ISOLATION AND DETERMINATION OF ANTIBIOTIC SUSCEPTIBILITY OF *Enterococcus faecalis* FROM MALWA REGION

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In

Microbial Sciences

By

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DECLARATION

I declare that the project entitled “Isolation and determination of Antibiotic Susceptibility of *Enterococcus faecalis* from Malwa region." has been prepared by me under the guidance of Dr. Somesh Baranwal, Assistant Professor, and Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Dimple Garg has prepared her project report entitled “Isolation and determination of Antibiotic Susceptibility of *Enterococcus faecalis* from Malwa region” for the award of M.Sc. degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Biochemistry and Microbial Sciences (School of Basic and Sciences), Central University of Punjab, Bathinda.

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ABSTRACT

Isolation and determination of Antibiotic Susceptibility of *Enterococcus faecalis* from Malwa region.

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About 20 isolates of *Enterococcus faecalis* is isolated from six different places of Malwa region. They include isolate from Animal waste soil, Agriculture soil, Sewage soil and public place soil also. Isolation of *Enterococcus faecalis* from environment causes concern for diseases *i.e.* Endocarditis, UTI, neonatal infections due to maintaince of unhygienic conditions. The biochemical tests are performed and measure as presumptive results for *Enterococcus faecalis*. Antibiotic susceptibility test is performed to observe the susceptibility of strains for different antibiotics. Six different types of antibiotics such as Ampicillin, Piperacillin, Tetracycline, Rifampin, Streptomycin, and Gentamycin are used for Antibiotic Susceptibility test. Antibiotic Susceptibility test analyse that Gentamycin and Streptomycin are found more resistant towards Enterococcus faecalis than others in Malwa region. The susceptibility of strains for antibiotics are different due to the IS elements which are horizontal transfer to the other *Enterococcus faecalis* also formed biofilm which makes it more antibiotic resistant due to slower growth rate, metabolic activities and poor penetration rate.

(Dimple Garg) (Dr. Somesh Baranwal)

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"The woods are lovely, dark and deep; however, I have promises to keep, and miles to go before I sleep"- Robert Frost (Stopping by Woods on Snowy Evening).

These lines from Robert Frost's poem have always been inspirational for me, and so are they now. It has been wonderful two years in CUPB and adventurous 8 months. Now when I am ready to submit my project dissertation report, I would like to take this opportunity and acknowledge the effort, trust and support I have received all through my M.Sc. program, be it the staff, faculty members of the University or the students, both my batch mates and seniors.

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Our lives are filled with people around us who enrich our lives one way or the other. Time may perish our association but our contributions to one another will remain intact in our memories till the end of our times. Days may past and clocks may run by to their last, a promise made to a friend by a friend your memories would remain somewhere in corner of my heart from this day, till the day I last.

(Dimple Garg)

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviations
1	Central nervous system	CNS
2	Urinary tract infections	UTI
3	Multi-drug resistant	MDR
4	Open reading frame	ORF
5	Insertion sequence	IS
6	Enterococcal surface protein	ESP
7	Aggregation substance	AS
8	Vancomycin resistant *Enterococci*	VRE
9	World health organisation	WHO
10	Extracellular polymeric substance	EPS
11	Micro litre	µl
12	Negative	-
13	Positive	+
14	*Enterococcus* differential Agar base	EDAB
15	Brain heart infusion broth	BHI
16	Potential of hydrogen	PH
17	Hours	HRS
18	Penicillin binding protein	PBP
19	Optical density	OD
20	Ribonucleic acid	RNA

Chapter 1

Introduction

1.1 INTRODUCTION

Historically, *Enterococcus* genus has originated from *Streptococcus* genus. Previously, *Enterococcus* was classified as a gram+ve, enteric, lactic acid producing (Ramsey *et al., 2014)* cocci shaped bacteria and placed under *Streptococcus* genus. In 1906, *Streptococcus faecalis* was isolated from Endocarditis patient by Andrews and Horder. In 1919, Orla-Jansen discovered *Streptococcus faecium*. Previously, it assumed that *Streptococcus faecium* is a variant of *Streptococcus faecalis* but after that *Streptococcus faecium* was differentiated from *Streptococcus faecalis* on the basis of biochemical characteristics. In 1980's Schleifer and Klipper-Balz removed the *Enterococci* from the genus *Streptococcus* on the basis of genetic difference and placed it in their own genus i.e. *Enterococcus.*

This genus includes 37 species and broad range of habitats i.e. soil, water, air (Ramsey *et al., 2014).* It is a genus of gram +ve, size(0.6-2.0 × 0.6-2.5 mm), facultative anaerobe, chemo-organotroph, catalase-ve(show pseudocatalase on blood agar), low G+C content in DNA sequence and found in the gastrointestinal tract of mammals including human. They are oval in shape and occur as single cells, in pair or as short chains. They are sturdy and versatile in nature (Cesar *et al., 2013*). Many strains are motile by scanty flagella. They lack capsule. *Enterococcus* genus contains more than 19 species i.e *E.faecalis, E.faecium, E.casseliflavus, E.gallinarum, E.raffinosus*. Among them, *E.faecalis* and *E.facium* are nosocomial pathogens in humans and cause severe bacteraemia, endocarditis, urinary tract infections and neonatal infections.

Microscopically, *Enterococcus* appear as smooth, white and cream colored colonies on the solid media. They grow in broth containing 6.5% NaCl and hydrolize Esculin in presence of 40% bile salts. *E.faecalis* is non haemolytic on Sheep Blood Agar but it is beta haemolytic on media containing horse, rabbit and human blood. It appear as a maroon or red colonies on Enterocooccus Differential agar base and cream colonies on nutrient agar whereas *Enterococcus faecium* form cream colonies on the Enterococcus Differential Agar Base . They also survive in chemical disinfectants such as chlorine, glutaraldehyde and alcohol.

Starvation of nutrients or adverse conditions make the bacteria viable but in non-culturable state but when the environmental conditions are normal, then bacteria become metabolically active and regain normal state. Besides the fact that *Enterococcus* is non-spore formig, they survive for months on dry surfaces and prevent dessication.

Enterococci are involved in human clinical infections has been reviewed by Murray (Hardie *et al*., 1997). Among various species of *Enterococci* the two nosocomial species i.e*. E.faecalis* and *E.faecium* are present as commensal in human intestine. They show low level of virulence in human and animals, being present as natural colonizers of gastro-intestine. They are also found in vagina and oral Cavity (Hardie *et al*., 1997). Root canal treated teeth prevalence value has increased from 30% to 90%. Root canal treated teeth are harbouring 9 times more *Enterococcus faecalis* than primary infections. It causes urinary tract infections, bacteraemia, bacterial sepsis, and endocarditis etc. They are 100% isolated from faecal samples (Hardie *et al., 1997). Enterococcus* carriage rate has increased in hospitalized patients and long term haemodialysis patient. *Enterococci are* involved in 10% urinary tract infections and 16% in nosocomial urinary tract infections (Hardie *et al*., 1997). *Enterococci* are responsible for 5-10% of bacterial endocarditis. Among all diseases, bacteraemia is more common than others and is caused in more elderly patients and immune-compromised patients. It rarely caused neonatal, CNS, respiratory tract infections (Hardie *et a*l., 1997).

Biofilm formation by *Enterococcus faecalis* makes the bacteria more resistant towards the antibiotics. Firstly, the biofilm was seen by *"Anton Von Leeuwenhoek*" in the tooth scraping and the "Biofilm" term was coined by *"Bill Costerton"* in 1978. Biofilm is a microbially derived sessile community irreversibly attached to the substratum. Biofilm has the capability of shedding individual bacteria and sloughed pieces of biofilm into surrounding tissues and circulatory system and produce acute illness even after the vigorous treatment by antibiotics. Biofilm cells are physiologically different, with reduced growth and metabolic rate of bacteria, reduced penetration of antibiotic and secrete exopolymeric substance which provides protection to the biofilm from the various antimicrobial agents.

