Extraction of compatible solutes from saltpan bacteria and their applications

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Extraction of compatible solutes from saltpan bacteria and their applications**" is based on the results of investigations carried out by me in the MSc Biotechnology at the discipline of biotechnology, School of Biological Sciences and Biotechnology (SBSB) at Goa University under the Supervision of Prof. Dr.Savita S.Kerkar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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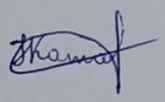

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "Extraction of compatible solutes from saltpan bacteria and their applications" is a bonafide work carried out by Ms. Nabila Kamal under my supervision in partial fulfilment of the requirements for the award of the Master's degree in the Discipline of Biotechnology at the School of Biological sciences and Biotechnology Goa University

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PREFACE

Compatible solutes are a class of low-molecular-weight organic compounds readily synthesized by halophilic organisms in response to the osmotic stress imposed by their high-salinity environments. These solutes play a vital role in protecting the cell's integrity and function under such conditions. Given their ability to maintain high intracellular hydration levels, compatible solutes have emerged as promising candidates for the development of novel moisturizing agents in the cosmeceutical industry. This study investigates the potential of bacteria isolated from the Ribandar river in Goa as a source for high-yield production of compatible solutes with application as moisturizing agents. The focus lies on identifying suitable bacterial isolates and exploring the utility of their compatible solutes for human skin hydration, with a particular emphasis on achieving high efficacy through the application of minimal concentrations. This approach aims to minimize production costs, reduce the risk of allergic reactions, and simultaneously achieve a pronounced moisturizing effect.

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ABREVIATIONS USED

Entity	Abbreviation
Molarity / Millimolar	M / mM
Weight per volume	w/v
Ultra-violet	UV
Gram / Milligram	g / mg
Optical density	OD
Nanometer	nm
Room temperature	RT
Zobell Marine Broth	ZMB
Liter / Milliliter / Microliter	L / mL / µL
Revolutions per minute	RPM
Celsius	°C
Seconds	sec
Minute	min
Thin Layer Chromatography	TLC

Volume per volume	v/v
Retardation factor	Rf
Red Blood Cells	RBCs
White Blood Cells	WBCs
Enzyme-linked immunosorbent assay	ELISA
Dimethyl sulfoxide	DMSO
Centimeter	cm

ABSTRACT

Halophiles, a class of extremophiles, thrive in high-salt environments due to their ability to synthesize or accumulate compatible solutes. These solutes play a critical role in maintaining cellular integrity and function under osmotic stress. Compatible solutes encompass a diverse range of molecules, including amino acids, polyols, sugars, and carbohydrates and their derivatives. Beyond their benefit to halophiles, compatible solutes offer promising applications for humans, serving as therapeutic agents, pharmaceuticals, and cosmetic actives. The primary focus of this study lies on characterizing the isolates, followed by evaluation of the toxicity of their derived compatible solutes on human white blood cells (WBCs). Finally, the study culminates in assessing the most crucial aspect: the moisturizing properties of these compatible solutes.

The minimal negative effects observed in toxicity tests suggest the safe utilization of ectoine on human skin. Furthermore, patch tests yielded positive results, indicating ability of ectoine to enhance skin hydration.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Halophilic bacteria and archaea abundant in hypersaline environments live in extremophilic environments, and this encounters challenges related to water activity arising from varying salt concentrations in their surroundings. To regulate their water activity, these microorganisms accumulate ions and compatible solutes. Additionally, they have the ability to synthesize compatible solutes through various pathways. The concept of compatible solutes was first introduced by Brown et al. in 1972 during their research on yeast and its sugar tolerance. They were defined as highly water-soluble, low-molecular weight osmoregulatory compounds that do not cause any disturbance in biological systems. They are also known as ‘‘protective solutes,’’ ‘‘osmotic stabilizers", or ‘‘compensatory solutes’’. Compatible solutes include amino acids, carbohydrates, and their derivatives, as well as sugars and polyols (Galinski et al., 1997).

Halophilic archaea typically utilize a constant intake of ions, such as K+ to counterbalance the elevated salt concentration in the external cell environment. Whereas halophilic bacteria generally produce or amass compatible solutes to uphold osmotic equilibrium in reaction to the high-salt external conditions. They regulate cell volume, turgor pressure, and internal electrolyte concentrations. This ensures the cytoplasm maintains an optimal level of hydration, enabling cell growth even in osmotically challenging conditions (Costa et al., 1998). Along with their stabilizing effect, compatible solutes possess multiple physiological, potential and biotechnological applications, starting from stabilizers of biomolecules and stress protective agents to therapeutic agents and active compounds in cosmetics (Shivanand et al., 2011).

1.1) HALOPHILIC BACTERIA

Nature encompasses diverse habitats, with some being highly conducive to a variety of living organisms, while others may be inhospitable for most life forms due to extreme physical factors such as temperature, pressure, radiation, and oxygen levels or geochemical conditions such as pH, salt, and water content. Nevertheless, these challenging environments are favorable to a category of organisms called extremophiles, which have existed in nature for a considerable period of time. They include different types such as thermophiles, psychrophiles, alkaliphiles, acidophiles, halophiles, barophiles, xerophiles and others. All of these extremophiles have different uses in biotechnology due to their ability to survive under harsh conditions.

One class of these extremophiles is known as halophiles, which are known to be salt-loving organisms, which show the best growth at salt concentrations of 0.85-8 M. For extreme halophiles, high salt concentrations are mandatory for their growth; however, halotolerant organisms are the ones who can survive in the presence or absence of salt and can even tolerate comparatively high salt concentrations in their environment. Based on their salt requirements, halophiles can be categorized slight halophiles (1.2–3%), moderate halophiles (3–15%), and extreme halophiles (> 15% NaCl) (Ventosa et al., 1998).

The world of halophiles is highly diverse, these microorganisms are found in three domains of life: Archaea, Bacteria, and Eukarya. Hypersaline habitats like saltern pond brines and natural salt lakes consist of relatively low diversity. These three domains have adapted to grow in extreme salinity, but as salt concentration increases, the diversity of microorganisms decreases (Oren, 1999). Namyslowski (1913) was the first to disclose that the brines in the Wieliczka salt mine in Poland

harbored a multitude of halophilic microorganisms. Heinz Dombrowski and Paul Tasch (1963) were the first to isolate microorganisms from rock salt. Reiser and Tasch (1960) successfully isolated diplococci observed in fluid inclusions in Permian salt (Kansas, USA), demonstrating their ability to thrive in a sodium chloride concentration ranging from 0 to 30% (w/v) (Dombrowski et al., 1963). Bibo et al., (1983) endeavored to replicate Dombrowski's isolations, incorporating numerous controls and surface sterilization tests. They successfully isolated extreme halophiles, characterized as Gram-positive cocci and rod-shaped spore formers, from 'primary' Permian Zechstein salt cores.

Halophiles are widely distributed across the globe, thriving in hypersaline environments that range from natural hypersaline brines in arid, coastal, and deep-sea locations to artificial salterns designed for salt extraction from the sea. The unique traits and ability for extensive cultivation render halophiles potentially valuable in the field of biotechnology (Bibo et al., 1983).

1.2) OSMOREGULATION

Within the microbial world, there are two fundamentally distinct strategies that microorganisms utilize in order to deal with the osmotic stress resulting from high salt concentrations in their environment. These methods are called:

1. Salt-in strategy: cells maintain a high intracellular salt concentration, osmotically in equilibrium with the extracellular concentration.
2. Compatible solute strategy: cells maintain a low salt concentration in the cytoplasm and attain equilibrium using compatible solutes.

Salt-in strategy

This strategy is used by extreme halophiles, including halophilic archaea and halophilic bacteria. These microorganisms do not synthesize compatible solutes to maintain osmotic pressure, instead, their adaptation involves the uptake of ionic solutes (K+, Mg2+, and Na+) in high concentration. The structural components and enzymes of these cells are adapted to high ion concentrations.

Compatible solute strategy

This strategy is predominantly utilized by moderately halophilic and halotolerant bacteria, certain yeasts, algae, and fungi. In this approach, these microorganisms uphold a low concentration of salt in the cytoplasm to regain turgor pressure. This is achieved by accumulating ionic solutes in lower concentrations as well as non-ionic solutes, referred to as compatible solutes, which can also be synthesized by the cell. These solutes enable the cell to attain high internal pressure without the inhibitory effects of high concentrations of ionic solutes. Rather, compatible solutes, being highly water-soluble, exert minimal to no interference with crucial cellular metabolic processes. Instead, they facilitate appropriate protein folding within the cell (McLaggan et al., 1994).

The ability of compatible solutes to maintain high levels of cellular hydration has garnered significant interest in the cosmeceutical industry. Ectoine, betaine, and inositol are some prominent examples currently utilized by companies like RonaCare™ and Merck KGaA. However, high concentrations of these solutes can potentially induce adverse effects, including allergic reactions, in susceptible individuals. Additionally, the cost of incorporating such solutes at high levels can be a limiting factor. Therefore, research efforts are directed towards the isolation and application of compatible solutes naturally abundant in halophiles. Utilizing these solutes at minimal concentrations could offer a two-

fold benefit: reduced production costs and a minimized risk of allergic reactions and other adverse consequences. Furthermore, investigations into the isolation of bacteria from unique environments, such as those found in Ribandar, Goa, could hold promise for the discovery of novel compatible solutes with superior production potential compared to traditionally employed bacteria.

1.3) AIM AND OBJECTIVES

Aim: Evaluate the potential of compatible solutes isolated from halophilic bacteria for use as a moisturizing agent in cosmetic formulations.

Objectives:

- Determination of the salinity tolerance of each isolate
- Characterization of the isolates
- Isolation of compatible solutes
- Determination of salt concentration resulting in the highest production of compatible solutes.
- Investigate the potential of compatible solutes for use as a moisturizing agent.

CHAPTER 2

REVIEW OF LITERATURE

2.1 TYPES OF COMPATIBLE SOLUTES

Galinski in 1993 identified 150 isolates from diverse environments such as salt lakes, salinas, salt mines, and other locations with elevated salinity. This exploration unveiled the complete spectrum of compatible solutes present in nature. Further, these osmoprotectants were classified into three major categories, known as zwitterionic, non-charged, and anionic solutes.

Examples of each category, along with their occurrence, are mentioned in the table given below.

