DECLARATION

I hereby declare that the data presented in this Dissertation report entitled, "**COMPATIBLE SOLUTES IN HALOPHILIC BACTERIA**" is based on the results of investigations carried out by me in the same (MSc Marine Biotechnology) at the School of Biological Sciences and Biotechnology, Goa University under the Supervision/Mentorship of Prof. Dr Savita 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 not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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02/05/23

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CERTIFICATE

This is to certify that the dissertation entitled "**COMPATIBLE SOLUTES IN HALOPHILIC BACTERIA**" is a bonafide work carried out by Shubham Dilip Salve under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree Master of Science in the Discipline Marine Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

Date: 2/5/2023

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COMPATIBLE SOLUTES IN HALOPHILIC BACTERIA

A Dissertation Report for

Code: MBC 381

Credits: 8

Submitted in partial fulfilment of Master's Degree in Marine Biotechnology

By

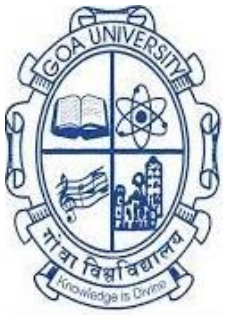
SHUBHAM DILIP SALVE

21P050018

Under the Supervision of

PROF. DR. SAVITA KERKAR

School of Biological Sciences and Biotechnology



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Place: Goa University

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LIST OF ABBREVIATIONS

g	Grams
mL	Milli litre
%	Percentage
L	Litre
Rpm	Revolution per minute
µL	Microlitre
nm	Nanometer
ZMA	Zobell Marine Agar
ZMB	Zobell Marine Broth
°C	Degree Celsius
Rf	Retardation factor
TLC	Thin Layer chromatography
cm	Centimetre
M	Molar
N	Normal
H	Hour

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CONTENTS

CHAPTER 1: INTRODUCTION

Introduction:

Halophiles are microorganisms that are found in hypersaline environments and have the ability to maintain osmotic pressure and resist the harmful effects of salt. Ex: - *Aphanothece halophytica*, and *Dunaliella salina* are examples of properly adapted and widely found halophilic microorganisms.

All continents and most countries hahalophyticve hypersaline ecosystems. They are categorized as those derived from **seawater** and so contain sodium chloride as the major salt, and those derived from **non-seawater** sources and have other ion ratios. This is common in soda lakes where carbonate is the major anion. The former is known as **thalassohaline**, whereas the latter is known as **athalassohaline**. Other instances of natural inland salt lakes can be observed in various ecosystems, such as Wadi Natrun, the soda lakes of Antarctica, Big Soda Lake, and Mono Lake in California. These lakes are dominated by sodium chloride and are a result of natural processes. Natural inland salt lakes can arise in sodium chloride-dominated ecosystems. Great Salt Lake in Utah is one example, but salt mine drainage waters, playas, natural coastal splash zones and tide pools, brine springs from subterranean salt deposits, and solar salterns are more examples.

There are strategies evolved by halophilic bacteria, as an osmoadaptation to hypersaline conditions:

(1) the salts-in-cytoplasm strategy and

(2) the organic-osmolytes strategy.

To deal with osmotic stress, halophiles and certain types of methanogenic archaea employ the organic-osmolyte method, which entails creating organic substances such as polyols and sugars, amino acids and their derivatives to combat the stress. These compounds, known as compatible solutes, are easily dissolved in water and do not interfere with metabolic processes, even at high cytoplasmic levels.

Even at high cytoplasmic concentrations, these non-ionic, highly water-soluble substances do not disrupt metabolism and are hence referred to as compatible solutes.

Ex: - Trehalose, Proline, Ectoine, Betaine etc.

This type of osmoadaptation by production or accumulation of compatible solutes is preferred by most moderately halophilic bacteria.

Aim: - Detecting the types of compatible solutes produced by the selected halophilic bacterial cultures.

Objectives: -

1. Isolation of halotolerant and halophilic bacteria from the salt pans of Goa.
2. Screening the salinity range of selected isolates.
3. Extraction of compatible solutes from these bacteria.
4. Partial purification and detection of these compatible solutes.

CHAPTER 2: LITERATURE REVIEW

Halophiles are extremophilic salt-loving microorganisms that can survive in extremely high levels of salinity (10-30% NaCl). They belong to three groups: - bacteria, archaea, and eukaryotes. Because of specific cellular adaptations such as the salts-in approach , compatible solute approach and enzymatic adaptations, halophiles can withstand increased salt concentrations. They require sodium ions for development and metabolism (Corral et al., 2022). Thus, based on the NaCl optimal requirement for growth the halophiles are classified into three different categories: 1. slightly halophilic (1-3%); 2. moderately halophilic (3-15%); and
3. extremely halophilic (15-30%). Halotolerant bacteria are found to be growing in the salinity range 3-5%.

The first halophilic microorganism came into account in 2700 BC (Bass-Becking, 1931) and was found in hypersaline areas. The first genome sequence has been studied of *Halobacterium* NRC-1 (Aparna et al., 2000).

2.1: Osmoadaptation in halophilic bacteria:

Two important mechanisms have evolved by halophiles, as an osmotic adaptation to hypersaline conditions:
(1) The salt-in-cytoplasm mechanism.
(2) The organic-osmolyte mechanism.
Organisms opting for the salt-in-cytoplasm strategy adapt the inner protein chemistry of the cell to higher salt concentrations. The cells survive by increasing the salt content in the cytoplasm to that of the surrounding environment.