Enterococcus faecalis has various virulence factors i.e. aggregation factors, cytolysin, protease, hemolysin, gelatinase etc. They show antibiotic resistance to various antibiotics i.e. clindamycin, cephalosporin and aminoglycosides. Antibiotic resistance is more in hospital environment than in community. It is caused by antibiotic stress in environment. Enterococcus transfer genetic mobile elements such as transposons and plasmid to other species and acquire antibiotic sensitivity against many antibiotics (Upadhaya et al., 2009). It is very difficult to treat antibiotic resistant *Enterococci* and prevalence rate is also increasing day by day.

Chapter 2

Review of literature

2.1 REVIEW OF LITERATURE

As it has been mentioned above in the introduction, *Enterococci* group was described as a group in 1984, whereas reclassified it from *Streptococci* to *Enterococci*. They are oval shaped and form chains of various lengths. They are sturdy and versatile (Cesar *et al., 2013*). They also survive in the environment of chemical disinfectants i.e. chlorine, glutaraldehyde and alcohol. Among various species of *Enterococci* the two nosocomial species i.e. E.*faecalis* and *E.faecium* is present as commensal in human intestine. They show low level of virulence in the human and animals, and are present as natural colonizers of gastro-intestine and also used as in probiotics for human and farm animals (Cesar *et al., 2013).* But nosocomial and biofilm form of bacteria causes bacteraemia, endocarditis, urinary tract infections and also neonatal infections.

Enterococcus faecalis shows resistance against different antibiotics like Vancomycin, Ampicillin, Clindamycin, Cephalosporin and Aminoglycosides, due to the presence of capacity to acquire and disseminate the determinants of antibiotic resistance, malleable gene and increasing number of genome in hospital, taking various antimicrobial drugs cause drug resistant micro-organism.

2.1.1 Metabolism and Physiology

Enterococci are sturdy and versatile (Cesar *et al., 2013*). They grow on high salt concentration i.e. 6.5%. High salt concentration tolerance makes them different from *Streptococci*. They grow in the temperature range of 10˚C-40˚C (Optimum temperature 35˚C) and also survive at 60˚C for 30 minutes. *Enterococcus* is grown at pH 9.6.

Enterococci have metabolic versatility (Ramsey *et al., 2014*). They can metabolize different types of substrates and form different types of products and thus, they are able to colonize on different places of host.

Sugar metabolism involved 13 types of sugar being metabolized by all and more 30 types of sugars by *Enterococci faecalis* and *Enterococci faecium* (Ramsey *et*

al., 2014). New carbon source metabolism mechanisms are under research and they have shown capabilities to share the new carbon metabolism mechanisms between each other which are present on mobile elements.

Ethanol production is obtained upon metabolism of glucose or mannitol by *Enterococcus faecalis* under nutrient limited conditions. Growth of *Enterococci* on excess of glucose, under reducing conditions forms the lactate (Ramsey *et al*., 2014). It is generated by reduction of pyruvate to regenerate NAD+ for occurring glycolysis. Glycerol metabolism involved the synthesis of lipids and teichoic acid. Glycerol can be metabolized under aerobic as well as anaerobic conditions. Citrate metabolism is commercially valuable. It contributes flavour compound to different types of cheeses. Citrate metabolism produced acetate and formate as major products.

2.1.2 Clinical features

Most common UTI infections are cystitis and pyelonephritis, and they include prostatitis and perinephric abscesses. Bacteremia is caused by bacteria from different types of sources i.e. urinary tract, intravascular catheters. Polymicrobial Bacteremia included Enterococcal infections and other microbial infections which increases the severity of enteric infection.

2.1.3 Pathogenicity and virulence

Enterococcus faecalis is nosocomial pathogen and causes serious and life threatening infections. Eight lakh cases of Enterococcal infections are occurring in USA alone (Upadhaya *et al., 2009*). Infections due to the MDR and virulence factors have become worst. Infections are occurring by native flora or nosocomial infections. Native flora obtains intrinsic antibiotic resistance which is common for host but in nosocomial infections, intrinsic and acquired antibiotic resistance causes transmission by hands of health care providers and also with medical devices *i.e.* catheters etc. Enterococcal infections are life threatening like

conditions of Bacteremia and endocarditis. Infections are caused due to the presence of virulence factors and MDR.

Virulence factors:

Enterococcus faecalis has different types of virulence factors. *Enterococcus faecalis* strain V583 has been sequenced and is known to contain total 3182 ORF (Upadhaya *et al., 2009*). 25% of genome contains mobile and exogenously acquired DNA which contains conjugative and composite transposons, pathogenicity islands, integrated plasmid genes, phage regions and high number of IS elements (Upadhaya *et al., 2009*). *Enterococcus faecalis* contains intrinsic resistance due to the chromosome or transfer of plasmid which confers resistance to other bacteria. The transfer of plasmid encoding virulent factors to other non-virulent bacteria enables bacteria to acquire newer traits and colonize it in more new areas in host and cause infections.

These different types of virulent factors are haemolysin i.e. a cytolytic protein which has the ability to lyse erythrocytes of human, horse and rabbit (Upadhaya *et al., 2009*). Haemolysin producing strains are virulent in many animal models and causes severe infection when associated with other bacterial infections. Haemolysin expression is regulated by two component regulatory system through quorum sensing.

Gelatinase virulence factor is a protease produced by bacteria which has the ability of hydrolysing gelatine, collagen, casein, haemoglobin and other peptides (Upadhaya *et al., 2009*). Gelatinase virulence factor contribute in endocarditis's virulence. It is shown that 141 *Enterococcus faecalis* blood isolates out of 219 are positive for gelatinase production. Surface adhesion (Enterococcal surface proteins ESP) involves cell wall associated protein in *Enterococcus faecalis.* The frequency of gene coding for ESP is higher in clinical isolates than commensal isolates of *Enterococcus faecalis* (Upadhaya *et al., 2009*). ESP helps the organism to adhere the epithelium of urinary bladder with specific component of urinary bladder wall i.e. mucin. So, ESP increases the persistence of *Enterococcus faecalis* in the urinary bladder during UTI.

Aggregation substances are pheromone inducible surface protein of *Enterococci* which helps in aggregation during bacterial conjugation (Upadhaya *et al., 2009*). AS are known to mediate the donor-recipient contact to promote plasmid transfer. AS have different types of functions i.e. promoting cell-cell contact, promote adhesion to host cells, also promote adhesion to ECM protein, increased cell surface and hydrophobicity. AS producing strains are resistant to phagocytosis. The role of AS in endocarditis is based upon the rabbit endocarditis model. MSCRAMM Ace is a collagen binding protein and structurally related to the staphylococci DNA adhesion (Upadhaya *et al., 2009*). MSCRAMM Ace is present in both commensal and pathogenic and expressed during infection in humans. Human derived antibodies are blocking the adherence of MSCRAMM Ace to extracellular matrix proteins in vitro.

Capsular polysaccharides and cell wall carbohydrates virulent factors are operon encoding and expressed more into the clinical isolates of *Enterococcus faecalis*. The purified fraction contains glycerol phosphate, glucose and galactose residues. Extracellular superoxide formation helps the *Enterococcus faecalis* to survive in in-vivo environment with the mixed infection of *Bacteroides fragilis* in subcutaneous infection.

2.1.4 Antibiotic resistance

Antibiotic resistance is more in the hospital environment than the community due to the antibiotic stress in the environment. Mechanism of antibiotic resistance involves inactivation of drugs, prevention of drug to reach its target site, reduction of target susceptibility, and acquisition of new and less sensitive targets which produces intrinsic and acquired resistance. Acquired drug resistance is due to the mutation and transfer of genetic mobile elements i.e. transposons and plasmids. *Enterococci* transfer these mobile elements to both gram+ve and gram-ve bacteria and make the situation worst (Upadhaya *et al., 2009*).

Vancomycin is given to treat the enterococci infection but due to the excessive use of vancomycin, the vancomycin resistant bacteria have developed and now to treat

VRE, linezolid is used (Upadhaya *et al., 2009*). But now linezolid resistant *enterococci* have also developed which is another challenge for the medical science. Resistance to vancomycin increases the double chances for bacterial infection. It is the only gram +ve bacteria which cause high chances of death during Bacteremia (Upadhaya *et al., 2009*). Death cases by Bacteremia are 12- 68% and death cases by bacterial sepsis are 4- 50%. Reaction of body against infection causes injury to own cells.