Class	**Type**	**Halophile**	**Reference**
ZWITTER IONIC SOLUTES	Betaine	*Methanohalophilus* strain FDF 1 *Actinopolyspora halophila* *Ectothiorhodospira halochloris,* Synechococcus sp. strain DUN 52	Roberts et al.,1992 Nyyssölä et al.,2000 Imhoff et al.,1984
	Ectoine	*Halomonas elongata* *Chromohalobacter salexigens* *Bacillus psychrophilus* *Marinococcus halophilus,* *Salibacillus salexigens* *Bacillus halodurans* *Virgibacillus pantothenticus*	Ono et al.,1999 Salvador et al.,2018 Kuhlmann et al.,2002

		Ectothiorhodospira halochloris *Methylobacter alcaliphilus* *Methylarcula marina* *Methylarcula terricola*	Galinski et al.,1985 Khmelenina et al.,2000 Doronina et al., 2000
	Hydroxyectoine	*Halomonas salina*, *Halomonas elongata* *Salibacillus salexigens* *Chromohalobacter salexigens*	Liu et al., 2021 Kuhlmann et al.,2002 Argandona et al.,2021
	N-γ-acetyldi-aminobutyrate	*Halomonas elongata CHR63*	Cánovas et al.,1999
	N-ε-acetyl-β-lysine	Methanohalophilus strain FDF1 *Methanohalophilus portucalensis*	Roberts et al.,1992 Martin et al.,1999
	β-glutamine	Methanohalophilus strain FDF1 *Methanohalophilus portucalensis*	Roberts et al.,1992 Robinson et al.,2001
UNCHARGED SOLUTES	α-Glucosylgly-cerol	-	-
	α-Mannosylgly-ceramide	*Rhodothermus marinus*	Silva et al., 1999

	Trehalose	*Chromohalobacter salexigens* Ectothiorhodospira sp. *Desulfovibrio halophilus* *Actinopolyspora halophila*	Reina-Bueno et al., 2012 Lippert et al., 1993 Welsh et al.,1996 Nyyssölä et al., 2001
	Sucrose	*Methylobacter alcaliphilus* 7G *Thiocapsa roseoparsarcina* *Thiocapsa halophila* *Ectothiorhodospira marisrnortui*	Khmelenina et al., 2000 DasSarma,2001 Galinski et al.,1991
	N-α-carbamoyl- Lglutamine 1-amide	*Ectothiorhodospira marisrnortui*	Galinski et al.,1991
	N-acetylgluta- minylglutamine	*Chromatium glycolicum,* *Chromobacterium violaceum* *Chromohalobacter salexigens*	DasSarma,2001

ANIONIC SOLUTES			
Carboxylate	L-α-glutamate	*Methanobacterium thermoautotrophicum* *Methanobacterium igneus*	Robinson et al.,2001
	β-glutamate	*Methanohalobium evestigatum,* *Methanohalophilus portucalensis* *Methanosalsum zhilinae*	Oren,2014
	Poly-β-hydroxy-butyrate	*Halomonas boliviensis,* *Haloferax mediterranei* *Methylarcula marina* *Methylarcula terricola*	Quillaguaman et al., 2006 Doronina et al., 2000
	α-glucosylgly-cerate	*Methanohalophilus portucalensis* FDF1 *Halomonas elongata* CHR63	Goudecet al.,2004
	α-mannosylgly-cerate	*Rhodothermus marinus* *Rhodothermus obamensis*	Silva et al., 1999
Sulphate	sulfotrehalose	*Natronococcus occultus* *Natronobacterium spp.*	Desmarais et al., 1997

Table 2.1 – Types of compatible solutes in halophiles

No halophiles were found to synthesize or accumulate the phosphate anionic compatible solutes therefore halotolerant microorganisms are mentioned in the table below

Class	**Type**	**Halotolerant**	**Reference**
Phosphate	α-Diglycerol phosphate	*Archaeoglobus fulgidus*	Lamosa et al., 2000
	Di-myo-inositol-1,1'-phosphate	*Archaeoglobus fulgidus*	Lamosa et al., 2000
	mannosyl-DIP	*Thermotoga maritima Thermotoga neapolitana*	Martins et al.,1996
	Cyclic-2,3-diphospho-glycerate	*Methanobacterium thermoautotrophicum δH* *Methanopyrus kandleri*	Ciulla et al.,1994

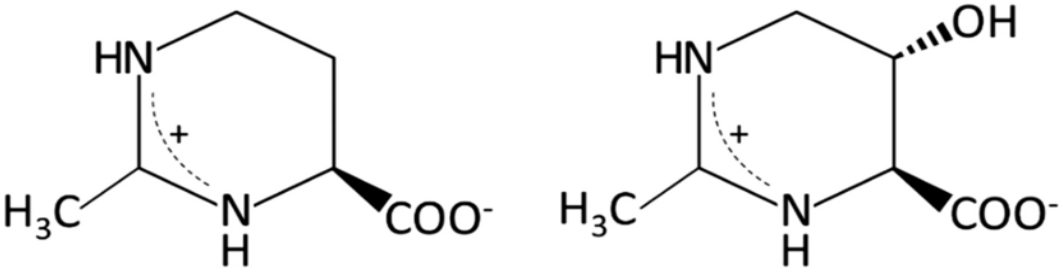
Table 2.2 – Types of compatible solutes in halotolerant organisms

This research focuses on evaluating the potential application of compatible solutes for human health. A comprehensive literature review is conducted to identify compatible solutes with documented protective effects against various stresses relevant to human health. For this study specific compatible solutes were chosen named as ectoine, hydroxyectoine and choline chloride.

2.2) ECTOINE AND HYDROXYECTOINE

Halophilic or halotolerant organisms that live in environments with high external osmolarity regulate osmotic balance by managing intracellular concentrations of compatible solutes, such as ectoine or hydroxyectoine.

Ectoine was initially identified in the anoxygenic photosynthetic halophile *Ectothiorhodospira halochloris* strain DSM 1059 (Galinski et al., 1985).It is considered one of the most abundant compatible solutes in halophiles. Ectoine has the important feature of not just maintaining the osmotic activity of the cell but also being able to protect biomolecules of the cell, such as proteins, enzymes, and membranes, from dehydration, dryness, heat and freezing (Chen et al., 2017). It is done due to the ability of ectoine to form a protective hydration shield around these biomolecules through its robust affinity for water molecules. This mechanism is referred to as "preferential exclusion."That results in the restriction of the structural fluctuation of biomolecules and improves their kinetic stability (Yu et al., 2007).



ECTOINE **HYDROXYECTOINE**

Fig.2.1- Structure of Ectoine and Hydroxyectoine molecules

Ectoine and hydroxyectoine share similar chemical structures; nonetheless, hydroxyectoine distinguishes itself from ectoine due to the inclusion of a hydroxyl group at one of the carbon atoms in the aromatic ring. Hydroxyectoine operates similarly to ectoine but exhibits increased polarity, enhanced solubility, and a more robust protective effect on intracellular macromolecules. When external osmotic pressure reaches a level beyond the cells' tolerance range, ectoine undergoes conversion into hydroxyectoine (Chen et al., 2023)

Ectoine is categorized as a compensating substance, signifying its role in shielding cells from alterations prompted by osmotic stress. Additionally identified as a kosmotropic substance, ectoine comes into play when considering the dynamic effects of salt ions on water. Salt ions can exhibit either a kosmotropic effect, which stabilizes the quasi-crystalline water structure, or a chaotropic effect, disrupting this structure and perturbing the polar arrangement of water molecules. This phenomenon arises from the intricate interplay of a free pair of electrons with cations and hydrogen atoms with anions, resulting in the formation of electrostatically stabilized hydration shells around the ions (Kunte et al., 2014).The immobilized water molecules within these hydration shells become inaccessible to macromolecules displaying hydrophobic characteristics, such as proteins. This is due to the stronger binding of the polar solvent (water) by the electric field of ions with a higher charge-to-radius ratio. Ectoine, serving as a kosmotropic substance, showcases the remarkable property of stabilizing water molecules in this complex interplay. This kosmotropic effect, elucidated by ectoine, manifests as a discernible reduction in the contact surface between water molecules and phospholipids within cell membranes (Goraj et al., 2019).

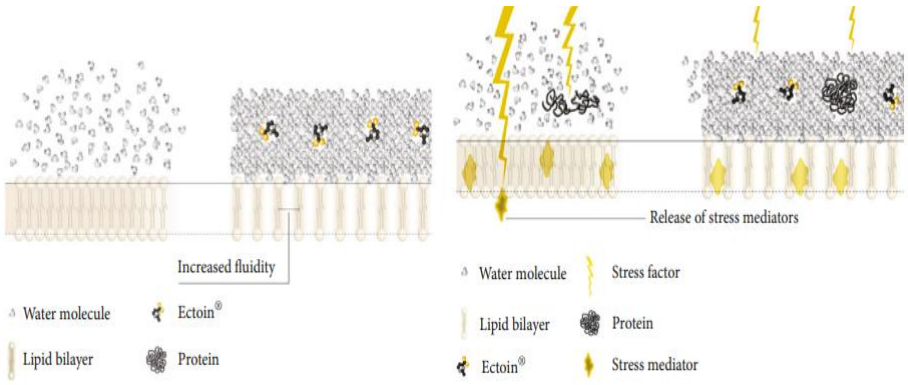


Fig.2.2 Mechanism of action of ectoine (Bilstein et al.,2021)

2.2.1) APPLICATIONS OF ECTOINE AND HYDROXYECTOINE

Various beneficial properties of ectoine make this amino acid multifunctional and have a variety of applications in different industries, such as biotechnology, pharmacy, cosmetology, and medicine. In the cosmetics industry, the use of this molecule is primarily based on its ability to protect skin from dehydration, UV radiation, and aging, as well as relieve inflammation. Ectoine can also be used as a therapeutic for Alzheimer's disease, HIV, intestinal, oral, immunological, and other diseases (Bownik et. al., 2016)

1) Moisturizing property

Our skin is a barrier between the body and its environment, therefore, it exposes exogenous as well as endogenous factors that might have destructive effects on it. Exogenous factors might include extreme climate factors such as cold, heat, drought, exposure to chemical agents like detergents or solvents, UV radiation, or mechanical damage. On the other hand, endogenous factors might include anatomical defects, mutations, aging, chronic eczema, psoriasis, etc. The stratum corneum has a double function in skin hydration. Firstly, keratinized cells form a hydrophobic barrier to prevent the

entry of water into the body through the skin. Secondly, the natural moisturizing factor (NMF) that is present in this layer prevents water evaporation from the skin and therefore maintains hydration of skin. After applying ectoine, it caused keratin dispersion, which led to improved hydration kinetics in the stratum corneum (Bow et al., 2021). It has been shown that addition of 2% ectoine to a skin care product improves its care properties, leads to better skin hydration, and improves elasticity and regeneration of skin structure (Heinrich et al., 2007).

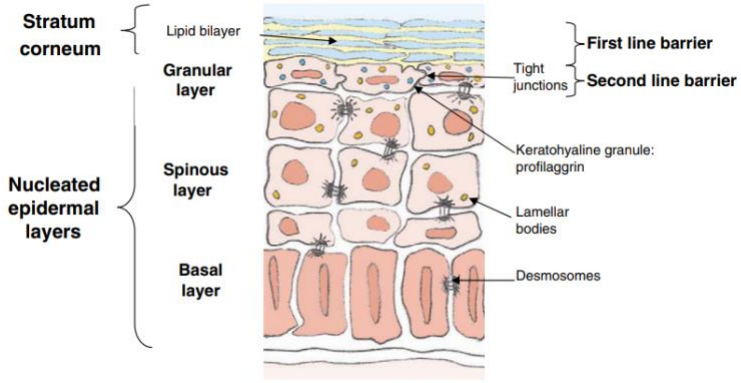


Fig.2.3 Structure of skin epidermis (Baroni et al., 2012)

2) Treatment of allergic rhinitis and rhinoconjunctivitis

Ectoine is employed as a therapeutic agent for addressing allergic conditions in the mucous membranes of the eyes, nose, and respiratory tract. Comparative analysis indicates a statistically significant improvement of approximately 16–25% in patients when contrasted with outcomes observed with placebo (Kocherovets, 2019). There was also significant improvement observed in patients with ocular symptoms of allergic rhinoconjunctivitis, postoperative secondary eye syndrome, ocular re-epithelialization after surgery, and nonspecific eye irritation or inflammation caused by physical damage (Bilstein et al., 2021).

3) Treatment of oral mucositis

Research that was conducted by Dao et al. in 2018 on sixty patients who had been using mouthwash containing calcium phosphate and ectoine and, after a period of time, mouthwash containing ectoine showed significant results. Therefore, ectoine was found to be effective against chemotherapy-induced oral mucositis.

4) Protection against UV-A radiation

In vitro efficacy studies have revealed that ectoine effectively mitigates the consequences of UVA-induced and accelerated skin aging across various cellular levels. Utilizing a UVA stress model, it was demonstrated that ectoine safeguards the skin from the adverse effects of UVA-induced cell damage through multiple mechanisms. In vitro, ectoine exhibits the potential to protect the mitochondria of human fibroblasts against UVA radiation-induced mutagenesis. Furthermore, exposure of dermal keratinocytes to UVA radiation activates the transcription factor AP-2, enhancing its binding capacity to the promoter region of UVA-inducible pro-inflammatory genes. Significantly, pretreatment of cells with 1 mM ectoine suppresses the induction of the transcription factor AP-2 following UV-A exposure (Buenger et al., 2004).

