Investigation of Bacterial Diversity in Cosmetics on the basis of 16S rRNA Sequencing

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A thesis submitted in the partial fulfilment of the requirements for the degree of

MASTER OF PHILOSOPHY

IN

BIOTECHNOLOGY

Department of Biotechnology

Faculty of Biological Sciences

Quaid-i-Azam, University

Islamabad, Pakistan

2022

In the name of Allah, The Most Gracious, The Most Merciful

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Certificate of Approval

This is to certify that the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan accepts the dissertation entitled "**Investigation of Bacterial Diversity in Cosmetics on the basis of 16S rRNA Sequencing**" submitted by **Ms. Tazeen** in its present form as satisfying the dissertation requirement for the Degree of Master of Philosophy in **Biotechnology**.

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DEDICATION

I dedicate my work wholeheartedly to my beloved parents, siblings and my niece Mantasha who have always been source of inspiration, motivation and strength for me whenever I thought of giving up and they continually provided me their moral, spiritual, emotional and financial support.

TAZEEN

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Acknowledgement

All praises for the **Almighty Allah**, the most Gracious, the most Merciful and respect for **Prophet Muhammad** (Peace Be Upon Him) who is forever a model of guidance and knowledge for the humanity.

First and foremost, I would like to express my deepest gratitude and appreciation to the chairman of the department of Biotechnology Quaid-i-Azam University; **Prof. Dr. Bilal Haider Abbasi** whose encouragement and moral support helped me achieving my goal.

It is my privilege to acknowledge the total support and standards of excellence and guidance provided by my supervisor; **Dr. Muhammad Ali**. It is due to his constructive criticism, encouragement, and great contribution to my work that I was able to complete my research. Dr. Ali's professional knowledge, valuable suggestions and unfailing courtesy enabled me to tackle the problems more meaningfully on the subject and provide an easy access to work seriously to pursue my objectives. I am very fortunate to have the assistance and encouragement of my seniors, **Ms. Lubna Rahman**, **Ms. Sidra Rahman** and especially **Muhammad Haris** and **Rizwan Abbas** who really helped me and guided me at every point of my research work. I am also thankful to my batchmates **Syed Ahsan Shahid**, **Sajid Ali Shah** and my junior lab fellows **Asia**, **Iffat**, **Hanbal, Asim** and **Adnan.** I am extremely grateful to my friends **Ms. Kinza Malik**, **Ms. Tehreen Tariq**, **Ms. Maham Irfan** and **Ms. Tahira Younas.** I am thankful to my supportive roommates **Aleesha Asghar** and **Badar-un-Nisa.**

Last but not the least, my acknowledgement cannot be completed without paying my gratitude to my loving parents, my sister **Ms. Mahnoor** and my brother **Lakht-e-Hasnain** for their immense love, support and prayers which made me able to achieve this target. I would acknowledge **Abdur Rahman** who made me believe in myself and supported me emotionally and morally that I can do this.

Tazeen

List of Abbreviations

DNA	Deoxyribonucleic Acid
BLAST	Basic Local Alignment Search Tool
TSA	Tryptic Soya Agar
SDA	Sabouraud dextrose agar
ng/µL	Nanogram per microliter
rRNA	Ribosomal RNA
FDA	Food and drug administration
SIM	Sulfur, Indole, Motility
PCR	Polymerase chain reaction
NCBI	National Center for Biotechnology Information
g	Gram
ml	Milli liter

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Abstract

Cosmetics products are one of the most essential and frequently used components in our daily life. Besides improving human health, they provide us healthy lifestyle and boost up our self-esteem. Globally cosmetics market is projected from 287 billion USD in 2021 to 415 billion USD 2022. This research study aims at the isolation, identification and characterization of bacterial strains isolated from cosmetics. 14 bacterial colonies were isolated by inoculating different cosmetics products on Tryptic Soya Agar. All the strains showed optimum growth at 37°C. Different approaches were adopted for the identification of bacterial strains which include biochemical testing and molecular characterization. All strains were assessed through biochemical tests and were further proceeded for nucleotide sequencing. We identified six strains through Sanger sequencing. Phylogenetic analysis showed different bacterial strains were revealed in cosmetics products including *Sphingomonas Paucimobilis, Cytobacillus oceanisediminis, Robertmurraya andreesnii, Cytobacillus Firmus, Falsibacillus pallidus*, and *Acinetobacter junii. Sphingomonas* has potential of bioremediation and can be utilized for degrading toxic compounds to make the environment better. Similarly, *Cytobacillus* is found to be involved in bio mineralization and also aids in fermentation. Our results have shown that there is dire need to assure the strict safety regulations regarding cosmetics. Improper manufacturing practices can lead to the contamination of cosmetics which could lead to the severe consequences deteriorating the quality of health. Further studies are needed to explore the potential of these isolates so that they can be utilized to improve our health as well as environment.

1 Introduction

Personal care products are frequently utilized by people in daily life and getting popular across the globe due to extensive use. They are purchased without any hurdle as they do not lie under the same regulation as those of medicines (Jairoun et al. 2020). The word "Cosmetic" is derived from the Greek word "*Kosmetike tekhne*" meaning "Technique of dressing and decoration". Cosmetics are defined as the substances which are applied to the external surface of human body for the purpose of altering the appearance to look attractive, to improve the texture of skin, to keep the body clean, to smell good and for skin protection. There are seven main categories of cosmetics which include oral care products, skin care products, and body care products, products of hair care, sun care, fragrance products i-e perfumes and decorative cosmetic products (Draelos and Z.D. 2012).

Cosmetics are comprised of combination of chemicals ingredients derived from natural as well as chemical sources. The vital ingredients of cosmetics include water, emulsifiers, preservative, thickeners, pigments, glitters and fragrances etc. However, these ingredients act as medium for the transport of pathogens in the daily lives of people because mostly these components encourage the growth of microbes. Microorganisms can survive at suitable temperature, pH moisture and metabolites. Almost all cosmetics products fulfil these requirements and harbor the growth of microbes. Mostly cosmetics contain growth stimulators, organic as well as inorganic components and stored in humid atmosphere which stimulates the growth of microorganisms. The microbial contamination of these personal care products deteriorate their quality ultimately affecting the human health resulting in severe consequences (Schneider et al. 2000).

The cosmetics products become prone to contamination either during manufacturing in a container or during use by the consumer. In the first case, the manufacturer of the product must adopt all the safety protocols to avoid contamination so that best quality of the product must be ensured. While in the second scenario, the user must keep the products in safe place to avoid health issues. The most important concern about cosmetics is that they are not labelled with their manufacturing and expiring date due to which chemicals used for preservation degrade at their specific time. Ultimately, the contamination will occur and people keep on using such contaminated products without being in their knowledge. The microbial contamination of

cosmetics results in the production of such toxins which cause severe irritation and allergy on the skin (Michelutti et al. 2020).

From extensive survey studies it has become evident that allergies caused by cosmetics are increasing day by day. Approximately 10% of total population experience severe allergic reactions after exposure to cosmetics products. Severity of allergy depends upon the ingredient which becomes contaminated as well as time of exposure to a particular product. The use of cosmetics on damaged or inflamed skin aggravates the allergic reactions and make them more severe and serious (Żukiewicz-Sobczak et al. 2013). Urticaria constitutes group of disorders that causes itchy scars ranging in size from millimeters to centimeters and become coalescent. It is caused by utilization of contaminated cosmetics and their repetitive use (Wedi et al. 2009).

The use of cosmetics also results in anaphylaxis which causes severe swelling in mucous membrane of skin followed by difficulty in breathing and nausea. The contamination of cosmetics causes contact dermatitis which results in red patches on skin, swelling and itching. It occurs mostly in immune compromised people whose immune systems have already become sensitized to microbes and allergens (Goossens, A. 2011).

The US FDA mentions clearly that cosmetics do not need to be sterile but they should be free of pathogenic microorganisms and the concentration of microbes which are non-pathogenic should also be minimum. A proper monitoring and management system should be established for the regulation of cosmetics to ensure best quality of the raw material used in their manufacturing and best hygiene facilities (Kim et al. 2020). There should be proper declaration by the manufacturers that they have tested the product and it does not contain any microbe. Such monitoring is essential to minimize the infection caused by contaminated products because those people whose immune system is compromised they are prone to every kind of infection and get lethal effects. Heavy metals concentration should be checked properly as their concentration beyond threshold level causes toxicity and serious issues. Our skin is the largest and most sensitive organ so it needs to be treated carefully. The personal care products recalls are occurring widely so these issues need to be addressed seriously so to ensure best health and microbe free products (Jairoun et al. 2020).

1.1 Aims and Objectives:

- To isolate and culture bacteria from different brands of cosmetics.
- To perform biochemical and molecular characterization of bacterial isolates.
- To assess the health risk or potential impacts associated with isolated strains.

2 Review of Literature

2.1 Significance and usage of cosmetics:

Cosmetics have been widely utilized in daily life of people and due to large scale consumption of these products, the cosmetics industry is expanding and cosmetics have become one of the most active trade globally. Cosmetics have been used by people since long, even in the Stone Age to protect themselves from excess heat and light. In ancient time people used to paint themselves using oils, different mixture of clays and plant materials to protect themselves and minimize the effects caused by heat and cold weather. However, as societies became more civilized and their esthetic sense developed with the passage of time, people developed more interest in the usage of cosmetics for the purpose of personal hygiene, to look more attractive, to enhance tranquility, to prevent aging, to protect skin from harmful UV radiations and other harmful environmental factors. Cosmetics have been divided into the main categories depending upon purpose of application which include cosmetics for skin care, makeup, cosmetics for body protection, cosmetics for hair care, oral use cosmetics, fragrances etc. (Draelos and Z.D. 1995).

Skin care cosmetics are termed as "facial cosmetics" as they are mainly applied on the face for the purpose of cleansing, protection and maintaining the texture of skin. Makeup cosmetics include those products which are mostly applied on face and can be base makeup as well as point makeup. Body care cosmetics include those products that protect us from sun and tan. They also include hair remover, insect repellent, soaps, hand care, moisturizers etc. Cosmetics for hair care include shampoos, hair damage protection products, conditioners, hair dyes and scalp treatments etc. Oral care cosmetics are mouth wash and toothpastes etc. Fragrances are applied on the body to smell good and include perfumes and deodorants (White et al. 2006).

The cosmetics products should be useful in physicochemical, physiological and psychological aspects. In terms of physicochemical aspect, the product should be effective in preventing skin from harmful effects like UV rays and it should be capable of covering spots and freckles on skin. While in physiological aspect, the cosmetics products should alleviate the roughness of skin giving smooth texture, preventing hair loss and perfectly confer protection to physiological features of hair as well as skin. In terms of psychological usefulness, the cosmetics should give

the user more confidence and it has been elucidated from the research that working efficiency increases after applying makeup (Nduka et al. 2019).

Table 2.1. Classification and usage of cosmetics

Classification	**Usage**				**Main Products**
Skin care cosmetics	Cleansers	Conditioners		Protectors	Cleansing creams, lotions, moisturizers, Milky lotions
Makeup cosmetics	Base makeup	Point makeup		Nail care	Foundations, Face powders, Lipsticks, Brushes, Nail enamels
Body care cosmetics	Bath	Sun care	Insect repellents	Bleaching creams	Soaps, Sunscreens, Insects repellent lotions, Bleach creams
Hair care cosmetics	Cleansing	Hair color	Scalp remover	Permanent waves	Shampoo, Hair bleaches and dyes, Dandruff free hair shampoo, hair wave lotion
Oral care cosmetics	Toothpastes		Mouth washes		Toothpastes and mouth washes
Fragrances	Fragrances				Perfumes and sprays

2.2 Microbial contamination of cosmetics:

Microbial contamination of cosmetics products is a global health issue and causing nuisance among consumers, manufacturing industries and clinicians. The cosmetics become prone to contamination due to impurity of raw material, due to use in contaminated atmosphere or poor personal hygiene (Kim et al. 2020). The microbes not only modify the physical features of products like color, viscosity, flavor and scent but also degrade the crucial components of products which result in severe consequences. The microbial interference may produce certain

toxic compounds and metabolites which cause severe allergic reaction to skin (Budecka et al. 2014).

2.3 Side effects of contaminated cosmetics:

The risks associated with contaminated products can have significant impact on human health ranging from mild to severe diseases. Pathogenic microbes have been isolated from cosmetics products which include ***Staphylococcus aureus*** and ***Pseudomonas aeruginosa***.

2.3.1 Staphylococcus aureus side effects:

Staphylococcus aureus causes infection of skin as well as soft tissues such as abscess formation, formation of pus in hair follicles, blebs, cysts, inflammation, impetigo which involves large sized blisters which exude fluids resulting in the formation of crust having color like honey. Staphylococcus also causes scalded skin syndrome which is characterized by fever, rash and formation of blisters. It also causes severe infection of blood stream called bacteremia resulting in fever and low blood pressure. Staphylococcus is also reported to cause septic arthritis resulting in pain and swelling in joints along with fever (Noble and W.C. 1998).

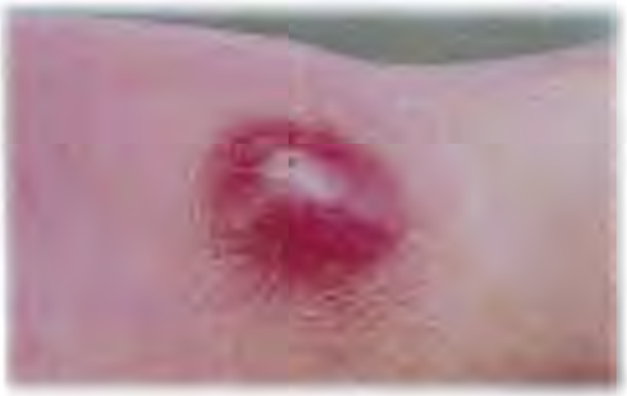

Figure 2.1 Skin infection caused by Staphylococcus aureus (Adapted from Noble and W.C. 1998).

2.3.2 Pseudomonas aeruginosa side effects:

Pseudomonas aeruginosa causes folliculitis involving itchy patches filled with pus around hair follicles. It also gives rise to otitis which is severe ear infection and characterized by ear pain, swelling, itching and discharge of fluid from ear. Pseudomonas is also causative agent of pneumonia followed by high fever, chills and difficulty in breathing. Numerous eye infections

have been reported to be caused by Pseudomonas causing inflammation and pus formation in eyes resulting in impaired vision (Wu et al. 2011).

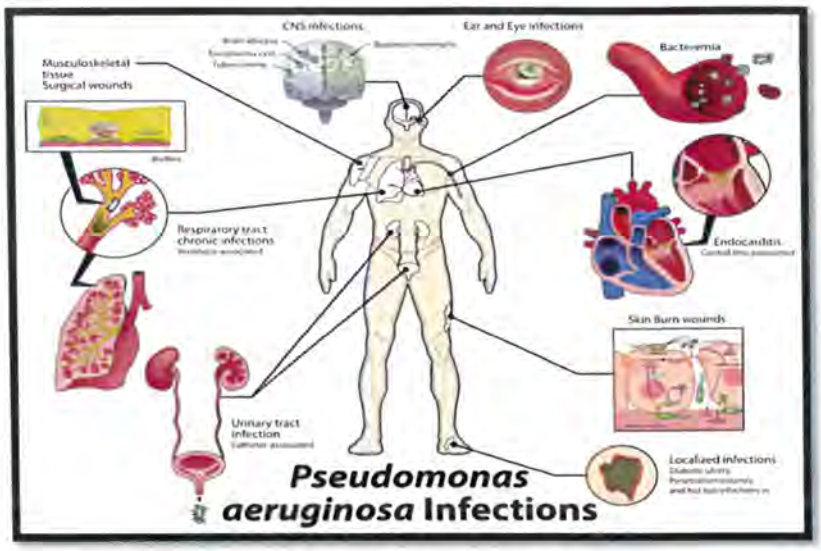

Fig.2.2. Types of Acute *P. Aeruginosa* Infections. (Proctor et al., 2021) *P. aeruginosa* is prevalent in skin and soft tissue infections (top right) including trauma, burns, and dermatitis. It also commonly causes swimmer's' ear (external otitis), hot tub folliculitis, and ocular infections, bacteremia and septicemia, especially in immunocompromised patients, and endocarditis associated with IV drug users and prosthetic heart valves (bottom right). *P. aeruginosa* can also cause central nervous system (CNS) infections such as meningitis and brain abscess (top left), bone and joint infections, including osteomyelitis and osteochondritis, respiratory tract infections, and hospital-acquired urinary tract infections (UTIs; bottom left). *P. aeruginosa* is also resistant to many common antibiotics (Adapted from Proctor et al., 2021).