First case of Ampicillin resistance was found in 1980 at US. Ampicillin resistance are due to the low affinity of penicillin binding proteins towards the beta-lactam. Glycopeptide resistant enterococci were found in Europe and in all over the world by 1986. Vancomycin resistant enterococci infections have increased from 0 to 28.5% in US Between 1989 to 2003. Prevalence rate of vancomycin resistance Enterococci is less in Europe, than in US due to the less use of vancomycin in Europe.

2.1.5 Biofilm production by *Enterococci*

Biofilm caused diseases are colitis, vaginitis, urethritis, conjunctivitis, otitis, gingival infection and loss of periodontal attatchment. It also colonizes the medical devices i.e. urinary, venous, arterial catheters, shunts, respirators, contact lenses, artificial implants, urinary prosthesis and orthopaedic prostheses which have been found to be infected with biofilm. Biofilm acquires different characteristics, and they are highly resistant to antimicrobial agents and immune killing. It has capability to shed individual bacteria into surrounding and cause acute illness after the vigorous treatment of antibiotics.

Biofilm produces saline and polysaccharides which is the main component of biofilm. Biofilm formation is affected by the media composition like presence of carbohydrate and depletion of iron contributes biofilm formation. Different types of factors that regulate the biofilm production are transcriptional regulators i.e. Bop D (biofilm on plastic regulator D), fsr (gene product of quorum sensing locus

Enterococcus faecalis regulator), Gel E (gelatinase), Ebp (Endocarditis and biofilm associated Pilli).

Biofilm reduces the growth rate and respiration rate of bacteria. EPS provides protection from natural and artificial agents. It reduces the penetration of antimicrobial agents. When biofilm is treated with any antibiotic, it kills only at the margins and deep micro-colonies forms the NIDUS and continually spread infections. Mostly planktonic form is susceptible to antibiotic than biofilm. Over perception of drugs produces antibiotic resistance microbes.

Chapter 3

Material and methods

3.1 METHODS AND METHODOLOGY

The methods used during the project have been adapted, going by the conditions and availability of resources present with us. The methods of identification, sampling has been framed by reading and adopting standard protocols from various globally authentic sources such as CDC, WHO, and various research papers.

TABLE: - 3.1 LISTS OF MEDIA USED

S.NO.	Name of media	Himedia catalogue no.	Use
1	EDAB	M1896	Selective isolation and differentiation *of E.faecalis* and *E.faecium.*
2	Nutrient Agar	M001	Cultivation of less fastidious microorganisms.
3	BHI Broth	M210	Used for propagation of fastidious pathogenic cocci.
4	Urea Agar	M112	Detection of urease production.
5	Tryptophan Broth	M463	Detection of indole production

3.1.2 Method of Sampling

In total six soil samples are collected from the six different sites of Malwa region. 1 gm of soil sample is taken in 9.0 ml distilled water. Dilutions are made from 10^{-1} to 10^{-4} by serial dilution method and centrifuge it. After that, 100µl of diluted sample is spread on the EDAB media from each dilution tube and after that kept in incubation for about 24 hrs at 37˚C.

TABLE:- 3.2 SITE OF SAMPLING

Sr.No.	No. of strains	Site of sample collection
1	1 to 4	Outside of CUPB (30.173708N,74.964995E)
2	5 to 7	Hospital soil of village Bajakhana (30.457677N,74.982693E)
3	8 to 10	Agriculture soil of village Bajakhana (30.457677N,74.982693E)
4	11 to 14	Sewage soil of CUPB (30.171868N,74.965907E)
5	14 to 16	Village malla (30.171868N,74.965907E)
6	17 to 20	Animal waste soil of village Bajakhana (30.457677N,74.982693E)

3.1.3 Biochemical Identification

A) GRAM STAINING

Gram staining is a differential staining procedure in microbiology to differentiate between Gram+ve bacteria and Gram-ve bacteria. These bacteria are distinguished on the basis of composition of cell wall. Cell wall composition affects the type of stain uptake or release.

Procedure: Take the clean glass slide. Label the underside of the slide with marker. Slightly heat the slide to kill germs present on the slide. Take broth culture which is grown overnight and make the smear with the help of inoculation loop. Dry for few minutes and after that heat fix the bacteria. Place the slide on the staining tray. Pour crystal violet on smear and stand for 1 minute. Tilt the slide and gently wash the slide with distilled water. Pour 95% alcohol drop by drop on the smear for 5 to10 seconds. Again tilt the slide and wash with distilled water. Pour safranin on the smear and stand for 45 seconds. Again tilt the slide and wash with distilled water. Dry the slide with blotting paper and see under the light microscope with oil immersion.

B) UREASE TEST

Urea is structurally a diamide of carbonic acid. It hydrolysed to form ammonia and carbon dioxide. Ammonia treated with carbon dioxide and water to form ammonium carbonate which converts the medium alkaline and indicator phenyl red change its colour from original orange to bright pink.

Procedure: Prepare the urea agar slant in test tube for urease test. Now take the colony which is 18-24 hrs. Grown previously on plate are streak on the urea agar. Cover test tube with aluminium foil to avoid urea agar to breakdown from light which give the false results. Put the loose cap on it and incubate for 35-37˚C for 48 hrs.

C) INDOLE TEST

Indole is produced by reductive deamination of tryptophan via formation of intermediate molecule called indolepyruvic acid. Reaction is catalyses by tryptophanase, during which the amino group of tryptophan is removed and pyridoxal phosphate is required as cofactor. When indole is combine with Kovac's reagent then colour changes from yellow to cherry red because amyl alcohol is not water soluble and red colour is formed on oily layer at the top of the broth.

Procedure: Prepare tryptophan broth and pour into the sterile test tubes. Take the colony which is 18-24 hrs. Grown previously on plate and inoculate the tryptophan broth with inoculation loop. Now incubate for 35-37˚C for 24 hrs. After that 0.5 ml Kovac's reagent is added to observe the ring formation.

D) OXIDASE TEST

The test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain.Oxidase test was conducted with fresh growth from BHI medium.

Procedure: Mark a sterilized slide with a marker. Now take an oxidase disc with the help of sterlized spatula and placed on the slide and now pick the colony from the EDAB plate which is 18-24 hrs. previously grown on plate. Now placed the colony on the oxidase disk and obsereve to turn colour into purple within 15 sec.

E) CATALASE TEST

This test is based on the presence of catalase enzyme which releases the oxygen by breakdown of hydrogen peroxide and this test differentiate the catalase producing enzyme or non-catalase producing enzyme.

Procedure: Take the sterile test tube and pour 2-3 ml freshly prepared 3% hydrogen peroxide and after that pick the colony from previously 24-28˚C grown petri plate with the help of sterile inoculation loop and put it into the test tube and observe the bubble formation produced by oxygen formation.

DISC DIFFUSTION ANTIBIOTIC SUSCEPTIBILITY TEST

Disc diffusion antibiotic susceptibility test (Kirby-Bauer test) used the antibiotic disc to determine the whether bacteria is susceptible or not to the antibiotic. Different types of antibiotic disc are used. I have chosen six antibiotics i.e. ampicillin, rifampin, tetracycline, Pipercillin, streptomycin and gentamycin. **Ampicillin** is broad-spectrum, semi-synthetic, beta-lactam penicillin, bactericidal activity. Ampicillin binds to the PBP and inactivates it which is present on the inner membrane of the bacteria. **Gentamycin** is broad –spectrum aminoglycoside used to treat many gram-ve infections and some gram+ve infections. It binds to ribosome and inhibits protein synthesis. **Streptomycin** is an aminoglycoside antibiotic and act as both bacteriostatic and bactericidal against bacteria. It is also second line anti-tuberculosis agents. It is also bind to the ribosome and inhibit protein synthesis. **Tetracycline** is broad spectrum and semisynthetic derivative of *Streptomyces actinobacteria*. It binds to the 30s subunit of ribosome and inhibit protein synthesis. Human cells are less susceptible. **Rifampin** is a synthetic derivative of the natural products of bacterium *S.mediterranei*. Rifampin is mainly used for the treatment of mycobacteria tuberculosis. It inhibits the DNA dependent RNA polymerase of mycobacteria. **Piperacillin** is a fourth generation antibiotic which used to treat several types of diseases.