In the organic-osmolyte mechanism, the organisms keep the cytoplasm free of NaCl to a larger extent, although the design of the cell's interior stays mostly intact. The archaeal osmoadaptation process involves a commonly recognized mechanism known as the salts-in-cytoplasm strategy. But potassium accumulates in the cell and is visible in the cytoplasm in molar proportions with Cl-.

Because the intracellular concentration of K+ is a hundredfold greater than the outside, a portion of the proton motive force must be used to keep the ion gradient stable. Evidence suggests that halophilic anaerobic bacteria are different in their approach because they contribute minimally to maintain ion gradients. As haloanaerobium praevalens cells grow exponentially, K+ becomes the most abundant cation but there are also increased levels of Na+ present. When the cells enter the stationary phase, they start replacing K+ with Na+. (Kunte, 2006).

2.2: Organic osmolyte strategy:

Halophiles generally collect organic compounds such as amino acids and their derivatives or sugars and polyols from their environment in response to osmotic stress. These substances, which are highly soluble in water and do not affect metabolism even at high concentrations in the cytoplasm are known as compatible solutes. Halophiles that use the organic-osmolyte mechanism are more adaptable than organisms that use the "salts-in cytoplasm strategy" because, while they can tolerate a wide range of salts, they can also grow in low salt conditions.

Halophiles that use the organic-osmolyte mechanism are more adaptable than organisms that use the "salt-in cytoplasm strategy" because, while they can tolerate a wide range of salts, they can also grow in low salt conditions.

Compatible solutes are effective stabilisers of proteins and even whole cells, in addition to maintaining osmotic equilibrium across the cell membrane.

They can protect against heat, desiccation, freezing and thawing, as well as denaturants like urea and salt. The preferential exclusion model explains why these chemical substances are compatible with metabolism and can even act as stabilisers of labile biological structures at the molecular level.

According to this view, suitable solutes are absent from protein surfaces due to structurally dense water bound to the protein. Compatible solutes prefer the less dense free water fraction in the cytoplasmic environment. They stabilise the two water fractions by fitting into the free water lattice and allowing the formation of hydration clusters, making protein unfolding and denaturation thermodynamically unfavourable (reinforcing the hydrophobic effect).

This explains why organisms evolved to low water-potential habitats benefit from the beneficial qualities of compatible solutes (Kunte et al., 2006).

Many of these compatible solutes are negatively charged because archaea store negatively charged compatible solutes, whereas other species store compatible solutes in neutral forms or zwitterion forms. Organic osmolytes fall into three general chemical categories:

(i) zwitterionic solutes, (ii) noncharged solutes, and iii) anionic solutes.

2.2.1: Zwitterionic Solutes

Free polar amino acids in cells play a role in osmotic balance. However, neutral amino acids are not accumulated to high concentrations, presumably because they are intermediates in protein biosynthesis. High and varying concentrations of these compounds could affect diverse cell pathways. Instead, many bacterial and archaeal cells synthesize and accumulate a few zwitterionic molecules derived from amino acids as compatible solutes (Roberts, 2005).

2.2.1.1 Betaine:

Glycine is found in halophilic bacteria of various phylogenetic affiliations and with primary amine methylation to create a quaternary amine. Some halophiles can produce betaine via choline oxidation or glycine methylation.
Ex: - *Actinopolyspora halophila* and *Halomonas elongata* and one methanogen (*Methanohalophilus portucalensis* FDF1) (Roberts, 2005).

2.2.1.2: Ectoine and hydroxyectoine:

Ectoine, a cyclic tetrahydro pyrimidine (1,4,5,6-tetrahydro- 2-methyl-4-pyrimidinecarboxylic acid) can almost be considered a marker for halophilic bacteria. (Roberts, 2005)
Ex: - *Methylarcula marina*, *M. terricola*, and *Methylophaga* sp.
A variant of this solute, hydroxy ectoine, has been detected in halotolerant *Sporosarcina pasteurii* grown in a high osmolarity medium.

2.2.1.3: Nε-acetyl-β-lysine and β-glutamine:

Methanogens generally accumulate several β-amino acids for osmotic balance. These solutes provide an excellent strategy for producing a compatible solute since β- amino acids are not incorporated into proteins or other macromolecules. At high external NaCl (>1 M), two zwitterionic β-amino acids have been shown to accumulate in response to external NaCl. Nε-acetyl-β-lysine has been detected in a wide range of mesophilic and a few thermophilic methanogens (Roberts, 2005).
Ex: - *Methanohalophilus* sp

2.2.2: Non-charged solutes:

Few polar compounds with no specific charges have been discovered as osmolytes in halophiles while being abundant in eukaryotes.

Ex: - Glycerol is a common osmolyte in both marine and halophilic dunaliella. In halophilic bacteria and archaea, polar noncharged solutes have been found as an osmolyte.

However, **negatively charged derivatives** of both **glycerol** and **inositol** are accumulated by archaea. The few uncharged solutes that are used by halotolerant bacteria and archaea include several carbohydrates and an amino acid/dipeptide modified to neutralize all charged groups. (Roberts, 2005)

2.2.2.1: Carbohydrates

α-Glucosylglycerol is accumulated by a member of the Proteobacteria, *Stenotrophomonas*. The non-reducing glucose disaccharide trehalose is used by organisms to counteract drying, but it also serves as an osmolyte.
(Roberts, 2005)

2.2.2.2: Uncharged Amino Acids and Peptides:

Two solutes in this class have been identified as osmolytes:
(i) a **carboxamine**, and (ii) an **acetylated neutral glutamine dipeptide**.
In both solutes, modifications mask the charged α-amino and α-carboxyl groups. N-α-Carbamoyl- L-glutamine 1-amide is an unusual amino acid derivative which is accumulated by the halophilic phototrophic bacterium *Ectothiorhodospira marismortui* (also known as *Ectothiorhodospira mobilis*) (Roberts, 2005).

