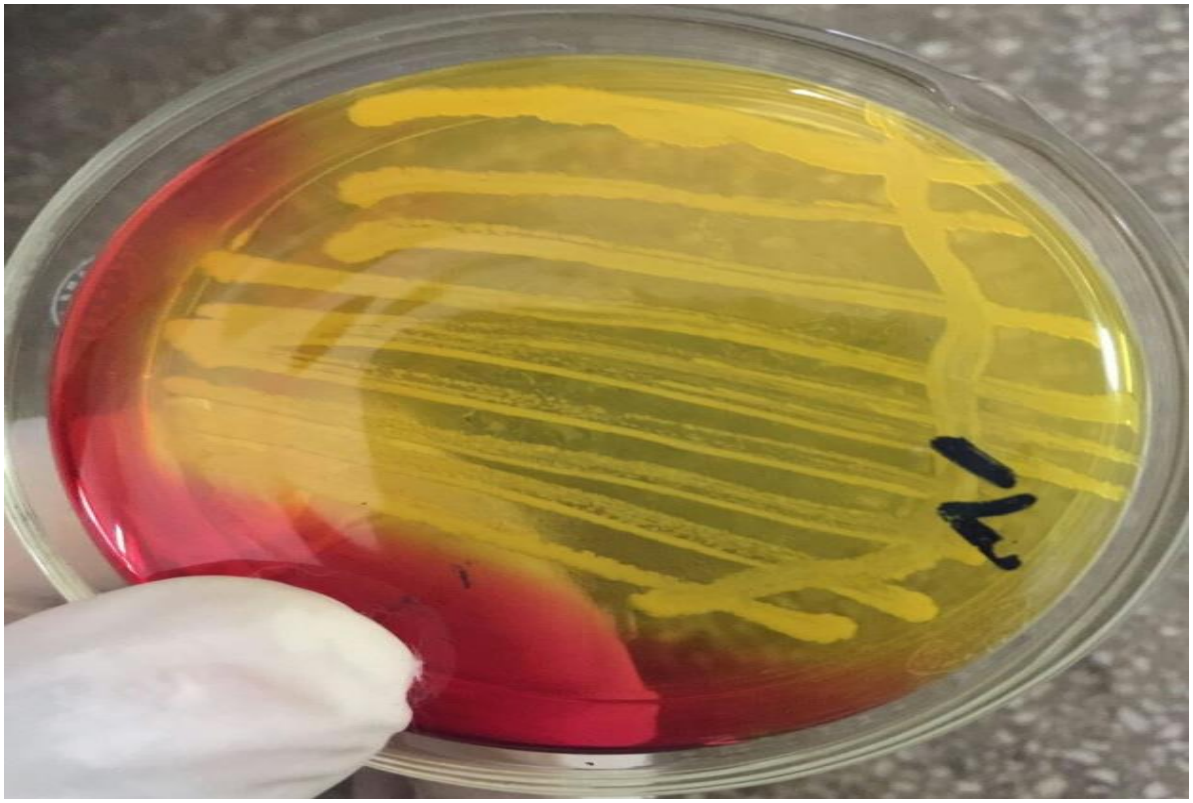

Fig 4.4 Colony morphology of *S. aureus*.

4.5 Growth characteristics of isolated *Stephylococcus aureus* on Steph-110 medium
Yellowish white color like colonies were obtained on Staph-110 medium (Fig 4.5).

