Synthesis of Polyhydroxyalkanoates by Halophilic Archaea and Bacteria and their Osmoadaptation

THESIS

Submitted in partial fulfilment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

by

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Under the Supervision of
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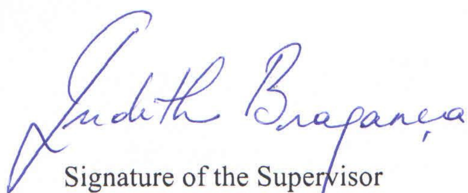
BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI
2015

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

This is to certify that the thesis entitled "**Synthesis of Polyhydroxyalkanoates by Halophilic Archaea and Bacteria and their Osmoadaptation"** submitted by **Ms. Salgaonkar Bhakti Balkrishna,** ID No **2010PHXF810G** for award of Ph.D. of the Institute embodies original work done by her under my supervision.



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Acknowledgement

It is my pleasure to convey my gratitude to all those who supported and helped me in many ways during the completion of this thesis.

I am greatly indebted to my guide, Prof. Judith Maria Bragança, Assistant Professor, Department of Biological Sciences, BITS Pilani, K K Birla Goa Campus, for her guidance, valuable suggestions, constructive criticism, patience, help and constant encouragement. Her scientific experience as well as effective writing skills and suggestions have influenced me throughout the successful completion of this research investigation.

I would like to thank Prof. B. N. Jain Vice-Chancellor; Prof K. E. Raman, Director; Late Prof S. K. Aggarwal, Ex-Director; Prof. P. K. Das, Associate Dean ARD; Prof. S. Bhand, Dean SRCD; Prof. S. D. Manjare, Former-In-Charge RCED; for all their support.

I am thankful to my Doctoral Advisory Committee (DAC) members, Prof. Utpal Roy and Prof. Meenal Kowshik, Head, Department of Biological Sciences BITS Pilani K K Birla Goa Campus for their valuable insights, help and guidance throughout my thesis.

.My sincere thanks to the faculty members of the Department of Biological Sciences, Prof. Srikanth Mutnuri, Prof. Dibakar Chakrabarthy, Prof. Vijayashree Nayak, Dr. Anasuya Ganguly, Dr. Angshuman Sarkar, Dr. Sumit Biswas, Dr. Malbika Biswas, Dr. Kundan Kumar, Dr. Sukanta Mondol, Dr. Indrani Talukdar, Dr. Raviprasad Aduri, Dr. Arnab Banerjee, Dr. Veeky Baths and Mr. Gautam Krishnan for their cooperation throughout this study.

I am grateful to Prof. Irene Furtado, Professor, Department of Microbiology, Goa University, for introducing me to the fascinating field of halophiles and developing a research enthusiast in me.

My special thanks to Dr. Hiroaki Minegishi, Bio-Nano Electronics Research Center Toyo University, Japan and Dr. Mohammad Ali Amoozegar, Iranian Biological Resource Center, University of Tehran, Iran, for their immense help and experience in sequencing of the 16S rRNA genes of the halophilic bacterial and archaeal isolates.

I thank Dr. Shyamprasad M. (Scientist-G) and Mr. Areef Sardar (SEM-Incharge), National Institute of Oceanography (NIO), Dona Paula, Goa for their timely help with Scanning Electron Microscopy Analysis.

I would like to thank Dr. Lisette D'souza, Chief Scientist, National Institute of Oceanography (NIO), Dona Paula, Goa and Sophisticated Instrumentation Facility (SIF),

Chemistry Division, Vellore Institute of Technology (VIT), Tamil Nadu, for helping me with FT-IR and NMR analysis.

I am grateful to Prof. N. N. Ghosh, Associate Professor, Department of Chemistry, BITS Pilani, K K Birla Goa Campus, and his students Dr. Bhanudas Naik, Mr. Subhanjit Hazra and Mr. Barun Kumar Ghosh, for their valuable help in thermal analysis and TEM analysis of the biopolymers.

I would like to thank Prof. Halan Prakash, Associate Professor, Department of Chemistry, BITS Pilani, K K Birla Goa Campus, and his students Ms. Priyadarshini Parakh and Mr. Gokulakrishnan S., for helping with HPLC analysis.

I owe my deepest gratitude to my colleague Mr. Kabilan Mani, for his timely help, support, encouragement and motivation throughout this research.

My special thanks to my dear friends Ms. Kshipra Naik, Dr. Vidhya Prabhudessai, Mr. Kabilan Mani, Ms. Priyadarshini Parakh and Ms. Geethanjali R. for all the good times and fun we had together, especially the birding and evening walks, apart from the unconditional support and motivation.

I am deeply indebted to the research scholars for their timely help, especially Dr. Sivaraman C., Dr. Vilas Desai, Dr. Raeesh Mohd Sheikh, Mr. Chandrashekar, Ms. Shruti, Mr. Ajay Ghosh, Mrs. Purnima, Ms. Archana, Ms. Ramya, Ms. Deepthi, Mr. Yogesh, Mr. Meghanath, Mr. Rajesh, Mrs. Pallavee, Ms. Akriti, Mr. Monsoor, and all the research scholars of the Biological Sciences Department.