2.3.3 Side effects of Enterobacter infection:

Enterobacter has been reported to be present in cosmetics. It causes a variety of ailments which include eye infection, skin infection as well as soft tissue infection, pneumonia, blood infection and urinary tract infection. Meningitis is also reported to be caused by Enterobacter in which meninges become prone to inflammation resulting in headache, fever, cramps in neck, vomiting and anorexia etc. (Ramirez et al. 2021).

Fig. 2.3. Cellulitis and edema related with skin and soft tissue infection.

2.3.4 Infection caused by Klebsiella pneumoniae:

Klebsiella pneumoniae is one of the most common bacteria isolated from cosmetics. It has capability to infect lungs, brain, liver, eyes, blood and wounds. Klebsiella causes severe pneumonia resulting in fever, chills, chest pain, coughing. It is also a causative agent of UTI resulting in frequent urination, fever, vomiting and nausea. When it invades the skin barrier and gain access to skin tissues, it causes cellulitis, dermatomyositis, redness on skin along with inflammation and fatigue. Endophthalmitis is one of the severe consequences of Klebsiella infection which is characterized by inflammation in white of eye leading to the eye pain, redness in eye, oozing of yellow or white fluid from eye and blurred vision (Elemam et al. 2009).

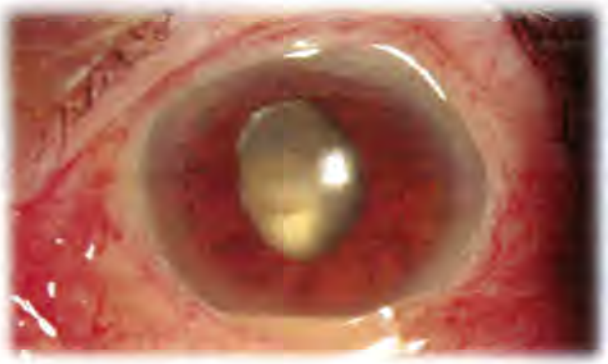

Fig.2.4 Endophthalmitis following intravitreal injection (Adapted from Vaziri et al. 2015)

2.3.5 Pseudomonas putida infection:

Another study has shown that *Pseudomonas putida* has also been isolated from mascara and eye liner products (Noor et al. 2020). It is a pathogenic microbe and cause of high rate of morbidity among immunocompromised individuals. It has caused severe blood infection among people

having soft tissue infection which further extends to the multi organ failure ultimately leading to the death of individual (Thomas et al. 2013).

Fig 2.5. Infection caused by *Pseudomonas putida* (Adapted from Wu et al. 2011).

Table 2.2. Classification of bacterial strains isolated from cosmetics, their characteristics and diseases caused by them

Bacterial strains	**Characteristics**	**Diseases**
Staphylococcus aureus	Gram positive, round shape, Facultative anaerobe, Member of Firmicutes	Abscess formation, Hair folliculitis, scalded skin syndrome, Bacteremia, Septic arthritis.
Pseudomonas aeruginosa	Gram negative, strict aerobe, encapsulated, rod shaped	Folliculitis, Otitis, Pneumonia, Eye infection
Enterobacter	Gram negative, rod shaped, facultative anaerobe, flagella	Eye infection, Skin infection as well as soft tissue infection, Pneumonia, Blood infection, Urinary tract infection and Meningitis
Klebsiella pneumoniae	Gram negative, non-motile, encapsulated, facultative anaerobe, lactose fermenting	Infection of lungs, Brain, Liver, Eyes, Blood and wounds, Pneumonia, Cellulitis, Dermatomyositis, Redness on skin, Endophthalmitis
Pseudomonas putida	Gram negative, rod shape, saprotrophic, non-fermenting	Blood infection, Multi organ failure leading to death.

2.4 Contact dermatitis:

Contact dermatitis is one of the most common skin disorder which is caused by cosmetics. The individual may have sensation of burning and stinging while in severe case it results in swelling, red patches on skin, itching and peeling of skin (Fonacier et al. 2010).

There are two main categories of contact dermatitis:

- Irritant contact dermatitis
- Allergic contact dermatitis

2.4.1 Irritant Contact Dermatitis:

Irritant Contact Dermatitis is one of the most common types of contact dermatitis and constitutes about 80% of all contact dermatitis. It is a type of non-allergic reaction that happens when the substance in cosmetics or any other chemical damages the outermost protective layer of the skin. It is a kind of non-specific response of skin receptors to the direct exposure of chemical which triggers the release of inflammatory mediators from epidermal cells (Litchman G et al. 2022). The irritant substance may be soaps, detergents, solvents, hair dye, makeup, solvents and insecticides etc. Some people get adverse reaction after exposure for the first time. While others may get affected by repetitive exposure even to the mild substance and some people become tolerant to the irritant over time (Bains et al. 2019).

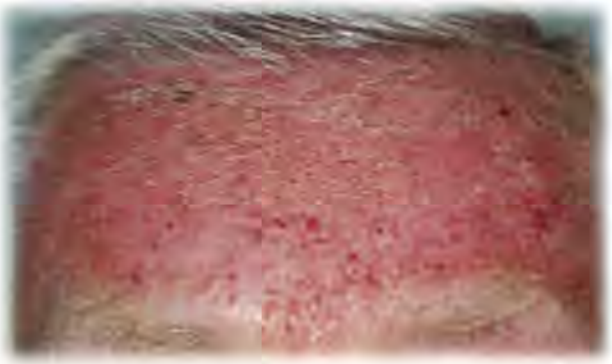

Fig.2.6 Acute irritant contact dermatitis on the forehead 1 -week after the application of diclofenac gel (twice daily) for the treatment of actino keratoses. The patient had skin type I and

very sensitive skin all his life. Patch testing with diclofenac gel as well as a repetitive open application test on the forearm for 1 -week was negative (Adapted from Frosch and John. 2011).

2.4.2 Allergic Contact Dermatitis:

Allergic Contact Dermatitis is a type of delayed hypersensitivity reaction which occurs after one or two days after exposure to an allergen (Litchman G et al. 2022). It produces effect only on the area of contact to the specific allergen and appears as rash on that specific area. For example, after exposure to poison ivy, the body gives rise to an immense inflammatory reaction to the plant oils and produces rash and itching on the skin. The most common allergens which are causative agent of allergic contact dermatitis include personal care products like deodorants, nail paints, body washes, hair dyes, mouth wash, antibiotic creams and sunscreens etc. (Kostner et al. 2017).

Fig.2.7. Skin infection due to allergic contact dermatitis (Adapted from Murphy et al. 2018).

2.5 Diagnosis of Bacterial infections:

The accurate and early-stage diagnosis of a disease is crucial to avoid the long-lasting effects and complications. The precise diagnosis of infection improves the effectiveness of the treatment required to alleviate that infection and prevents from unnecessary practices and medication. The precise diagnosis of a disease prevents the outbreak of that disease and minimizes the development of antibiotic resistance. Conventionally, bacterial infections are diagnosed with the help of culture methods however, due to certain limitations like some bacteria are difficult to grow and their growth requirements are different and time-consuming procedure do not make it an ideal method for bacterial diagnostics. Over the several decades, the nucleic acid testing has revolutionized the diagnosis of disease and it is more fast, accurate and sensitive than the

traditional culture methods (Muthukumar et al. 2008). The pros and cons of diagnosis methods are described below in the table 2.3 and 2.4.

2.5.1 Comparison of culture and nucleic acid testing methods:

Table 2.3. Pros and cons of culture- based diagnosis (Adapted from Muthukumar et al. 2008).

Pros	Cons
Least instrumentation required	Biosafety concern arises
High specificity	Low sensitivity
Less expensive	Time consuming for slow growing bacteria
Antibiotics sensitivity can be tested	Non-viable bacteria cannot be tested
Biochemical characterization can be performed	The contradiction among biochemical phenotype and genotype may occur.
Morphology of colonies can be visually inspected	

Table 2.4. Pros and cons of nucleic acid based diagnosis (Adapted from Muthukumar et al. 2008).

Pros	Cons
High sensitivity	Chances of false positive results
Detection of nonviable bacteria	Genotype and phenotype contradiction
High resolution analysis	Additional training required
Reduced biosafety concerns	Special instrumentation required
Detection of certain antibiotic resistant strains without culturing	Few antibiotics sensitivity tests available
Rapid turnaround time	Interference of false negative occur and hinders the amplification process

2.6 Molecular and biochemical identification of bacterial strains isolated from cosmetics:

S.aureus is one of the most frequently occurring bacteria worldwide and is reported to infect large number of cosmetics users worldwide. Molecular methods like PCR have been extensively used for the identification of ***S.aureus***. In Egypt, five colonies of S.aureus have been isolated from cosmetics and preserved in falcon tubes for further identification. The isolates were identified through various biochemical tests like dnase, catalase, mannitol etc. Results showed that 95% isolates were dnase positive. 48% of strains could ferment mannitol and 90% isolates were catalase positive. The presence of S.aureus was further confirmed through PCR in which the electrophoresis profile indicated that the given strains are of *S.aureus* (Ezzeldeen et al. 2011).

P. aeruginosa is an opportunistic bacterium and causes severe infections in immunocompromised patients. It has been isolated from various products of cosmetics and conventional culture methods are very time consuming for its detection. However, accurate and rapid identification of isolates has been performed through PCR. 100 samples of products were collected and inoculated on media for growth. After obtaining their pure colonies, the DNA was extracted and PCR was performed based on 16srRNA gene. The primers were designed for ToxA and exoS genes and PCR reaction mixture volume constituted 20µl. After PCR confirmation, samples were processed for Sanger sequencing and the results indicated that 61 samples were *P.aeruginosa* positive. The Tox A gene was detected in 69% isolates while exoS gene was present in 49% isolates (Shaebeth et al. 2019).

Enterobacter is one of the most common pathogens which cause neonatal diseases. The identification methods based on biochemical profiles do not produce satisfying results due to their resemblance with the other members of *Enterobacteriaceae*. However, PCR has been proven to be the most accurate method for their identification based on 16S rRNA gene sequencing. 60 samples from various brands were collected for the detection of enterobacter species. The DNA extraction, PCR and sequencing were done for confirmation. The results indicated the presence of *K. pneumoniae, E. cloaceae, Acenitobacter vivianii, E.coli* and *K.quasipneumonae*. (El-zamkan et al. 2018).

K. pneumoniae isolated from cosmetics has been characterized. 39 isolates have been obtained at a hospital of Mexico. The biochemical tests indicated all of them as *K. pneumoniae* whereas Multiplex PCR identified 36 of them as *K. pneumoniae*. One of them as *K. variicola*, and rest of them were identified as *K. quasivariicola* through phylogenetic analysis (Garza-Ramos et al. 2018).

Pseudomonas putida has been identified through various phenotypic as well as molecular methods. Pseudomonas was collected from cosmetics products in New York and firstly characterized through oxidase, catalase and gram staining methods. The results indicated it as gram negative, oxidase and catalase positive. Further 16S rRNA sequencing was carried out to confirm the pseudomonas strains which showed the presence of *P. putida, P. fluorescens, P. marginalis, and P .tolasi*. Hence it is evident that biochemical profile does not classify and distinguish microorganisms accurately. The molecular techniques have revolutionized the diagnosis and identification of pathogens due to which accurate treatment can be provided to the patients on time. (Wiedmann et al. 2000).

2.7 Treatment of diseases caused by the use of contaminated cosmetics:

S. aureus infection is one of the most prevalent bacterial infections among consumers of cosmetics products which poses serious health threats. Treatment is recommended to the patients according to the type of infection, its severity and location. Antimicrobial agents are used to tackle *S. aureus* infection for example in case of impetigo topical antibiotics are given like mupirocin, retapamulin. In abscess formation, antibiotics are not necessarily given, rather incision and drainage are preferred. In bacteremia, intravenous therapy is prescribed and for MRSA vancomycin or daptomycin while in MSSA cefazolin or nafcillin are administered. In case of pneumonia, the treatment is a long procedure comprising of 3 weeks duration in which antibiotics with best bioavailability are administered with optimum range of dose. Linezolid is one of the best antibiotic given to the S.aureus infection patients. Doxycycline has shown the best results for patients having S.aureus infection and it cannot be given to the children below 8 years (Creech et al. 2015).

The infection of ***Pseudomonas aeruginosa*** is treated with different combinations of compounds having beta lactam and aminoglycosides. Carbapenems with quinolones are used in combination

with aminoglycosides to treat its infection largely. Cephalosporins are used for patients having bacteremia, meningitis, fever and neutropenia. Fluoroquinolones are administered to the patients having intra- abdominal infections and osteomyelitis. Polymyxins are used for the patients having meningitis and ocular infection (Ibrahim et al. 2020).

The fourth-generation compounds are used extensively to treat the infection caused by ***Enterobacter*** species. They have proven to be much better than the third-generation compounds in stability and resistance pattern. Cefepime is a fourth-generation antibiotic and has been administered successfully in patients diagnosed with enterobacter infection. Broad spectrum penicillin has also been used for the treatment of enterobacter infection. Carbapenem is the best choice in patients having bacteremia. Meropenem is the useful candidate for treating meningitis (Villegas et al. 2002).

The infection of ***Klebsiella pneumonia*** is treated with different antibiotics. In case of UTI, piperacillin is the preferred compound administered to treat the infection. Tazobactam is given to the patients having intra-abdominal infection. Intravenous drugs are given in skin infection along with topical creams and ointments. Aminoglycosides and cephalosporins are used to treat soft tissue infection caused K. pneumonia (Lee et al. 2012).

Dermatitis is treated by the application of topical steroids. Antibiotics are orally given to the patients along with topical lotions. In severe infection, steroids are orally prescribed for five to seven days. Azathioprine is mostly given to the patients of dermatitis. Primecrolimus is administered in conjunction with corticosteroids to reduce severity of rashes and allergy. Cleansers which are soap free are recommended by the doctors to the patients to use on daily basis. Photo therapy is one of the best treatments and has proven most effective to cure the severe atopic dermatitis. The secondary infections of skin are known to be treated best by anti-staphylococcal drugs (Frazier et al. 2020).

2.8 Pattern of antibiotics susceptibility and resistance in bacterial isolates from cosmetics:

Antibiotics susceptibility refers to the effectiveness of an antimicrobial agent and its tests are performed by medical technologist to determine the best antibiotic for a patient. It is performed on bacteria or fungi that are relevant to an individual infection. The results are expressed in the

form of MIC (Minimum inhibitory concentration) that refers to the minimum concentration of the drug required to retard the growth of microbes (Van Belkum et al. 2020). Antibiotic resistance refers to the loss of susceptibility of pathogens to the drugs which kill them or interfere with their growth. It has become a global issue which has led to the severe consequences and complication in the treatment of patients resulting in the increased rate of morbidity (Ventola and C.L. 2015).