2.3) CHOLINE CHLORIDE

Choline chloride, a quaternary ammonium white crystalline salt, comprises choline cations ([(CH3)3NCH2CH2OH]+) and chloride anions (Cl−), initially investigated by Senko & Templeton in 1960. It is practically neutral in aqueous solutions, making it water-soluble and recognized as vitamin B4. Choline chloride serves as an osmoprotectant, shielding cyanobacteria, animals, and

numerous plant species from stressful conditions. In animals, it plays a crucial role as a component of membrane phospholipids such as phosphatidylcholine and sphingomyelin. This presence enhances membrane stability and fluidity while regulating metabolism and fat conversion, thus preventing fat accumulation in the liver and deterioration of liver and kidney tissues. This molecule is often given as a supplement for livestock, poultry, and fish. In plants, choline chloride contributes to structural stabilization. Due to the nontoxicity of choline chloride for living beings and environment it can easily be added to food, water, and soil. Its formation as a quaternary ammonium salt can occur through the reaction of ethylene oxide, hydrogen chloride, and trimethylamine or by treating trimethylamine with 2-chloroethanol. (Riaz et al., 2021).

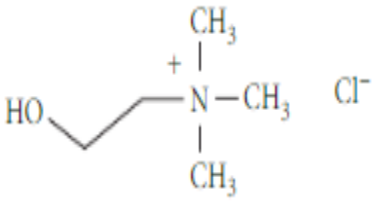


Fig.2.4 Structure of choline cation and chloride anion (Isaifan et al., 2018)

2.3.1) APPLICATIONS OF CHOLINE CHLORIDE

This organic compound can be administered via oral, intramuscular, or intravenous routes, after which it is absorbed into the lumen of the small intestine.

1) Treatment of cerebellar and spinocerebellar ataxia

In a clinical trial involving 20 patients, choline chloride was orally administered in either a high dose (12 g/day) or a low dose (6 g/day) over two consecutive 6-week treatment periods. The trial revealed

positive effects on upper and lower limb coordination in certain patients with Friedreich's ataxia, mixed spinocerebellar ataxia, and cerebellar ataxia. Interestingly, results indicated that the higher dose elicited a more favorable response in patients while also demonstrating no high accumulation in blood serum (Livingstone et al., 1981). The enhancement of coordination could potentially be attributed to the elevated levels of the neurotransmitter acetylcholine facilitated by the presence of choline chloride, which is a precursor of this neurotransmitter (Cohen et al., 1975).

2) Treatment of asthma

Choline chloride significantly improved symptoms of asthma and improved PC20 FEV1 levels, which indicates reduced sensitivity and less hyperreactivity in patients. Choline therapy also led to a notable decrease in levels of inflammatory mediators like IL-4, IL-5, and TNF-a, along with reductions in blood eosinophil count and total IgE levels. Isoprostanes, which are reliable biomarkers indicating the presence of oxidative stress, have decreased in patients consuming choline chloride. Lastly, the given study has shown a decrease in cysteinyl leukotriene and leukotriene B4 levels, which are the main factors of pathogenicity in the airways. Overall, the study has proved that choline chloride has a positive impact on patients with asthma by reducing oxidative stress and inflammation of various airway obstructions (Mehta et al., 2010).

3) Treatment of Tardive Dyskinesia

Different trials have been conducted among patients with tardive dyskinesia and showed positive effects when compared with patients taking placebo. It has been hypothesized that choline chloride is cholinomimetic, which means it has the ability to mimic the neurotransmitter acetylcholine and therefore improve movement and coordination in patients (Berger et al., 1980).

4) Treatment of hepatic abnormalities

A lack of choline plays a significant role in the development of liver disease associated with total parental nutrition (TPN). Choline chloride was shown to be useful in reversing hepatic abnormalities associated with TPN when given orally in the form of supplements over a period of 24 weeks. Prevention of hepatic steatosis, which can lead to the death of TPN-dependent patients (Buchman et al., 2001),

CHAPTER 3

METHODOLOGY

3.1) STUDY OF HALOPHILIC BACTERIA

3.1.1. Sample collection

Natural salt samples were obtained from a salt pan located in Ribandar, Goa, India. The sample was then transported to the laboratory and stored in sealed screw-cap bottles as an ingredient in the medium. The isolates used in the present study were cultivated on high-salt agar.

3.1.2. Salinity test

All four isolates, designated NSK3, NSK6, NSK10, and SH2, were selected for this study. To verify their halophilic nature, a salinity test was carried out on these isolated cultures. All four isolates were cultivated in Media D (Kerkar et al., 2011), which consisted of 1.5% tryptone and 0.5% soya peptone. The media was supplemented with varying concentrations of natural salt to achieve salinity levels of 0% (no salt), 5%, 10%, 15%, 20%, 22%, and 25%. All flasks were incubated at RT for a period of 24 hours, followed by the measurement of OD at 600 nm.

3.1.3. Optimization of salt concentration for a higher yield of compatible solutes

3.1.3.a) *Media preparation*

Zobell Marine Broth (ZMB) was supplemented with varying concentrations of sodium chloride (NaCl) derived from a crude salt sample. Individual flasks containing sterile distilled water were amended with the crude salt to achieve final natural salt concentrations of 10%, 15%, 20%, 22%, and

25%. Subsequently, the resultant solutions were passed through filter paper to remove the particulate debris in the solution.

3.1.3.b) *Cultivation Conditions*

The four isolates, designated NSK2, NSK6, NSK10, and SH2, were cultivated in individual 250-mL shake flasks. Each flask contained 100 mL of media prepared with five distinct salt concentrations. Subsequently, all flasks were incubated at RT for a period of 48 hours.

3.2) PHENOTYPIC CHARACTERIZATION OF MICROORGANISMS

Various tests were carried out in order to characterize the probable genus of the halophilic bacteria used in the present work.

3.2.1) Cell and Colony Morphology

The isolated cultures were streaked on separate media plates to obtain isolated colonies. Colony characteristics and morphological observations were noted. Gram staining revealed Gram-type (positive or negative), while colony morphology, including shape, elevation, margin, etc., was documented. The motility test was also carried out to assess the ability of isolates for self-movement.

3.2.2) Biochemical tests

Biochemical tests reveal a microbe's unique enzymatic machinery. By assessing its ability to utilize specific substrates or produce certain enzymes. This method allows one to differentiate between various species and narrow down the identity of unknown microorganisms.

1. *IMViC*: KB001 HiIMViC ™ Biochemical Test Kit (HiMedia) was used for this test, which included the following tests: Indole,Methyl Red, Voges-Proskauer's and Citrate Utilization Test. Tests for sugars such as glucose, Adonitol, Arabinose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose, and sucrose were successfully performed.
2. *Carbohydrate Utilization:* The KB009 HiCarbo™ Test Kit (HiMedia) was used to observe the ability of isolates to utilize 35 different carbohydrates such as Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose, Inulin, Sodium Gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbitol, Mannitol, Adonitol, Arabitol, Erythritol, alpha-Methyl-D-glucoside, Rhamnose, Cellobiose, Melezitose, alpha-Methyl-D-Mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Citrate, Malonate, and Sorbose.
3. *Catalase test:* Slants of Nutrient Agar (HiMedia) supplemented with 0.05% KCl and 10% NaCl were streaked with the selected cultures and incubated overnight at RT. A few drops of 3% H_2O_2 were added to the slants, and the presence or absence of effervescence was observed immediately.
4. *Amylase test*: isolates streaked on Norberg-Hofstein media supplemented with 1% starch and incubated at RT overnight. After pouring iodine, the presence of clear zones around cultures was observed.

5. *Gelatin liquefaction*: Culture tubes were prepared using Nutrient broth media supplemented with 0.05% KCl, 10% NaCl, and 0.5% gelatin. Culturing was done by the stab method, followed by incubation at 25 degree Celsius overnight. The tubes were then kept for an hour in the fridge. Following incubation, the cultures were examined for gelatin liquefaction.
6. *Urease test*: The isolates were inoculated on Christensen's Urea Agar slants and incubated at RT overnight. Slants were then checked for a color change from yellow to pink.
7. *Oxidase test*: An actively growing 24-hour old culture was aseptically transferred on sterile oxidase discs soaked in Kovac's reagent. Discs were observed for purple coloration.
8. *Protease test*: isolates streaked on Norberg-Hofstein media supplemented with 1% skimmed milk powder and incubated at RT for 48 hours. The cultures were examined for the presence of a zone of clearance.
9. *Hydrogen sulfide test:* Culture tubes of SIM media supplemented with 10% NaCl were prepared for the test. Culture was inoculated by the stab method, followed by incubation at RT for a period of 24 hours. Tubes were analyzed for the presence of black precipitate.

3.3) MOLECULAR CHARACTERIZATION

Traditional methods for identifying bacteria rely on growth and morphological characteristics and biochemical tests. Molecular characterization offers a more precise and objective approach. By analyzing a specific gene, like the 16S r-RNA gene, we can directly compare the bacteria's DNA sequence to known databases, revealing its identity with much greater accuracy.

3.3.1) *DNA isolation*

1. Culture was inoculated in ZMB supplemented with 10% NaCl for 24 hours.
2. Broth with actively growing isolates was transferred into a sterile centrifuge tube and centrifuged at 9500 RPM at 4 degrees Celsius for 10 minutes.
3. The pellet was re-suspended in 200µL Tris-HCl buffer (pH 8.5) and a mixture of phenol: chloroform: isoamyl alcohol in ratio 25:24:1.
4. The tube was centrifuged at 9500 RPM at 4 degrees Celsius for 10 minutes.
5. The aqueous layer was taken into another sterile tube and filled with 1/10th volume of 3M sodium acetate.
6. To this mixture, twice the amount of 99.9% cold ethanol was added and incubated overnight at -20 degrees Celsius for DNA precipitation.
7. The tube was then centrifuged at 12000 RPM at 4 degrees Celsius for 15 minutes.
8. The precipitated DNA was re-suspended in 500µL of 70% ethanol and centrifuged at 12000 RPM at 4 degrees Celsius for 10 minutes.
9. The pellet was re-suspended in 50µL of TE buffer.

3.3.2) PCR

The 16S r-RNA gene fragment was amplified using a universal forward primer (27F) (5′-AGAGTTTGATCCTGGCTCAG-3′) and a reverse primer (1492R) (5′-TACGGTTACCTTGTTACGACTT-3′) via a standard polymerase chain reaction (PCR) protocol using isolated genomic DNA as a template. In each PCR reaction mixture, 2µL of 10x Taq buffer, 2mM MgCl2, 1µL of dNTPs, 1µL of both reverse and forward primers, 1µL of Taq polymerase, 1µl

of template DNA, and Milli-Q water to make volume up to 50µL were added. Initial denaturation for 2 min at 95 °C was followed by denaturation (30 sec at 95 °C), annealing (30 sec at 51.9 °C), and extension (7 min, 33 sec at 72 °C) in total, making 35 cycles, with a final extension for 8 min at 72 °C to bring the reaction to an end. To verify the successful amplification, gel electrophoresis was performed to observe the presence of the band.

3.4) EXTRACTION OF COMPATIBLE SOLUTES

The extraction of osmoprotectants was carried out using two methods:

- Methanol: chloroform; water method
- Ethanol: chloroform method

3.4.1.) Methanol: chloroform: water method

1. Culture media containing different concentrations of natural salt were dispersed in centrifuge tubes and centrifuged at 10000 rpm for 10 minutes.
2. The pellet was then re-suspended in a mixture of methanol: chloroform: water (10:1:4) and incubated at room temperature for 4 hours with regular, gentle shaking at hourly intervals.
3. Tubes were centrifuged at 10000 rpm for 5 minutes.
4. The supernatants were transferred to new tubes, followed by the addition of 1 mL of chloroform and 1 mL of water.
5. Tubes were vortexed and centrifuged at 10000 rpm for 5 minutes.