I thank Dr. Prasath R. Department of Chemistry, BITS Pilani, K K Birla Goa Campus, for his timely help. I am thankful to Mr. Athiappan, Salem, Tamil Nadu, for helping me in procuring sago starch waste for my agro-industrial waste studies.

I express my sincere thanks to Mrs. Jebel (Saltpan owner), Siridao, Goa, Mr. Kedilliappan, Ezumalayan salt works, Vedaranyam, Tamil Nadu, and all the salt pan workers who helped me with my sample collection and providing me with all the minute details on salt production processes.

I am grateful to financial support provided by various funding agencies, University Grant Commission (UGC) for the Junior Research Fellowship (JRF) on Major Research Project, Council of Scientific and Industrial Research (CSIR) for awarding me the Senior Research Fellowship (SRF) and Department of Biotechnology (DBT-CTEP) for the travel grant for attending the 9th International Congress on Extremophiles 2012 (Sevilla, Spain).

I thank Mrs. Kamna Uphadhay, Mr. Mahadeo Shetkar and Mr. Mahaling Lamani for their timely help during the study.

Finally, I would not have been able to complete this thesis without the understanding and support of my parents and family. Thank you all and I will be forever grateful to you all.

Above all, I praise God, the almighty for providing me this opportunity and granting me the capability to proceed successfully.

Salgaonkar Bhakti Balkrishna

Dedicated to my parents

who valued education above all…

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Abstract

Halophilic microorganisms were isolated from sediment and water samples from solar salterns of Goa and Tamil Nadu, India. The microorganisms were able to grow over a wide range of NaCl concentrations (0.5-30 %, w/v) and were segregated into moderately and extremely halophilic bacteria / archaea. Isolates were characterized morphologically by Gram staining and scanning electron microscopy (SEM) and showed varying cell morphologies such as rods in chains, cocci in groups, irregular cocci in groups, involuted oval discs, etc. The isolates were characterized by studying their response to antibiotics, metals, presence of specific polar lipids and carotenoid pigments. The halophilic archaea were susceptible to novobiocin and rifampicin and showed the presence of characteristic signature glycolipids and phospholipids such as sulfated diglycosyl diether (S-DGD), phosphatidylglycerol (PG), sulfated triglycosyl/tetraglycosyl diether (S-T/TeGD), phosphatidylglycerosulfate (PGS) and phosphatidylglycerophosphate (Me-PGP) when separated by thin layer chromatography (TLC). They harboured pink to orange to brick red pigment having characteristic absorption maxima at 496 nm and two shoulder peaks at 471 and 528 nm, characteristic of archaeal C-50 α-bacterioruberins and C-40 carotenoids. Most of the halophilic bacteria and archaea were able to utilize carbohydrates such as D-glucose, sucrose, D-fructose, D-mannitol, D-maltose, glycerol and produced catalase and oxidase whereas some were able to hydrolyse substrates such as starch, skimmed milk, gelatin, cellulose and olive oil confirming their ability to produce extracellular enzymes such as amylase, protease, gelatinase, cellulase and lipase.

Thirty one moderately halophilic and forty four extremely halophilic isolates were screened for accumulation of polyhydroxyalkanoates (PHA) by Sudan black B, Nile Blue A and Nile Red stains. Cells of PHA positive microorganisms showed the presence of brown black granules when stained with Sudan black B and bright orange-red PHA granules, when stained with Nile Red. Three moderately halophilic isolates H15, H16 and H26 showed the best PHA accumulation and were identified as *Bacillus megaterium* based on phenotypic and genotypic characterization. H16 was able to accumulate PHA in the presence and absence of NaCl. In the absence of NaCl, PHA of 40.0% (w/w) of cell dry weight (CDW) was accumulated at 42 hrs of growth, whereas in presence of 5% (w/v) NaCl, the culture

showed longer lag phase of up to 24 hrs and accumulated a maximum PHA of 39.0 % (w/w) of CDW at 54 h of growth. Fourier transform infra red (FT-IR), X-ray diffraction (XRD) and nuclear magnetic resonance (NMR) confirmed the polymer to be homopolymer of 3-hydroxybutyrates (PHB). Thermogravimetric analysis (TGA) confirmed the polymer to be stable up to a temperature of 160°C. This isolate could be exploited further for the production of PHA at an industrial scale due to its tolerance and growth in presence of up to 5% NaCl.