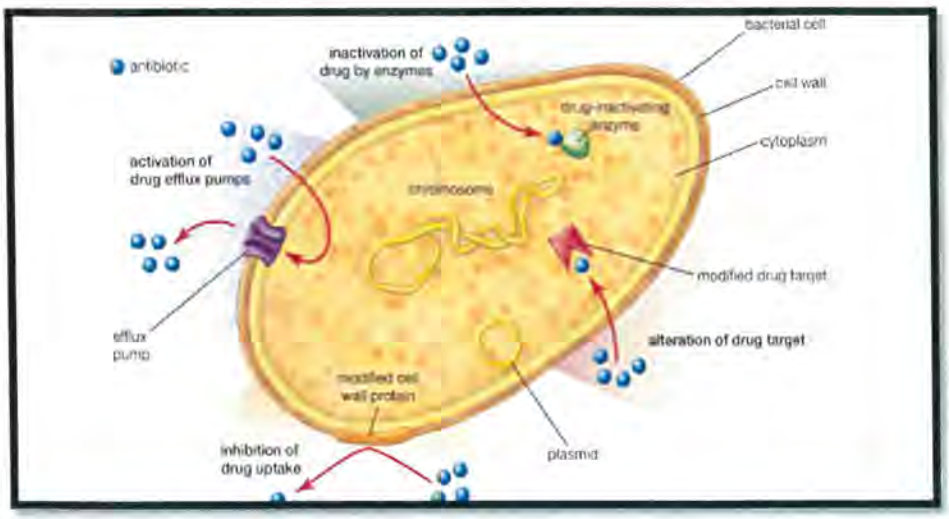

Fig.2.8 Different Mechanisms of antibiotic resistance which include interference of bacterial enzymes with drugs causing inactivation, Activation of efflux mechanism for drug expulsion, change in drug target, the drug uptake by cell ceases (Adapted from Morier. 2021).

Total of 10 cosmetics products from different brands were collected which included mascara, eye shadow, eye liner in West Bangal and India. The bacterial strains were isolated and identified. Later on, the antibiotics susceptibility for strains was determined by disk diffusion method. The Mueller Hinton agar was inoculated with fresh strains and incubated with antibiotic discs which included Ampicillin, Ciprofloxacin, Meropenem, Vancomycin, Cefpodoxime, Gentamycin and Cefotaxime. The results showed that Bacillus was sensitive to all the antibiotics whereas Pseudomonas showed resistance to five antibiotics which included Ampicillin, Vancomycin, Cefpodoxime, Ciprofloxacin, Nalidixic acid and Cefotaxime. The chromobacterium violaecium showed resistance to the Ampicillin and Cefpodoxime. Listeria monocytogenes was resistance to Vancomycin and Nalidixic acid (Shayamapada and M. 2016). The enterobacter was susceptible to Cefotaxime, Ampicilin and resistant to erhthromycin and kanamycin. Pseudomonas putida

was susceptible to the Ampicilin, Novobiocin and resistant to Cefotaxime, Penicillin and Erythromycin (Orus et al. 2015). Staphylococcus aureus has been found to be resistant to the vancomycin and daptomycin (Foster and T.J. 2017). Klebsiella pneumoniae was found to be resistant against Meropenem, Ceftazidime, Ciprofloxacin and Gentamycin (Navon-Venezia et al. 2017). There is dire need to overcome the multifactorial and complex process of antibiotic resistance as it is a global threat to health and development. A huge number of infections have become hard to treat and cure due to inability of drugs to stop microbial growth. Immediate efforts are needed to minimize these threats and mortality caused by antibiotic resistance (Meerza et al. 2021).

2.9 Beneficial bacteria as components of cosmetics:

The revenue of international market of cosmetics has accretion up to 429.8 billion dollars by 2022. This significant progress in cosmetics industry is achieved by adopting aggressive strategy of marketing by different international brands through the production of ecofriendly cosmetics. These biological products have appealed every kind of consumer acquainted with ayurvedic and biocompatible ingredients. People have become keen to the use of cosmetics products derived from biological origin providing maximum benefits. The addition of organic ingredients in cosmetics have minimized the risks and side effects which are caused by chemical components in cosmetics. As microbes exist in huge diversity, so they act as potential candidates for biotechnological and cosmetics products in future (Gupta et al. 2019).

Microbes are considered favorable candidates for the production of metabolites and they are capable of producing the product in large quantity at very reasonable price making the process cost effective. Microbes also have the ability to survive in different set of conditions and environments which make them distinct from organisms and are able to produce diverse bioactive compounds. They are enriched in compounds like fatty acids omega 3-6, enzymes, carotenoids, lipopolysaccharides and ceramides etc. which possess extensive application in cosmetics industry. These compounds are involved in the production of esters, aromatic compounds and active components used in manufacturing of cosmetics. Bacteria secrete compounds of immense commercial value like Vitamins, Bio surfactants, Exopolysaccharides and peptides etc which serve the purpose of enhancing beauty as well as improving the health of consumer (El-Enshasy et al. 2016).

2.9.1 Bacterial strains used in cosmetics:

2.9.1.1 Oligosaccharides:

Cyclodextrins are ring structured compounds that are made up of fused moieties of oligosaccharides which have remarkable benefaction in formulation of cosmetics. The bacterial strains which are extensively used for the production of cyclodextrins are *Bacillus subtilis 313, Microbaterium terrae* KNR 9 and species of *Brevibacterium*. They are frequently used for the reduction of esters volatility in perfumes leading to the enduring aromatic effect. They are also used in diapers and sanitary pads to reduce and minimize the odor (Gupta et al. 2019).

2.9.1.2 Bio surfactants:

The most important and significant source of bio surfactants are *Bacillus subtilis, Bacillus lichenoformis, Bacillus amyloliquefaciens* and *Bacillus pumilus.* Due to being biodegradable and non-toxic biosurfactants are extensively used as agents for skin hydration, foaming agents, detergents as well as emulsification agents. One of the most distinguished and appealing class of biosurfactants is Mannosylerythritol lipid (MEL) which are extensively used in the production of eye shades, eye liner, lipsticks, body sprays, soaps, nail care and body massage kits. One of the main uses of biosurfactants in cosmetics is in the developments of anti-aging creams and cleansing agents. Biosurfactants have enormous dermatological applications and used in topical formulations on large scale (Del Valle et al. 2004).

2.9.1.3 Exopolysaccharides:

Exopolysaccharides are the prominent class of skin hydrating agents which maintain the skin in appropriate environment. Dextran is the most frequently used class of exopolysaccharides which is produced from *Leuconostoc mesenteriodes* and *Streptococcus mutants*. Dextran is mainly employed as smoothening as well as brightening agent for skin due to promoting glow and fairness of skin and reducing the wrinkles. Besides skin brightening agents, dextrans also act as anti-inflammatory agents and improve the rate of blood flow. Xanthomonas produce another class of EPS called Xanthan which act as moisturizing agents in skin formulations (Yildiz et al. 2018).

2.9.1.4 Hyaluronic acid:

Streptococcus and Bacillus species act as synthetic machinery for the production of hyaluronic acid which is dermal filling agents in cosmetics. They are also used as moisturizing agents in lotions. HA improves the texture of skin by retaining its moisture content and minimizing the wrinkles on it (Sze et al. 2016).

2.9.1.5 Proteins and peptides:

Bacillus species are mainly used for the production of peptides in cosmetics applications. These peptides are used in gel preparations, moisturizers and powders. Some insoluble peptides are used in the preparation of facial mask. Penta-peptides are frequently used as skin smoothening and anti-aging agents (Lods et al. 2000).

2.9.1.6 Enzymes:

Enzymes have been widely used in cosmetics industry since ancient times and have produced stable and compatible products. Superoxide dismutase and peroxidase are the main enzymes used in this industry and they were secreted from *Marinomonas* species as well as from extremophiles such as *Thermus thermophiles* in earlier times. With the technological advancements lactic acid bacteria have been genetically engineered which produce these enzymes in higher quantity having maximum stability. These enzymes act as free radical scavengers and block the UV light reaching the surface of skin (Lodz et al. 2000).

Proteases are another important class of enzymes which hydrolyze the peptide bonds of many types of skin tissues like keratin, elastin and collagen. These proteases have been isolated from Bacillus licheniformis, Thermoanaerobacter, Thermococcus, Lysobacter, Kocuria, Vibrio, Stentrophomonas etc. Protease have been used to treat skin disorders such as psoriasis (inflammation and flaking of skin), xerosis (skin dryness) and scaly skin disorder called ichthyoses. Keratinases are used to cure stretch marks and regeneration of epithelial cells to augment healing process. They are also used in topical ointments like lotions for heels and knees. Keratinases are extensively used in delaying the growth of hair as well as hair removing process (Gupta et al. 2013).

2.10 Preservation of cosmetics:

Besides oil and water, cosmetics contain such components which encourage growth of microbes due to which they become prone to contamination easily. Therefore, it becomes indispensable to add preservatives which confer long lasting protection against putridness and rottenness due to bacterial interference in cosmetics from multiple sources. Contamination which arises due to microbes during production is termed as primary contamination and it typically arises due to presence of bacteria in water (Gram negative and rod-shaped bacteria). The contamination which occurs during usage of product is called secondary contamination and it usually occurs due to bacteria in environment (Gram positive rod and round shaped bacteria). Primary contamination can be minimized by adopting manufacturing practices and products are not acceptable to the consumers if products are not made according to the good manufacturing practices. Secondary contamination can be avoided by taking care of hygiene conditions and using the products with utmost care. It is necessary to add preservatives to prevent the contamination which suppress the growth and proliferation of microbes on time and thus deterioration of product can be avoided. The most commonly used preservatives are parabens and they are also used for preservation of food. Disinfectants are also added to the cosmetics products to sterilize the skin surface and maintain the texture of skin. They are mostly used to suppress acne, dandruff and odor in skin. Most important disinfectants are benzalkonium chloride and trichlorocarbanilide (Mitsui et al. 1997).

2.11 Requirements of an ideal antimicrobial agent in cosmetics:

Not every kind of antimicrobial agent is added to the cosmetic as all of them do not possess desirable features. So, an ideal antimicrobial agent should have following salient characteristics:

1. Antimicrobial agent should be highly safe without causing any kind of irritation.
2. It should have efficacy against wide range of microorganisms.
3. It should be easily miscible in ingredients of cosmetics.
4. It should be neutral having no alteration in pH.
5. It should not have any impact on effectiveness of ingredients of product.
6. It should maintain the appearance of the product.
7. It should be economical and readily available.

2.12 Evaluation of safety of cosmetics:

The most important aspect of cosmetics is their safety assessment because more than half of the world is depending upon the usage of cosmetics. The consumption of cosmetics is directly linked with consumers and their health, so it is most crucial to consider the safety of products and ensure it at every point (Turnbull and S.E. 2018).

2.12.1 Evaluation methods for safety of cosmetics:

The key concept of each method of safety assessment is that cosmetics are the products to be used for long terms so they should not contain such constituents which cause any kind of irritation and toxicity after use. Though it is necessary to test the products on human but mostly animals are used as models to test the safety and efficacy of the product (Rogier et al. 2020).

2.12.1.1 Skin irritation:

The most important and foremost thing about safety of cosmetics is that it should not cause allergic reaction on skin when applied. The allergic reaction is not always caused by the ingredients of the products but also by the misuse of product and secondary contamination. Skin allergy is caused by the direct toxic action of chemicals on the skin cells and blood vessels. This kind of response is observed in the people which are exposed to strong acids and bases. Rabbits and pigs have been used as test models due to similarity in response to that of humans and the test material is applied on the skin of model once or repeatedly and thus product safety is evaluated (Panico et al. 2020).

2.12.1.2 Sensitization:

The allergic reaction may also occur when body is exposed to the chemicals repeatedly which have sensitization potential. Such chemicals when administered through skin it is called contact allergy or sensitization. It is a delayed type of hypersensitivity response due to delay in inflammatory reaction. The mouse is used as test model but mostly used animal is guinea pig to test cosmetics ingredients. The adjuvant and patch test methodologies are used to evaluate the final product. Some impurities have been reported in the preservatives, fragrances and coloring agents and classified as allergenic substance. So, it is extremely important to do sufficient tests at the developmental stage of the compounds to avoid harmful impacts of the substances (Monnot et al. 2020).

2.12.1.3 Photosensitization:

Photosensitization is a type of allergic response which only occurs in the presence of light. Photosensitivity has been reported for a variety of materials which include UV absorbers, fragrances and bactericidal substances. So, it is most important thing to assure that neither component of cosmetics are photo allergenic as cosmetics products applied on the skin are exposed to sunlight directly in routine. The sunscreens and UV absorbents should be evaluated on the basis of photosensitivity which are most commonly used in UV light. The process of photosensitization results in the activation of material by the light which changes the functions of cells of immune system consequently, changing the interaction among the material applied and cells of immune system. Mice and pigs are used as test models in which photosensitization is induces and application of product is carried out and response is monitored (Mujtaba et al. 2020).

2.12.1.4 Eye irritation:

Some cosmetics like mascara, eye shades, kajal and shampoos may enter the eye, so it is important to assure the safety of these products so that they do not cause any kind of irritation and allergy in eyes. Rabbit has been used as test model in which product is applied to one of the eyes of the rabbit and the effect on cornea and iris is monitored over time. It is necessary to consider the safety assessment of every kind of product thoroughly and minimize the irritants in the cosmetics (Lebrun et al. 2021).

2.12.1.5 Toxicity:

2.12.1.5.1 Acute toxicity:

Acute testing aims at investigation of the side effects and consequences of ingestion of cosmetics accidentally. In this test, product is administered to the rodents' like mice and toxicity level is determined from lethal dose. Substances are administered through various routes i-e oral, transdermal and subcutaneous etc. (Chinedu et al. 2013).

2.12.1.5.2 Subacute and chronic toxicity:

This kind of testing aims at determination of toxic effects on the organs caused by the long term use of cosmetics. The tests are performed by administering the product to the rodents continuously over a time periods from weeks to months. Various parameters like body weight,

intake of food and growth are monitored and biochemical tests are carried out. As the particular time period ends then weight of each organ is measure and histopathological examination is carries out and effect on over all body is also determined (Gupta et al.2012).

2.12.1.5.3 Reproductive testing:

This testing determines that cosmetic has potential of causing toxic effects on reproduction or not. The testing product is administered to the rodents multiple stages which include before pregnancy, during organogenesis, during lactation stage (Parker and R.M. 2006).

2.12.1.5.4 Testing on humans:

Many types of side effects ranging from mild to severe have been reported from the application of cosmetics products. Before launching a product in the market it is important to assure the safety and efficacy of the product .Various kinds of safety assessment tests are being conducted on animal models but it becomes very challenging to predict the likelihood of sensory response like burning, irritation, stinging and itching etc. using animal models. Thus patch and stinging tests should be necessarily carried out on the human volunteers in an ethical manner to ensure the safe and best use of the products (Pistollato et al. 2021).

3 Material and Methods

Different products of cosmetics from various brands were purchased for biochemical and molecular characterization of persistent microbes. Different biochemical tests were performed along with phenotype analysis for the identification of bacterial strains isolated from cosmetics. The molecular characterization was performed for the confirmation of strain at *Molecular Systematic and Applied Ethnobotany Lab (MoSAEL),* Department of Biotechnology, Quaid-i-Azam University, Islamabad. A schematic representation of current research is shown in fig.3.1.