2.2.3: Organic anionic solutes:

Cells have a negative potential inside and often quite high intracellular K+.
Negatively charged solutes could serve to balance high intracellular K+ as well as counteract osmotic pressure. At lower external NaCl, many bacteria and archaea use L-α-glutamate as an osmolyte (Roberts, 2005).

2.2.3.1: β-Glutamate:

The negatively charged glutamates are accumulated when the external NaCl is less than 1 M. In that case, the total intracellular glutamates occur at concentrations comparable to the intracellular K+. (Roberts, 2005)
Ex:- *Nocardiopsis halophila*

2.2.3.2: Choline chloride:

Choline chloride is a water-soluble colourless compound with vitamin-like properties. (McDonald *et al*., 2011). Choline is amino ethyl alcohol and has three methyl groups on the nitrogen atom, chemically termed as (2-Hydroxyethyl) trimethylammonium. The chemical formula of choline is $C_5H_{14}NO^+$ and choline chloride is ($HOCH_2CH_2N$ (CH_3)3HCl) (Chaudhari et al., 2017).

APPLICATIONS OF COMPATIBLE SOLUTES

Ectoines are used to increase the safety and quality of foods by stabilising dietary components, as well as to cure the eye's mucous membrane. It is used as an ingredient in moisturisers and other cosmetics to combat the effects of UV-A-induced and accelerated skin ageing. (Pooja S et al., 2011) Betaine is an excellent cryoprotectant and has been used to boost product yield and specificity in PCR amplification of GC-rich DNA templates. (Pooja S et al., 2011)
Mannosylglycerate also has applications as a cryoprotectant and is used in PCR. Trehalose has too had an application as a cryoprotectant as well as is used to preserve microbes without damaging their cell walls. (Pooja S et al., 2011)

CHAPTER 3: MATERIALS AND METHODS

3.1: Isolation of halophilic bacteria from salt crystals of salt pans:

3.1.1: Sample collection:

The crude salt sample was collected from a salt pan from Ribandar, Panaji, Goa. The salt samples were brought to the laboratory in a sealed ampule and further processed for the isolation of halophilic bacteria.

3.1.2: Isolation of bacteria:

3.1.2.1: Serial dilutions of the sample:

Crude salt (1gm) was weighed and added to 10 ml of sterile seawater (diluent) and vortexed. The sample was then serially diluted tenfold to 10^{-6}. Dilutions were performed as follows:

Sr. No.	Dilution	Volume of sample	Volume of seawater (mL)
1.	10^{0}	1g sample	10ml
2.	10^{-1}	1mL	9mL
3.	10^{-2}	1mL	9mL
4.	10^{-3}	1mL	9mL
5.	10^{-4}	1mL	9mL
6.	10^{-5}	1mL	9mL
7.	10^{-6}	1mL	9mL

Table 1 - Serial dilution table

3.1.2.2: Screening for halophilic bacteria and determining their salinity range:

The samples were cultured on the following two types of media to isolate halophilic bacteria: -

1. Zobell Marine Agar media
2. Nutrient Agar media

All the above media were prepared with 15%, 20% and 23% saline water.

0.1ml of 10^{-1}, 10-^{2}, 10^{-3}, 10^{-4} and 10^{-5} serially diluted samples were spread plated on all the above media plates and incubated at 37 °C for 2 weeks for observation of growth. The plates showing growth were then sub cultured on the respective media plates from which they were isolated.

Media	15%					20%					23%				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
ZMA	-	+	-	-	-	-	+	+	-	-	-	-	+	+	-
NA	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-

Table 2 :- Growth table (Key: '+'- Growth, '-'- No growth)

3.1.2.3: Salinity test:

All 5 bacterial cultures (ShclWC1, ShclTr2, ShclY3, ShclR4, ShclP5) thus obtained were inoculated in 0%, 5%, 10%, 15%, 20%, 23% saline Zobell marine broth and incubated at 37 °C in a 120-rpm shaker incubator for 48 hrs.

3.2: Extraction of compatible solutes:

For extraction of compatible solutes, all cultures (ShclWC1, ShclTr2, ShclY3, ShclR4, ShclP5) were inoculated in 18% Zobell marine broth and incubated at 37 °C on a 120-rpm shaker incubator for 48hrs.

For extraction of compatible solutes, a phase separation method was performed: Methanol: Chloroform: Water Extraction

METHANOL: CHLOROFORM: WATER EXTRACTION

1. Bacterial culture was inoculated in ZMB at 18% salinity and incubated at 37 °C for 48hrs in a 120 rpm shaker incubator.
2. Bacterial cell growth was further processed by centrifugation at 9500 rpm for 10 minutes at 4 °C to obtain a pellet. The pellet was then washed by adding 5mL of sterile 18% saline water (at a concentration identical to the growth medium to prevent the solute release and remove the impurities). The cell suspension was then vortexed for one minute and centrifuged again at 9500 rpm for 10 minutes at 4 °C. The pellet was collected and stored at 4 °C until further processing.