Procedure: Prepare the agar petri plates carefully. Plates are formed very carefully not over dry the agar plates. Inoculums are prepared by inoculation of micro-organism into broth medium and culture it from 16-24 hrs. growth of micro-

organism are not cross the log phase of bacteria so be careful about the growth of bacteria. After the growth of micro-organism, the optical density is checked by spectrophotometer. The OD is taken on the 600 nm. The absorbance will come near to the 0.5 which is the good indication of growth. After that make the concentration 0.1%, take the previously prepared agar plates and take 200 µl with micropipette and pour on agar plates and spread on it. Put that plates in incubator for 30 min. after that placed antibiotic disc on the agar plate with the help of sterile tong such as Gentamycin, Ampicillin, Rifampin, Streptomycin, Tetracycline, and Piperacillin. Press little bit to disc for fixing the disc. Put the petri plates in incubator for 24 hrs.

BIOFILM FORMATION

Biofilm is group of micro-organisms which are stick together and produce extra-polymeric substance. Biofilm producing bacteria are physiologically distinct and show resistance towards various antibiotics. Biofilm formation is passes through the various steps. Each step makes the micro-organism more attached to the surface and protect from the cleaners and sanitizers.

Step 1: Attachment

When the bacterial cell is come near to the surface, the motion of bacteria become slowly and they reversibly attached to the surface or pre-attached bacteria. The solid-liquid environment provides the best environment for the growth of bacteria. Presence of loco motor structure on cell surface facilitates biofilm formation.

Step 2: Micro-colony formation

Micro-colony is formed after the attachment of bacteria on to the physical surface. Bacteria are multiplied and produce a threshold signals for the production of EPS. After that bacterium is multiplied and form a micro-colony.

Step 3: Three dimensional structure formation and maturation

After micro-colony formation, the gene associated with EPS production is activated and EPS are produced. Extracellular matrix formation is triggered by attatchment of

bacteria to the surface. Extracellular matrix formation followed by water filled channels formation which is act as a circulatory system to reach the nutrition to each cell of biofilm and also used to dispose the waste out from the biofilm.

Step 4: Detachment

Detachment of bacteria from biofilm is regular basis process. When bacteria are unable to form the EPS or the new formed cells are used to detach the bacteria from the biofilm. Bacteria are detached in the form of planktonic form. A well-established colony shed the planktonic form bacteria into the surrounding and causes contamination on surface.

Procedure: Take the overnight growing bacterial culture in broth. Bacteria should be in log phase. Take a sterilized round bottom 96- well plate. Make the culture: media is 1:200 and take the 200µl in well. Put it into incubator for 24 hrs. After 24 hrs wash the 96-well plate with distilled water three time and dump out the left media. Avoid direct washing of the wells. Now take the 125 µl of 0.1% crystal violet with the help of micropipette to stain the biofilm and put in incubator for 30 min. at 37˚C. After that wash the 96- well plate with distilled water again three times and dump out the left media. After that take 125 µl of 30% acetic acid to solubilize the biofilm with the help of micropipette and put the 96-well plate in incubator for 2 hrs. at 37˚C. Take 100 µl with the help of micropipette into the new round bottom 96-well plate to take the reading on micrototre plate reader at 550 and 595 nm.

CHAPTER 4

RESULTS

4.1 RESULTS AND DISCUSSION

4.1.1 ISOLATION OF *Enterococcus faecalis:*

Samples were collected near from the Central University of Punjab Bathinda and rural areas of the Malwa region. The samples collected were brought to the lab within three hours from collection. They were treated accordingly going by the protocol for sampling and enrichment. once the probable colonies were obtained on a EDAB medium, the sample isolates were further subjected to Biochemical analysis and other identification assays.The tests yielded following results.

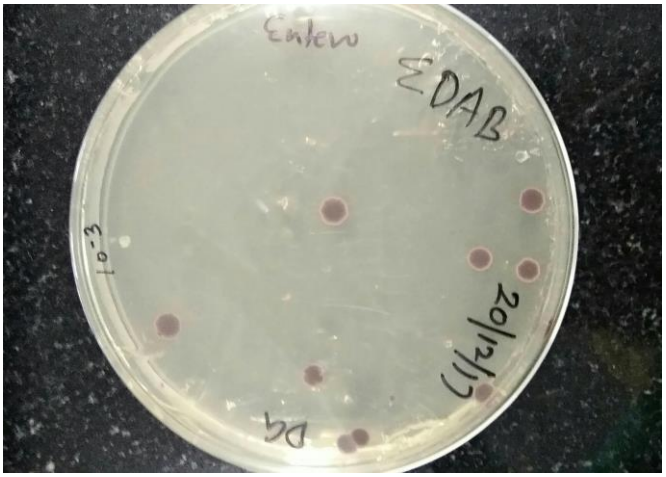

fig:- 4.1.1 Isolated E.faecalis

(Maroon colonies or red centred with creamy outer layer on the EDAB media confirms the presence of *Enterococcus faecalis* bacteria on the plate.)

4.1.2 SAMPLE 1

The sample are grown over the EDAB media to isolate colony of desired bacteria. Out of ten maroon colonies, 4 potent colnies were screened out on the basis of size and shape.

TABLE NO:-4.1.2 RESULT OF BIOCHEMICAL TEST OF SAMPLE 1

Strain no.	Gram staining	Catalase test	Oxidase test	Indole test	Urease test
1	+	–	–	–	–
2	+	–	–	–	–
3	+	–	–	–	–
4	+	–	–	–	–

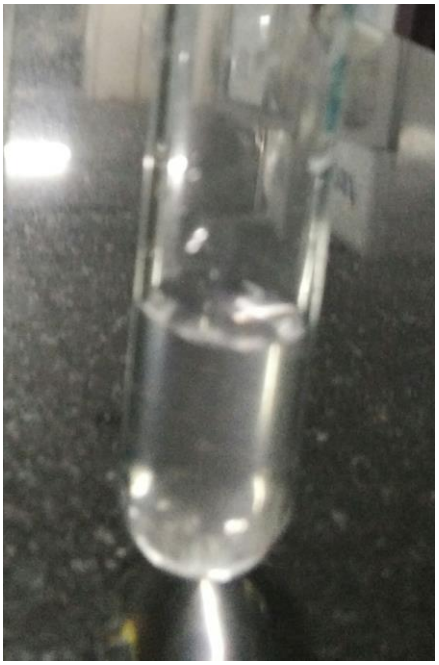

fig:- 4.1.2.1 CATALASE –VE

(No bubble formation confirmed *Enterococcus faecalis* bacteria is catalase-ve)

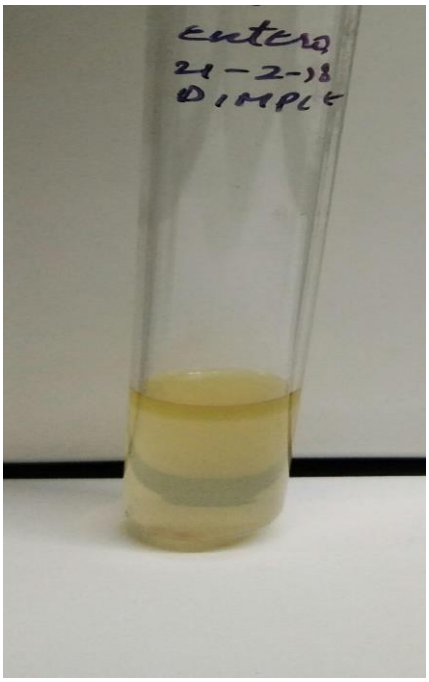

fig:- 4.1.2.2 INDOLE –VE

(No red coloured ring formed on top shows that *Enterococcus faecalis* is indole-ve)

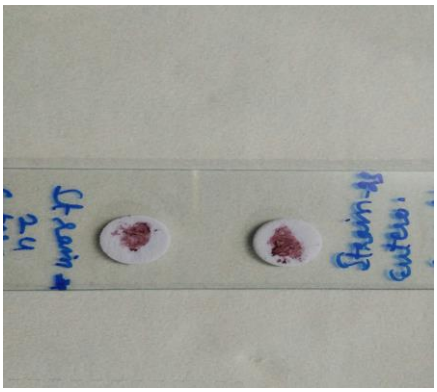

fig 4.1.2.3:- OXIDASE –VE

(Purple colour not formed in 15 Seconds show that *Enterococcus faecalis* is oxidase-ve)

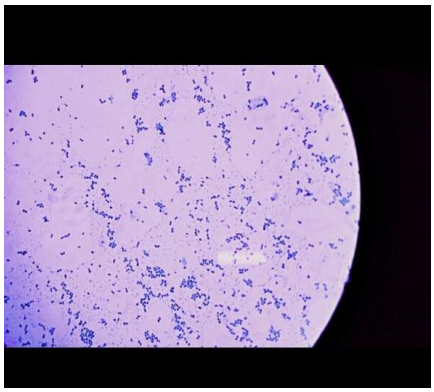

fig 4.1.2.4-: GRAM+VE

(Purple colour of cells shows that *Enterococcus faecalis* is gram+ve bacteria)

fig 4.1.2.4:- UREASE –VE

(No magenta colour appeared on the urease agar shows that *Enterococcus faecalis* Is urease-ve)

4.1.3 SAMPLE 2

The sample are grown over the EDAB media to isolate colony of desired bacteria. Out of ten maroon colonies, 3 potent colnies were screened out on the basis of size and shape.