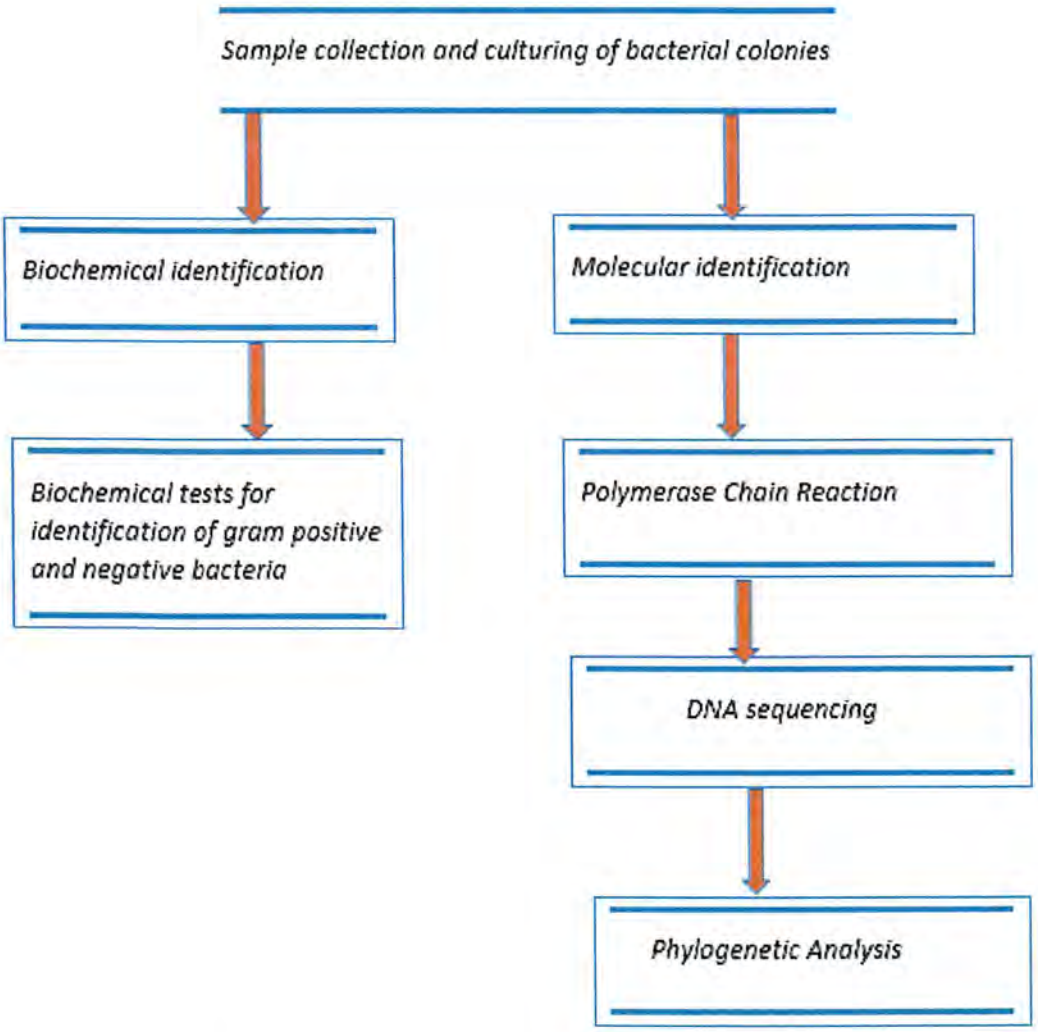

Fig.3.1 Schematic representation of research study

3.1 Isolation of bacterial strains:

The isolation of bacterial strains is accomplished by providing them suitable nutrients, temperature and environment. Bacteria are isolated successfully by growing them on nutrient media. Thus, bacterial strains were obtained after testing different products of cosmetics.

3.2 Media Preparation:

Different types of media are used for bacterial growth and study. General use media facilitates the growth of a large variety of bacteria. For example, TSA (Tryptic Soya Agar) while selective media encourages the growth of selective microbes by inhibiting the growth of other microorganisms for example, MSA (Mannitol Salt Agar) is used for the growth of *Staphylococcus*. Differential media consists of components that distinguishes microbes visually on the basis of their appearance like form of colony or media. MacConkey Agar is used for the selection of gram negative bacteria. Normally, a solid nutrient media contains protein components like peptone, tryptophan and salts etc. along with the addition of agar. Agar is the most important component of nutrient media that is used frequently for the isolation and purification of cultures.The solid media contains 1.5-2% Agar and due to its physical structure bacterial colonies are obtained in suitable form. Thus, solid media is employed for the isolation of bacteria and to study its colony characteristics.

3.3 Supplies:

- Autoclave machine
- Glass reagent bottles
- Autoclaved petri plates.
- Ingredients of media according to the recipe (Already prepared media powder was used).
- Graduated Cylinder to measure distilled water.
- Heat tolerant gloves for handling hot reagent bottles.
- 70% ethanol for the purpose of disinfecting working area
- Weighing balance for weighing media powder
- Spatula for handling media powder

3.4 Procedure of media preparation:

To prepare media, the media powder was measured on weighing balance. Then Agar was weighed on the balance. Both of these reagents were then added to properly label sterile glass reagent bottles. Distilled water was then added in the bottle to make the mixture. Different types of media were prepared including TSA ((Tryptic Soya Agar), SDA (Sabouraud dextrose Agar), MacConkey Agar, SIM (Sulfur, indole, motility), Simmons citrate agar, Urea base agar.

Media Composition

Table 3.1. Different types of media utilized for culturing and testing bacteria

Sr.No.	Media type	Amount of Media Powder	Amount of water	Purpose
1-	TSA (Tryptic Soya Agar) Lab Protocol	30g 12g	1L 400ml	Isolation of bacteria.
2-	SDA(Sabouraud dextrose Agar)	65g 26g	1L 400ml	Isolation of fungi
3-	Simmons citrate agar	24g 4.8g	1L 200ml	Biochemical test
4-	SIM	30g 6g	1L 200ml	Biochemical test
5-	Urea base agar	2.4g 1.27	95ml 50ml	Biochemical test
6-	MacConkey agar	51.5 10.25	1L 200ml	Biochemical test

3.4.1 Sterilization of media:

The lids of reagent bottles containing media in them were kept loose and placed in autoclave machine to minimize the contamination of atmosphere or hands. The autoclave machine was maintained at the temperature of 121 ° for sterilization at the pressure of 15 psi for duration of 20

minutes. The temperature of 121° kills the microbes which cause contamination leaving the components of media intact and safe.

The sterilization cycle has four steps:

- Heating up autoclave chamber at temperature 121°.
- Penetration of heat in medium container at temperature ≤100°-121°.
- Holding time of 20 minutes at the temperature of 121°.
- Cool down of the chamber from 121° to 80°.

3.4.2 Pouring of media into plates:

- Before pouring the media, the laminar hood was sterilized properly by using 70% ethanol.
- The sterile petri plates were placed on the clean surface and were properly labelled.
- The media was kept to be cooled enough to be handled properly.
- Then the lid of bottles containing media was opened to pour media.
- The 15-20 ml of media was poured into each petri plate in such a way that bubble formation should be minimum.
- The plates were then placed for a few minutes to let the media solidify and then the plates were inverted to avoid moisture on the lid of plates.
- The plates were then kept overnight at the room temperature to check contamination.
- The media was ready to be used for culturing or stored in refrigerator in a tight plastic bag for future use.

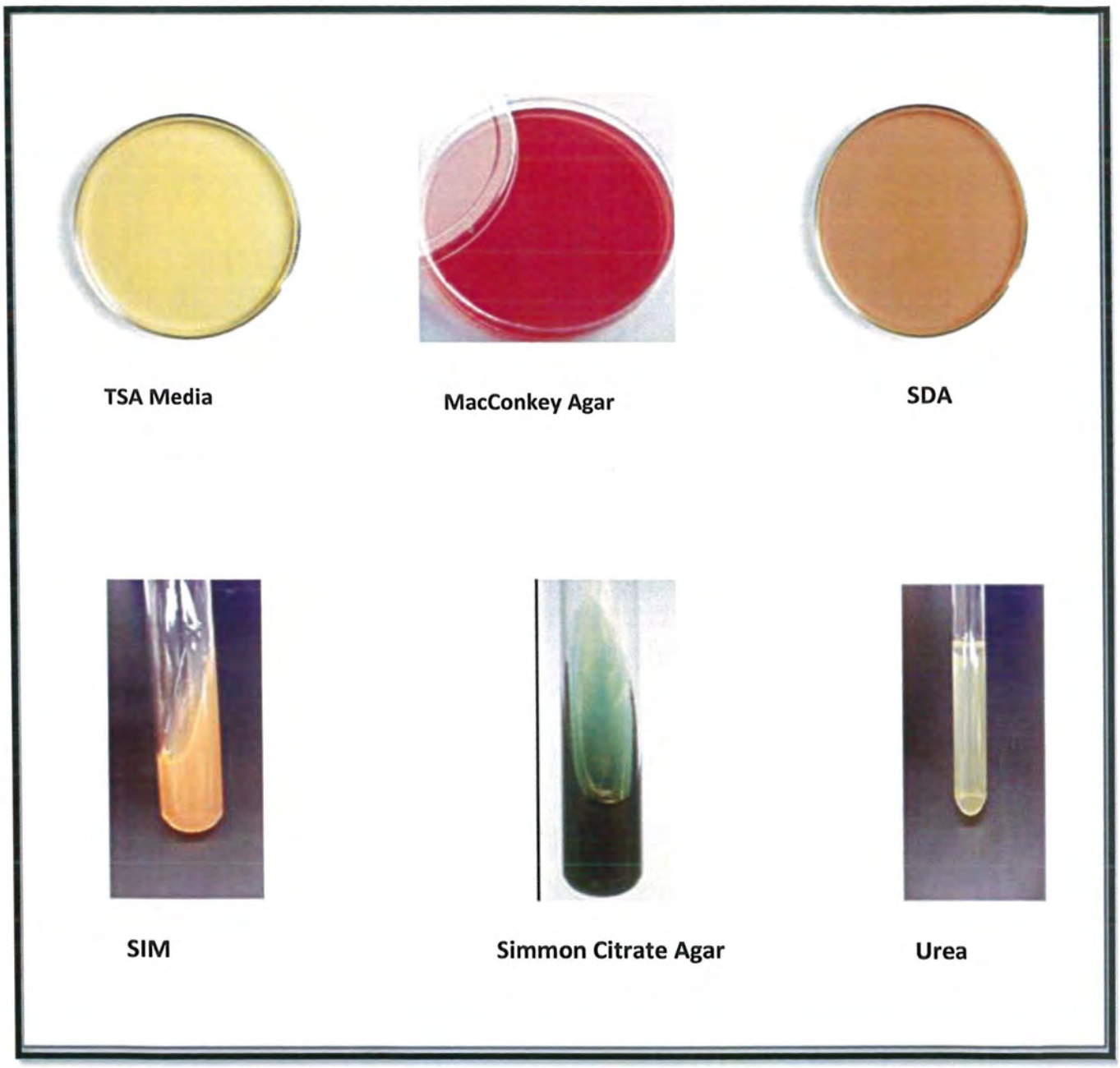

Fig 3.2. Different media prepared for testing purpose

3.5 Sample processing and inoculation:

The cosmetics products used as samples were labeled properly. The serial dilution was performed for each sample. Each product of cosmetics was dissolved in autoclaved distilled water and DMSO (Dimethyl sulfoxide). Each product was dissolved in 1ml solvent. The samples were then inoculated on the TSA and SDA media plates for culturing.

3.5.1 Culturing bacteria on media plates:

A drop of each labeled liquid sample was taken with the help of sterile cotton swab and spread on the media plate. While the dry powdered sample was taken with the help of sterilized inoculation loop. The inoculation loop was sterilized by keeping it in the flame of spirit lap till it became red hot and then it was allowed to cool. The loop and cotton swab both were streaked gently over the quarter of media plate in back and forth motion. The loop was again kept in flame and then cooled down and streaking was done in second quarter of the plate. The streaking was repeated again and again to cover the whole area of the plate. The streaked plates were then kept in the incubator at 37°C for 24 hours so that bacterial growth can occur significantly.

3.5.2 Sub-culturing bacteria on media plates:

Bacterial colonies were sub cultured for further analysis. A sterile inoculating loop was used to pick single colony from the culture plate and streaked onto the new media plate. Streaking was done at the different angles to cover whole area of the plate. Then plates were placed in bacterial growth incubator at the temperature of 37°C for 24 hours to facilitate the growth of bacterial colonies to great extent.

3.6 Biochemical testing:

Various biochemical tests were performed in order to further recognize the bacterial strains. For this purpose, different media were prepared which included MacConkey Agar, SIM (Sulfur, indole, motility), Simmons citrate agar, Urea base agar. The prepared media were poured in the test tubes and sealed with sterile cotton. Then single colony from overnight grown bacteria was picked and suspended in agar slant. The tubes were then incubated at 37°C for 24 hours to check the result.

Table 3.2. Optimized quantity of different media and their purpose of testing

Sr.No.	Media	Optimized quantity of powder	Optimized quantity of water	Purpose of test	
1-	Simmons citrate agar	4.8g	200ml	Identification of gram-negative bacteria on the base of citrate utilization	
2-	SIM	6g	200ml	Sulfur	Identification of gram negative bacteria on the basis of Sulfur reduction
				Indole	Identification of gram-negative bacteria on the basis of Indole production
				Motility	Motile
3-	Urea base agar	1.27	50ml	Hydrolysis of urea	
4-	MacConkey agar	10.25	200ml	Gram negative bacteria grow on it	

3.7 Molecular Identification:

The molecular characterization is done in following steps as depicted below diagrammatically and each step is explained separated as well.

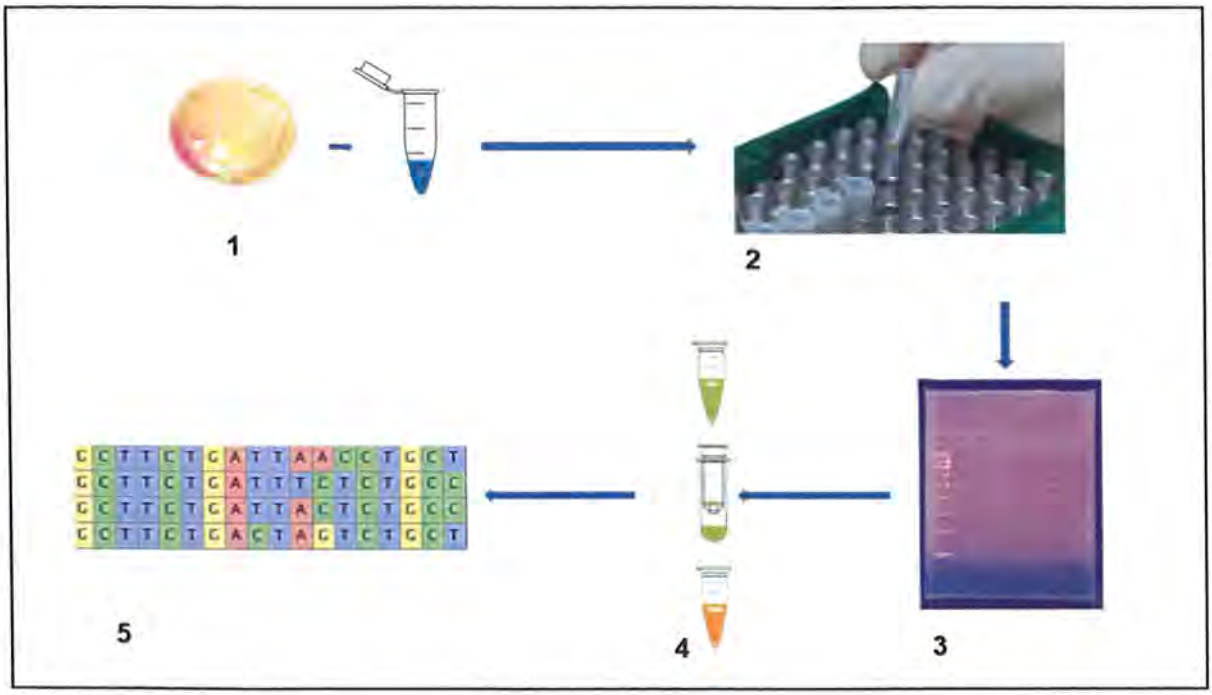

Fig.3.3 Procedure of molecular characterization performed at Biotechnology department, QAU. 1. DNA extraction of pure bacterial isolates using plain boiling method 2. Polymerase Chain Reaction (PCR) using universal primers for the amplification of 16S rRNA gene. 3. Agarose gel electrophoresis for the confirmation of amplified product. 4. Purification of amplified product from agarose gel electrophoresis. 5. Sanger sequencing and post sequence analysis using bioinformatics tools.