Among the forty four extremely halophilic isolates screened, TN9 and E3 grew fast with the accumulation of PHA within 3–5 days, followed by strains BBK2, TN4–7 and TN10 within 5–8 days. Based on phenotypic, chemotaxonomic and genotypic characterization, the isolates were grouped in five different genera namely *Halococcus*, *Haloferax*, *Haloarcula, Haloterrigena* and *Halogeometricum* of the family *Halobacteriaceae*. TN9 and E3 were able to synthesize PHA of 14.0 and 73.5% (w/w) of CDW in NGSM supplemented with 0.2% nitrogen source and 2% carbon source. The crotonic acid assay, FT-IR, XRD, TGA-DTA and ^{1}H NMR analysis indicated the polymer produced by *Hgm. borinquense* strains TN9 to be a homopolymer of PHB while strain E3accumulated co-polymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)]. Industrial processes require organisms with standing harsh physicochemical conditions like high salt concentration analogous to high ionic strength and low water activity (aw).

Optimization of culture conditions for *Hgm. borinquense* strain E3 showed that it could grow over a range of glucose concentrations, pH, temperature and salinity but the best cell mass to PHA yield was 5.8 ± 0.4 (g l^{-1}) and 4.0 ± 0.045 (g l^{-1}) respectively was obtained at 2.0% glucose, pH 7.0, 37°C and 20% NaCl. Chemical extraction using sodium hypochlorite (NaClO) with 4 % (w/v) chlorine could extract maximum PHA from the cell mass of 77.85 ± 0.3% (w/w) of CDW and soxhlet extraction using $CHCl_3$ gave PHA yield of 72.4 ± 3% (w/w) of CDW, respectively. The soxhlet extraction for polymer recovery is advantageous as up to 95% of $CHCl_3$ employed can be recovered by rotary evaporator. ^{1}H NMR analysis of the [P(HB-*co*-HV)] revealed it to have 21.4% hydroxylvalerate (HV) units which on decolourization with NaClO/acetone slightly reduced to 19.39 / 19.51% HV units.

Since India's economy is dominated by agriculture and agro based industries, large amounts of agro industrial wastes are being generated. Ability of extremely halophilic archaeon *Hgm. borinquense* strain E3 to use the agro-industrial wastes for the production of PHA was investigated. *Hgm. borinquense* strain E3, was able to grow well on renewable agro-industrial wastes such as sugarcane bagasse (SCB), coconut oil cake (COC) and sago starch waste (SSW). SCB was selected for further studies as the culture showed the best tolerance and grew well in NaCl synthetic medium (NSM) with 25% and 50% (v/v) SCB hydrolysate. The time-course of growth of *Hgm. borinquense* strain E3 in NSM with 25% and 50% (v/v) SCB hydrolysate showed maximum PHA of 50.4 ± 0.1% and 45.7 ± 0.19% (w/w) of CDW on the 7th day. The DSC thermogram showed two melting endotherms in range of Tm_1 = 136.59 - 142.69°C and Tm_2 = 149.4 - 155.27°C. ^{1}H NMR analysis of the polymer obtained from SCB and SSW hydrolysates revealed it to be a co-polymer of [P(HB-*co*-HV)] comprising 13.29 and 19.65 % HV units.

Osmoadaptation in halophilic microorganisms was studied by growing halophilic bacteria and archaea in E2 and NGSM/NH medium with varying NaCl concentrations. *Halomonas elongata* strain M4 showed the best resistance and growth in E2 medium over a range of NaCl concentrations, from 5% to 25% (w/v). The high performance liquid chromatography (HPLC) profile along with ^{1}H NMR spectra of the cell extracts concluded that, *H. elongata* strain M4 when grown in E2 medium at low osmolarity (5% NaCl) produces ectoine, trehalose and traces of γ-aminobutyrate (GABA). Whereas, at high osmolarity of 20% NaCl, the osmolyte ectoine and trehalose were accumulated in lower amount and GABA was not accumulated. In moderately halophilic medium (MHM), at high osmolarity (20% NaCl) the osmolyte glycine betaine (GB) was accumulated more than ectoine, whereas at low osmolarity (5% NaCl) the concentration of ectoine was higher than GB. No accumulation of osmolytes was seen in the total cell extracts of the haloarchaeal isolates *Haloferax* strain BBK2, *Halococcus* strain BK20 and *Halorubrum sp.* strain BS17. On the other hand, an intracellular K^+ accumulation of 4.77, 8.14 and 10.12 of K^+/protein (μM/mg) was observed in halophilic archaeon.

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List of Abbreviations and Symbols