TABLE 4.1.3: RESULTS OF BIOCHEMICAL TESTS OF SAMPLE 2

Strain no.	Gram Staining	Catalase test	Oxidase test	Indole test	Urease test
5	+	–	–	–	–
6	+	–	+	–	–
7	+	–	–	+	–

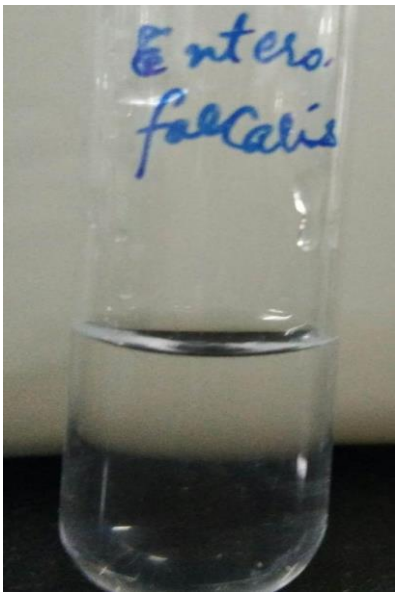

fig:-4.1.3.1 CATALASE TEST –VE

(No bubble formation confirmed that *Enterococcus* faecalis bacteria catalase-ve)

fig:-4.1.3.2 UREASE TEST -VE

(No magenta colour appeared On the urease agar shows is that Enterococcus *faecalis* is urease-ve)

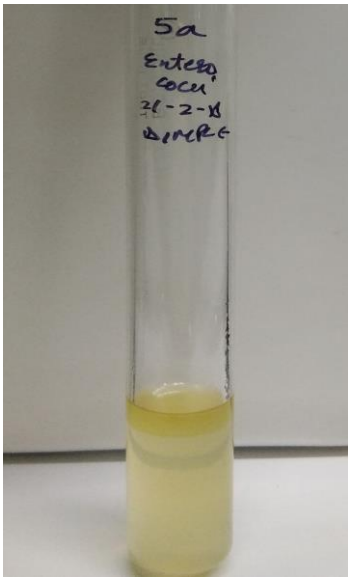

fig:-4.1.3.3 INDOLE TEST -VE

(No red coloured ring formed on top shows that Enterococcus faecalis *faecalis* is indole-ve)

fig:-4.1.3.4 OXIDASE TEST -VE

(Purple colour not formed in 15 Seconds show that *Enterococcus faecalis* is oxidase-ve

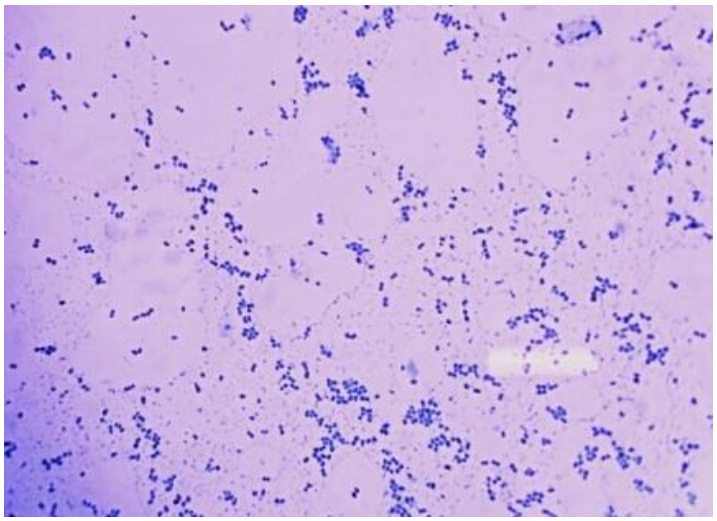

fig:- 4.1.3.5 GRAM'S STAINING

(Purple colour of cells shows that *Enterococcus faecalis* is gram+ve bacteria)

4.1.4 SAMPLE 3

The sample are grown over the EDAB media to isolate colony of desired bacteria. Out of ten maroon colonies, 3 potent colnies were screened out on the basis of size and shape.

TABLE 4.1.4: RESULTS OF BIOCHEMICAL TESTS OF SAMPLE 3

Strain no.	Gram staining	Catalase test	Oxidase test	Indole test	Urease test
8	+	–	–	–	–
9	–	–	–	–	–
10	+	–	–	–	–

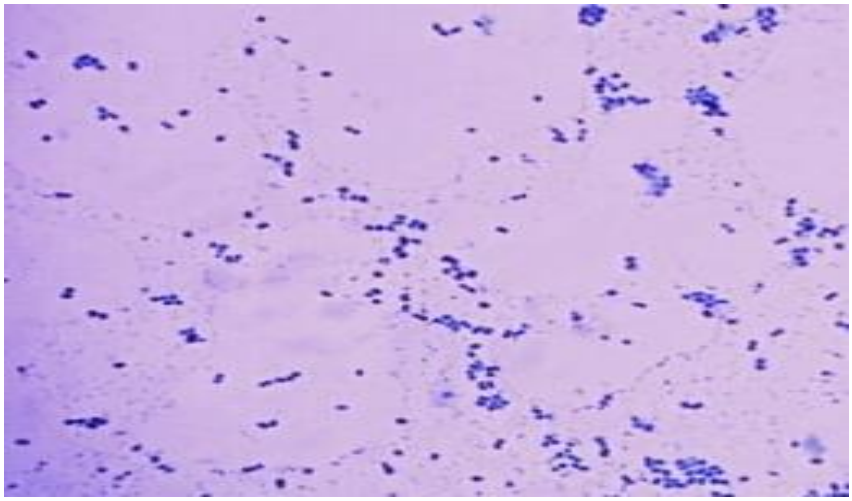

fig:- 4.1.4.1 GRAM'S STAINING

(Purple colour of cells shows that *Enterococcus faecalis* is gram+ve bacteria)

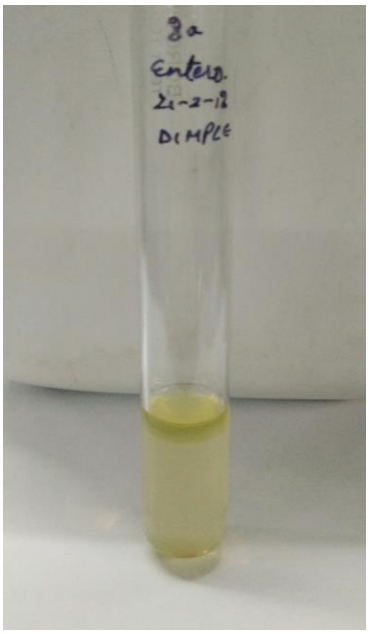

fig:- 4.1.4.2 INDOLE TEST –VE

(No red coloured ring formed on top shows that *Enterococcus faecalis* is indole-ve)

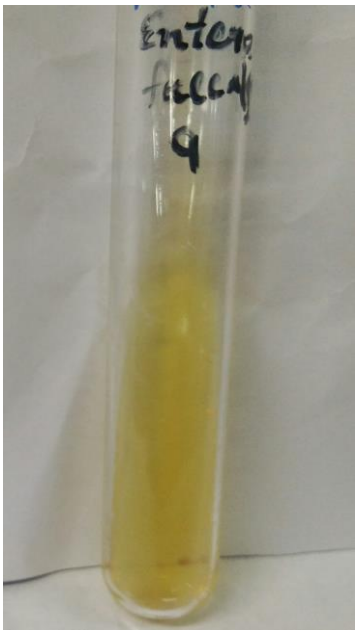

fig:- 4.1.4.3 UREASE TEST –VE

(No magenta colour appeared on the urease agar shows that *Enterococcus faecalis* is urease-ve.)