3.8 DNA Extraction by Plain Boiling Method:

To extract DNA from bacterial colonies, simple plain boiling method was used to higher yield of DNA. For this purpose, single colony from over- night grown bacteria was picked with micro-pipette and dissolved in 1ml distilled water in an Eppendorf tube. Then each Eppendorf tube containing bacterial colony and distilled water was kept at 95°C in water bath for 10 minutes. After boiling in water bath, each tube was centrifuged at 1000 rpm for 5 minutes. The supernatant having the bacterial DNA was shifted to another separate tube and stored at -20°C. The pellet containing the residues was discarded.

3.9 Polymerase Chain Reaction:

3.9.1 Ingredients of PCR:

- Dream Taq Green PCR Master Mix (2X) (Thermo scientific)
- Forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3)
- Reverse Primer 1492R (5'- GGTTACCTTGTTACGACTT-3')
- Template DNA
- Nuclease Free PCR water (Thermo scientific)

All the components of reaction mixture were added in an optimized quantity making the total volume of 20 µl in PCR tube.

Table 3.3. Different components of PCR reaction mixture with optimized quantity

Sr. No.	Components for Master Mix	Optimized Quantity
1-	Dream Taq Green PCR Master Mix (2X) (Thermo scientific)	10µl
2-	Forward primer 27F	1.5µl
3-	Reverse Primer 1492R	1.5µl
4-	Template DNA	5µl
5-	PCR water	2µl
6-	**Total volume of reaction mixture**	**20µl**

3.9.2 Polymerase chain reaction procedure:

Template DNA of 5µl was added in each PCR tube separately comprising the total reaction mixture of 20µl. The primers used for 16S rRNA gene in the PCR reaction mixture were 27F (5'-AGAGTTTGATCCTGGCTCAG-3) and 1492R ((5'- GGTTACCTTGTTACGACTT-3'). After adding all the ingredients, the PCR tubes were vortexed for short duration so that all the components are distributed uniformly. Then PCR tubes having reaction mixture were placed in the thermal cycler which was set at the particular conditions for 3 hours. After the completion of

PCR cycle, the amplified DNA was obtained and further analyzed on the gel through gel electrophoresis.

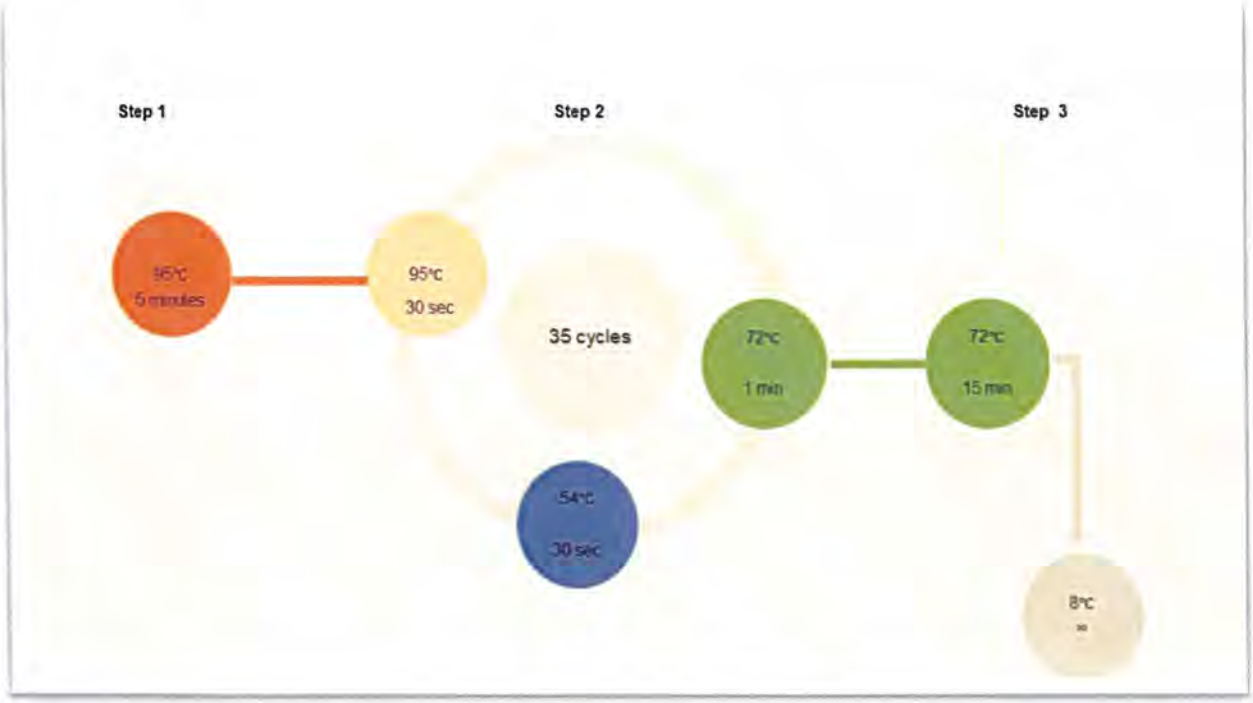

Fig 3.4. PCR conditions optimized for bacterial genome. Step 1 explains initial denaturation. While step 2 includes denaturation, annealing and extension. Step 3 proceeds towards final extension and storage.

3.10 Gel electrophoresis:

3.10.1 Set up for gel preparation:

Before preparing gel solution, the gel casting tray was prepared. Appropriate comb was placed and set in the tray and both ends were sealed with the help of tape. 0.6 g agarose powder was weighed and taken in the flask. Then 40ml of 1X TAE buffer was poured in the flask to prepare gel mixture. Then buffer/gel mixture was heated in the oven for 30 seconds to melt the powder properly. After heating in the oven, the mixture was kept to be cooled till the temperature falls to 50°C. After cooling down, 4µl ethidium bromide was added in the flask having gel mixture. Then agarose solution was poured into the gel tray and it was kept for 30-40 minutes to let it solidify at room temperature.

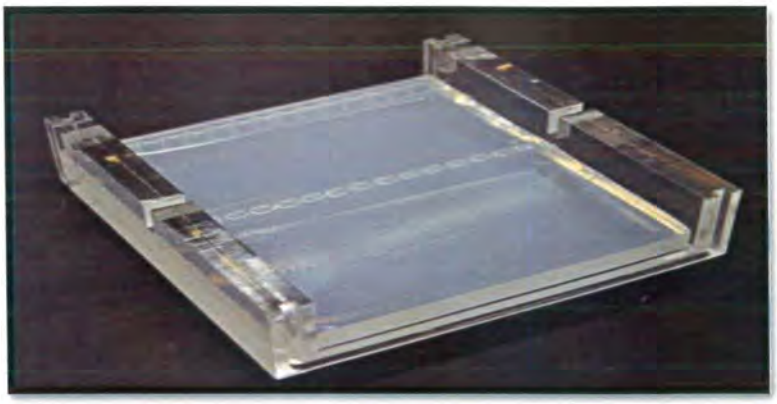

Fig 3.5. Gel prepared for gel electrophoresis

After solidification, the comb was removed and gel was placed in the gel tank. Then samples were loaded into the wells. 10µl of each sample was loaded into the wells.

1kb ladder GeneRuler™ was also loaded. Enough quantity of running buffer was added to cover the surface of gels. Same running buffer was used which was used to prepare gel. Then the leads of gel box were attached to the power supply. The power supply was set at the 90 voltage and gel was run for 40 minutes.

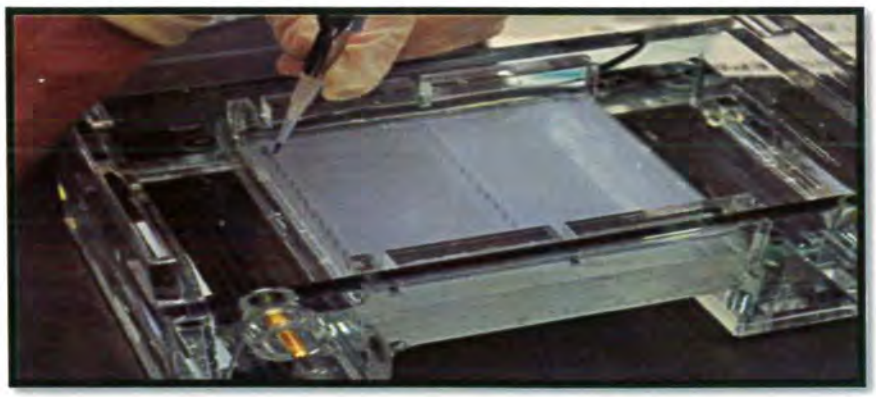

Fig 3.6. Gel tank and samples loading into the wells

Table 3.4. Components for gel preparation with optimized concentrations

Sr. No.	Gel ingredients	Quantity
1-	Agarose powder	0.6g
2-	1X TAE Buffer	40ml
3-	Ethidium Bromide	4µl

3.11 Visualization of DNA fragments:

After the completion of gel electrophoresis, power supply was turned off and the lid of gel tank was removed. Then gel was removed from the gel tray and unnecessary buffer was removed from the surface of gel. Gel was exposed to UV light. UV trans- illuminator was used to visualize DNA bands. The gel slice was cut at the position where sharp and clear bands appeared. Pictures were captured and gel was carefully discarded.

Fig 3.7. Cutting of gel slice having desired band

3.12 Gel Purification of PCR Products:

Thermo Scientific GeneJET PCR Purification Kit was used for the purification of PCR products. Following steps were performed to purify PCR products:

- First of all, the weight of gel slice having was determined and then binding buffer was added in 1:1 to the completed PCR mixture.
- Then PCR tubes were placed in hot plate at the temperature of 65°C for 10 minutes to melt the gel completely. Inverted mixing was done to homogenize the mixture. Then mixture was vortexed briefly.
- The mixture was then transferred to the GeneJET purification column and centrifuged for 30-60 seconds. Flow trough was discarded.
- 700µl Wash Buffer was added to the GeneJET purification column and then centrifuged for 30-60 seconds. The flow through was discarded and purification column was placed back into the collection tube.
- Empty GeneJET purification column was again centrifuged for additional 1 minute to completely remove the residue material.
- The GeneJET purification column was transferred to sterile micro centrifuge tube having 1.5ml capacity.
- 30µl elution buffer was added to the center of GeneJET purification column membrane and centrifuged for 1 minute.
- The GeneJET purification column was then discarded and purified DNA was stored.

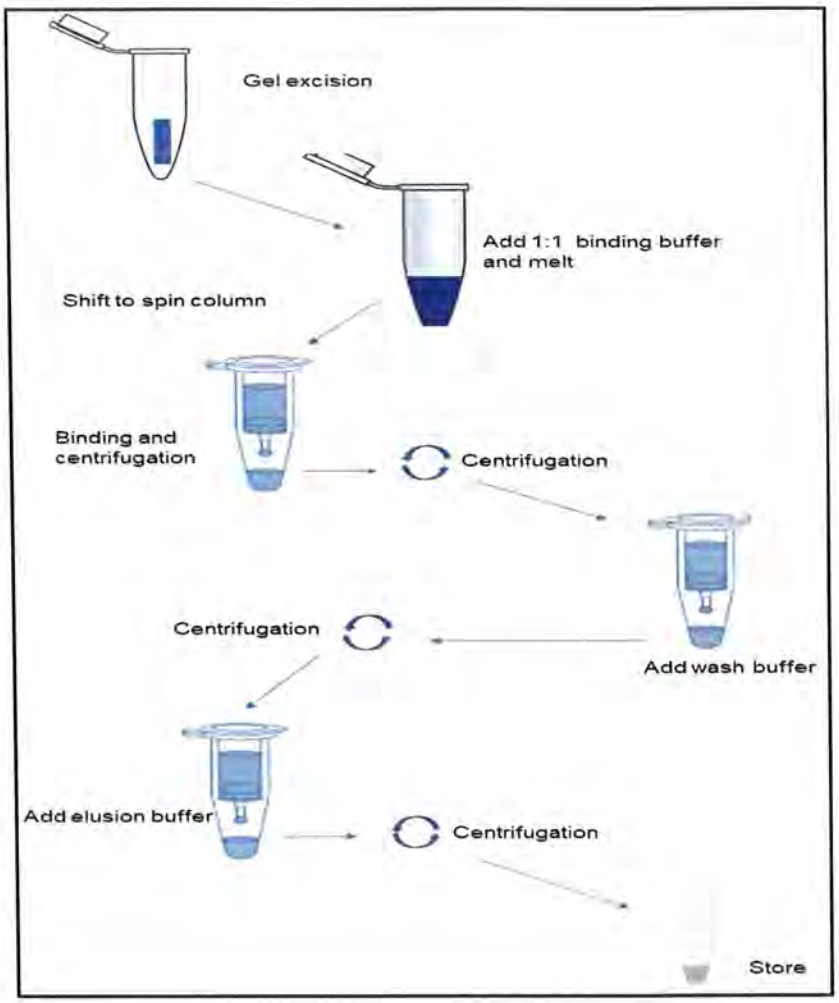

Fig 3.8. Schematic representation of gel purification protocol

3.13 Sequencing of 16S rRNA gene:

After the purification of PCR products, they were processed for sequencing to CEMB in Lahore. The sequencing was carried out using forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3) by the Sanger Sequencing Method.

3.14 Phylogenetic analysis:

After 16S rRNA gene sequencing, the sequences were compared to the reference sequence retrieved from the NCBI (National center for Biotechnology Information) for phylogenetic analysis. BLAST tool was used for the comparison of similarity index. The most similar sequences having highest similarity index were selected and aligned using the software MEGA X. The phylogenetic tree was constructed using the same software by using maximum likelihood

method at 1000 bootstrap value. The maximum likelihood method predicted the evolutionary relationship of the strains to the closely related strains.

4 Results

In this study, we revealed the diversity of bacteria isolated from six cosmetic products of various brands. Total n=14 bacterial colonies were obtained through culturing which were identified on the basis of biochemical as well as molecular characterization. The tested products were taken from famous brands. The names of brands are not mentioned in the study due to commercialization issues.

Table 4.1. Products used for study and bacteria isolated from them

Sr. No.	Cosmetic products	Bacterial isolates
1-	Foundation	T12,T1,T2
2-	Eye shadow 1	T3,T4
3-	Eye shadow 2	T11,T7
4-	Lip gloss	T8,T6,T112
5-	Sunscreen	T10,T103
6-	Primer	T14,T9

4.1. Observation of strains on TSA plates:

Different bacteria were grown on plates having TSA media after 24 hours of incubation at 37°C. No contamination was observed in negative control and plates inoculated with samples. Multiple bacterial colonies were obtained after 24-48 hours of incubation. Bacterial growth was optimum at temperature 37°C.