ABC	ATP-binding cassette
ATCC	American Type Culture Collection
Bé	Baumé
bp(s)	base pair(s)
BR	Bacterioruberin
$CDCl_3$	deuterochloroform
CFU	Colony forming unit
cm	Centimeter
CHNS	Carbon, Hydrogen, Nitrogen, Sulphur
COD	Chemical Oxygen Demand
DGD	Diglycosyl Diether
DMSO	Dimethyl sulfoxide
DNSA	3,5-Dinitrosalicylic acid
DSC	Differential Scanning Calorimeter
EDTA	Ethylene Diamine Tetra-Acetic acid
EtBr	Ethidium bromide
GB	Glycine Betaine
$g\ L^{-1}$	Grams per Liter
$g\ kg^{-1}$	Grams per Kilogram
Har	*Haloarcula*
Hcc	*Halococcus*
Hgm	*Halogeometricum*
hr	hour(s)
Htg	*Haloterrigena*
H_2SO_4	Sulphuric acid
IDR	Isopentenyldehydrorhodopin
APHA	American Public Health Association
BABR	Bisanhydrobacterioruberin
BLAST	Basic Local Alignment Search Tool
C	Celsius
CDW	Cell Dry Weight
CI	Chloroform-isoamyl alcohol
$CHCl_3$	Chloroform
C/N	Carbon by Nitrogen
COC	Coconut Oil Cakes
CS	Compatible Solute
DDBJ	DNA Data Bank of Japan
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTA	Differential Thermal Analysis
EHM	Extremely Halophilic Medium
EMBL	European Molecular Biology Laboratory
FT-IR	Fourier Transform Infra Red
GPC	Gel Permeation Chromatography
HA	Hydroxyalkanoates
HB	Hydroxybutyrate
Hfx	*Haloferax*
HPLC	High Performance Liquid Chromatography
Hrr	*Halorubrum*
HV	Hydroxyvalerate
IR	Infra Red

JCM	Japan Collection of Microorganisms
LC	Liquid Chromatography
µ	Growth rate
MCC	Microbial Culture Collection
mcl	medium chain length
Me-PGP	Methyl ester of Phosphatidyl Glycero Phosphate
MHM	Moderate halophilic medium
µl	Microlitre
MR	Methyl red
NA	Nutrient agar
NaClO	Sodium hypochlorite
NGSM	NaCl Glucose Synthetic Medium
NMR	Nuclear magnetic resonance
NT	NaCl Tri-Sodium Citrate
nt/s	Nucleotide/s
PBS	Poly(butylene succinate)
PCR	Polymerase Chain Reaction
PG	Phosphatidyl Glycerol
PHA	Polyhydroxyalkanoate
P(HB-*co*-HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PLA	Poly Lactic Acid
ppm	Parts Per Million
PPP	Poly(*p*-phenylene)
Rf	Retention Factor
rpm	revolutions per minute
scl	Small chain length
SDS	Sodium dodecyl sulphate
kb	kilobase pairs
LCM	Lignocellulose material
LPS	Lipopolysaccharide
MABR	Monoanhydrobacterioruberin
MEGA	Molecular Evolutionary Genetics Analysis
µg	Micro gram
MHz	megahertz (10^6 Hz)
min	minute
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NCBI	National Center for Biotechnology Information
nm	Nanometer
NSM	NaCl Synthetic Medium
NTYE	NaCl Tryptone Yeast Extract
OD	Optical Density
PCI	Phenol-chloroform-isoamyl alcohol
PE	Polyethylene
PGS	Phosphatidyl Glycero Sulfate
PHB	Polyhydroxybutyrate
PHBHV4HB	Poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate-co-4-hydroxybutyrate]
PTT	Poly(trimethyl terephthalate)
RNA	Ribonucleic acid
rRNA	ribosomal RNA
SCB	Sugar Cane Bagasse
S-DGD	Sulfated Diglycosyl Diether
sec	second

SEM	Scanning Electron Microscopy
SIM	Sulfide Indole Motility
spp.	species
STA	Simultaneous Thermal Analysis
S-TeGD	Sulfated Tetraglycosyl Diglycosyl Diether
TE	Tris-EDTA
TFA	Tri Fluoro Acetic acid
TGD	Triglycosyl Diglycosyl Diether
TKN	Total Kjeldahl nitrogen
T_m	Melting Temperature
TS	Total Solids
UFLC	Ultra Fast Liquid Chromatography
VP	Voges−Proskauer
VS	Volatile Solids
wt%	weight percent
w/w	weight by weight
SET	Sucrose EDTA Tris
SPDM	Single Photon Detector Module
SSW	Sago Starch Waste
S-TGD	Sulfated Triglycosyl Diglycosyl Diether
T_d	Degradation Temperature
TEM	Transmission Electron Microscopy
TGA	Thermo Gravimetric Analysis
θ	Theta
TLC	Thin Layer Chromatography
TNTC	Too Numerous To Count
TSA	Trypticase Soy Agar
UV	Ultra Violet
vol	volume
v/v	volume by volume
wt	weight
w/v	weight by volume
XRD	X-ray diffraction

Chapter I

Introduction and Literature Review

1.1 Halophilic Microorganisms

1.1.1 Hypersaline environments

Hypersaline environments are regions with high salinity and therefore referred to as extreme environments. Organisms inhabiting these eco-niches are termed as extremophiles. Hypersaline environments can be classified as thalassohaline with predominant Na^+ and Cl^- ions (marine composition) or athalassohaline environments with predominant other ions such as Mg^{2+} and K^+ ions (non marine composition) (Montoya et al., 2013; DasSarma and Arora, 2002). Thalassohaline environments originate from sea water and include high-salt environments such as the solar salterns, brine wells, saline soils, salt mines, halite, Great salt Lake, Dead Sea, saline lakes, Soda lakes, deep-sea brines, etc (Mani et al., 2012a; Fukushima et al., 2007; Xiang et al., 2008; Waino et al., 2000; Chen et al., 2007; Fish et al., 2002; Ma, et al., 2010; Vander Wielen et al., 2005; Pagaling, et al., 2009; Bodaker, et al., 2010). Athalassohaline environments are aquatic hypersaline environments which are inland and include the Tirez lagoon in Spain, Chaka lake in China, lakes of the Atacama Desert in Chile, etc (Demergasso et al., 2004; Montoya et al., 2013; Jiang, et al., 2006). Among the two, thalassohaline environments are best characterized for its microbial biodiversity (Makhdoumi-Kakhki et al., 2012).