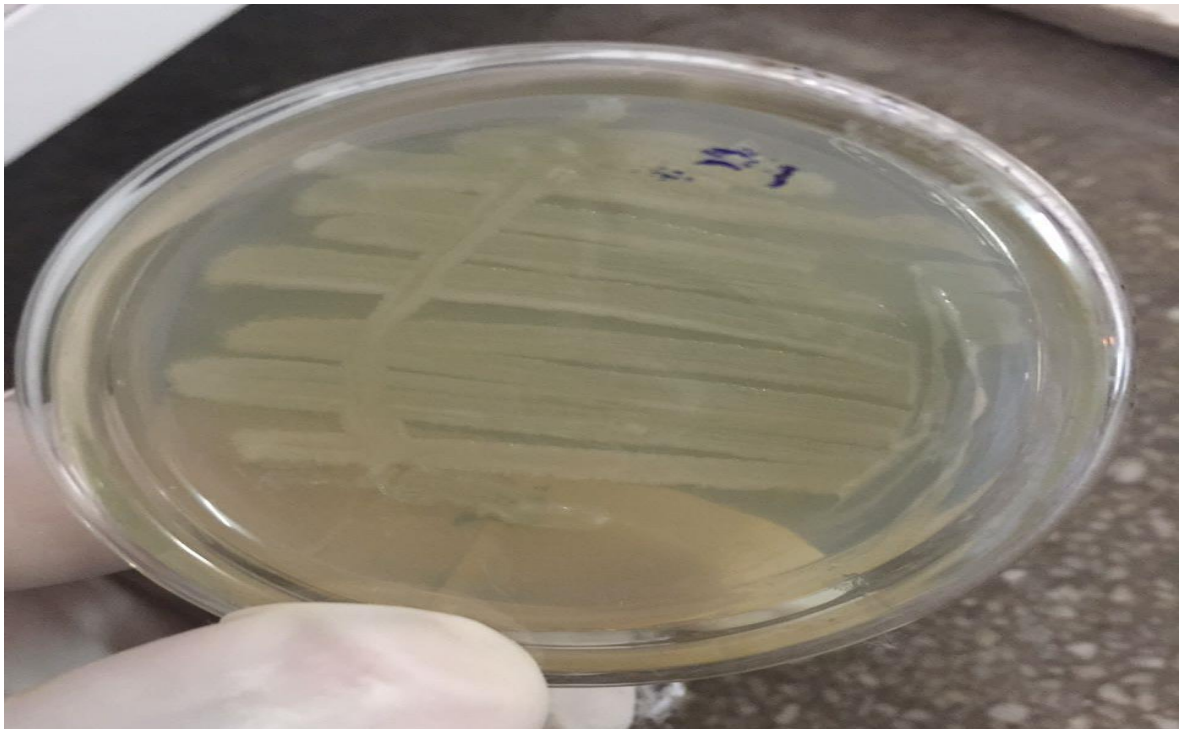

Fig 4.5 Colony morphology of *S. aures*.

Fig 4.6 B-hemolysis by *S. aureus*.

4.6: Growth characteristics of isolated *Stephylococcus aureus* on Blood agar and their hemolytic properties

These isolates were streaked on the Blood agar. Growth appeared as golden color colonies. Hemolytic properties of the isolates were as follows: (Fig 4.6).

4.7 Microscopic appearance of isolated *Stephylococcus aureus*

Under microscope these isolates were appeared as Gram-positive cocci, grapes like clusters form. Their microscopic features are as follows:

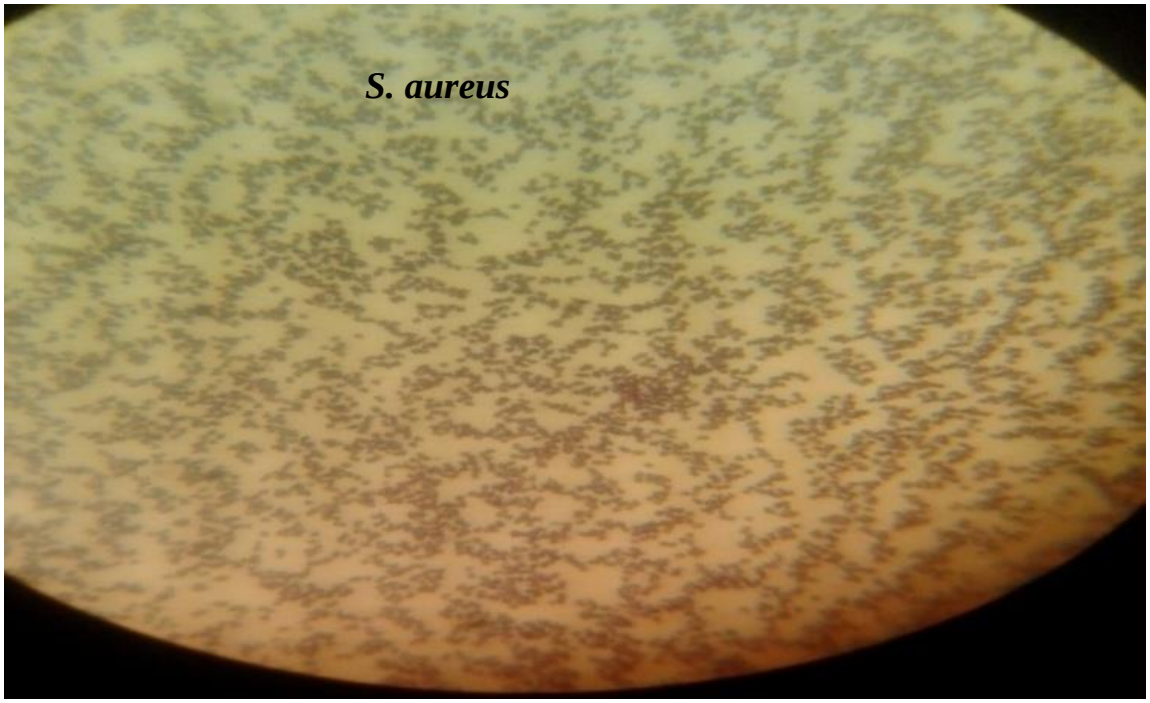

Fig 4.3 Microscopic features of *S. aureus* (Cocci in clusters).

4.8 Screening of isolates for lipase activity

Tributyrin agar is the specific media for identification of lipase enzyme. Afetr resolved *Stephylococcus aureus* was cultured on Tributyrin agar.By the hydrolysis of tributyrin agar,*S.aureus* produced zone of clearance(Fig 4.8)