Fig 4.1 A represents un-inoculated plate of TSA plate

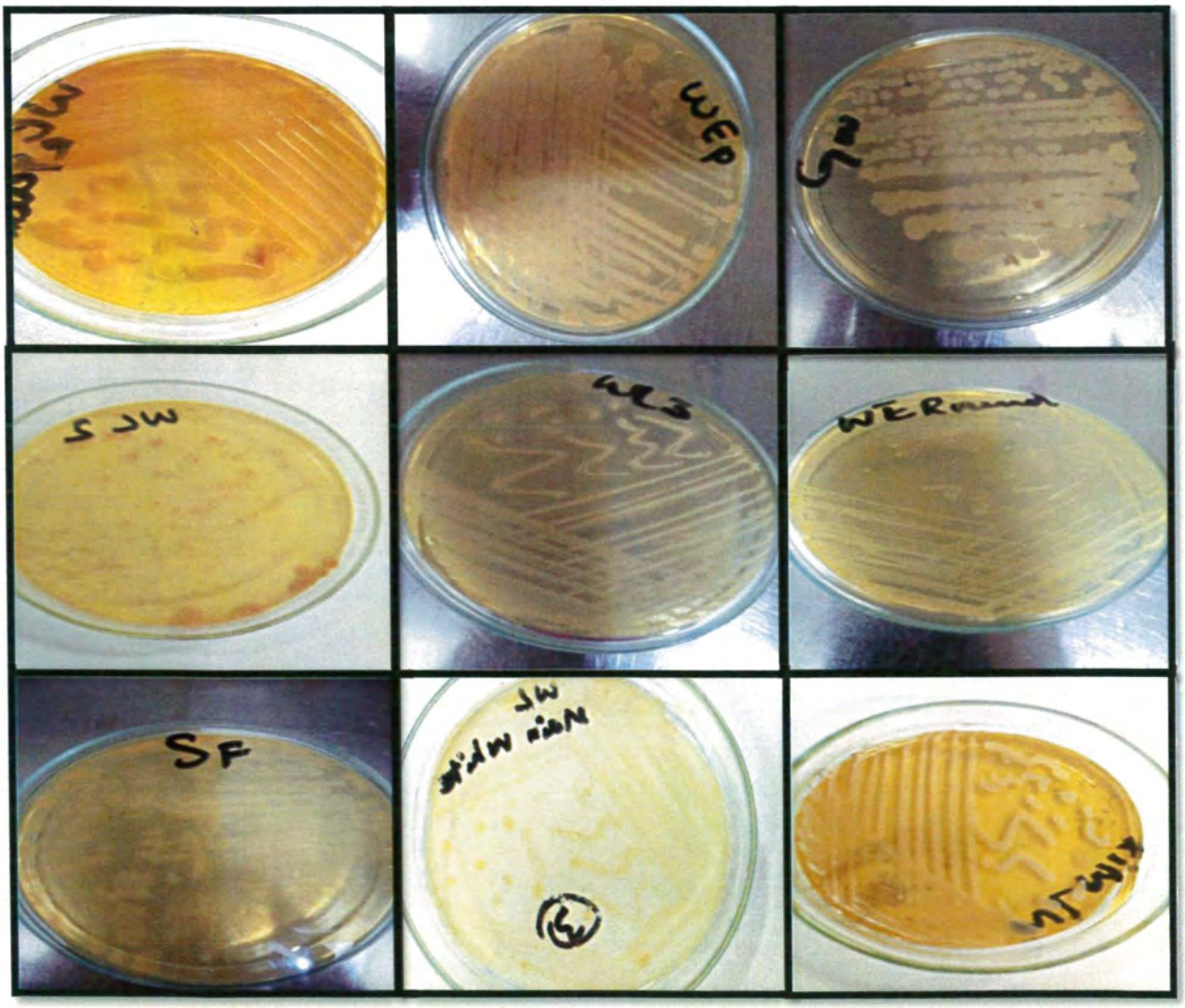

Fig 4.1B represents the bacterial colonies obtained after inoculation were mostly yellow, brown, and white in color and rest were transparent

4.2. Biochemical tests:

Various biochemical tests were performed to identify bacterial isolates which included SIM (Sulfur, Indole, and Motility), MacConkey Agar, Simmons citrate Agar, and Urease and Starch hydrolysis test.

4.2.1. SIM test:

4.2.1.1. Sulfur test:

Sulfur test was done to identify isolates which were gram negative enteric bacillus based on the production of hydrogen sulfide gas which caused the formation of black precipitates. Four isolates showed positive Sulfur test while all other isolated were negative.

A B

Fig 4.2. A represents the sulfur positive isolates while B represents the sulfur negative isolates

4.2.1.2. Indole test:

This test aimed at the differentiation of gram negative bacteria based on the production of hydrolysis of tryptophan. Indole positive bacteria produced pink to red color ring on the top surface of media while no color change was observed for indole negative bacteria. Five bacteria gave indole positive test while rest of them gave negative test.

Fig. 4.3. Represents the bacteria which showed indole positive test.

4.2.1.3.Motility test:

Motility test identified the strains which were motile and could move from those which were non-motile. The motile isolates produced turbidity and cloudiness in medium while non-motile isolated grew along the stab line only and did not produce any turbidity.

A B

Fig.4.4. Represents the results of motility test

Table 4.2. Represent the bacterial isolates which showed positive and negative sulfur, motility and indole test.

Sr. No.	Bacterial isolate	Sulfur test	Motility test	Indole test
1-	T1	Negative	Positive	Negative
2-	T2	Positive	Positive	Positive
3-	T3	Positive	Positive	Negative
4-	T4	Positive	Negative	Negative
5-	T112	Negative	Positive	Negative
6-	T6	Negative	Positive	Positive
7-	T7	Negative	Positive	Positive
8-	T8	Negative	Positive	Negative
9-	T9	Negative	Positive	Positive
10-	T10	Negative	Positive	Positive
11-	T11	Positive	Positive	Negative
12-	T12	Negative	Positive	Negative
13-	T103	Positive	Positive	Positive
14-	T14	Negative	Negative	Positive

4.2.2. Simmons Citrate test:

Simmons citrate test was used to identify gram negative bacteria on the basis of citrate utilization. It was typically used to differentiate *Enterobacteriaceae*. Citrate positive bacteria changed the color of media from green to bright blue while citrate negative bacteria did not change the media color. Four isolates were found to utilize citrate as energy source while rest of isolates were unable to do so.

Fig.4.5 Results of Simmon citrate Agar test

4.2.3. MacConkey Agar test:

MacConkey agar test was used for the differentiation of gram negative bacteria on the basis of their ability to ferment lactose. Gram positive bacteria did not grow on MacConkey agar. Lactose fermenting bacteria grew as pink to red colored isolates while some bacteria grew as colorless colonies due to their inability to ferment lactose. Only two bacteria appeared as pink colonies and were lactose fermenting whereas rest of them were non-lactose fermenting.

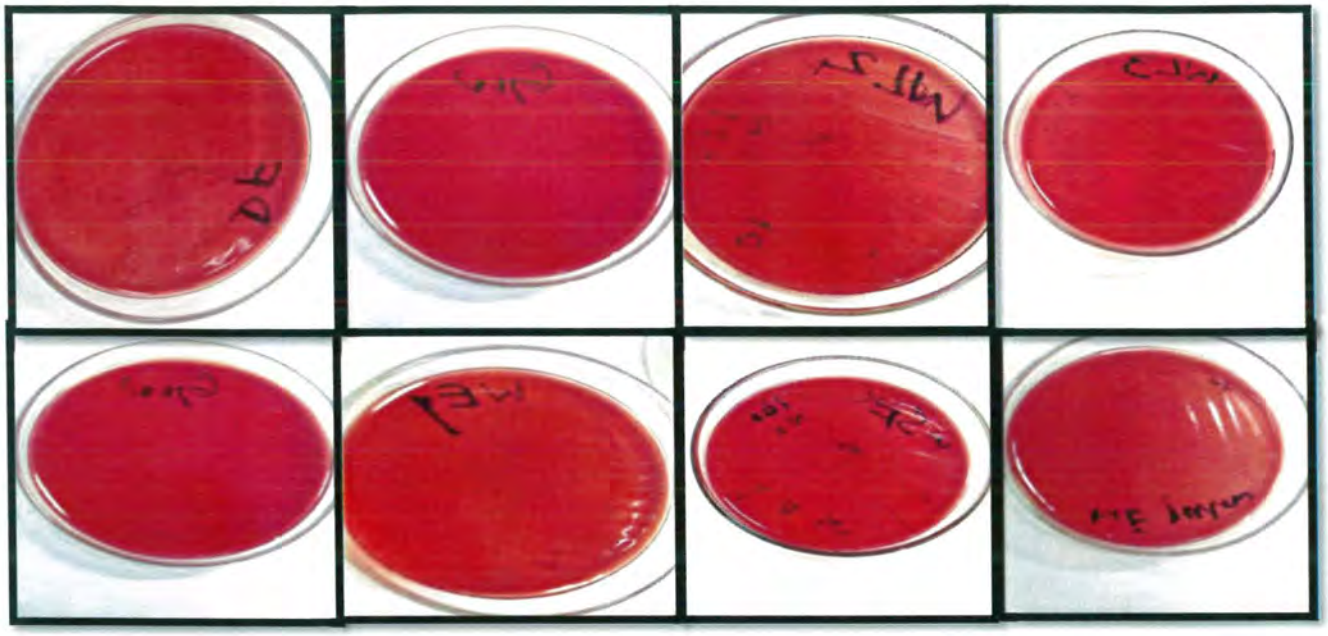

Fig.4.6. Results of MacConkey Agar test which showed that almost all bacteria grew transparent as non-lactose fermenting while two strains were pink and lactose fermenting.

Table. 4.3. Genomic DNA quantification obtained by plain boiling method

Sr.No.	Isolates	ng/µL	260/280
1-	T1	62.5	1.46
2-	T2	60.59	1.38
3-	T3	56.5	1.45
4-	T4	67.56	1.76
5-	T5	50.75	1.78
6-	T6	55.32	1.87
7-	T112	46.6	2.06
8-	T7	62.2	1.52
9-	T10	55.67	1.47
10-	T12	49.5	1.98
11-	T14	63.55	1.63

4.2 Gel Electrophoresis:

After the completion of PCR, the products were visualized on 1.5% agaose gel. 5-10µl PCR products were loaded into the wells and run for 30-45 minutes at 90V. 1kb ladder was used for the comparison of product size.

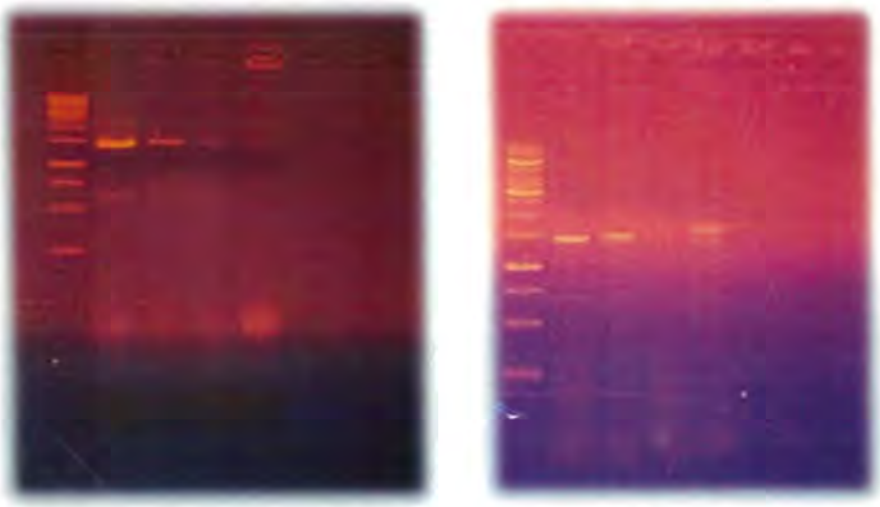

Fig.4.7. represents the PCR products of 1500bp comapred with ladder of 1kb size.

4.3.Sequencing of PCR Product:

PCR products were purified and processed for sequencing using single universal primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3) based on 16S rRNA gene. The sequencing results were analyzed after generation of chromatogram and through phylogenetic tree construction.

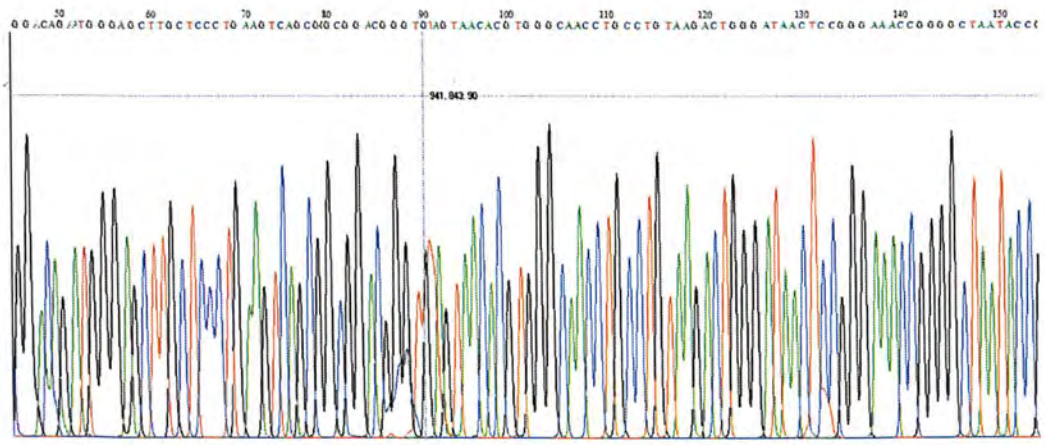

Fig.4.8. Representation of chromatogram of isolate T12 using bioedit

4.4.Sequencing analysis:

After sequencing, the BLAST was used for comparing the sequences with reference sequences. The most similar sequences were retrieved from NCBI. Sequencing results indicated one strain of *Cytobacillus oceanisediminis* and another identified as *Sphingomonas paucimobilis*. Others included *Cytobacillus firmus, Acinetobacter junii, Robertmurraya andreesnii* and *Falsibacillus pallidus.*

4.5.Construction of Phylogenetic tree:

Mega X was used to analyze the sequence of nucleotides of the bacteria isolated from cosmetics products in comparison with the reference sequence of nucleotides of bacteria from all over the world. Maximum likelihood method was used to evaluate the evolutionary history of strain. Phylogenetic analysis showed that isolate QAUT10R showed similarity to the *Acinetobacter junii* strain isolated from China while QAUT7 strain showed most similarity to the *Cytobacillus oceanisediminis* strains found in Russia and China. QAUT11 had shown evolutionary relation with bacterium *Falsibacillus pallidus* isolated from China. QAU 112 isolate was significantly similar to the *Cytobacillus* strain found in China. QAU103 strain showed great similarity to *Sphingomonas paucimobilis* and also with bacillus strains found in China and America. QAUT12 was found to be greatly related to the *Robertmurraya andreesnii* isolated from America.