6. Two separate layers were observed. The organic layer is at the bottom, and the aqueous layer is on top. The aqueous layer was collected into sterile tubes and kept at -80°C for further analysis (Kunte et al., 1993).

3.4.2.) Ethanol extraction method

1. Culture media containing different concentrations of natural salt were dispersed in centrifuge tubes and centrifuged at 10000 rpm for 10 minutes.
2. The pellet was re-suspended in 80% ethanol and kept at 50°C until dried.
3. Cells were then re-suspended in chloroform and water in a ratio of 2:1 and mixed well.
4. The aqueous layer was taken into separate sterile tubes for further analysis and kept at 4°C (Santos et al., 2006).

3.4.3) Screening of compatible solutes

To verify the presence of the desired compatible solutes, Thin Layer Chromatography (TLC) was used. Standards of ectoine, hydroxyectoine, and choline chloride were employed for comparison. Samples underwent multiple spotting applications, with thorough drying in between. The modified solvent system comprised of n-butanol, acetic acid, and water in a 12:5:3 (v/v) ratio was used (Oka et al., 1980). For visualization of choline chloride dried from the solvent system, a TLC plate was placed in a chamber containing iodine crystals, which, on sublimation, form fumes that interact with choline chloride to form a spot. After visualization of the choline chloride plate, a spot is marked, and the plate is allowed to evaporate iodine. The next visualization step is done for amino acids; for that,

the TLC plate is sprayed with a ninhydrin solution and placed in the oven at 120 degrees Celsius for 15 minutes to promote the reaction. Spots were marked, and Rf (Retardation factor) values were calculated for standards and samples.

3.5) Toxicity tests

3.5.1.) Hemolysis Test

All four bacterial cultures were streaked onto blood agar plates supplemented with 5% NaCl. Following incubation at 37°C for 24-48 hours, the plates were examined for hemolysis patterns.

3.5.2) Comet assay

To assess the potential genotoxicity of the compatible solutes, a comet assay was employed on human white blood cells.

To prepare a whole blood culture 50 mL of RPMI 1640 medium was mixed with 10 ml of 10% heat-inactivated fetal bovine serum, 1 mL of PHA, 4 mL of antibiotic solution (HiMedia-Penicillin 5000 units and 5 mg Streptomycin), 200 mM L-glutamine, and 5 mL of blood sample. 5 mL of prepared media was transferred to 13 sterile glass vials (Scarfi et al., 1994).

Commercially available ectoine (Sigma-Aldrich) was used to prepare a series of solutions with varying final ectoine concentrations. A stock solution of known concentration (e.g., 10 mg/mL) was prepared. Aliquots of this stock solution were then carefully measured and added to separate tubes containing diluent to achieve the desired final ectoine concentrations expressed per liter: 1 mg/L, 5 mg/L, 10 mg/L, 20mg/L, 30 mg/L, 40 mg/ L, 50 mg/L, 60 mg/L, 70 mg/L, 80 mg/L, 90 mg/L, and

100 mg/L. A control tube containing only 5 mL of blood cell culture media without the addition of ectoine was used as a control. Glass vials were incubated at 37 degrees Celsius for 24 hours.

The frosted slides were used for the comet assay. 500µL of 1% agarose was added to each slide and covered with a coverslip until it solidified. After removing coverslips, 200µL of low melting agarose (0.7%) was mixed with 100µL of blood cell culture media and added to the slide as the second layer, covered with coverslips. After this layer was solidified, another 200µL of low melting agarose was added as the third layer to fix cells. After slides were prepared, they were kept in lysis buffer overnight at 4 degrees Celsius. Slides were then kept in the electrophoresis buffer in the gel electrophoresis unit for 20 minutes, followed by electrophoresis, which was set at 20 V and 200 mA for 20 minutes. The next step included placing slides into the neutralization buffer for 10–15 minutes.

Samples were visualized using fluorescence microscopy following the addition of 3 drops of ethidium bromide (EtBr) stain, covered with a coverslip, and observed for intact cells and ‘comet-like structures'.

3.5.3) MTT Assay

The potential cytotoxicity of the compatible solute was evaluated using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay adapted for suspension cell cultures. Blood Cell Culture Media, previously used in cell viability experiments, was employed for this assay. To account for the potential interference of red blood cells (RBCs) with the assay, the protocol was modified. Briefly, culture media from the previous assay was transferred to sterile centrifuge tubes and centrifuged at 2500 RPM to pellet the cells. ELISA plates were not used in this step since this assay relies primarily on the metabolic activity of viable white blood cells, whereas RBCs present in

culture media can adhere to the plate and reduce WBC interaction with the MTT reagent, leading to falsely low readings. Following centrifugation, the supernatant was carefully removed, and the cell pellet was resuspended in phosphate-buffered saline (PBS). Subsequently, 20µL of a 2 mg/mL MTT reagent was added to each tube, and the cultures were incubated at 37°C for 4 hours. The tubes were then centrifuged again at 2500 RPM, the supernatant was discarded, and the cell pellet containing the formazan crystals was resuspended in DMSO. The tubes were covered with aluminum foil and shaken on an orbital shaker for 1 hour to ensure complete formazan crystal dissolution. Finally, the tubes were centrifuged to pellet the cells, and the supernatant containing the dissolved formazan was transferred to an ELISA plate in triplicate wells. The absorbance was measured at 595 nm, and cytotoxicity was calculated using the formula:

Cytotoxicity level (%) = (Control-Sample) ✕100%

To avoid errors Blank readings (DMSO) were subtracted from the sample and control readings.

3.6) APPLICATIONS

Compatible solutes are known for their interaction with water molecules. This interaction facilitates the formation of a protective hydration layer around biomolecules. This hydration layer offers a significant advantage: it helps prevent dehydration and safeguards protein function even under stressful conditions. Recognizing this protective property, we explored the potential application of compatible solutes in the cosmetic industry. Our primary focus was to investigate their ability to protect human skin from dehydration when put into moisturizer. To assess this potential, a series of experiments were conducted.

3.6.1) Antimicrobial test

The potential antimicrobial properties of the isolated compatible solutes were investigated against a panel of common skin pathogens. These pathogens included *Streptococcus spp.*, *Proteus vulgaris*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

The experiment employed a disc diffusion assay. Briefly, aliquots of Nutrient agar were inoculated with each bacterial strain to create lawns of growth. Sterile filter paper discs were then prepared. For each compatible solute sample, a disc was saturated with 5µL aliquots of the crude extract, allowing for complete drying between applications. A total of four 5µL additions were used per disc. Subsequently, these discs were placed onto the Mueller Hinton Agar (HiMedia) plates containing the respective bacterial cultures. Following incubation at 37°C for 24 hours, the plates were examined for the presence of zones of growth inhibition surrounding the discs. The diameter of these inhibition zones would be indicative of the antimicrobial activity of the tested compatible solutes.

4.6.2) Assessment of Skin Hydration Following Patch Application

Evaluating the ability of the formulated moisturizer to enhance skin hydration was a crucial step in this investigation. Three designated regions on the hand were marked for patch application:

1) Control: This area served as a baseline and received no treatment. Changes in hydration level measured here would reflect natural fluctuations.

2) Secondary Control: aloe vera gel alone was applied to this region. The average increase in hydration level measured here would represent the inherent moisturizing properties of aloe vera itself. It allows us to isolate the compatible solute's effect on hydration by subtracting the average hydration

increase from the aloe vera patch (secondary control) from the test patch (both aloe vera and solute), we then determine the hydration improvement due solely to the compatible solute.

3) Test Patch: The formulated moisturizer containing both aloe vera and the compatible solute sample was applied to this designated area.

This patch testing experiment spanned a period of two weeks. Skin hydration levels at all three marked locations were evaluated using the Skin Detector (Dealsure SG-5D, China). To ensure accurate results, triplicate readings were recorded at each site, and the average value was recorded for analysis. Following the experiment, the presence or absence of significant improvements in skin hydration was determined by comparing the average readings across the control, secondary control, and test patch areas.

4.6.3) Comparative Evaluation of Moisturizers on Skin Hydration

An evaluation of the moisturizing capabilities of commercially available moisturizers compared to Ectoine was conducted. Four readily obtainable market products were selected: Foxtale, Pure Sense, Nivea, and Pond's. Each product was applied to designated areas on the hand along with Ectoine moisturizer. An additional control area received no moisturizer application. Following a 30-minute incubation period, skin moisture content was measured in triplicate for each designated area. Data analysis involved subtracting the control value from the observed values of each product to account for baseline moisture levels. The results were subsequently visualized through a graphical representation for comparison

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1) SCREENING FOR OPTIMUM SALINITY TOLERANCE

The isolated bacterial cultures exhibited the most significant growth, as measured by optical density (OD) at 600 nm, in media with salinities ranging from 5% to 15%. This observation suggests their halophilic nature, specifically categorizing them as moderate halophiles.

Salinity	**NSK3**	**NSK6**	**NSK10**	**SH2**
0%	0.007	0.058	0.050	0.009
5%	0.256	0.090	0.260	0.191
10%	0.347	0.145	0.210	0.280
15%	0.280	0.175	0.199	0.256
20%	0.259	0.135	0.163	0.226
22%	0.186	0.064	0.115	0.180
25%	0.115	0.034	0.090	0.048

Table 4.1 –Absorbance of growth of various isolates at 600nm

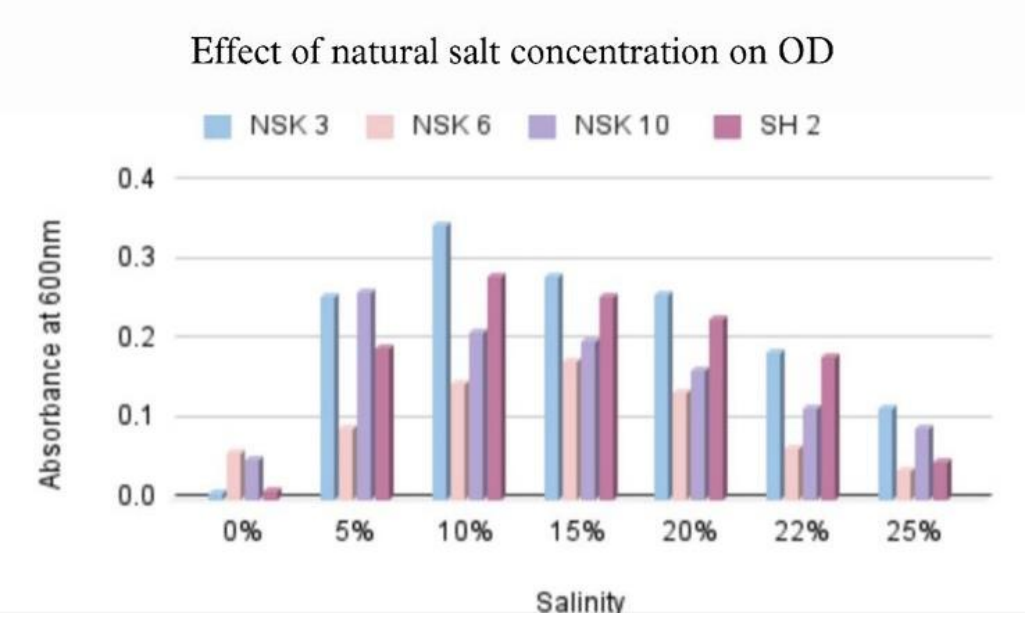


Fig.4.1 – Absorbance of bacterial growth at 600nm vs Salinity (%)