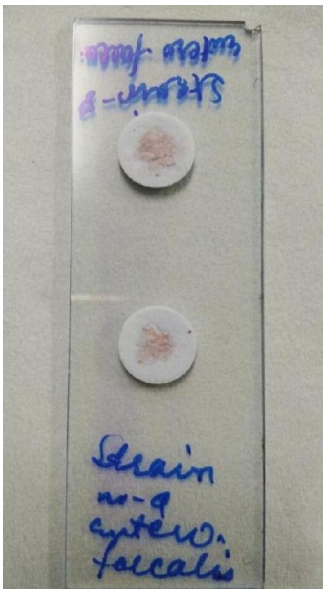

Fig:- 4.1.4.4 OXIDASE TEST –VE

(Purple colour not formed in 15 Seconds show that *Enterococcus faecalis* is oxidase-ve)

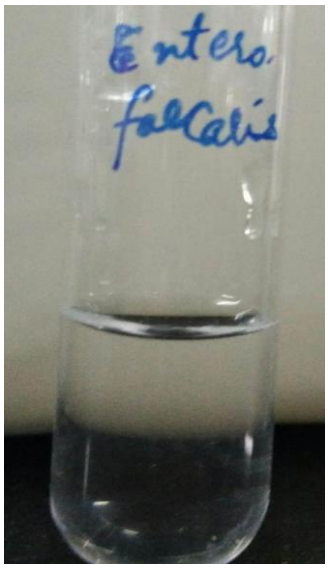

fig:- 4.1.4.5 CATALASE TEST –VE

(No bubble formation confirmed *Enterococcus faecalis* bacteria is catalase-ve)

4.1.5 SAMPLE 4

The sample are grown over the EDAB media to isolate colony of desired bacteria. Out of ten maroon colonies, 3 potent colnies were screened out on the basis of size and shape.

TABLE 4.1.5: RESULTS OF BIOCHEMICAL TESTS OF SAMPLE 4

Strain No.	Gram staining	Catalae test	Oxidase Test	Indole test	Urease test
11	+	–	–	–	–
12	+	–	–	–	–
13	+	–	–	–	–

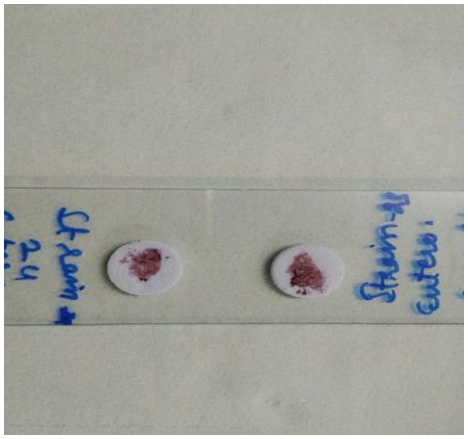

fig:- 4.1.5.1 OXIDASE TEST-VE (Purple colour not formed in 15 Seconds show that *Enterococcus faecalis* is oxidase-ve)

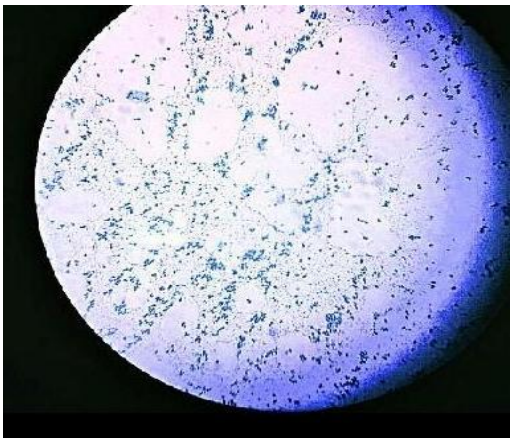

fig:-4.1.5.2 GRAM'S STAINING (Purple colour of cells shows that *Enterococcus faecalis* is gram+ve bacteria)

fig:- 4.1.5.3 UREASE TEST –VE

(No magenta colour appeared On the urease agar shows that Enterococcus *faecalis* is urease-ve.)

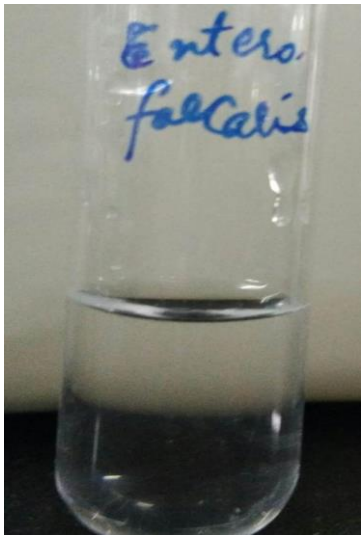

fig:-4.1.5.4 CATALASE TEST –VE

(No bubble formation confirmed that *Enterococcus* faecalis bacteria is catalase-ve)

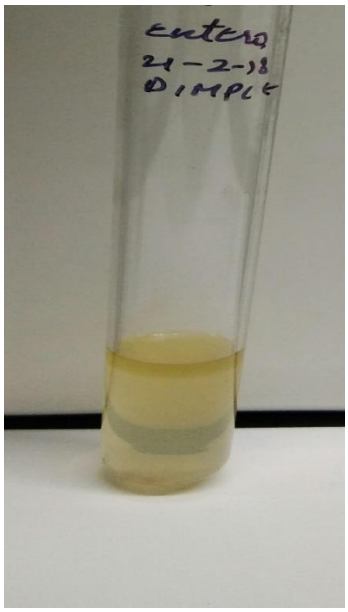

fig:-4.1.5.5 INDOLE TEST -VE

(No red coloured ring formed on top shows that *Enterococcus faecalis* is indole-ve)

4.1.6 SAMPLE 5

The sample are grown over the EDAB media to isolate colony of desired bacteria. Out of ten maroon colonies, 3 potent colnies were screened out on the basis of size and shape.

TABLE 4.1.6: RESULTS OF BIOCHEMICAL TESTS OF SAMPLE 5

Strain no.	Gram staining	Catalase test	Oxidase test	Indole test	Urease test
14	+	–	–	–	–
15	+	–	–	+	–
16	+	–	–	+	–

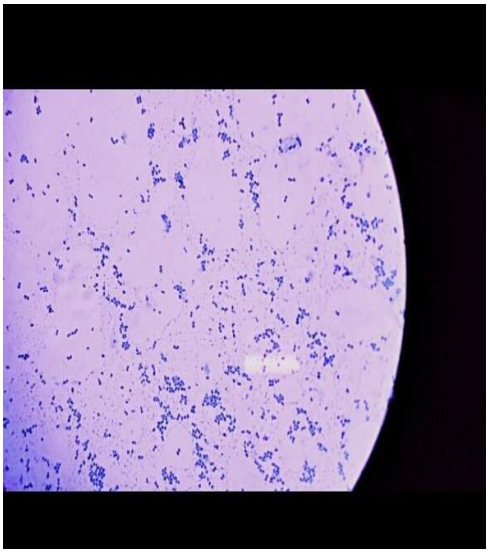

fig:-4.1.6.1 GRAM'S STAINING

(Purple colour shows that *Entero-coccus faecalis* is gram+ve bacteria)