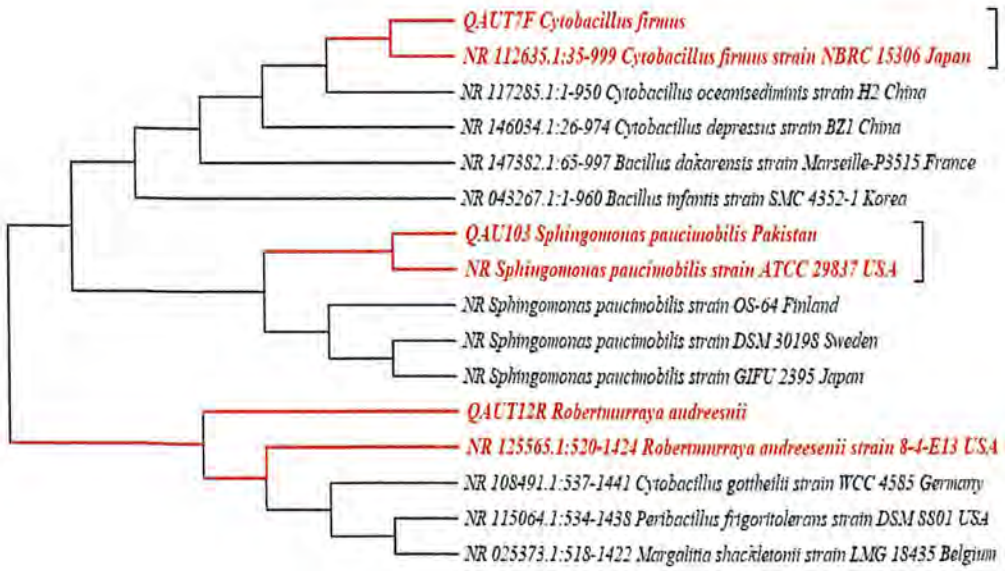

Fig 4.9. Representation of phylogenetic tree of isolates constructed through maximum likelihood method (from n=14). The reference sequences were obtained from Genbank by using BLAST. QAUT7F which was identified as *Cytobacillus firmus,* QAU 103 identified as *Sphingomonas paucimobilis*, QAU 12R identified as *Robertmurraya andreesnii.* Only the most relevant search results were included in the references.

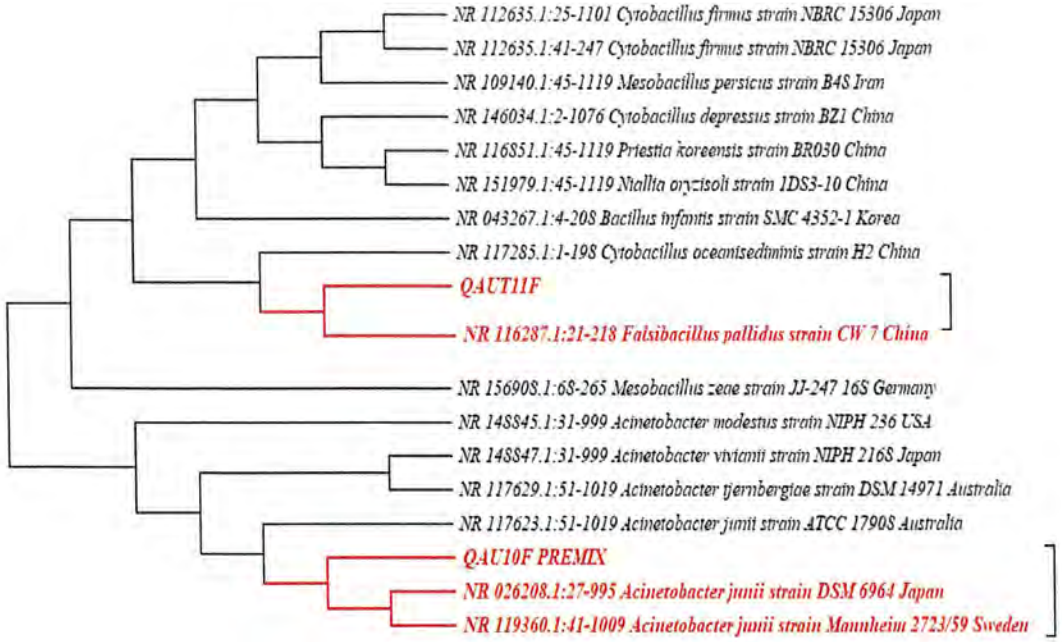

Fig 4.10. Representation of phylogenetic tree of isolates constructed through maximum likelihood method (from n=16). The reference sequences were obtained from Genbank by using BLAST. QAUT11F was identified as *Falsibacillus pallidus* and QAU10F was identified as *Acinetobacter junii.*

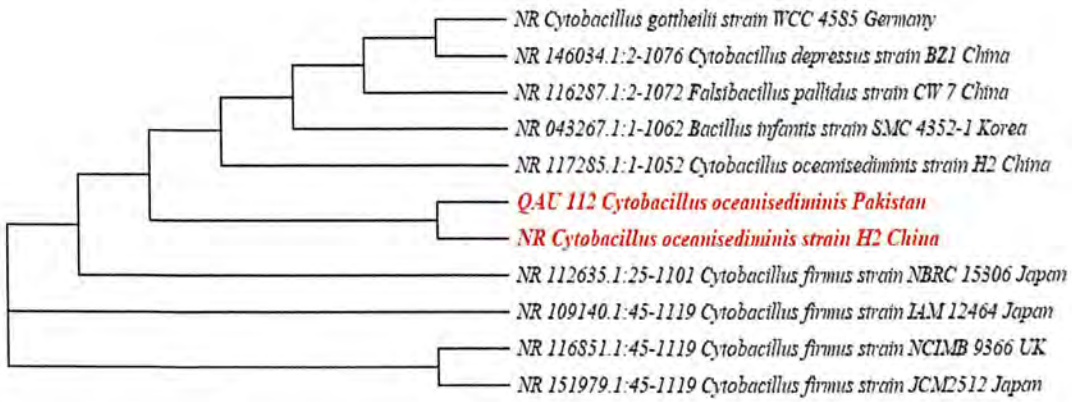

Fig 4.11. Representation of Phylogenetic tree of isolate QAU112 (identified as *Cytobacillus oceanisedminis*) constructed through maximum likelihood method (from n=10). The reference sequences were obtained from Genbank by using BLAST.

Discussion

The cosmetic products are inhabitable companion of everyone in daily life which enhances the elegance of the personality. The use of cosmetics has become indispensable as they are not merely used to improve the appearance but also to keep the body in healthy state. They are used for various purposes such as to keep the body clean, for skin protection, for protection from odor and to keep mucosal membrane intact (Goossens, A. 2011). Cosmetics also act as important vehicle for transmission of pathogens to humans due to which concern about their use and safety is rising with passage of time. They harbor a wide variety of microbes due to contamination from various sources which cause mild to severe allergic reactions leading to complicated life-threatening infections.

According to the recent research in Aston University, scientists have detected the harmful bacteria in cosmetics like *E.coli* and *Staphylococcus* in mascara, eye liner, creams and different applicators. More than 450 types of cosmetics products were analyzed and approximately 79 to 90% contamination level was detected due to presence of *C.freundii, Enterobacter, Pseudomonas* and *Staphylococci.* These bacteria when gain access to the body fluids after invading the skin barrier cause severe infections like diarrhea, infection of wounds and blood poisoning . Several studies have shown the most abundant strains found in cosmetics were *Bacillus, Pseudomonas* and various fungi and molds. However, after testing on different products the researchers did not find any dangerous bacteria but normal skin flora which do not have any kind of side effects. Some of the bacteria and molds which cause severe infections were isolated for example, *S.epidermidis, S. hominis, B. circulans, P. lateus*. (Kim et al. 2020).

The diagnosis of bacteria isolated from cosmetics is quite a challenging task as different methods have been used for their identification. The identification of infectious bacteria is important for the management of patient health and control of disease transmission. The gold standard for the identification of bacterial pathogens in diagnostics lab is culture and biochemical testing. They are used for evaluating and tracing the outbreak of a particular disease based on morphology, metabolite production and colony growth. It is most inexpensive and easy protocol for bacterial identification in diagnosis. But it has certain limitations due to difficulty in culturing many bacteria such as strict anaerobe bacteria are difficult to culture and most of the bacteria exhibit

same morphology and patterns due to belonging to the same family, so it becomes quite difficult to differentiate them on the basis of their shape and growth patterns. The metabolic and phenotypic profile of some bacteria get change due to fluctuation in external factors. Thus, culture and biochemical testing approach is not ideal one for bacterial identification. (Bullock et al. 2013).

Molecular characterization is the most ideal and accurate method for identification of bacterial isolates. Generally, identification is carried out on the basis of 16s rRNA gene, rpoB gene, 23s and 5s rRNA gene. The identification based on 16s rRNA gene is one of the best methods for the identification of novel and non-cultivable bacteria. This method requires only DNA of the bacteria to be tested and accurately identifies those bacteria which are misidentified from conventional approaches. 16s rRNA gene is persistent and conserved in all bacteria, also its universal presence and size make it the best methodology for diagnosis. (Muhammad Rizal et al. 2020).

Current research studies have revealed bacterial diversity in cosmetics in terms of pathogenic as well as beneficial impacts. *Sphingomonas paucimobilis* is one of the bacteria isolated from cosmetics in my research work. It is a gram negative, aerobic and opportunistic pathogen and causes infections in immunocompromised individuals (Toh et al. 2011). It causes soft tissue infection characterized by fluid and puss exudates from deep tissues. It is also reported to cause symptoms of pneumonia resulting in severe difficulty in breathing (Bayram et al. 2013). It is not just significant in clinical terms but it has significant importance in environment as it is the fundamental agent used in bioremediation. This bacterium has ability to degrade the aromatic compounds and it is an effective and ecofriendly tool for the degradation of pollutants and carcinogenic compounds from the soil. These bacteria can be used for in-situ bioremediation after enhancing the production of biomass in bioreactors. Further work and research is needed to explore and uncover the capacity of this bacteria to perform the bioremediation and make our environment free of harmful substances. Recombinant DNA technology can be used to identify the genes responsible for bioremediation and recombinant strains can be developed to remediate the environment (Jaafar and R.S. 2019).

Another bacterial strain isolated from cosmetics in my research work is *Cytobacillus oceanisediminis*. It is a gram positive, rod shaped and aerobic bacteria and found in marine

sediments. It is not known either it is pathogenic or not pathogenic. It helps in the fermentation by the production of acetate, lactate and ethanol. They play an important role in biomineralization. It is a novel strain reported to be isolated from marine system. Further research is needed to explore its potential and its effects on humans and animals (Zhanng et al. 2010).

Acenitobacter has been isolated from cosmetic products. It is a gram negative bacteria and coccobacillus in morphology. It is a well-known infectious agent and has ability to colonize the skin, gastrointestinal tract and respiratory pathways. It has been reported to cause severe form of pneumonia associated with ventilator. It is a notorious pathogen and also a causative agent of blood infection as well as urinary tract infection (Visca et al. 2011). It is one of the pathogens involved in eye infections and damages the eye epithelium which leads to the corneal ulcer. Corneal ulcer is one of the most common diseases caused due to use of contaminated mascara and eyeliner (Broniek et al. 2014).

Robertmurraya andreesnii strain is another bacterium isolated in my research work. It is a gram positive, motile and rod shaped bacterium. It is an opportunistic bacterium and causes gastrointestinal infections. It is reported to cause endocarditis, blood infection, pneumonia and skin infection. It causes morbidity in immunocompromised patients and in people having diabetes or serious injuries (Esmkhani et al. 2022).

Bacillus firmus has been isolated from one of the cosmetics samples. It is a gram positive, aerobic bacterium. It is not known to have pathogenic effects on humans and does not cause any significant disease to humans. It has significant importance in promoting plant growth by interfering and inhibiting the nematode and cysts growth (Huang et al. 2021). It is also used for the preparation of animal feed as probiotic supplements which have played significant role in improving animal health (Mingmongkolchai et al. 2018).

Conclusion

Different bacteria have been isolated from cosmetics products which include *Sphingomonas paucimobilis, Cytobacillus oceanisediminis, Falsibacillus pallidus*, *Acinetobacter, Bacillus firmus and Robertmurraya andreesnii*. Some of these bacteria are pathogenic and have capacity to cause numerous kinds of infectious diseases. Whereas beneficial bacterial components have been derived from these bacteria which have the potential to give maximum benefits to us and environment as well. The bacterial count should be zero in these products because at certain stage they may cause serious harms to human health. However, some strains are still unknown that whether they are pathogenic are not and there is dire need to explore and check the metabolic profile of these bacteria so that they can employed for therapeutic purpose.

Future prospects:

Bacterial diversity in cosmetics has immense significance in terms of health as well as in conferring benefits to the environment. Although a large number of pathogenic bacteria have been reported to be isolated from cosmetics in different countries but on the other hand, a large number of beneficial bacterial components have been introduced in cosmetics to attain maximum benefits. Bacterial components are now being utilized to manufacture skin friendly cosmetics which are used to treat certain skin disorders. Bacillus species are widely used to make best quality moisturizers for skin and in anti-aging creams. Topical ointments have been prepared with the addition of certain bacteria which make pleasant and smooth texture of skin and minimize the scars and wrinkles. *Sphingomonas paucimobilis* could be utilized for remedy of environment. It can be modified in the way that its pathogenicity is reduced to the minimum level and thus can be used for the bioremediation.

Cytobacillus oceanisediminis can make significant contribution in production of important metabolites and aid in bio-mineralization process. Bacillus strains isolated from cosmetics can give benefits in terms of production of animal feed and probiotics. They are also successfully used for promoting the health of plants. However, any pathogenic strain can compromise the desired beneficial effects of the cosmetics.

Recommendations:

The safety of cosmetics should be assured at every point and should not be compromised. There should be proper labeling of manufacturing and expiry dates as well on the products. Necessary tests should be conducted on the products to assure that it is safe to use and apply and will not cause any kind of infection. Proper protocols and precautionary measures should be adopted by the manufacturing companies to minimize the risk of contamination in products. Personal hygiene measures should also be followed by the user to ensure the best health of skin. Regulatory authorities should assess and monitor the safety of cosmetics as they are the most important part of our daily routine. The molecular methodologies are best and ideal approaches for genome-based identification of bacteria rather than traditional approaches. 16S rRNA gene sequencing is the most suitable approach for identification of different bacterial strains and carried out with skills and expertise within short time.