4.2) CELL AND COLONY MORPHOLOGY

4.2.1) NSK3

Characteristics	NSK3
Colony Shape	Circular
Elevation	Flat
Margin	Entire
Surface	Smooth
Opacity	Opaque
Texture	Moist
Cell Shape	Bacilli
Color	Off-white
Gram Character	Negative
Motility	Peritrichous

Table 4.2 - Cell and colony morphology of NSK3

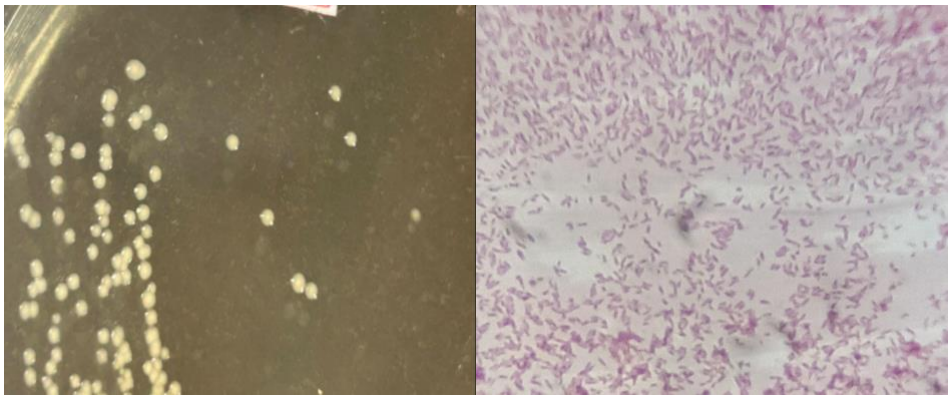


Fig.4.2 Colony and Cell Morphology of NSK3

4.2.2) NSK6

Characteristics	**NSK6**
Colony Shape	Punctiform
Elevation	Flat
Margin	Entire
Surface	Smooth
Opacity	Transparent
Texture	Mucoid
Cell Shape	Cocci
Color	Orange
Motility	Motile
Gram Character	Positive

Table 4.3 - Cell and colony characteristics of NSK6

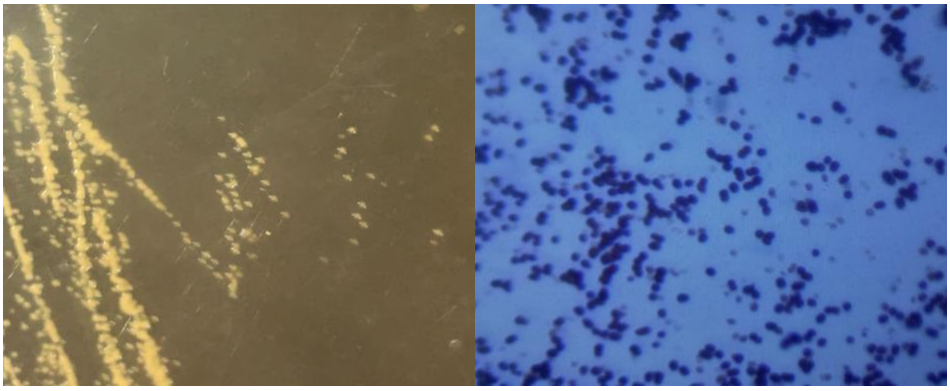


Fig.4.3 Colony and Cell Morphology of NSK6

4.2.3) NSK10

Characteristics	**NSK10**
Colony Shape	Circular
Elevation	Flat
Margin	Entire
Surface	Smooth
Opacity	Opaque
Texture	Moist
Cell Shape	Bacilli
Color	Off-white
Motility	Monotrichous
Gram Character	Negative

Table 4.4 - Cell and colony morphology of NSK10

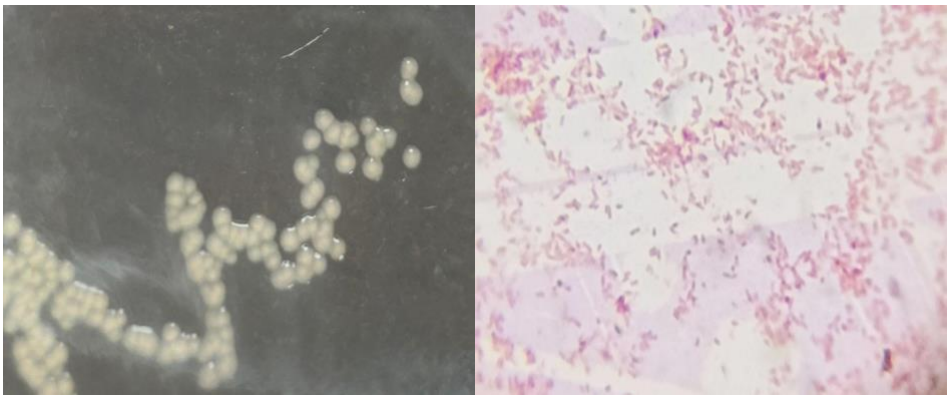


Fig.4.4 Colony and Cell Morphology of NSK10

4.2.4) SH2

Characteristics	**SH2**
Colony Shape	Circular
Elevation	Flat
Margin	Entire
Surface	Smooth
Opacity	Transparent
Texture	Mucoid
Cell Shape	Bacilli
Color	Off-white
Motility	Motile
Gram Character	Negative

Table 4.5 - Cell and colony morphology of SH2

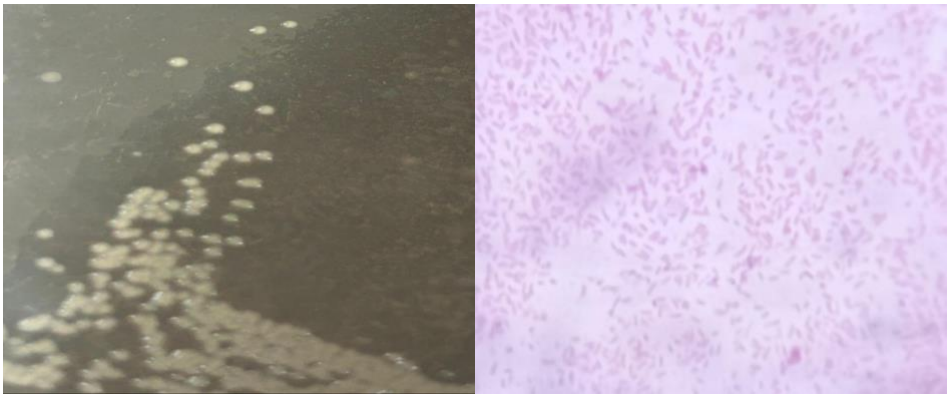


Fig.4.5 Colony and Cell Morphology of SH2

4.3) BIOCHEMICAL TESTS

4.3.1) IMViC Test

Sr. No.	Tests	NSK3	NSK6	NSK10	SH2
1	Indole	-	-	-	-
2	Methyl red	-	-	-	+
3	Voges Proskauer's	-	-	-	-
4	Citrate utilization	+	-	+	-
5	Glucose	-	-	-	+
6	Adonitol	-	-	-	-
7	Arabinose	-	-	-	+
8	Lactose	-	-	-	-
9	Sorbitol	+	-	-	-
10	Mannitol	+	-	-	-
11	Rhamnose	-	+	-	-
12	Sucrose	+	-	-	-

Table 4.6 - IMViC test Interpretation chart {Key: '+' – Positive; '-'- Negative}

4.3.2) Carbohydrates Utilization Test

Sr.No	Carbohydrate	NSK3	NSK6	NSK10	SH2
1	Lactose	+	-	+	-
2	Xylose	+	-	+	+
3	Maltose	+	-	-	-
4	Fructose	-	-	+	-
5	Dextrose	+	-	+	+
6	Galactose	+	-	+	-
7	Raffinose	-	-	-	-
8	Trehalose	-	-	-	-
9	Melibiose	+	-	-	-
10	Sucrose	-	-	-	-
11	L-Arabinose	+	-	+	-
12	Mannose	+	-	+	+
13	Inulin	-	-	-	-
14	Sodium gluconate	-	-	-	-

15	Glycerol	-	-	-	-
16	Salicin	-	-	-	-
17	Dulcitol	-	-	-	-
18	Inositol	-	-	-	-
19	Sorbitol	-	-	-	-
20	Mannitol	-	-	-	-
21	Adonitol	-	-	-	-
22	Arabitol	-	-	-	-
23	Erythritol	-	-	-	-
24	alpha-Methyl-D-glucoside	-	-	-	-
25	Rhamnose	-	-	-	-
26	Cellobiose	-	-	-	-
27	Melezitose	-	-	-	-
28	alpha-Methyl-D Mannoside	-	-	-	-
29	Xylitol	-	-	-	-
30	ONPG	+	+	-	-

31	Esculin	-	-	-	-
32	D-Arabinose	+	-	+	+
33	Citrate	-	-	-	-
34	Malonate	+	-	+	-
35	Sorbose	-	-	-	-

Table 4.7 - Carbohydrate utilization tests Interpretation chart {Key: '+' – Positive; '-'- Negative}

4.3.3) Enzyme Assay

Sr.No.	**Enzymes**	**NSK3**	**NSK6**	**NSK10**	**SH2**
1	Catalase	+	+	+	+
2	Amylase	-	+	-	-
3	Gelatinase	-	-	-	-
4	Urease	-	-	-	-
5	Oxidase	+	+	+	+
6	Protease	+	+	+	+

Table 4.8 - Enzyme assay Interpretation chart {Key: '+' – Positive; '-'- Negative}

4.3.4) Hydrogen sulfide test

None of the isolated bacterial cultures demonstrated the formation of a black precipitate within the media. This observation suggests the absence of hydrogen sulfide (H_2S) gas production by these isolates.