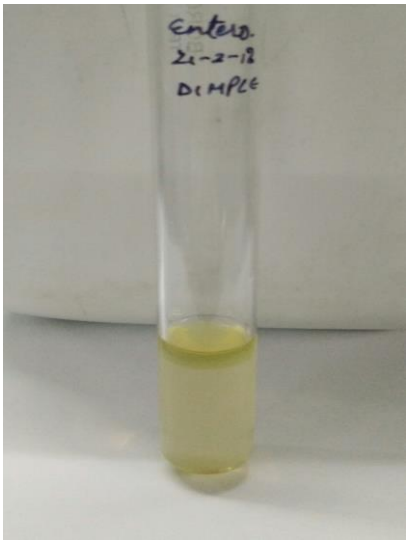

fig:-4.1.6.2 *I*NDOLE TEST-VE

(No red coloured ring formed on top shows that *Enterococcus faecalis* is indole-ve)

fig:-4.1.6.3 UREASE TEST –VE

(No magenta colour appeared On the urease agar shows that Enterococcus *faecalis* Is urease-ve)

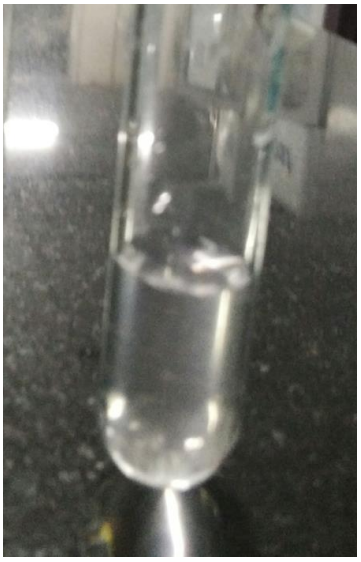

fig:-4.1.6.4 CATALASE TEST -VE

(No bubble formation confirmed that *Enterococcus* faecalis bacteria is catalase-ve)

fig:-4.1.6.5 OXIDASE-VE

(Purple colour not formed in 15 Seconds show that *Enterococcus faecalis* is oxidase-ve.)

4.1.7 SAMPLE 6

The sample are grown over the EDAB media to isolate colony of desired bacteria. Out of ten maroon colonies, 3 potent colnies were screened out on the basis of size and shape.

TABLE 4.1.7: RESULTS OF BIOCHEMICAL TESTS OF SAMPLE 6

Strain no.	Gram staining	Catalase test	Oxidase test	Indole test	Urease test
17	+	–	–	–	–
18	+	–	–	–	–
19	+	–	–	–	–
20	+	–	–	–	–

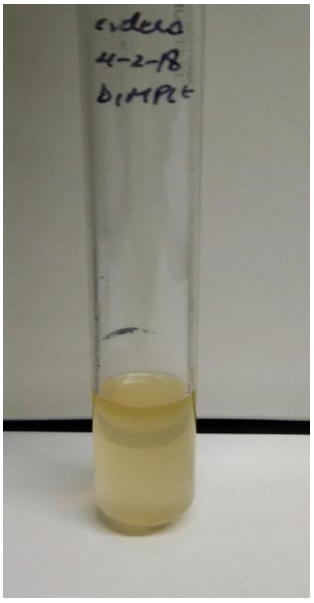

fig:-4.1.7.1 INDOLE TEST –VE

(No red coloured ring formed on top shows that *Enterococcus faecalis* is indole-ve)

fig:-4.1.7.2 UREASE TEST –VE

(No magenta colour appeared on the urease agar shows that *Enteroc-occus faecalis* is urease-ve.)

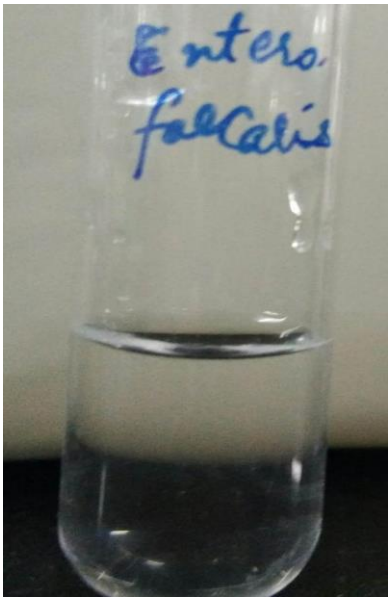

fig:-4.1.7.3 CATALASE TEST-VE

(No bubble formation confirmed that *Enterococcus* faecalis bacteria is catalase-ve)

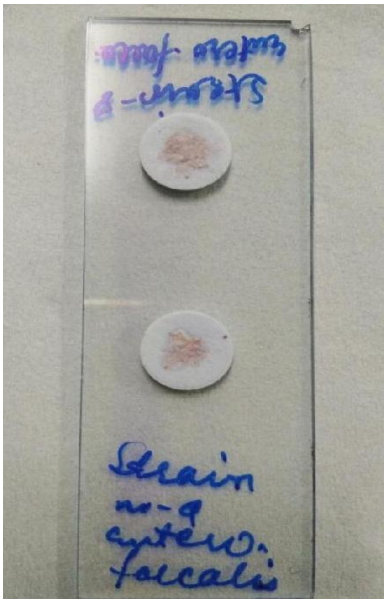

fig:-4.1.7.4 OXIDASE TEST-VE

(Purple colour not formed in 15 Seconds show that *Enterococcus faecalis* is oxidase-ve)

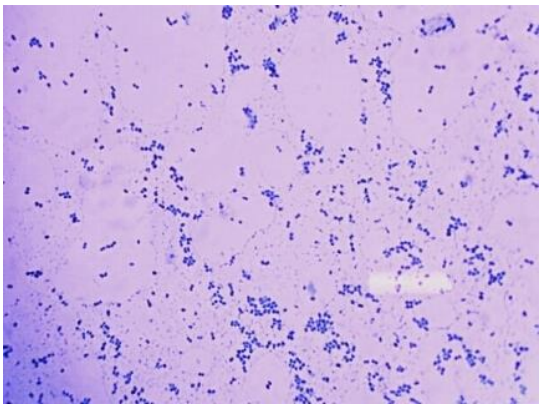

fig:-4.1.7.5 GRAM'S STAINING

(Purple colour of cells show that *Enterococcus faecalis* is gram+ve bacteria)

4.1.8 DISC DIFFUSION ANTIBIOTIC SUSCEPTIBILITY BASED RESULTS

TABLE 4.1.8: RESULTS OF ANTIBIOTIC SUSCEPTIBILITY TESTS.

Strain No.	Piperacillin (cm)	Tetracycline (cm)	Rifampcin (cm)	Ampicillin (cm)	Streptomycin (cm)	Gentamycin (cm)
1	0	1	1.5	0	0.5	0.2
2	1	1.3	2	1	1.9	0.6
3	1.1	1.7	1.1	0.8	1.5	1.3
4	1.8	1.6	1.2	1	1.2	0
5	2	1.1	0	0.9	2	1
6	1.1	1.5	1.6	1.9	0	0.9
7	0.6	0.8	1	1	0	0
8	0.4	0.2	1	0.6	0	0.1

fig:-4.1.8.1 PIPERACILLIN AND TETRACYCLINE SUSCEPTIBILTY

(Clear zone around the disc shows that *Enterococcus faecalis* is susceptible towards Piperacillin and Tetracycline antibiotics.)

fig:-4.1.8.2 PIPERACILLIN AND TETRACYCLINE SUSCEPTIBILTY

(Clear zone around the disc shows that *Enterococcus faecalis* is susceptible towards Piperacillin and Tetracycline antibiotics.)

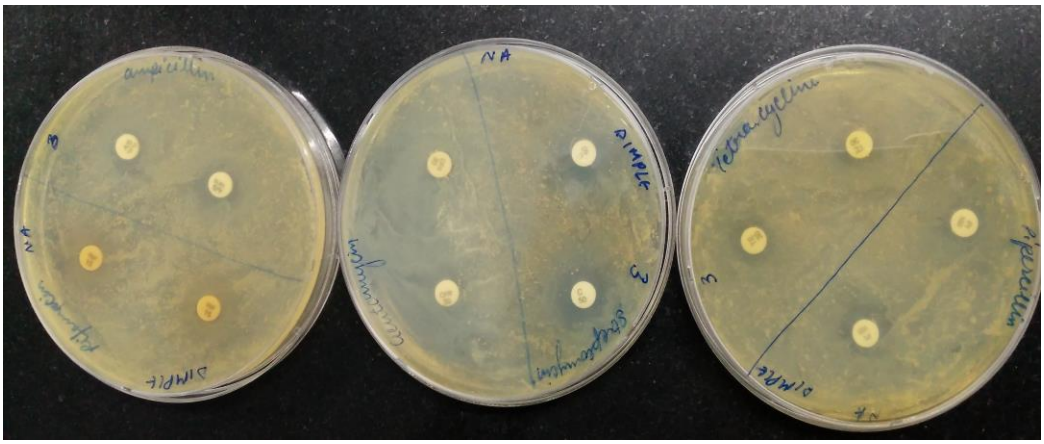

fig:-4.1.8.3 ANTIBIOTIC SUSCEPTIBILITY TEST

(Clear zone around the disc shows that *Enterococcus faecalis* is susceptible towards different antibiotics with different extent such as Ampicillin, Rifampcin, Gentamycin, Streptomycin, Tetracycline,Piperacillin etc.)