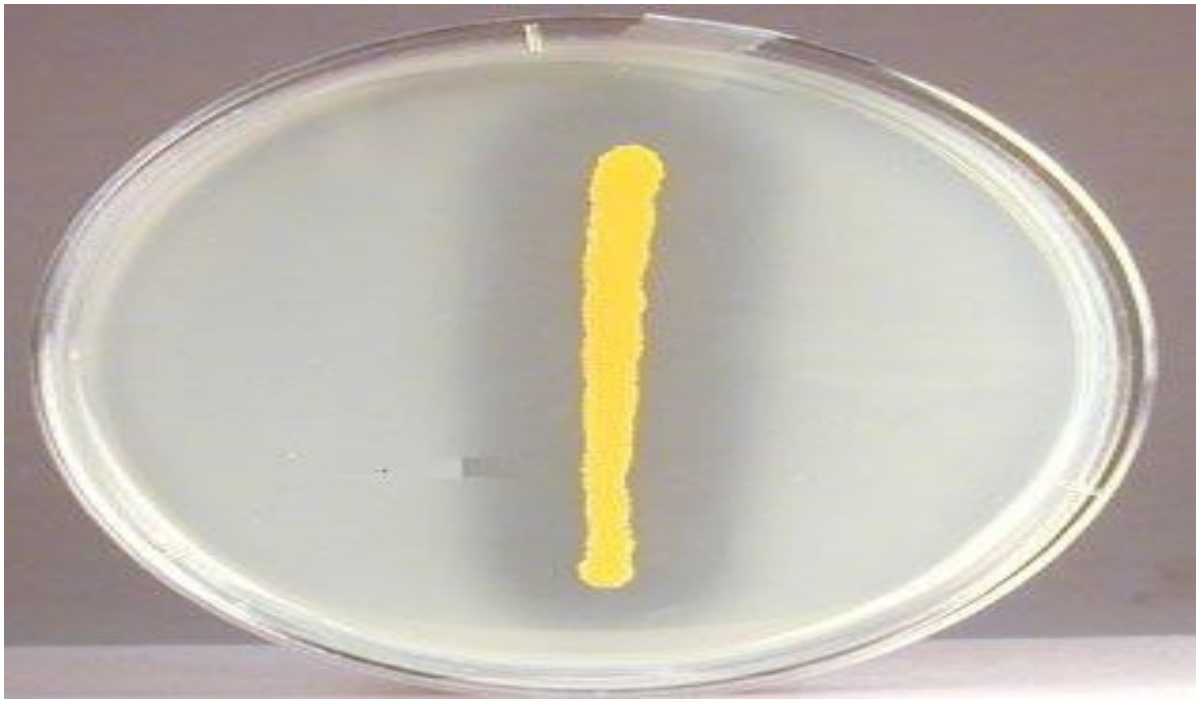

Fig 4.8 *S.aureus* produced zone of clearance

4.9 Results of Cultural/Morphological Characterization of Isolates

Table 4.9: Results of Cultural/Morphological Characterization of Isolates

No.of samples	Source Oil contaminated waste	General Media	Selective/Differential Media	Morphology	Positive for *Steph.aureus*
S1	Zaiqa Ghee mil	Nutrient Media	S-110 Mannitol salt agar Blood agar	Whitish yellow, glistening, Golden color colonies	S1 and S3 were +ve
S3		Nutrient Media	S-110 Mannitol salt agar Blood agar	Whitish yellow, glistening, Golden color colonies	
S12	Darja-e-awal Ghee mil		S-110 Mannitol salt agar Blood agar	Whitish yellow, glistening, Golden color colonies	S12 and S20 were +ve
S20			S-110 Mannitol salt agar Blood agar	Whitish yellow, glistening, Golden color colonies	

4.10 Biochemical Tests

Catalase test, Coagulase tests were performed for confirmation of *Stephylococcus aureus* (Table 4.10).

Table 4.10 Biochemical Tests for *Stephylococcus aureus*

Biochemical Tests	*S. aureus*
Catalase	+ve
Coagulase	+ve
Mannitol Fermentation	+ve
B-Hemolysis	+ve
Lipase	+ve

10.1 Catalase Test

Catalase test was performed to determine the catalytic activity of all isolates. Interestingly, all the isolates were found positive for catalase production which confirms that all the isolates are of *Stephylococcus aureus* (Table 4.10, Fig 4.10.1).

Fig 4.10.1 Catalase Test

4.10.2 Coagulase Test

Coagulase test was performed to confirm group *Stephylococcus aureus* among the *Steph.*spp. (Table 4.10, Fig 4.10.2).

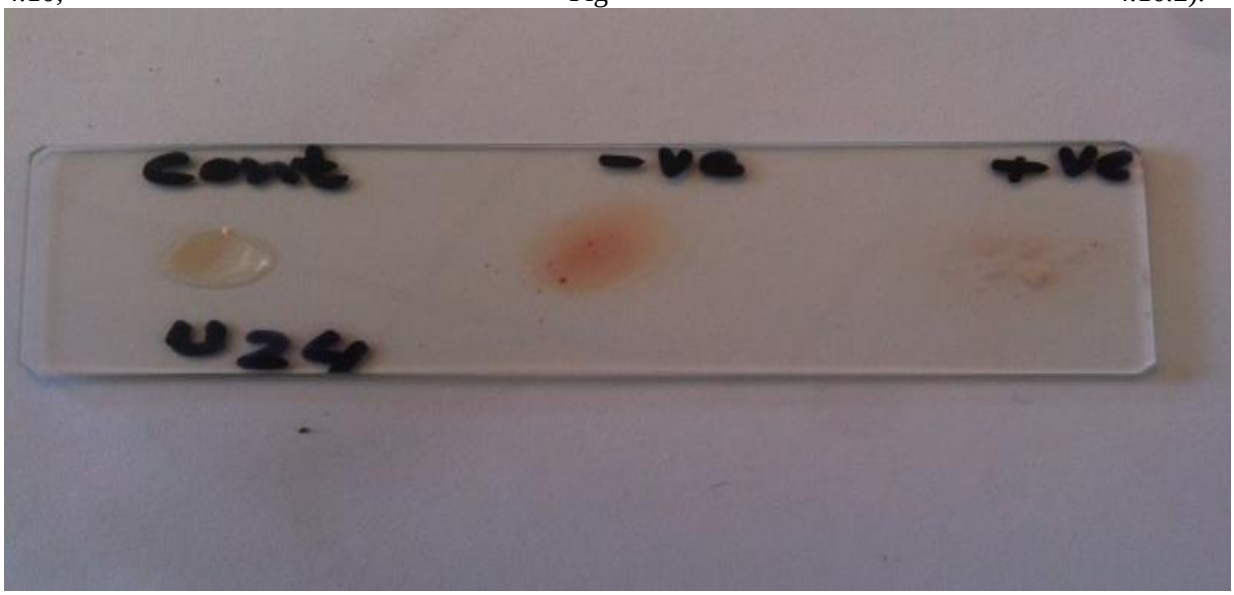

Fig 4.10.2 Coagulase Test

DISCUSSION:

The purpose of this study was to carry out the isolation and identification of lipase-producing bacterium *staphylococcus aureus*. For this study, soil samples of oil contaminated wastes were collected from two different industries, Darja-e-awwal oil mill and Zaiqa Ghee mill for the isolation of lipase-producing bacteria. About 10 represented sites of each factory were used for sample collection. Sample collection was handled by taking solid or liquid wastes into plastic bag and kept them at 4ºC before use. A total of 4 isolates were found positive for *Stephylococcus aureus* out of 20 samples during proposed study. The overall prevalence of *Stephylococcus aureus* was 20%. After the cultivation of wastes effluents samples, *Stephylococcus* was obtained by culturing on Nutrient agar. Large, yellowish white, glistening colonies of *S. aureus* were obtained on Nutrient agar. Total samples collected were twenty out of which four were positive for *Stephylococcus aureus*. It means that about 20 % of isolates for *Stephylococcus aureus* had shown positive results as compared to remaining samples.

These results are in line with Reza *et al.*,(2014) and Uhlman and Chapadgaonkar, (2013) who stated that the soil samples were successively diluted and were protected for isolation purpose i.e. nutrient agar and on their selective media like Staph 110, Ceirlmide agar, Bacillus selective agar. Fungus was full-grown on sabouraud agar. Pure cultures attained were divided for lipolytic micro-organisms on nutrient agar having olive oil, sabouraud agar having olive oil, rhodamine B agar have olive oil and spirit blue agar. When screening was completed then many micro-organisms such as Staphylococcus specie, Bacillus specie, Pseudomonas specie, and Aspergillus specie formed lipase *doe* for the development of clearing zones round the colonies on nutrient agar having olive oil. Rhodamine B agar and sabouraud agar consisting olive oil and spirit blue agar. Screening methods has been acquainted by Veerapagu *et al.*,(2013) and Kumar *et al.*,(2012a). Greater region for hydrolysis was noticed for *Bacillus ikheniformis* tracked by *Staphylococcus aureus, pseudomonas aeruginosa* and *Aspergiihts. Mot. Bacillus and non-farm's* formed extracellular lipase enzyme.

Identification was completed by biochemical, morphological and cultural features of the remote species as these methods were also described by Reza *et al.*,(2014) and Kanimozhi *et al.*,(2013). Gram-negative bacteria recognized were *pseudomonas acruginosa. Staphylococcus aureus* and *Bacillus licheniformis* was Gram +ve. *Bacillus licheniformis* identified as aerobic bacteria, spore forming and rod-shaped, was +ve for sugar fermentation tests also +ve for citrate utilization test, catalase test and voges proskauer test but -ve for indole test, coagulase test and methyl red test.

Description of lipase enzyme was taken @ different ranges of pH, temperature, incubation duration and dissimilar substrates. PH showed very significant role for the making of lipase because it effects the lipase activity and progress of micro-organisms. Extreme lipase yield from *Bacillusiidatniformis* @ 8 PH and lowest yield was @ 4 pH. It might be possible that theengine was sluggish in very basic medium due to variation in hydrogen ion concentration and it prevents the active site of the caloric and caused reduced activity of lipase. The

enzymes like *aeruginosa* and *pseudomonas* showed maximum yield @ 8 pH. *Staphylococcus aureus* showed highest yield @ 7 PH andmaximum production highest yield from *Aspergillus niger* was observed @ pH 7.

Effect of temperature was also studied on enzyme production. It was concluded that extreme enzyme action of *Bacillus licheniformis* was observed @ 60ºC temperature. It means that *Bacillus licheniformis* acted as a heat stable bacteria and formed heat stable enzyme with great industrial status e.g it used in any pharmaceutical industry. At very high and very low temperature enzymatic activities and cell biomass was inhibited and moisture % was also decreased @ very high temperature. Extreme lipase yield andenzymatic activities of *Pseudomonas aeruginosa* was observed @ 40°C. For *Staph: aureus* ideal temperature was observed and that was also @ 40°C and the temperature for *Aspergillus niger* yield was 30˚C. these results were described by Gaur *et al.*,(2012), Couto and Sanrornan, (2006), Padmapriya *et al.*,(2011), Naqvi *et al.*,(2012) and Kumar *et al.*,(2012b).