1.1.2 Diversity of Halophiles

Carl Woese (1974), reclassified the prokaryotes into two distinct domains namely bacteria and archaea, by analysing their ribosome (Woese and Fox, 1977). As of January 2015, the domain archaea comprises of five phylogenetically distinct phyla namely, *Crenarchaeota*, *Euryarchaeota*, *Korarchaeota*, *Nanoarchaeota*, and *Thaumarchaeota* with two more proposed novel Phyla namely *Aigarchaeota* and *Diapherotrites* (Woese et al., 1990; Huber et al., 2002; Fuhrman et al., 1992; Elkins et al., 2008; Brochier et al., 2005; Brochier-Armanet et al., 2008; Nunoura et al., 2011; Youssef et al., 2015). Halophilic archaea are catagorised in the the phylum *Euryarchaeota* (Greek for "broad old quality") which contains methanogens, class *Methanococci* and extreme halophiles, class *Halobacteria*.

Halophiles are a highly diverse group of organisms that thrive over an extended range of salt concentrations (Ma et al., 2010, Grant, 2004). Among these microorganisms, the dominant ones are bacteria and archaea, inhabiting saline and hypersaline environments

(Baati et al., 2010; Lichfield and Gillevet, 2002). The term “Halophiles” comes from the Greek word, meaning "salt-loving". Most halophiles are classified within the domain archaea such as members of the family *Halobacteriaceae* and some Methanogens (Oren, 2014). They also include some members of the domain bacteria like family *Halomonadaceae* (genus *Halomonas*) and *Bacteroidetes* (genus *Salinibacter*) (Oren, 2013c) and some are members of domain eukarya, such as family *Teratosphaeriaceae* (the black yeasts *Hortaea werneckii*), family *Dunaliellaceae* (unicellular green alga, *Dunaliella salina*), family *Wallemiaceae* (fungus *Wallemia ichthyophaga*) (Vaupotic et al., 2007; Schubert et al., 2010; Gunde-Cimerman et al., 2009). Ghozlan et al., (2006) studied the diversity of moderately halophilic bacteria in hypersaline environments of Egypt, and reported that the Gram-negative isolates obtained include members of the genus *Flavobacterium*, *Chromohalobacter* and *Halomonas* whereas the Gram-positive isolates grouped under the genera *Halobacillus* and *Staphylococcus*. Recent study by Baati et al, (2010), characterized and segregated the culturable moderately halophilic bacteria from Tunisian solar salterns in two groups, *Gamma-Proteobacteria* (90%) and *Firmicutes* (10%). Further, the group *Gamma-Proteobacteria* was subdivided into several subgroups namely *Halomonadaceae*, *Vibrionaceae*, *Alteromonadaceae*, *Idiomarinaceae*, and *Alcanivoracaceae* (Arahal et al., 2007). The diversity of halophilic bacteria identified is listed in Table 1.1 (de la Haba et al., 2011a).

Table 1.1 List of moderately halophilic microbial genus with representative species name within the various phyla, class, order/suborder and family (de la Haba et al., 2011a; 2011b).

Genera	Species	References
Phylum *Proteobacteria*		
Class *Alphaproteobacteria*		
Order *Rhodobacterales*		
Family *Rhodobacteriaceae*		
Yangia	*Y. pacifica*	Dia et al., 2006
Order *Rhizobiales*		
Dichotomicrobium	*D. thermohalophilum*	Hirsch and Hoffman, 1989
Rhodobium	*R. gokarnense*	Urdiain et al., 2008
Order *Rhodospirillales*		
Family *Rhodospirillaceae*		
Fodinicurvata	*F. fenggangensis*	Wang et al., 2009a
Class *Gammaproteobacteria*		
Order *Oceanospirillales*		
Family *Halomonadaceae*		
Aidingimonas	*A. halophila*	Wang et al., 2009b
Carnimonas	*C. nigrificans*	Garriga et al., 1998