   [This is the cell lysis step to obtain the compatible solutes by further processing]
3. To these pellets, Methanol: Chloroform: Water (solvent) was added in the ratio 10:5:4 and then subjected to vortex. After this step, they were kept for incubation at room temperature for 4 hours in sterile glass tubes.
4. After 4 hours of incubation, the content in the glass tubes was transferred to sterile centrifuge tubes and was centrifuged at 9500 rpm for 10 minutes at 4 °C.
5. The supernatant obtained was collected in sterile glass tubes, and to this, 1.25mL chloroform and 1.25mL water were added.
6. Then they were vortexed for one minute and the whole content was transferred to centrifuge tubes again for centrifugation at 9500 rpm for 10 minutes

   at 4 °C.

7. Two separate phases were obtained after centrifugation; one was the organic phase (lower part) and the second was the aqueous phase (upper part). Both phases were collected in separate sterile glass tubes and refrigerated at 4 °C until further processing.

3.3: Detection of compatible solutes:

To detect the presence of compatible solutes, biochemical tests were performed from the aqueous samples obtained after methanol: chloroform: water extraction.

3.3.1: Test for detection of Glycine: To determine the presence of glycine in the obtained aqueous sample, 5 drops of dilute HCL (appendix) and 1mL of 1M sodium nitrate solution (appendix) were added to 1mL of standard glycine. The generation of colourless gas with bubbles indicates the presence of glycine in the aqueous sample. (Roberts, 2006)

3.3.2: Test for detection of Proline: To determine the presence of proline, 1mL of aqueous sample and 1mL of the standard were mixed with 500µL of the ninhydrin reagent (appendix) and observed for yellow colouration. (Carillo P., 2011)

3.3.3: Test for detection of Glutamic acid: To determine the presence of glutamic acid in the sample, 1mL of the sample was mixed with 500µL of water and 500µL of NaOH (Appendix). To these 5 drops of ninhydrin are added along with 500µL of 3M sodium acetate (appendix) and boiled for 15 minutes in a water bath. Observation of a dark-bluish violet colour confirms the presence of glutamic acid in the aqueous sample. (Jayaraman, 2005)

3.3.4: Test for detection of Choline Chloride: To determine the presence of choline chloride in the sample, 1mL of aqueous sample and 1mL of the standard was dissolved in 100µL of iodine solution (appendix) and observed for red precipitate which dissolves in 1N NaOH. The appearance of yellow colour indicated a positive result. (Bowman K., 2009)

3.3.5: Test for the detection of Betaine Chloride: To determine the presence of betaine chloride in the aqueous sample, 1mL of the sample and 1mL of the standard were mixed with a few drops of Bromocresol green (appendix) indicator (yellow in acidic conditions and green in basic conditions). Observation of green colour indicates a positive test. (Yemm et al., 1955)

3.4: Confirmation of the presence of compatible solutes:

To confirm the presence of particular compatible solutes in the aqueous samples, Thin Layer Chromatography (TLC) was performed. The standard of choline chloride along with the aqueous samples was spotted on the TLC plate. TLC plate was kept in the solvent system: Butanol: Glacial Acetic acid: Water (B: A : W) (Jayaraman, 1981) in the ratio of 3: 1: 1 v/v.

Once the run was complete, the TLC plate was sprayed with the developing agent i.e., Ninhydrin solution (appendix). The Rf value, which is the ratio of distance travelled by solute/distance travelled by solvent was calculated for the standard and the samples.

Following are the Rf values for methanol: chloroform: water extracted samples:

Sr No.	Standard/ sample	Distance travelled by the solute (cm)	Distance travelled by the solvent (cm)	Rf value
1.	Choline Chloride (Std)	4.3	14	0.307
2.	ShclWC1	4.4	14	0.314
3.	ShclTr2	4.3	14	0.307
4.	ShclY3	4.4	14	0.314
5.	ShclR4	4.1	14	0.392
6.	ShclP5	-	-	-

Table 3 - Rf value table for Methanol: Chloroform: Water extraction

3.5: Studying the applications of compatibles solutes:

The applications of the compatible solutes obtained from the samples were studied successfully.

CHAPTER 4: RESULT AND DISCUSSION

4.1: ENUMERATION AND GROWTH CHARACTERISTICS:

After 48 hours of incubation following results were obtained:

Zobell Marine Agar (15% saline)

Sr. No	Dilution Factor	CFU/0.1mL	CFU/mL	Average CFU/mL
1.	10^{-1}	-		$13 * 10^4$
2.	10^{-2}	13	$130 * 10^3$	
3.	10^{-3}	-		
4.	10^{-4}	-		
5.	10^{-5}	-		

Table 4 -Results of enumeration on 15% ZMA
(Key: TMTC- Too many to count, TLTC- Too less to count, '-'– No growth)

Zobell Marine Agar (20% saline)

Sr. No	Dilution factor	CFU/0.1mL	CFU/mL	Average CFU/mL
1.	10^{-1}	-	-	$6 * 10^4$
2.	10^{-2}	6	$60 * 10^3$	
3.	10^{-3}	-	-	
4.	10^{-4}	-	-	
5.	10^{-5}	-	-	

Table 5 -Results of enumeration on 20% ZMA
(Key: TMTC- Too many to count, TLTC- Too less to count, '-'– No growth)

Zobell Marine Agar (23% saline)

Sr. No	Dilution factor	CFU/0.1mL	CFU/mL	Average CFU/mL
1.	10^{-1}	-	-	-
2.	10^{-2}	-	-	
3.	10^{-3}	TLTC	-	
4.	10^{-4}	TLTC	-	
5.	10^{-5}	-	-	

Table 6 -Results of enumeration on 23% ZMA
(Key: TMTC- Too many to count, TLTC- Too less to count, '-'– No growth)

Nutrient Agar medium (15% saline)