References

e, I. S., & Maibach, H. I. (2014). Irritant contact dermatitis. *Reviews On Environmental Health*, 29(3), 195–206. https://doi.org/10.1515/reveh-2014-0060

ins, S. N., Nash, P., & Fonacier, L. (2019). Irritant Contact Dermatitis. *Clinical Reviews In Allergy & Immunology*, 56(1), 99–109. https://doi.org/10.1007/s12016-018-8713-0

yram, N., Devrim, I., Apa, H., Gülfidan, G., Türkyılmaz, H. N., & Günay, I. (2013). Sphingomonas paucimobilis infections in children: 24 case reports. *Mediterranean Journal Of Hematology and Infectious Diseases*, 5(1), e2013040. https://doi.org/10.4084/MJHID.2013.040

een J. O. (2010). Skin and soft tissue infections in immunocompetent patients. *American Family Physician*, 81(7), 893–899.

roniek, G., Langwińska-Wośko, E., Szaflik, J., & Wróblewska, M. (2014). *Acinetobacter junii* as an aetiological agent of corneal ulcer. *Infection*, 42(6), 1051–1053. https://doi.org/10.1007/s15010-014-0647-8

udecka, A., & Kunicka-Styczyńska, A. (2014). Microbiological contaminants in cosmetics – isolation and characterization. *Biotechnology and Food Science,* 78(1), 15-23. https://doi.org/10.34658/bfs.2014.78.1.15-23

ullock, N. O., & Aslanzadeh, J. (2013). Biochemical profile-based microbial identification systems. *Advanced Techniques in Diagnostic Microbiology* (pp. 87-121). Springer, Boston, MA.

hinedu, E., Arome, D., & Ameh, F. S. (2013). A new method for determining acute toxicity in animal models. *Toxicology International*, 20(3), 224–226. https://doi.org/10.4103/0971-6580.121674

el Valle EM.Martin, "Cyclodextrins and Their Uses: A Review," *Process Biochemistry,* Vol. 39, No. 9, 2004, pp. 1033-1046. Doi: 10.1016/S0032-9592(03)00258-9

ing, J., Wu, B., & Chen, L. (2022). Application of Marine Microbial Natural Products in Cosmetics. *Frontiers in Microbiology*, 13, 892505. https://doi.org/10.3389/fmicb.2022.892505

raelos Z. D. (2012). Cosmetics, categories, and the future. *Dermatologic Therapy*, 25(3), 223–228. https://doi.org/10.1111/j.1529-8019.2012.01498.

emam, A., Rahimian, J., & Mandell, W. (2009). Infection with panresistant Klebsiella pneumoniae: a report of 2 cases and a brief review of the literature. *Clinical infectious diseases: An Official Publication of the Infectious Diseases Society of America*, 49(2), 271–274. https://doi.org/10.1086/600042

-Zamkan, MA, Mohamed, HMA. Molecular detection of Enterobacter spp. and other related species in powdered milk infant formula and milk powder. *J Food Saf.* 2018; 38:e12538. https://doi.org/10.1111/jfs.12538

smkhani, M., & Shams, S. (2022). Cutaneous infection due to Bacillus cereus: a case report. *BMC Infectious Diseases*, 22(1), 393. https://doi.org/10.1186/s12879-022-07372-9

zzeldeen, N. A., Mansour, H. A., & Ahmed, A. A. (2011). Phenotypic and molecular identification of Staphylococcus aureus isolated from some Egyptian salted fish. *World Appl. Sci. J,* 15(12), 1703-1712.

onacier, L. S., Dreskin, S. C., & Leung, D. Y. (2010). Allergic skin diseases. *The Journal Of Allergy and Clinical Immunology,* 125(2 Suppl 2), S138–S149. https://doi.org/10.1016/j.jaci.2009.05.039

oster T. J. (2017). Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. *FEMS Microbiology Reviews,* 41(3), 430–449. https://doi.org/10.1093/femsre/fux007

arza-Ramos, U., Barrios-Camacho, H., Moreno-Domínguez, S., Toribio-Jiménez, J., Jardón-Pineda, D., Cuevas-Peña, J., Sánchez-Pérez, A., Duran-Bedolla, J., Olguín-Rodriguez, J., & Román-Román, A. (2018). Phenotypic and molecular characterization of Klebsiella spp. isolates causing community-acquired infections. *New microbes and new infections*, 23, 17–27. https://doi.org/10.1016/j.nmni.2018.02.002

oossens A. (2011). Contact-allergic reactions to cosmetics. *Journal of Allergy*, 2011, 467071. https://doi.org/10.1155/2011/467071

upta, P. L., Rajput, M., Oza, T., Trivedi, U., & Sanghvi, G. (2019). Eminence of Microbial Products in Cosmetic Industry. *Natural Products and Bioprospecting*, 9(4), 267–278. https://doi.org/10.1007/s13659-019-0215-0

ıpta, R., Sharma, R., & Beg, Q. K. (2013). Revisiting microbial keratinases: next generation proteases for sustainable biotechnology. *Critical Reviews in Biotechnology,* 33(2), 216–228. https://doi.org/10.3109/07388551.2012.685051

ɔod, R.D. (Ed.). (2012). Developmental and Reproductive Toxicology*: A Practical Approach (3rd ed.). CRC Press*. https://doi.org/10.3109/9781841848211

ıang, M., Bulut, A., Shrestha, B., Matera, C., Grundler, F., & Schleker, A. (2021). Bacillus firmus I-1582 promotes plant growth and impairs infection and development of the cyst nematode Heterodera schachtii over two generations. *Scientific Reports*, 11(1), 14114. https://doi.org/10.1038/s41598-021-93567-0

afar, R. S. (2019). THE POTENTIAL ROLE OF SPHINGOMONAS PAUCIMOBILIS IN BIOREMEDIATION OF SOILS CONTAMINATED WITH HYDROCARBON AND HEAVY METAL: Bioremediation using *Sphingomonas paucimobilis*. *Malaysian Journal of Science,* 48-58.

im, H. W., Seok, Y. S., Cho, T. J., & Rhee, M. S. (2020). Risk factors influencing contamination of customized cosmetics made on-the-spot: Evidence from the national pilot project for public health. *Scientific Reports*, 10(1), 1561. https://doi.org/10.1038/s41598-020-57978-9

ostner, L., Anzengruber, F., Guillod, C., Recher, M., Schmid-Grendelmeier, P., & Navarini, A. A. (2017). Allergic Contact Dermatitis. *Immunology and Allergy Clinics of North America*, 37(1), 141–152. https://doi.org/10.1016/j.iac.2016.08.014

ɛbrun, S., Nguyen, L., Chavez, S., Chan, R., Le, D., Nguyen, M., & Jester, J. V. (2021). Same-chemical comparison of nonanimal eye irritation test methods: Bovine corneal opacity and permeability, EpiOcular™, isolated chicken eye, ocular Irritection®, OptiSafe™, and short time exposure. *Toxicology in vitro: An International Journal Published in Association with BIBRA*, 72, 105070. https://doi.org/10.1016/j.tiv.2020.105070

ɛe, G. C., & Burgess, D. S. (2012). Treatment of Klebsiella pneumoniae carbapenemase (KPC) infections: a review of published case series and case reports. *Annals of Clinical Microbiology and Antimicrobials*, 11, 32. https://doi.org/10.1186/1476-0711-11-32

chman G, Nair PA, Atwater AR, et al. Contact Dermatitis. [Updated 2022 May 8]. In: StatPearls [Internet]. *Treasure Island* (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK459230/

ds, L. M., Dres, C., Johnson, C., Scholz, D. B., & Brooks, G. J. (2000). The future of enzymes in cosmetics. *International Journal of Cosmetic Science*, 22(2), 85–94. https://doi.org/10.1046/j.1467-2494.2000.00012.x

ingmongkolchai, S., & Panbangred, W. (2018). Bacillus probiotics: an alternative to antibiotics for livestock production. *Journal of Applied Microbiology*, 124(6), 1334–1346. https://doi.org/10.1111/jam.13690

itsui, T. (1997). *New Cosmetic Science*. Amsterdam: Elsevier Science.

onnot, A. D., Towle, K. M., Warshaw, E. M., Fung, E. S., Novick, R. M., Paustenbach, D. J., & Drechsel, D. A. (2019). Skin Sensitization Induction Risk Assessment of Common Ingredients in Commercially Available Cleansing Conditioners. Dermatitis: contact, atopic, occupational, drug, 30(2), 116–128. https://doi.org/10.1097/DER.0000000000000445

uhamad Rizal, N. S., Neoh, H. M., Ramli, R., A/L K Periyasamy, P. R., Hanafiah, A., Abdul Samat, M. N., Tan, T. L., Wong, K. K., Nathan, S., Chieng, S., Saw, S. H., & Khor, B. Y. (2020). Advantages and Limitations of 16S rRNA Next-Generation Sequencing for Pathogen Identification in the Diagnostic Microbiology Laboratory: *Perspectives from a Middle-Income Country*. Diagnostics (Basel, Switzerland), 10(10), 816. https://doi.org/10.3390/diagnostics10100816

ujtaba, S. F., Masih, A. P., Alqasmi, I., Alsulimani, A., Khan, F. H., & Haque, S. (2021). Oxidative-Stress-Induced Cellular Toxicity and Glycoxidation of Biomolecules by Cosmetic Products under Sunlight Exposure. *Antioxidants* (Basel, Switzerland), 10(7), 1008. https://doi.org/10.3390/antiox10071008

urphy, P. B., Atwater, A. R., & Mueller, M. (2021). Allergic Contact Dermatitis. In StatPearls. StatPearls Publishing.

uthukumar, A., Zitterkopf, N. L., & Payne, D. (2008). Molecular tools for the detection and characterization of bacterial infections: A review. *Laboratory Medicine*, 39(7), 430-436. https://doi.org/10.1309/M6MBU1KGP0FF1C00

avon-Venezia, S., Kondratyeva, K., & Carattoli, A. (2017). Klebsiella pneumoniae: a major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiology Reviews*, 41(3), 252–275. https://doi.org/10.1093/femsre/fux013

oble W. C. (1998). Skin bacteriology and the role of Staphylococcus aureus in infection. *The British Journal of Dermatology*, 139 Suppl 53, 9–12. https://doi.org/10.1046/j.1365-2133.1998.1390s3009.x

oor, A. I., Rabih, W. M., Alsaedi, A. A., Al-Otaibi, M. S., Alzein, M. S., Alqireawi, Z. M., Mobarki, K. A., AlSharif, R. A., & Alfaran, H. S. (2020). Isolation and identification of microorganisms in selected cosmetic products tester. *African Journal of Microbiology Research*, 14(9), 536-540.

rús, P., Gomez-Perez, L., Leranoz, S., & Berlanga, M. (2015). Increasing antibiotic resistance in preservative-tolerant bacterial strains isolated from cosmetic products. *International microbiology: the official journal of the Spanish Society for Microbiology*, 18(1), 51–59. https://doi.org/10.2436/20.1501.01.234

anico, A., Serio, F., Bagordo, F., Grassi, T., Idolo, A., DE Giorgi, M., Guido, M., Congedo, M., & DE Donno, A. (2019). Skin safety and health prevention: an overview of chemicals in cosmetic products. *Journal of Preventive Medicine and Hygiene*, 60(1), E50–E57. https://doi.org/10.15167/2421-4248/jpmh2019.60.1.1080

stollato, F., Madia, F., Corvi, R., Munn, S., Grignard, E., Paini, A., Worth, A., Bal-Price, A., Prieto, P., Casati, S., Berggren, E., Bopp, S. K., & Zuang, V. (2021). Current EU regulatory requirements for the assessment of chemicals and cosmetic products: challenges and opportunities for introducing new approach methodologies. *Archives of Toxicology*, 95(6), 1867–1897. https://doi.org/10.1007/s00204-021-03034-y

octor, L. L., Ward, W. L., Roggy, C. S., Koontz, A. G., Clark, K. M., Quinn, A. P., Schroeder, M., Brooks, A. E., Small, J. M., Towne, F. D., & Brooks, B. D. (2021). Potential Therapeutic Targets

for Combination Antibody Therapy against Pseudomonas aeruginosa Infections. *Antibiotics* (Basel, Switzerland), 10(12), 1530. https://doi.org/10.3390/antibiotics10121530

zzatti, G., Lopetuso, L. R., Gibiino, G., Binda, C., & Gasbarrini, A. (2017). Proteobacteria: A Common Factor in Human Diseases. *BioMed Research International*, 2017, 9351507. https://doi.org/10.1155/2017/9351507

ɔgiers, V., Benfenati, E., Bernauer, U., Bodin, L., Carmichael, P., Chaudhry, Q., Coenraads, P. J., Cronin, M., Dent, M., Dusinska, M., Ellison, C., Ezendam, J., Gaffet, E., Galli, C. L., Goebel, C., Granum, B., Hollnagel, H. M., Kern, P. S., Kosemund-Meynen, K., Ouédraogo, G., ... Worth, A. (2020). The way forward for assessing the human health safety of cosmetics in the EU - Workshop proceedings. *Toxicology*, 436, 152421. https://doi.org/10.1016/j.tox.2020.152421

ıaebth, L. (2019). Molecular identification and sequencing of Pseudomonas aeruginosa virulence genes among different isolates in Al-Diwaneyah hospital. *Iraqi Journal of Veterinary Sciences,* 32(2), 183-188. Doi: 10.33899/ijvs.2019.153847

ze, J. H., Brownlie, J. C., & Love, C. A. (2016). Biotechnological production of hyaluronic acid: *A Mini review. 3 Biotech*, 6(1), 67. https://doi.org/10.1007/s13205-016-0379-9

ıomas, B. S., Okamoto, K., Bankowski, M. J., & Seto, T. B. (2013). A Lethal Case of Pseudomonas putida Bacteremia Due to Soft Tissue Infection. *Infectious Diseases in Clinical Practice* (Baltimore, Md.), 21(3), 147–213. https://doi.org/10.1097/IPC.0b013e318276956b

'oh, H. S., Tay, H. T., Kuar, W. K., Weng, T. C., Tang, H. J., & Tan, C. K. (2011). Risk factors associated with Sphingomonas paucimobilis infection. *Journal of Microbiology, Immunology, and Infection* = Wei mian yu gan ran za zhi, 44(4), 289–295. https://doi.org/10.1016/j.jmii.2010.08.007

an Belkum, A., Burnham, C. D., Rossen, J., Mallard, F., Rochas, O., & Dunne, W. M., Jr (2020). Innovative and rapid antimicrobial susceptibility testing systems. *Nature reviews. Microbiology*, 18(5), 299–311. https://doi.org/10.1038/s41579-020-0327-x

aziri, K., Schwartz, S. G., Kishor, K., & Flynn, H. W., Jr (2015). Endophthalmitis: state of the art. *Clinical ophthalmology* (Auckland, N.Z.), 9, 95–108. https://doi.org/10.2147/OPTH.S76406

ntola C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. P & T: *a peer-reviewed Journal for Formulary Management*, 40(4), 277–283.

sca, P., Seifert, H., & Towner, K. J. (2011). Acinetobacter infection--an emerging threat to human health. *IUBMB life*, 63(12), 1048–1054. https://doi.org/10.1002/iub.534

edi, B., Raap, U., Wieczorek, D., & Kapp, A. (2009). Urticaria and infections. *Allergy, asthma, and clinical immunology: Official Journal of the Canadian Society of Allergy and Clinical Immunology*, 5(1), 10. https://doi.org/10.1186/1710-1492-5-10

iedmann, M., Weilmeier, D., Dineen, S. S., Ralyea, R., & Boor, K. J. (2000). Molecular and phenotypic characterization of Pseudomonas spp. isolated from milk. *Applied and Environmental Microbiology*, 66(5), 2085–2095. https://doi.org/10.1128/AEM.66.5.2085-2095.2000

u, D. C., Chan, W. W., Metelitsa, A. I., Fiorillo, L., & Lin, A. N. (2011). Pseudomonas skin infection: clinical features, epidemiology, and management. *American Journal of Clinical Dermatology*, 12(3), 157–169. https://doi.org/10.2165/11539770-000000000-00000

ıldiz, H., & Karatas, N. (2018). Microbial exopolysaccharides: Resources and bioactive properties. *Process Biochemistry*.

ıang, J., Wang, J., Fang, C., Song, F., Xin, Y., Qu, L., & Ding, K. (2010). Bacillus oceanisediminis sp. nov., isolated from marine sediment. *International journal of Systematic and Evolutionary microbiology*, 60(Pt 12), 2924–2929. https://doi.org/10.1099/ijs.0.019851-0

ıang, Q., Li, J., Zhang, W., An, Q., Wen, J., Wang, A., Jin, H., & Chen, S. (2015). Acute and sub-chronic toxicity studies of honokiol microemulsion. *Regulatory Toxicology and Pharmacology*: RTP, 71(3), 428–436. https://doi.org/10.1016/j.yrtph.2014.11.007

ıkiewicz-Sobczak, W. A., Adamczuk, P., Wróblewska, P., Zwoliński, J., Chmielewska-Badora, J., Krasowska, E., Galińska, E. M., Cholewa, G., Piątek, J., & Koźlik, J. (2013). *Allergy to selected cosmetic ingredients. Postepy dermatologii i alergologii,* 30(5), 307–310. https://doi.org/10.5114/pdia.2013.3836

Appendix

MacCkonkey Agar Media

Ingredients	Quantity (g/L)
MacConkey Agar	51g

Simmon Citrate Agar

Components	Quantity (g/L)
Magnesium sulfate	0.2
Monoamonium phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Bromothymol blue	0.08
Agar	15
Final pH	6.8 to 7

10X TAE Buffer

Ingredients	Concentration
Tris-base	108g
Acetate Acid	55g
0.5M EDTA	40ml
pH	8.0

1.5% Agarose gel

Ingredients	Quantity in 40ml
1X TAE	40ml
Agarose	0.6g

thesis

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