Genera	Species	References
Chromohalobacter	*C. israelensis*	Arahal et al., 2001
Cobetia	*C. marina*	Arahal et al., 2002
Halomonas	*H. elongata*	Vreeland et al., 1980
Kushneria	*K. indalinin*	Sanchez-Porro et al., 2009b
Modicisalibacter	*M. tunisiensis*	Ben Ali Gam et al., 2007
Salinicola	*S. halophilus*	de la Haba et al., 2010
Halotalea	*H. alkalilenta*	
Order *Alteromonadales*		
Family *Alteromonadaceae*		
Marinobacter	*M. maritimus*	Shivaji et al., 2005
Idiomarina	*I. zobellii*	Ivanova et al., 2000
Melitea	*M. salexigens*	Urios et al., 2008a
Order *Chromatiales*		
Family *Ectothiorhodospiraceae*		
	Alkalilimnicola ehrlichii	Hoeft et al., 2007
Class *Deltaproteobacteria*		
Order *Deltaproteobacteria*		
Family *Desulfohalobiaceae*		
Deltaproteobacteria	*Desulfovermiculus halophilus*	Belyakova et al., 2006
Class *Epsilonproteobacteria*		
Order *Campylobacterales*		
Family *Campylobacteraceae*		
Arcobacter	*A. halophilus*	Donachie et al., 2005
Phylum *Firmicutes*		
Class *Bacilli*		
Order *Bacillales*		
Family *Bacillaceae*		
Bacillus	*B. persepolensis*	Amoozegar et al., 2009
Alkalibacillus	*A. halophilus*	Tian et al., 2009
Aquisalibacillus	*A. elongatus*	Marquez et al., 2008
Filobacillus	*F. milosensis*	Schlesner et al., 2001
Gracilibacillus	*G. halophilus*	Chen et al., 2008
Halalkalibacillus	*H. halophilus*	Echigo et al., 2007
Halolactibacillus	*H. alkaliphilus*	Cao et al., 2008
Halobacillus	*H. karajensis*	Amoozegar et al., 2003
Lentibacillus	*L. halophilus*	Tanasupawat et al., 2006
Oceanobacillus	*O. picturae*	Lee et al., 2006
Ornithinibacillus	*O. californiensis*	Mayr et al., 2006
Paraliobacillus	*P. quinghaiensis*	Chen et al., 2009c
Pontibacillus	*P. halophilus*	Chen et al., 2009b
Salimicrobium	*S. halophilum*	Yoon et al., 2007b
Salinibacillus	*S. aidingensis*	Ren and Zhou, 2005b
Salirhabdus	*S. euzebyi*	Albuquerque et al., 2007
Salsuginibacillus	*S. kocurii*	Carrasco et al., 2007
Sediminibacillus	*S. halophilus*	Carrasco et al., 2008
Tenuibacillus	*T. multivorans*	Ren and Zhou, 2005a
Thalassobacillus	*T. cyri*	Sanchez-Porro et al., 2009a
Virgibacillus	*V. sediminis*	Chen et al., 2009a
Family *Planococcaceae*		
Jeotgalibacillus	*J. alimentarius*	Yoon et al., 2001
Family *Staphylococcaceae*		
Salinicoccus	*S. iranensis*	Amoozegar et al., 2008
Class *Clostridia*		

Genera	Species	References
Order *Halanaerobiales*		
Family *Halanaerobiaceae*		
Halanaerobium	*H. fermentans*	Kobayashi et al., 2000
Halocella	*H. cellulosilytica*	Simankova et al., 1994
Halothermothrix	*H. orenii*	Cayol et al., 1994
Family *Halobacteroidaceae*		
Halobacteroides	*H. elegans*	Zhilina et al., 1997
Acetohalobium	*A. arabaticum*	Zhilina and Zavarzin, 1990
Halanaerobacter	*H. salinarius*	Moune´ et al., 1999
Halonatronum	*H. saccharophilum*	Zhilina et al., 2001
Natrionella	*N. acetigena*	Zhilina et al., 1996
Phylum *Actinobacteria*		
Orenia	*O. salinaria*	Moune et al., 2000
Selenihalanaerobacter	*S. shriftii*	Switzer Blum et al., 2001
Sporohalobacter	*S. marismortui*	Oren et al., 1987
Order *Actinopolysporineae*		
Family *Actinopolyporaceae*		
Actinopolyspora	*A. halophila*	Gochnauer et al., 1975
Order *Corynebacterineae*		
Family *Corynebacteriaceae*		
Corynebacterium	*C. halotolerans*	Chen et al., 2004
Order *Glycomycineae*		
Family *Glycomycetae*		
Haloglycomyces	*H. albus*	Guan et al., 2009
Order *Micrococcineae*		
Family *Bogoriellaceae*		
Georgenia	*G. halophila*	Tang et al., 2010b
Family *Micrococcaceae*		
Nesterenkonia	*N. halobia*	Stackebrandt et al., 1995
Family *Ruaniaceae*		
Haloactinobacterium	*H. album*	Tang et al., 2010c
Family *Promicromonosporaceae*		
Isoptericola	*I. halotolerans*	Zhang et al., 2005
Order *Pseudonocardianeae*		
Family *Pseudonocardiaceae*		
Amycolaptosis	*A. halophila*	Tang et al., 2010a
Praseurella	*P. marina*	Wang et al., 2010
Saccharomonospora	*S. saliphila*	Syed et al., 2008
Saccharopolyspora	*S. halophila*	Tang et al., 2009
Order *Streptosporangineae*		
Family *Nocardiopsaceae*		
Haloactinospora	*H. alba*	Tang et al., 2008
Nocardiopsis	*N. salina*	Li et al., 2004b
Streptomonospora	*S. salina*	Cui et al., 2001
Phylum *Spirochaetes*		
Spirochaeta	*S. halophila*	Greenberg and Canale-Parola, 1976
Phylum *Bacteroidetes*		
Class *Bacteroidia*		
Order *Bacteroidales*		
Family *Marinilabiaceae*		
	A. thermohalophila	Denger et al., 2002
Class *Flavobacteria*		
Order *Flavobacteriales*		
Family *Flavobacteriaceae*		