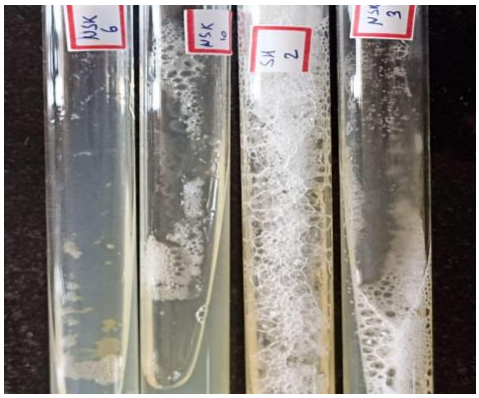


Fig.4.6 Catalase test

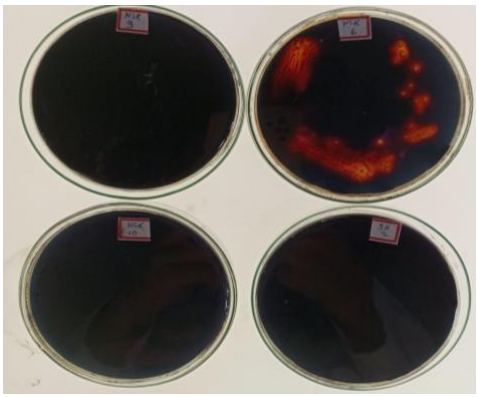


Fig.4.7 Amylase test

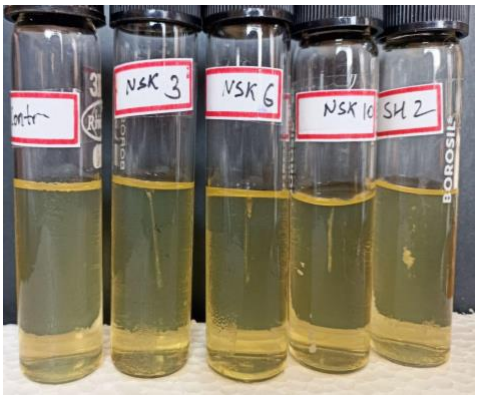


Fig.4.8 Gelatinase test

Fig.4.9 Urease test

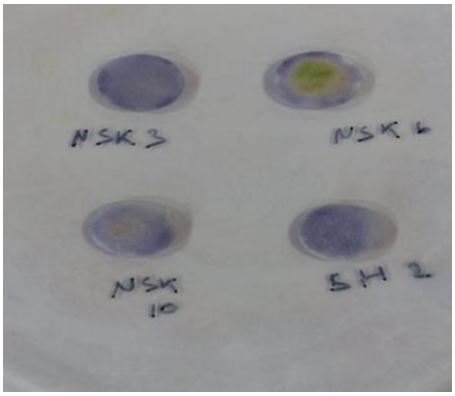


Fig.4.10 Oxidase test

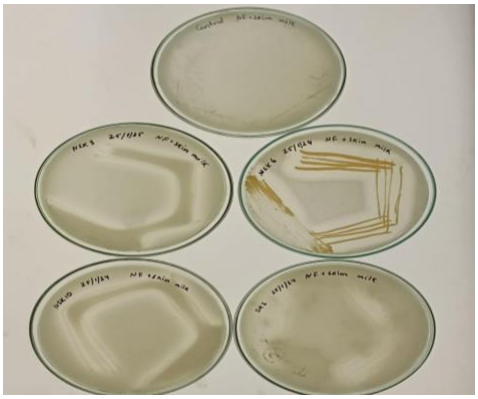


Fig.4.11 Protease test

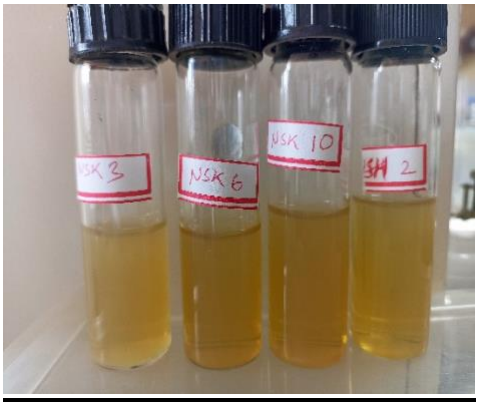


Fig.4.12 Hydrogen sulfide test

4.4) MOLECULAR CHARACTERIZATION

DNA was successfully extracted from three isolates (NSK3, NSK10, and SH2) using the phenol-chloroform method, a well-established technique for DNA isolation.Subsequent amplification of the 16S ribosomal RNA (r-RNA) gene was performed for these isolates. The 16S r-RNA gene is a highly conserved genetic marker widely used for bacterial identification.

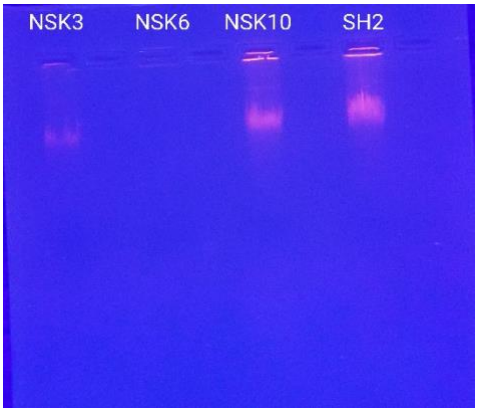


Fig.4.13 Isolated DNA on agarose gel

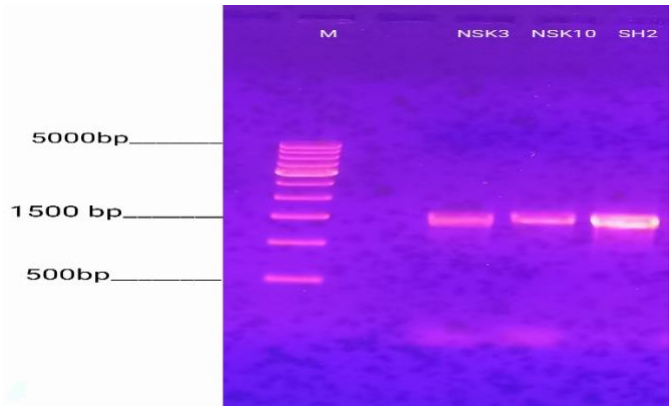


Fig.4.14 Bands of 16S r-RNA PCR product (bp - base pairs; M- Marker 500bp)

Unfortunately, analysis of the sequenced 16S r-RNA gene products from these isolates yielded data of insufficient quality, hindering definitive identification at the genus level.

Owing to the limitations of the sequencing data, a phenotypic identification approach was employed for isolates NSK3, NSK10, and SH2. This method utilizes Bergey's Manual of Systematic/Determinative Bacteriology, a standard reference for bacterial classification based on observable characteristics. Using this approach, NSK3 was tentatively identified as belonging to the genus *Alcaligenes.* Isolate NSK10 exhibited characteristics consistent with either *Pseudomonas* or *Alteromonas*. Isolate SH2 demonstrated close phenotypic similarity to the genus *Chromobacterium.* It is important to note that these identifications are presumptive based on phenotypic traits and may require further confirmation using additional methods.

Unlike the other isolates, DNA isolation using the phenol-chloroform method was unsuccessful for NSK6. Consequently, this isolate was directly sequenced in its pure form, bypassing the DNA isolation step. Sequencing analysis of NSK6 showed close similarity to *Halobacillus alkaliphilus* strain FP5 of 99.56%.

4.5) DETECTION OF COMPATIBLE SOLUTES BY TLC

The aqueous layers obtained after extraction of compatible solutes by methanol: chloroform: water and ethanol: water methods were spotted on TLC plates. Spots were visualized firstly when TLC plate was exposed to iodine fumes and after spraying ninhydrin reagent ,spots did appeared as brown spots on white background having Rf values close to one od standard ectoine spot indicating presence of ectoine in the samples.

1) Methanol: chloroform: water method

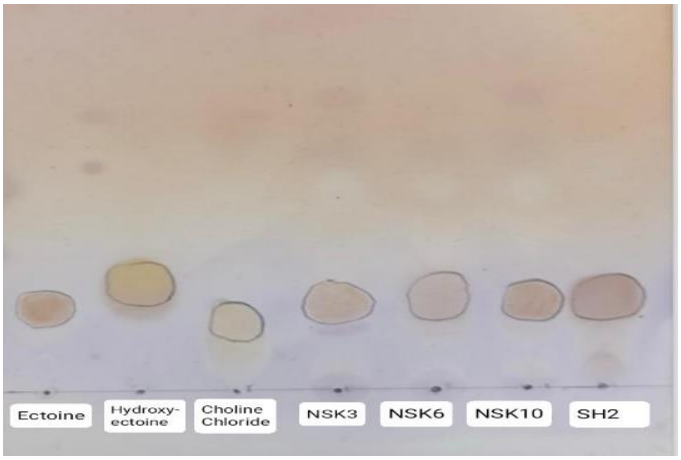


Fig.4.15 Compatibles solutes extracted using methanol: chloroform: water method

Sr. No.	Standard/Sample	Distance travelled by solute (cm)	Distance travelled by solvent (cm)	Rf value
1	Ectoine	1.55	8.4	0.184
2	Hydroxyectoine	1.90	8.4	0.226

3	Choline chloride	1.20	8.4	0.142
4	NSK3	1.52	8.4	0.180
5	NSK6	1.60	8.4	0.190
6	NSK10	1.50	8.4	0.179
7	SH2	1.55	8.4	0.184

Table 4.9 - Rf values of samples by methanol: chloroform: water extraction method

2) Ethanol: water method

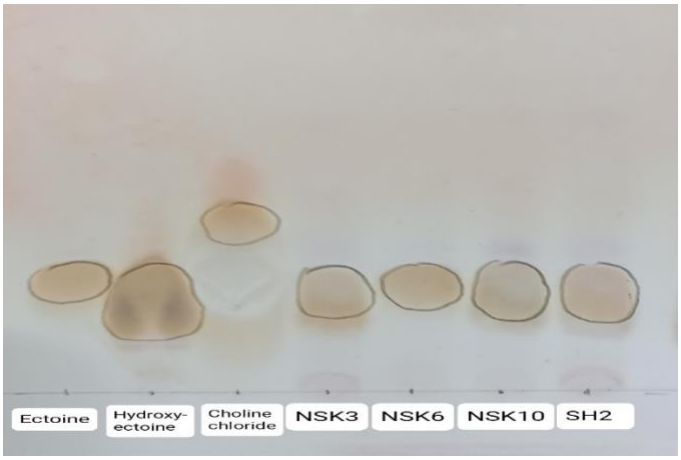


Fig4.16 Compatibles solutes isolated using ethanol: water method

Sr. No.	Standard/Sample	Distance travelled by solute (cm)	Distance travelled by solvent (cm)	Rf value
1	Ectoine	2.00	8.25	0.242
2	Hydroxyectoine	1.60	8.25	0.193
3	Choline chloride	3.10	8.25	0.37
4	NSK3	1.90	8.25	0.230
5	NSK6	2.00	8.25	0.242
6	NSK10	1.9	8.25	0.230
7	SH2	1.9	8.25	0.230

Table 4.10 - Rf values of samples by ethanol: water extraction method

Thin-layer chromatography (TLC) analysis of the bacterial extracts revealed Rf values for samples NSK3, NSK6, NSK10, and SH2 that closely matched the Rf value of the standard amino acid ectoine. This observation suggests the production of ectoine by these bacterial isolates when grown under high salt concentration conditions. Spots other than ectoine with observed on TLC plate of methanol: chloroform: water method indicating the presence of other solutes as well in bacteria. On the other side smear that appear on the plate indicated possible impurities in the samples.

Ectoine is a well-characterized compatible solute known for its role in osmoprotection in halophilic bacteria. Given its prevalence, it is reasonable to expect that ectoine might be commonly found alongside other compatible solutes in a significant number of halophilic bacterial species. This widespread occurrence could potentially facilitate its extraction for various applications.

Following the successful detection of ectoine in all four bacterial strains, attempts to quantify the specific concentration of this amino acid at each tested salinity level were unfortunately hampered by the lack of access to high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) instrumentation. However, drawing upon prior research findings, it can be postulated that the highest concentration of this osmoprotectant is likely to be produced at higher salinity levels. A decline in bacterial growth was observed at a salinity of 25%, potentially indicating a growth arrest or a significant reduction in viability. This suggests that the high salt concentration may be exceeding the tolerance limits of the bacteria, hindering their ability to proliferate effectively. In contrast, at a salinity of 22%, the bacteria exhibited continued active growth. .This suggests a potential upregulation of ectoine production as a cellular response to increased environmental salinity, with the highest production potentially occurring at a salinity of 22%.

4.6) HEMOLYSIS TEST

The plates streaked with four isolates were examined for hemolysis patterns which can be categorized as alpha, beta or gamma. This test helped to provide valuable data for identification of potential virulence of isolates. Following incubation, none of the isolates displayed notable clearing zones surrounding the colonies. This observation signifies gamma hemolysis, indicating that the bacteria do not produce hemolysins, the enzymes responsible for RBC lysis

Fig.4.17 Isolates growing on Blood Agar

4.7) COMET ASSAY

The potential genotoxic effects of ectoine on white blood cells (WBCs) were evaluated through fluorescence microscopy. Blood cell suspensions were exposed to varying concentrations of the amino acid ectoine. Following incubation, the cells were examined under a fluorescence microscope to assess the presence of comet structures, a hallmark indicator of DNA damage.

Observations revealed intact cells with distinct round shapes across all ectoine concentrations tested. Notably, no comet structures were observed within the cells. This finding suggests that ectoine, at the

concentrations employed in this study, does not induce genotoxicity in WBCs and therefore can be used for human applications such as moisturizer.