Optimal incubation period for *Bacillus licheniformis* was 48 hours, 72 hours for *Pseudomonas aeruginosa*, 48 hours and 72 hours for *Staphylococcus aureus* and *Aspergillus niger*, respectively reported by Kanimozhi *et al.*,(2013) and Kathirvan *et al.*,(2012).

Lipases are formed by microorganisms having excessive industrial status because of its selectivity, stability and vast substrate specificity (Gricbeler *et al.*,2011). In Pakistan, lipases from micro-organisms arc commercially very important (Hsu *et at.,* 2002). Bacteria which yield lipase are Gram-positive bacteria and Gram-negative bacteria, Gram-positive bacteria contains Bacillus which is rod-shaped bacteria, numerous species of Bacillus are Bacillus stearothermophillus, Bacillus megaterium, Bacillus subtilis, Bacillus cereus, Bacillus brevis, Bacillus acidocaldarius, Bacillus coagulans and Bacillus thermoleovorans etc reported by Kenneday and Rennarz, (1979), Nawani and Kaur, (2000), Gowland *et al.*, (1987), Janssen *et al.*, (1994), Lee *at al.*, (1999), Godtfredsen, (1990), Kim *et al.*, (1998), El- Shafei and Rezkallah, (1997) Holisto and Korpela, (1998), Hou, (1994), Rua *et al.*, (1998), Becker *et al.*, (1997) and Manco *et al.*, (1998).

Different species of bacteria Staphylococcus also formed lipase which contains Staphylococcus canosus, Staphylococcus epidermidis, Staphylococcus swarneri, Staphylococcus aureus and Staphylococcus hyicus reported by Tahoun *et al.*, (1985), Simons *et al.*, (1998), Lee and Yandolo, (1986), Van-Kampen *et al.*, (1998), Van-Oort *et al.*, (1989), Meens *et al.*, (1997) Farrell *et al.*, (1993) and Talon *et al.*, (1995). Sztajer *et al.*, (1988) reported that Streptococcus specie yielding lipase is Streptococcus lactic. Micro coccus lipase yielding specie is Micro coccus luteus (Hou, 1994). Diverse species of lipase yielding Propionibacterium are Propionibacterium granulosum and Propionibacterium acne (Sztajer *et al.*, 1988). Burkholderia mallei and Burkholderia glumae also yield lipase enzyme, Yeo *et al.*, (1998) and El-Khattabi *et al.*, (2000).

Many Gram +ve bacteria too yield lipase enzyme similar to many species of Pseudomonas which are Pseudomonas alcaligenes, Pseudomonas aeruginosa, Pseudomonas fluorescence, Pseudomonas fragi, Pseudomonas cepacian, Pseudomonas putida, Pseudomonas mendocina and Pseudomonas glumae reported by Hsu *et al.*, (2000), Mencher and Alford, (1967), Jaeger and Reetz, (1998), Frenken *et al.*, (1993), Lang *et al.*, (1998), Lee and Rhee, (1993), Lin *et al.*, (1995), Noble *et al.*, (1994), Guillou *et al.*, (1995) and Maragoni, (1994).

CONCLUSION:

It was concluded that the samples collected from the two factories showed significant results in the sense of distribution of *Staphylococcus aureus*, their characteristicson nutrient agar, characteristics onmannitol salt agar, growth characteristics on steph-110 medium and growth characteristics on blood agar and their hemolytic properties.There were about two isolates S1 and S3 from Zaiqa Ghee mil showed Positive signs for *Steph. aureus* while isolates S12 and S20 from Darja-e-awal oil mil also showed positive signs for *Steph. aureus.* Samples having Whitish yellow, glitering, golden color colonies in the all contaminated samples. These isolates were streaked on the Blood agar. Growth appeared as golden color colonies. Under microscope these isolates were appeared as Gram-positive cocci, grapes like clusters form. Tributyrin agar is the specific media for identification of lipase enzyme. After which *Staphylococcus aureus* was cultured on Tributyrin agar. By the hydrolysis of tributyrin agar, *S. aureus* produced zone of clearance. All the isolates were found positive by using catalase test for catalase production which confirms that all the isolates are of *Staphylococcus aureus.*

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