Sr.No	Dilution factor	CFU/0.1mL	CFU/mL	Average
1.	10^{-1}	8	$80 * 10^3$	$8 * 10^4$
2.	10^{-2}	TLTC	TLTC	
3.	10^{-3}	-	-	
4.	10^{-4}	-	-	
5.	10^{-5}	-	-	

Table 7 -Results of enumeration on 15% NA

(Key: TMTC- Too many to count, TLTC- Too less to count, '-' – No growth)

Nutrient Agar medium (20% saline)

Sr. No	Dilution factor	CFU/0.1mL	CFU/mL	Average
1.	10^{-1}	-	-	$20 * 10^4$
2.	10^{-2}	2	$20 * 10^3$	
3.	10^{-3}	-	-	
4.	10^{-4}	-	-	
5.	10^{-5}	-	-	

Table 8 -Results of enumeration on 20% NA

(Key: TMTC- Too many to count, TLTC- Too less to count, '-' – No growth)

The master plate/ or the ZMA plate on which 5 different halophilic bacterial cultures growth was observed was 15% saline and the serial dilution used to spread the plate was 10^{-2}.

The image of the plate is attached below: Fig 1 A] and 1 B] – 15% ZMA spread plate

Fig 1 A] and 1 B] – 15% ZMA spread plate

A]

B]

Five isolates were obtained from the above media plates after subculturing them. They had the following cultural characteristics:

4.1.1: Isolate ShclWC1:

Table 9: - Culture characteristics of WC1

TYPE	CHARACTERISTICS
Size	3mm
Shape	Circular
Elevation	Raised
Margin	Entire
Consistency	Mucoid
Opacity	Opaque
Colour	Creamish white

2]

Figure 2: - Culture plate of ShclWC1

3]

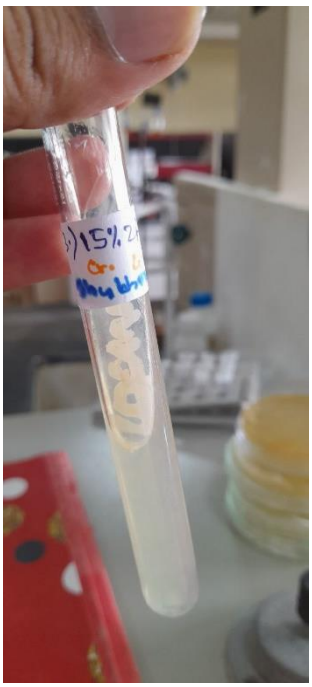


Figure 3: - Slant of culture culture ShclTR2

4.1.2: Isolate ShclTr2:

Table 10- Cultural characteristics of Tr2

TYPE	CHARACTERISTICS
Size	2mm
Shape	Circular
Elevation	Raised
Margin	Entire
Consistency	Mucoid
Opacity	Translucent
Colour	Colourless

4.1.2: Isolate ShclY3:

Table 11- Cultural Characteristics of Y3

TYPE	CHARACTERISTICS
Size	4mm
Shape	Circular
Elevation	Raised
Margin	Entire
Consistency	Mucoid
Opacity	Opaque
Colour	Yellow

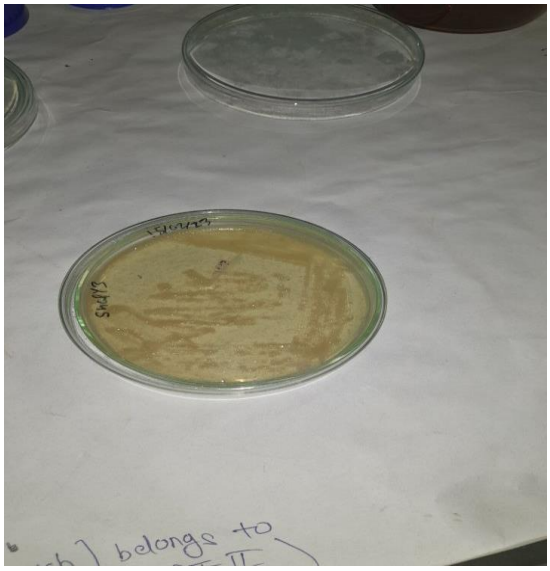


Figure 4: - Culture plate of culture ShclY3

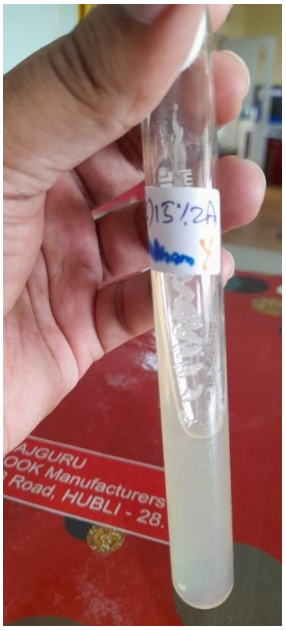


Figure 5: - Slant of culture ShclR4

4.1.4: Isolate ShclR4:

Table 12: - Cultural characteristics of R4

TYPE	CHARACTERISTICS
Size	2mm
Shape	Circular
Elevation	Raised
Margin	Entire
Consistency	Pulvinate
Opacity	Opaque
Colour	Red

4.1.5: Isolate ShclP5:

Table 13: - Cultural characteristics of P5

TYPE	CHARACTERISTICS
Size	3mm
Shape	Circular
Elevation	Raised
Margin	Entire
Consistency	Mucoid
Opacity	Opaque
Colour	Pink

Figure 6: - Slant of ShclP5 culture

Figure 7: - Plate of ShclP5 culture

4.2: SCREENING FOR OPTIMUM SALINITY TOLERANCE:

All the isolates that were inoculated in 0%, 5%, 10%, 15%, 20% and 23% zobell marine broth, showed growth after 48hrs of incubation. Isolates inoculated in 0% showed no growth but showed growth in all other concentrations of salinity.