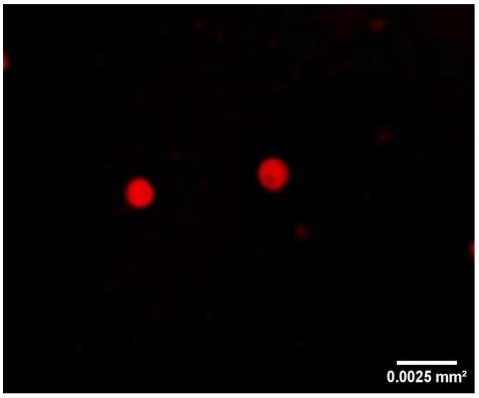


Fig.4.18 Control sample with no ectoine

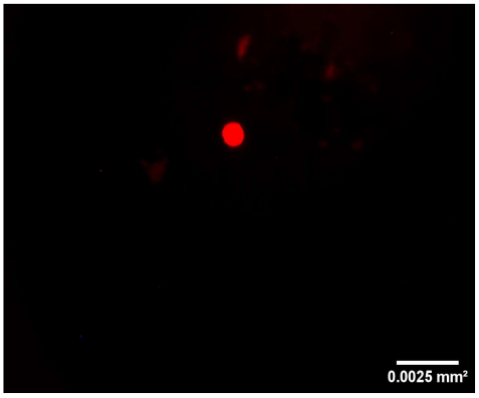


Fig.4.18.a Sample containing 5mg/L of ectoine

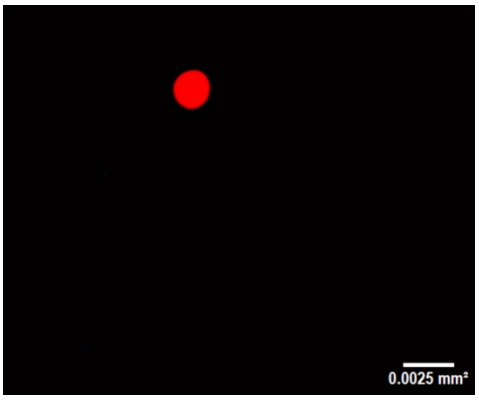


Fig.4.18.b Sample containing 10 mg/L of ectoine

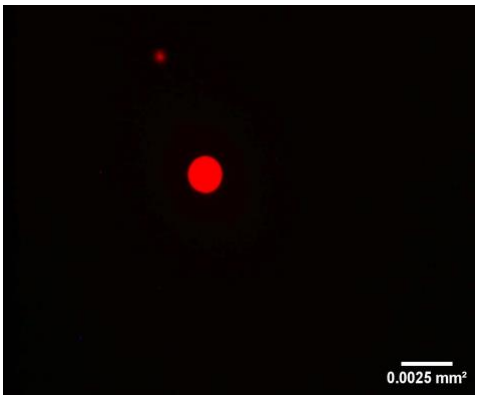


Fig.4.18.c Sample containing 30mg/L of ectoine

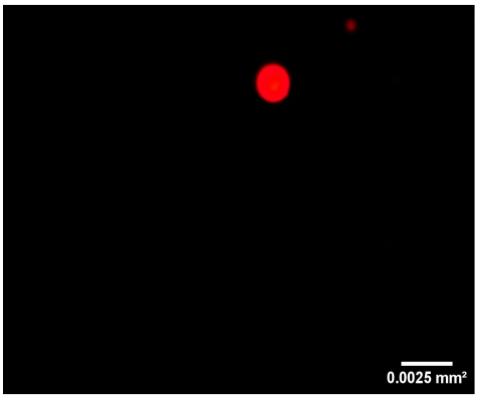


Fig.4.18.d Sample containing 50 mg/L of ectoine

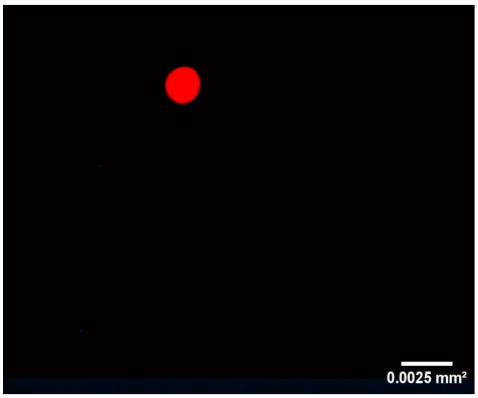


Fig.4.18.e Sample containing 80 mg/L of ectoine

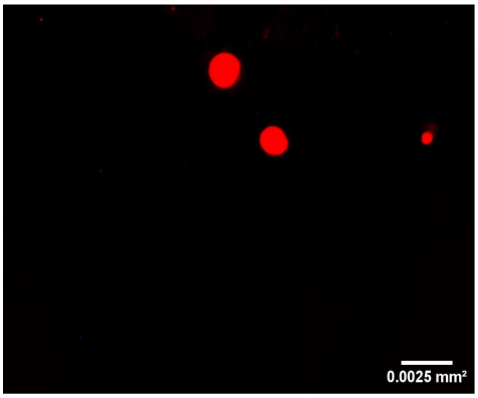


Fig.4.18.f Sample containing 100 mg/L of ectoine

4.8) MTT ASSAY

Absorbance at 595nm of samples containing various concentrations of ectoine was noted down and cytotoxicity level was calculated .Low level of cytotoxicity indicates safe use of ectoine for human applications such as in cosmetic products. Uneven variations of cytotoxicity might be due to pipetting errors, ELISA plate reader or incomplete dissolution of formazan crystals.

Concentration of Ectoine (mg/L)	**Absorbance at 595 nm (average)**	**Standard deviation**	**Cytotoxicity level (%)**
1	0.445	0.009899494937	1.4
5	0.446	0.008485281374	1.3
10	0.452	0.0113137085	0.7
20	0.44	0.008485281374	1.5
30	0.45	0.01697056275	0.9
40	0.448	0.01202081528	1.1
50	0.443	0.0113137085	1.6
60	0.44	0.01272792206	1.8
70	0.448	0.01414213562	1.1
80	0.447	0.0113137085	1.2
90	0.456	0.0113137085	0.3
100	0.453	0.007071067812	0.6
Control	0.459	0.0005773502692	

Table 4.11 - Absorbance at 5 95nm and cytotoxicity level at different Ectoine concentrations

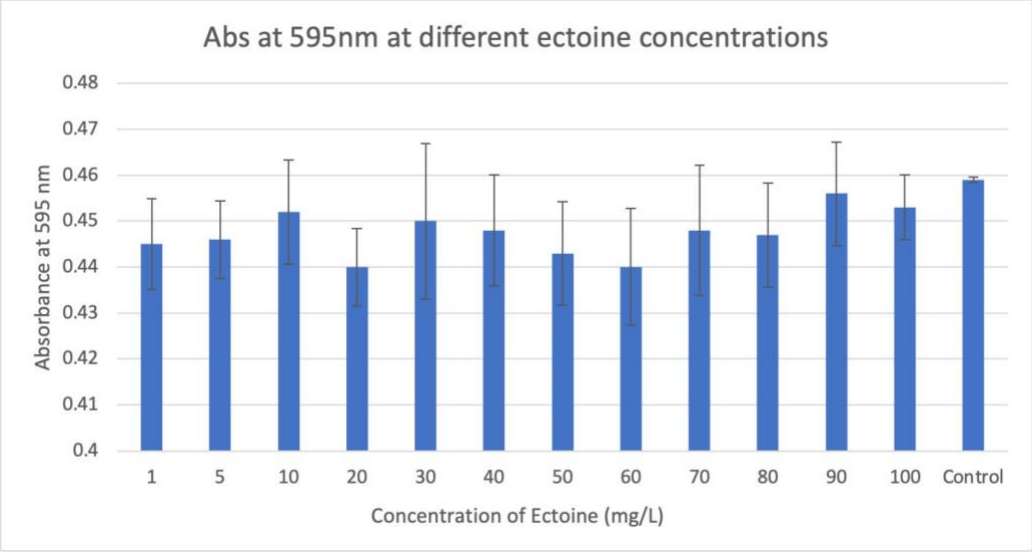


Fig.4.19 Absorbance at 595nm at various Ectoine concentrations

4.9) ANTIMICROBIAL TEST

The antimicrobial activity of the compatible solute was evaluated against four human skin pathogens: The assay employed agar diffusion methods with zones of inhibition around the solute discs indicating growth inhibition of the pathogens.

Following incubation, no zones of inhibition were observed for *Proteus vulgaris*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. This finding suggests that the compatible solute lacks inhibitory activity against these specific pathogens. Conversely, the plate containing *Streptococcus* sp. exhibited an unexpected result. Instead of a zone of inhibition, enhanced bacterial growth was observed in the region surrounding the compatible solute disc. This observation suggests a potential growth-promoting effect of the solute on *Streptococcus* sp., which may not be desirable for topical application on human skin, especially for individuals infected with this bacterium.

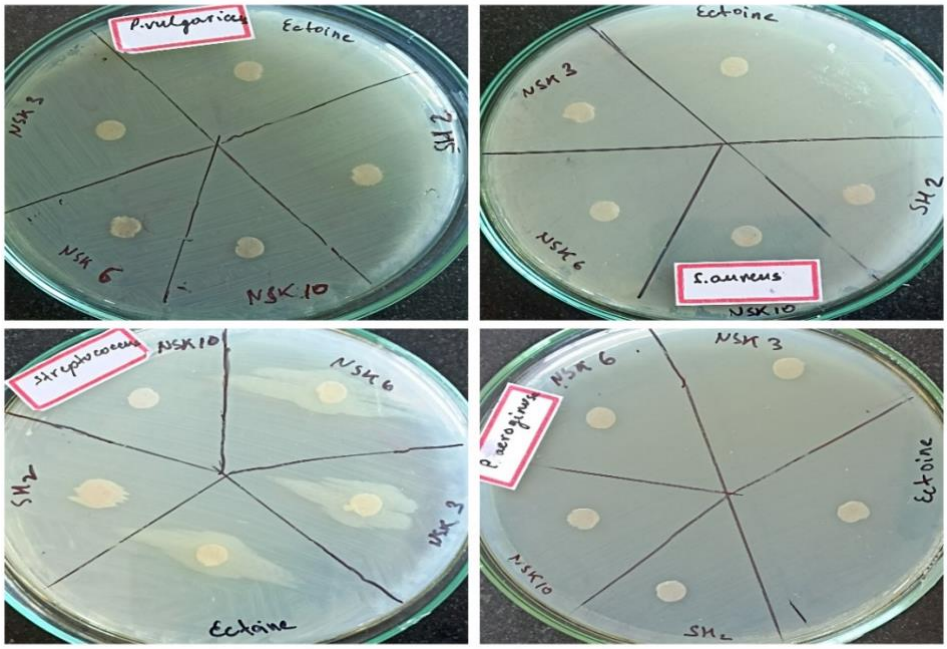


Fig.4.20 Antimicrobial Susceptibility Testing of skin pathogens using Disc Diffusion Method

4.10) ASSESSMENT OF SKIN HYDRATION FOLLOWING PATCH APPLICATION

Three spots were marked on skin named as Control, Secondary control and Patch Test Skin moisture content was documented in a table at defined intervals throughout the study. This data collection allowed for the assessment of potential changes in skin hydration levels induced by ectoine application compared to the control and secondary control.

The application of a moisturizer containing 10 mg/L ectoine in aloe vera gel resulted in a statistically significant increase in skin hydration levels. This improvement was sustained throughout the experimental period, demonstrating long-term efficacy. Moisture content measurements revealed a rise from average value of skin which is 40.97 with pure aloe vera gel to 52.9% and to 58.06% with

the ectoine-containing gel, representing a net increase of 5.16% compared to the secondary control. These findings suggest that ectoine possesses potent moisturizing properties at a relatively low concentration (10 mg/L).