	Genera	Species	References
	Maribacter		
	Polaribacter		
	Salimicrobium		
	Psychroflexus	*P. tropicus*	Donachie et al., 2004
	Gramella	*G. echinicola*	Nedashkovskaya et al., 2005
Class *Sphingobacteria*			
Order *Sphingobacteriales*			
Family *Rhodothermaceae*			
	Rhodothermus		Vaisman and Oren, 2009
	Salinibacter	*S. ruber*	Anton et al., 2002
	Salisaeta	*S. longa*	
Phylum *Thermotogae*			
Phylum *Cyanobacteria*			
	Halospirulina	*H. tapeticola*	Nubel et al., 2000
	Prochlorococcus	*P. marinus*	Chisholm et al., 1992
	Prochlorothrix		
	Rubidibacter	*R. lacunae*	Choi et al., 2008
Phylum *Tenericutes*			
Class *Mollicutes*			
Order *Haloplasmatales*			
Family *Haloplasmataceae*			
		H. contractile	Antunes et al., 2008a

1.1.3 Family *Halobacteriaceae* and its salient features

Gibbons (1974) first proposed the family *Halobacteriaceae*, subdivided as Phylum *Euryarchaeota*, Class *Halobacteria*, Order *Halobacteriales* (Grant et al., 2001). As of November 2014 it encompasses 49 genera and 182 species (genus name and recommended three-letter abbreviation): *Halobacterium* (Hbt), *Haladaptatus* (Hap), *Halalkalicoccus* (Hac), *Halarchaeum* (Hla), *Halarchaeobius* (Hab), *Haloarcula* (Har), *Halobaculum* (Hbl), *Halobellus* (Hbs), *Halobiforma* (Hbf), *Halococcus* (Hcc), *Haloferax* (Hfx), *Halogeometricum* (Hgm), *Halogranum* (Hgn), *Halolamina* (Hlm), *Halomarina* (Hmr), *Halomicrobium* (Hmc), *Halonotius* (Hns), *Halopelagius* (Hpl), *Halopenitus* (Hpt), *Halopiger* (Hpg), *Haloplanus* (Hpn), *Haloquadratum* (Hqr), *Halorhabdus* (Hrd), *Halorientalis* (Hos), *Halorubrum* (Hrr), *Halosarcina* (Hsn), *Halosimplex* (Hsx), *Halostagnicola* (Hst), *Haloterrigena* (Htg), *Halovenus* (Hvn), *Halovivax* (Hvx), *Halohasta* (Hht), *Halomicroarcula* (Hma), *Halorubellus* (Hrb), *Natrialba* (Nab), *Natrinema* (Nnm), *Natronoarchaeum* (Nac), *Natronobacterium* (Nbt), *Halorussus* (Hrs), *Natronococcus* (Ncc), *Natronolimnobius* (Nln), *Natronomonas* (Nmn), *Natronorubrum* (Nrr), *Salarchaeum* (Sar), *Salinarchaeum* (Saa), *Salinigranum* (Sgn), *Salinirubrum* (Srr), *Halapricum* (Hpr),

and *Natribaculum* (Oren, 2014; Song et al., 2014; Liu et al., 2014; Oren and Ventosa, 2013).

Haloarchaeal diversity studies of the family *Halobacteriaceae* have most identified isolates as aerobic with chemoheterotrophic metabolism. However, few isolates grow anaerobically (facultative) by fermentation, anaerobic respiration, or using bacteriorhodopsin to absorb light as an energy source (Oren, 2013a; Sharma et al., 2007). Most of the members have a high salt requirement with optimum being 150–200 g L^{-1} and most of the haloarchaeal strains (except *Halococcus*) undergo lysis when exposed to low salinity / osmolarity solutions (Oren, 2014; Fukushima et al., 2007; Mani et al., 2012a). They have unique physiological and chemotaxonomic features which include, the red-pigmented colonies whose cytoplasmic membranes contain bacterioruberin type of pigments (carotenoids) and retinal proteins such as bacteriorhodopsin, halorhodopsin and sensory rhodopsins. They also possess signature ether lipids (Oren, 2014).