4.1.9 BIOFILM FORMATION

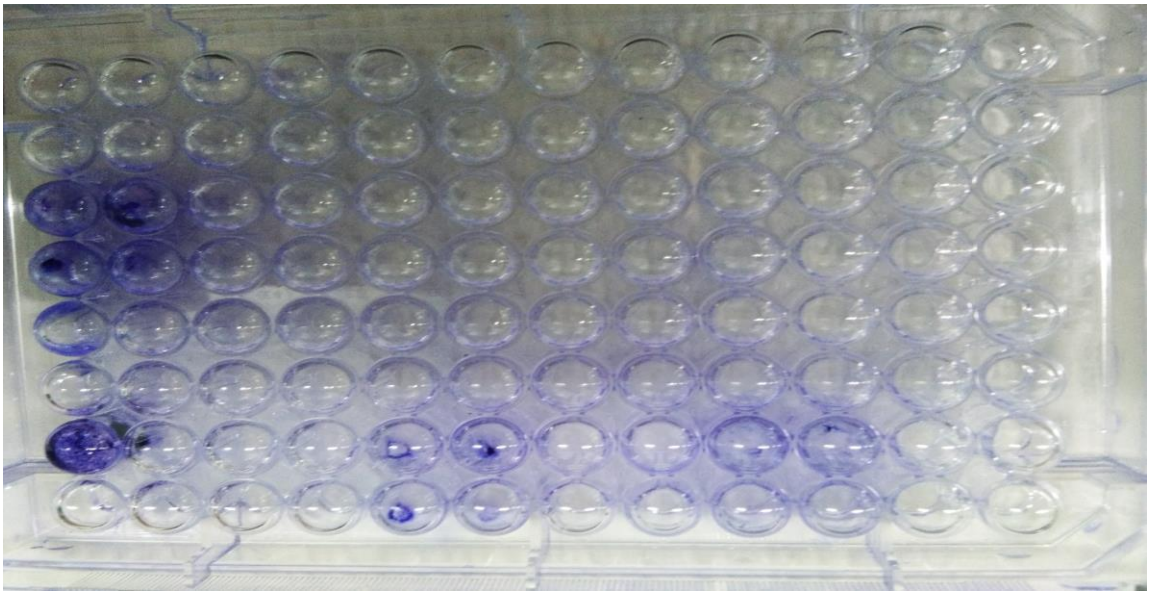

*fig:-*4.1.9.1 Biofilm formation by *Enterococcus faecalis*

TABLE 4.1.9: RESULTS OF BIOFILM FORAMTION

Sr. No.	Strains	Absorbance(595 nm)
1	BLANK	0.057
2	STRAIN 1	0.463
3	STRAIN 2	0.31
4	STRAIN 3	0.607
5	STRAIN 4	0.229
6	STRAIN 5	1.591
7	STRAIN 6	1.58

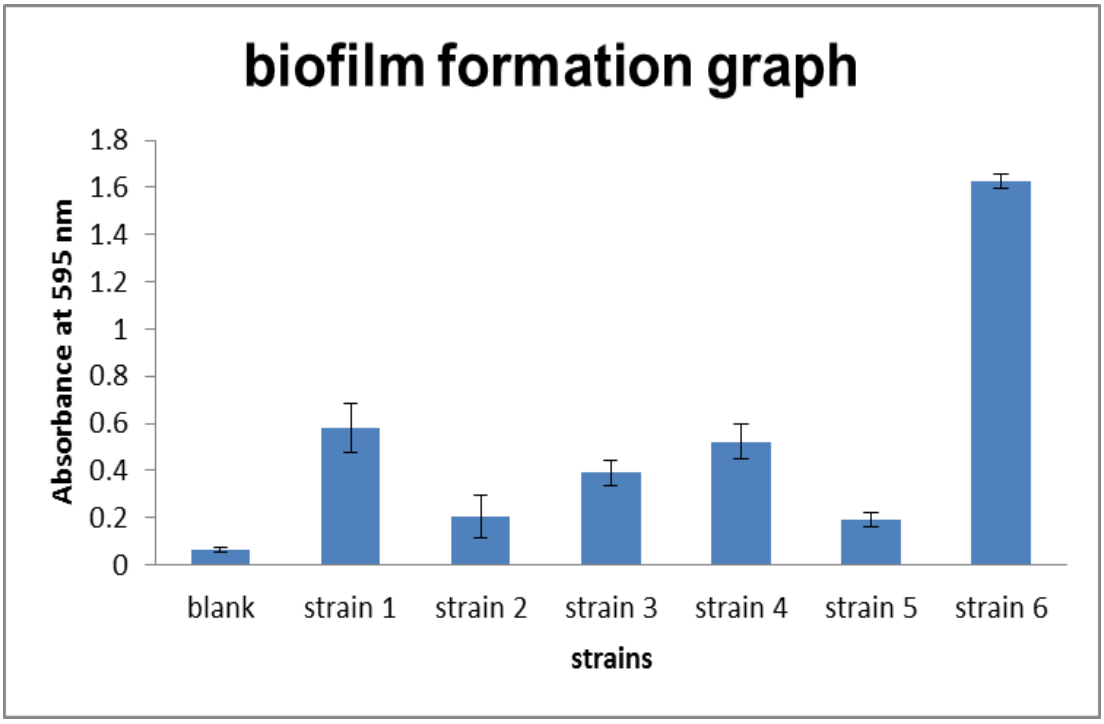

fig:-4.1.9.2 biofilm formation against different strains of *Enterococcus faecalis* with different absorbances.

Chapter 5

Discussion

5.1 DISCUSSION

The *Enterococcus faecalis* (non-enteric) are isolated from different places and also from different types of sources. They are isolated on the specific medium i.e. EDAB. Isolation of *Enterococcus faecalis* confirms the presence of *Enterococcus faecalis* in Malwa region. *Enterococcus faecalis* are hard to kill with alcohol and any sanitizers which causes concern against different diseases caused by the *Enterococcus faecalis* bacteria. The biochemical tests also confirmed the presence of *Enterococcus faecalis* from Malwa region. The biochemical tests for *Enterococcus faecalis* shows various characteristics of *Enterococcus faecalis*. Gram staining shows that *Enterococcus faecalis* is a gram +ve bacteria which show that cell wall of this bacteria is made up of peptidoglycan and with less content of lipids. Oxidase negative test shows the absence of cytochrome enzyme. Catalase negative test shows the absence of catalase enzyme used to breakdown of hydrogen peroxide. Urease negative test also show the absence of urease enzyme which is used to hydrolyze urea. Indole negative test shows that *Enterococcus faecalis* doesnot produce tryptophanase enzyme which hydrolyzes the tryptophan. *Enterococcus faecalis* bacteria also shows Antibiotic susceptibility towards different antibiotics. It shows that *Enterococcus faecalis* with non-enterococcal origin have ability to transfer mobile elements which are actually carrying resistance genes which are horizontally transferred to the another *Enterococcus faecalis* bacteria and also make that bacteria resistant towards the antibiotics and thus it becomes more virulent and infections become difficult to treat The isolated *Enterococcus faecalis* from Malwa Region shows more resistance against Gentamycin and Streptomycin as compared to other antibotics. *Enterococcus faecalis* also formed biofilm and showed more antibiotic resistance. Biofilm are hard to clear from any surface and difficult to treat due to the slow penetration rate and also exchange mobile elements which contain antibiotic resistance genes and thus there is a need to findout antibiotic for which *Enterococcus faecalis* are susceptible and do not produce resistant towards it easily.

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