The effectiveness observed at this concentration further highlights its potential as a moisturizing agent in cosmetic creams. Compared to other products in the market that might require higher concentrations of active ingredients to achieve similar results, the lower concentration requirement for ectoine could potentially translate to a cost advantage for these creams.

Fig.4.21 Assessment of skin hydration following patch application, Where:

1 – Control, 2- Secondary control, 3– Patch Test

Day	**Control (average)**	**Standard Deviation of control**	**Secondary Control (average)**	**Standard deviation of secondary control**	**Patch Test (average)**	**Standard deviation of patch test**
1	40.76	0.208167	48.56	0.51316	52.73	0.404145
2	41.26	0.351188	50.16	0.321455	53.63	0.61101

3	41.23	0.288675	50.2	0.4	54.4	0.43589
4	40.37	0.61101	50.43	0.378594	55.3	0.351188
5	40.76	0.550757	51.1	0.3	56.23	0.404145
6	40.9	0.52915	51.8	0.568624	56.96	0.152753
7	41.56	0.404145	52.9	0.351188	58.06	0.208167

Table 4.12 - Evaluation of effect of ectoine on skin moisture

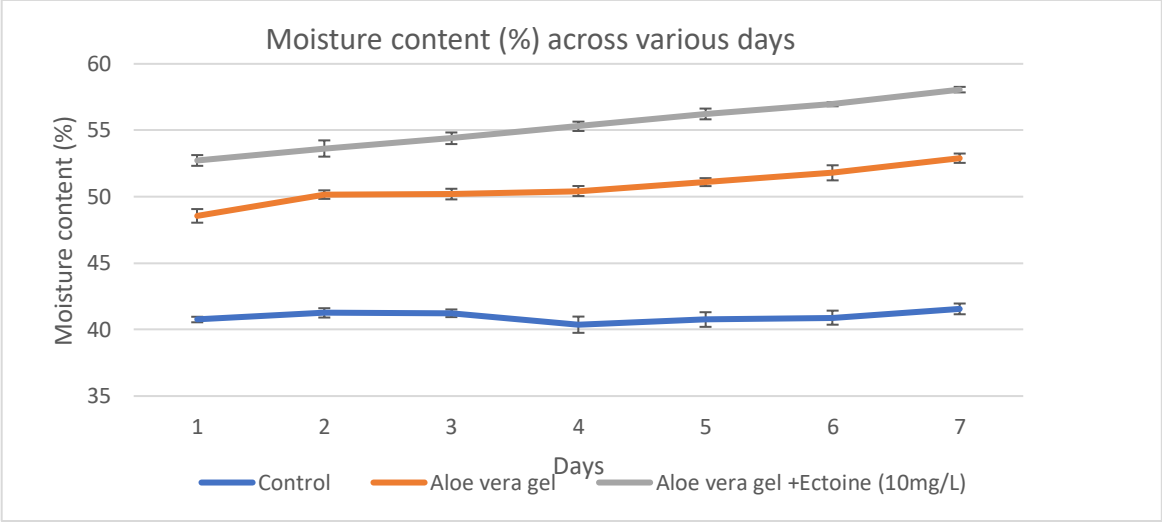


Fig.4.22– Moisture content observed during a period of 7 days

4.11) COMPARATIVE EVALUATION OF MOISTURIZERS ON SKIN HYDRATION

Various moisturizers were applied for a period of 30 minutes followed my measurement of moisture content. Results have shown least moisture in ectoine containing moisturizer, but difference between second last moisturizer is only 0.5 % which indicates that ectoine containing moisturizer is very close to commercially available popular moisturizers and therefore can be used by customers. Price of this moisturizer will be much lower than others products since concentration of ectoine required is very small yet giving good results.

Sr.No.	Product name	Moisture content after application (Reading 1, 2 and 3)(%)	Hydration property (average) (%)	Standard deviation
1	Foxtale	45.9 45.7 44.9	12.2	0.5291502622
2	Pure sense	49.9 50.1 51.3	17.13	0.7571877794
3	Nivea	52.3 52.9 52.3	19.2	0.3464101615
4	Pond’s	49.6 48.9 49.1	15.9	0.3605551275
5	Ectoine	44.8 45.3 45	11.73	0.2516611478
6	Control	32.6 34.1 33.3		

Table 4.13 Hydration property of different moisturizers

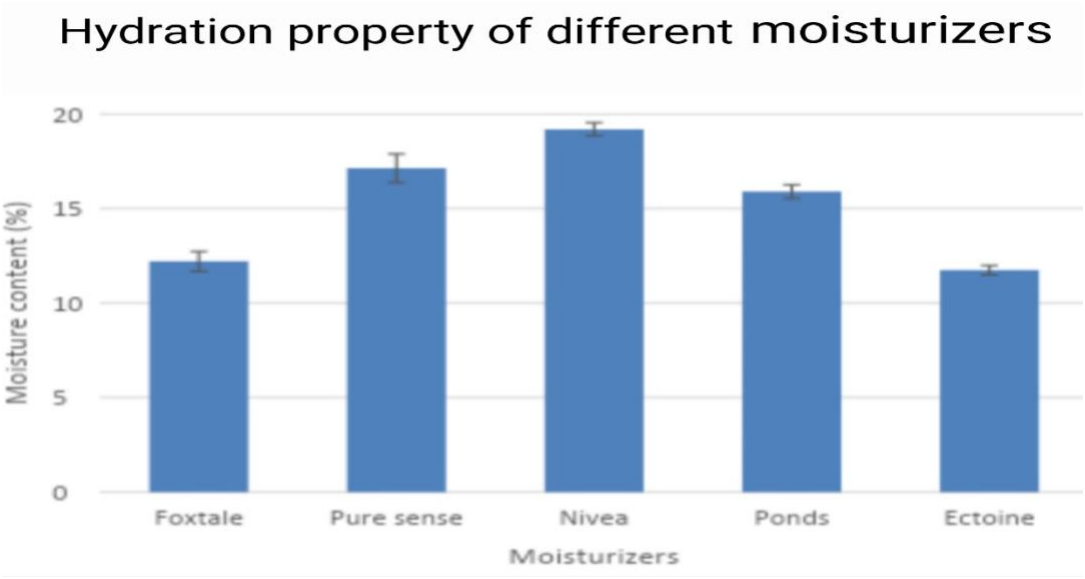


Fig.4.23 – Hydration property of different moisturizers

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APPENDIX I

MEDIA COMPOSITION

1. Zobell Marine Broth/ Agar 2216 (HiMedia M385)

Ingredients	**g / L**
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100

Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Sodium fluorate	0.0024
Distilled water	1000 mL
Agar (for ZMA only)	15
Final pH (at 25°C)	7.6±0.2

2, MD media

Ingredients	**g / L**
Soya peptone	5.000
Tryptone	15.000
Sodium chloride	As per requirement
Distilled water	1000 mL
Final pH (at 25°C)	7.2±0.2

3. Urea Agar Base (Christensen) (HiMedia M112S**)**

Ingridients	**g / L**
Dextrose	1.000
Peptic digest of animal tissue	1.500
Sodium chloride	5.000
Monopotassium phosphate	2.000
Phenol red	0.012
Agar	15.000
Final pH (at 25°C)	6.7±0.2

4. Norberg Hofstein media

Ingredients	**g / L**

Sodium chloride	100.000
Potassium chloride	5.000
Yeast extract	1.000
$MgSO_4 7H_20$	10.000
Final pH (at 25°C	7.0±0.2

5. Nutrient Agar (HiMedia M001)

Ingredients	**Gms / Litre**
Peptone	5.000
Sodium chloride	5.000
HM peptone B#	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

- Equivalent to Beef extract

6. Mueller Hinton Agar (HiMedia M173)

Ingredients	**Gms / Litre**
HM infusion B from #	300.000
Acicase ##	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

- Equivalent to Beef infusion from ## - Equivalent to Casein acid hydrolysate

7. Sheep Blood agar Base (HiMedia M1301)

Ingredients	**g / L**
Tryptone	14.000
Peptone	4.5000
Yeast extract	4.5000
Sodium chloride	5.000
Agar	12.500
Final pH (ar 25°C)	7.3±0.2

8. SIM media (HiMedia M181)

Ingredients	**Gms / Litre**

HM Peptone B#	3.000
Peptone	30.000
Peptonized iron	0.200
Sodium thiosulphate	0.025
Agar	3.000
Final pH (at 25°C)	7.3±0.2

- Equivalent to Beef extract

APPENDIX II

REAGENTS

1) **1 M HCl**	
Hydrochloric acid (conc)	88 mL
Distilled water	912 mL

2) **1 M NaOH**	
Sodium hydroxide	40 g
Distilled water	1000 mL

3) **Kovacs reagent**	
p-Dimethylaminobenzaldehyde	5g
Amyl alcohol	75 mL
Hydrochloric acid	25 mL

4) **Methyl Red**	
Methyl Red	0.1 g

Ethanol (95%)	300 mL
Deionized water	200 mL

5) **Barritt's reagent A:**	
a-naphthol	5 g
Absolute ethanol	100 mL

6) **Barritt's reagent B**	
Potassium hydroxide	40 g
Deionized water	100 mL

7) **3% H_2O_2**	
H_2O_2 (30%)	10 mL
Distilled water	90 mL

8) **Tris-HCl buffer**	
Tris base	1.21 g
Nuclease free water	10 mL
pH	8.5±0.1

9) **3M sodium acetate**	
Sodium acetate	2.461g
Distilled water	10 mL

pH (Adjust with glacial acetic acid)	5.2±0.1

10) **70% ethanol**	
Absolute ethanol	70 mL
Distilled water	30 mL

11) **80% ethanol**	
Absolute ethanol	80 mL
Distilled water	20 mL

12) **TE buffer**	
EDTA	0.0292 g
Tris-Cl	0.1576
Distilled water	200 mL
pH (Dissolve both chemicals separately, adjust pH to 8 and mix together)	8.0

13) **Ninhydrin solution**	
Ninhydrin	1.5 g
n-butanol	100 mL

Acetic acid	3 mL

14) **10% fetal bovine serum**	
Fetal bovine serum	10 mL
Dulbecco Modified Eagle Medium (DMEM)	90 mL

15) **0.85% Saline**	
Sodium Chloride	0.85 g
Distilled water	100 mL

16) **200mM L-glutamine**	

L-glutamine	58 mg
0.85% Saline	2 mL

17) **Phosphate- buffered saline**	
Sodium Chloride	80.1 g
Disodium phosphate	14.4 g
Potassium chloride	2 g
Potassium dihydrogen phosphate	2,7 g
Distilled water	1000 mL
pH	7.2±0.2

18) **Lysis buffer**	

Sodium chloride	14.602 g
Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA)	3.174 g
Tris-base	0.12 g
Sodium hydroxide	0.8 g
Distilled water	90 mL
10% DMSO	10 mL
1% TritonX	1 mL

19) **10 N NaOH**	
NaOH	20 g
Distilled water	50 mL

20) **200mM EDTA**	
EDTA	1.48 g
Distilled water	20 mL
pH	10

21) **Electrophoresis buffer**	
10 N NaOH	27 mL
200 mM EDTA	4.5 mL
DMSO	1.8 mL
Distilled water	867 mL

22) **Neutralization buffer**	
Tris-base	4.845 g
Distilled water	100 mL
pH	7.5

23**) Ethidium Bromide (10X)**	
EtBr	10 mg
Distilled water	50 mL

24) **TAE buffer (50x)**	
Tris-base	24.2 g
Glacial acetic acid	5.71 mL

500mM EDTA	10 mL
Distilled water	84 mL
pH	8

25) **0.7 % agarose**	
Low melting agarose	0.35 g
Distilled water	50 mL

26) 1% agarose	
Agarose	0.5 g
Distilled water	50 mL

27) **MTT reagent**	
MTT	4 mg
PBS	2 mL