Culture code	Salinity (%)						Type of culture
	0%	5%	10%	15%	20%	23%	
ShclWC1	-	+	+	+	+	+	Moderately Halophile
ShclTr2	-	+	+	+	+	+	Moderately Halophile
ShclY3	-	+	+	+	+	+	Moderately Halophile
ShclR4	-	-	-	+	+	+	Moderately Halophile
ShclP5	-	-	-	+	+	+	Moderately Halophile

Table 14 - Result of Optimum salinity tolerance

Culture code	O.D at 600nm				
	5%	10%	15%	20%	23%
ShclWC1	0.562	0.814	0.868	0.681	0.850
ShclTr2	0.525	0.677	0.339	0.250	0.520
ShclY3	0.806	0.709	0.640	0.358	0.527
ShclR4	0.071	0.060	0.690	0.100	0.640
ShclP5	0.075	0.080	0.359	0.670	0.100

Table 15 - Culture O.D at different saline concentrations

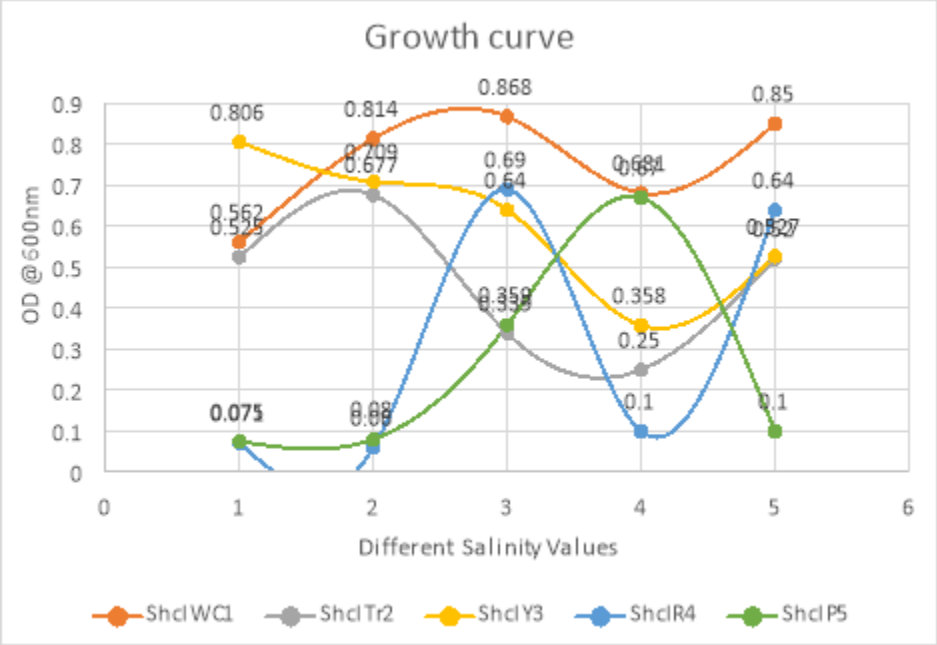


Figure 8: Graphical representation of the growth of various isolates in different salinity values Vs OD at 600 nm

The X-axis represents 1-> 5%, 2-> 10%, 3-> 15%, 4->20%, 5->23% salinity values.

The above graph represents the growth of five different halophilic bacterial cultures in zobell marine broth at six different salinity concentrations i.e., 0%, 5%, 10%, 15%, 20% and 23%, incubated at 37°C on an incubator shaker for 48hrs. This experiment was performed to check the optimum salinity range for the growth of each of the bacterial cultures. It was observed that ShclY3, ShclWC1 and ShclTr2 grow very fast in 5% saline ZMB and they also have fast growth in 10% saline ZMB. ShclP5 and ShclR4 show no growth in 5% and 10% saline ZMB. All five cultures show optimum growth in 15% saline ZMB. ShclP5 and ShclR4 also showed growth in 20% and 23% saline ZMB. In 0% saline ZMB, none of the cultures showed growth. The obtained O.D values indicate the intensity of growth of the halophilic bacterial cultures measured at 600 nm.

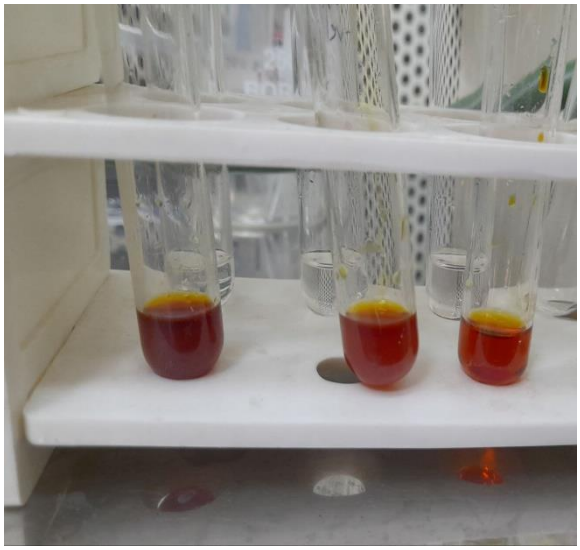
Detection of compatible solutes:

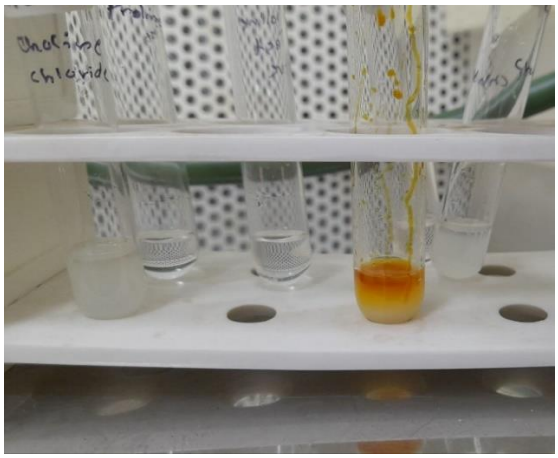
The biochemical tests performed were:

1. Proline Test
2. Betaine hydrochloride
3. Choline chloride
4. Glycine test

Positive results were obtained for only the choline chloride test of the samples thus obtained by methanol: chloroform: water extraction.

Figure 9 A] 9B]





9C]

Figure 9A], 9B], 9C]: - Results of Choline Chloride test

4.3: DETECTION OF COMPATIBLE SOLUTES BY THIN-LAYER CHROMATOGRAPHY:

Thin Layer Chromatography (TLC) of the aqueous samples obtained after extraction using the methanol: chloroform: water method was performed and the following results were obtained:

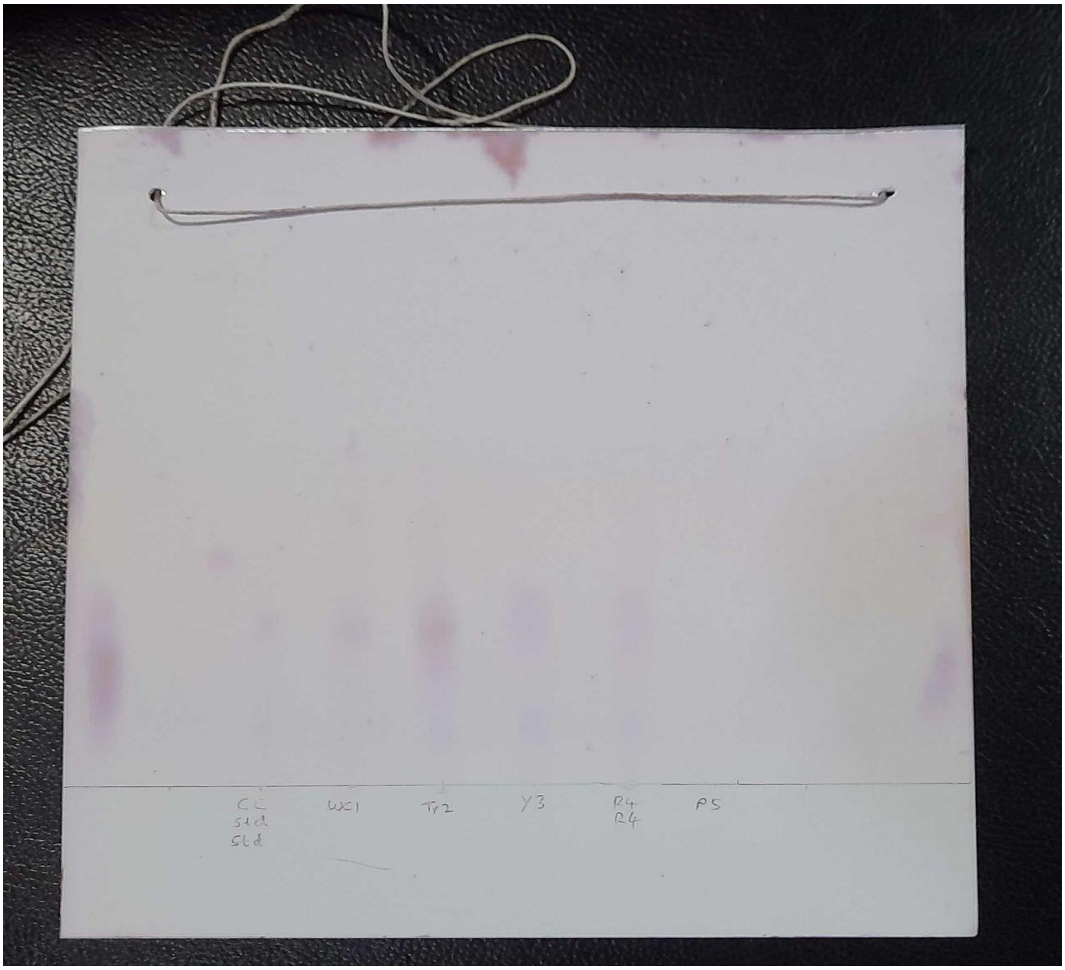


Figure 10: - Result of TLC

The Rf values of both, the samples and standard were calculated and compared. They are as follows:

Amino acid/amino acid derivative/sample	Retardation factor (Rf)
Choline Chloride (standard)	0.307
ShclWC1	0.314
ShclTr2	0.307
ShclY3	0314
ShclR4	0.292
ShclP5	-

Table 16:- Rf value table for Methanol: Chloroform: Water extracted samples

The Rf values of all the samples are close to the Rf value of choline chloride(standard).

Based on the biochemical tests and the TLC results of the obtained aqueous samples from the methanol: chloroform: water extraction; it was been confirmed that choline chloride is the compatible solute that is being synthesized by all the sample cultures except the ShclP5 culture, thus isolated from the crude salt crystals.

Following are the results thus obtained:

Isolates	Choline Chloride
ShclWC1	+
ShclTr2	+
ShclY3	+
ShclR4	+
ShclP5	-

Table 17 - Results of the confirmatory tests for detection of compatible solute.

4.4: APPLICATIONS OF CHOLINE CHLORIDE:

Choline chloride is widely used in the poultry sector. Young chickens are unable to synthesise choline at the required rate so they require a highly supplemented feed with choline chloride for their faster growth. Choline Chloride also increases egg production, feed conversion efficiency, the weight and lowers blood cholesterol in broilers. Choline chloride supplementation improves milk output and milk fat while decreasing blood serum NEFA's and cholesterols in dairy animals like buffalo, goat, sheep etc. Choline chloride is also used in deep eutectic solvents because of its excellent solubilization ability (Chaudhari et al., 2017). DESs have the potential as alternative new green dissolving agents. Choline chloride has the role of DES in the production and purification of biodiesel. Choline chloride also has a role as a growth promoter in plants (Kaila et al., 2017). Orally choline chloride is used for diseased liver including inflammation in liver and liver failure; hypercholesterolemia; depression; memory loss; Alzheimer's disease and dementia; schizophrenia; bodybuilding; delaying fatigue in endurance sports; preventing neural tubes defects; avoiding cancer; Huntington's chorea; Tourette's disease; cerebellar ataxia; complex partial seizures; asthma; and as a supplement in infant formulas. TPNs-associated hepatic steatosis, choline insufficiency, and foetal alcohol syndrome are treated with intravenous choline chloride.
(Yuen et al., 2019)

CONCLUSION:

From the experimental findings it can be concluded that the bacteria isolated from the crude salt crystals of Ribandar saltpans, Goa can cope with the persistent hypersaline conditions through the synthesis of compatible solutes.

It was found that all the cultures thus isolated: ShclWC1, ShclTr2, ShclY3, ShclR4 and ShclP5 are moderately halophilic and were found to produce choline chloride as a compatible solute in response to the hypersaline conditions.

This particular compatible solute has a high demand in the poultry industry as a growth promoter in chickens and for dairy cattle and buffaloes in their diet.

Alongside having an application in DES and biodiesel purification proves the potential of this particular compatible solute industrially.

FUTURE PROSPECTS

- Purification and applications of obtained compatible solute.
- Optimize the production of this compatible solute at a higher scale in order to find a better application.
- Molecular characterization and phylogenetic analysis of all 5 isolates.

Chapter 8: BIBLIOGRAPHY

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APPENDIX

A] MEDIA COMPOSITION

ZOBELL MARINE AGAR (ZMA) 2216

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
Sodium florate	0.0024
Ammonium nitrate	0.0016
Disodium Phosphate	0.008
Agar	15.000
Final Ph (at 25 °C)	7.6 ±0.2

B. ZOBELL MARINE BROTH (ZMB) 2216

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
Sodium florate	0.0024
Ammonium nitrate	0.0016
Disodium Phosphate	0.008

C] Nutrient Agar Ph 6.8

Ingredients	**Gms/litre**
Peptone	5.000
HM Peptone B#	3.000
Agar	15.000
Final Ph (at 25°C)	6.8±0.2

B] Reagents:

1. 80% Ethanol

Absolute Ethanol	80mL
Distilled water	20mL

2. Dilute HCL

Concentrated HCL	10mL
Distilled Water	90mL

3. 1M Sodium Nitrate

Sodium Nitrite	7.5g
Distilled Water	1000mL

4. 1N NaOH

NaOH	40g
Distilled Water	1000mL

5. 3M Sodium Acetate

Sodium Acetate	246.1g
Distilled Water	1000mL

6. Iodine Solution

Potassium Iodide	10g
Iodine	5g
Distilled Water	100mL

7. Bromocresol Green

Bromocresol green	0.1g
Absolute ethanol	100mL

8. Ninhydrin Reagent

Ninhydrin	0.2g
Acetic acid	0.5mL
Butanol	100mL
Distilled Water	4.5mL
Final Ph (at 25 °C)	7.6 ±0.2

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CHAPTER 1: INTRODUCTION

Introduction: Halophiles are microorganisms that are found in hypersaline environments and have the ability to maintain osmotic pressure and resist the harmful effects of salt. Ex: - Aphanothece halophytica, and Dunaliella salina are examples of properly adapted and widely found halophilic microorganisms. All continents and most countries hahalophyticve hypersaline ecosystems. They are categorized as those derived from seawater and so contain sodium chloride as the major salt, and those derived from non-seawater sources and have other ion ratios. This is common in soda lakes where carbonate is the major anion. The former is known as thalassohaline, whereas the latter is known as athalassohaline. Other instances of natural inland salt lakes can be observed in various ecosystems, such as Wadi Natrun, the soda lakes of Antarctica, Big Soda Lake, and Mono Lake in California.

These lakes are dominated by sodium chloride and are a result of natural processes. Natural inland salt lakes can arise in sodium chloride-dominated ecosystems. Great Salt Lake in Utah is one example, but salt mine drainage waters, playas, natural coastal splash zones and tide pools, brine springs from subterranean salt deposits, and solar salterns are more examples. There are strategies evolved by halophilic bacteria, as an osmoadaptation to hypersaline conditions: (1) the salts-in-cytoplasm strategy and (2) the organic-osmolytes strategy. To deal with osmotic stress, halophiles and certain types of methanogenic archaea employ the organic-osmolyte method, which entails creating organic substances such as polyols and sugars, amino acids and their derivatives to combat the stress. These compounds, known as compatible solutes, are easily dissolved in water and do not interfere with metabolic processes, even at high cytoplasmic levels.