1.1.3.1 Cell wall and surface layer of family *Halobacteriaceae*

Surface layers (S-layer) cell wall are present in most of the non-cocoidal members of the family *Halobacteriaceae* (Oren, 2013a). This S-layer is responsible for maintaining the native shape of cell and constitutes high-molecular-weight glycoprotein which is rich in acidic amino acid residues, containing attached acidic and neutral saccharide chains. It requires high NaCl concentrations for stability and undergoes denaturation and dissolution when suspended in water. Therefore, the cells of most species of haloarchaea lyse in the absence of salt. Nevertheless, cell wall of genus *Halococcus* comprises of a thick sulfated heteropolysaccharide constituting sulfated sugar residues such as glucose, galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine and different uronic acids. Therefore, to maintain its cell rigidity, it does not require high salt concentrations (Schleifer et al., 1982). On the other hand, genus *Haloferax* excretes exopolysaccharides mostly constituting glucose, mannose, and sulfated glucose units that form a slime layer around the cells (Poli et al., 2011). In some species, relatively high concentrations of divalent cations like magnesium and calcium (Mg^{2+}, Ca^{2+}) are required in addition to high NaCl concentrations to maintain the structural stability of the glycoprotein cell wall (Oren, 2013a).

1.1.3.2 Sensitivity of the family *Halobacteriaceae* to antibiotics

Various antibiotics are used to differentiate members of the family *Halobacteriaceae* from bacteria. Interestingly, halophilic archaea are resistant to antibiotics like penicillin, ampicillin, cycloserine, kanamycin, neomycin, polymyxin, and streptomycin which are bacteria specific (Oren, 2013b; Dornmayr-Pfaffenhuemer et al., 2011). Most species are sensitive to bacitracin and novobiocin. Rifampicin is also found to inhibit the growth of haloarchaea.

(i) **Bacitracin** is a mixture of related cyclic polypeptides, which acts by interfering with peptidoglycan synthesis. Moreover, it inhibits the incorporation of the high-molecular-weight saccharide into the cell wall glycoprotein of non-coccoid halophilic archaea and may also inhibit lipid biosynthesis in these organisms (Oren, 2013b).

(ii) **Novobiocin** belongs to the DNA interfearing antimicrobials of family aminocoumarin and is an inhibitor of *gyrB* subunit of DNA gyrase (type II DNA topoisomerase) and acts on the same target in archaea and bacteria (Gadelle and Forterre 1994).

(iii) **Rifampicin** belongs to family ansamycins and is a subclass of the DNA-interfearing antimicrobials. It acts by inhibiting DNA-dependent RNA polymerase thereby blocking the RNA synthesis (Campbell, et al., 2001). Rifampicin is known to inhibit many haloarchaeal members (Bonelo et al., 1984; Hilpert et al., 1981; Pecher and Bock, 1981). Bacterial RNA polymerase contains 5 different proteins whereas the RNA polymerase from methanogens and halophiles contains 8 proteins.

1.1.3.3 Lipids of family *Halobacteriaceae*

Presence of unique lipids based on branched phytanyl (20-carbon) and / or sesterterpanyl (25-carbon) chains bound to glycerol by ether bonds are one of the characteristic feature of the family *Halobacteriaceae* (Kamekura, 1993; Kates, 1978). These polar lipids are used as chemotaxonomic signatures in the taxonomic classification of certain genera and species of the halophilic bacteria (Oren et al., 2009). Polar lipids include phospholipids, sulfolipids and glycolipids, which are encountered in different representatives of the family (Oren et al., 2013a). All members of *Halobacteriaceae* contain diether derivatives of phosphatidyl glycerol (PG) and the methyl ester of phosphatidyl glycerophosphate (PGP-Me) (Kates et al., 1993). The diether derivative of phosphatidyl glycerosulfate (PGS) is present in many

species. The glycolipids are generally absent in the alkaliphilic members of the family, but are present in most of the neutrophilic representatives and these are diglycosyl diether (DGD), triglycosyl diether (TGD), and tetraglycosyl diether (TeGD) lipids.

1.1.3.4 Pigments of family *Halobacteriaceae*

Red-orange pigmentation is one of the key characteristics of almost all members of the family *Halobacteriaceae* except the two species, *Natrialba asiatica* and *Halorhabdus tiamatea* (Hezayen et al., 2001; Antunes et al., 2008b). This pigmentation is due to a high content of carotenoids in their cell membrane. The various types of carotenoid structures are represented in Fig 1.1. The most abundant carotenoids in halophilic archaea are the 50-carbon compounds α-bacterioruberin (BR) and its derivatives mono-anhydrobacterioruberin (MABR) and bis-anhydrobacterioruberin (BABR) (Yatsunami et al., 2014; Oren, 2009). Also, small amounts of 40-carbon carotenoids like lycopene and β-carotene are present. Carotenoids are derived from the terpenoid biosynthetic pathway and function as antioxidants, protect the cells against lethal UV light photodamage and also act as membrane stabilizers (Yatsunami et al., 2014).

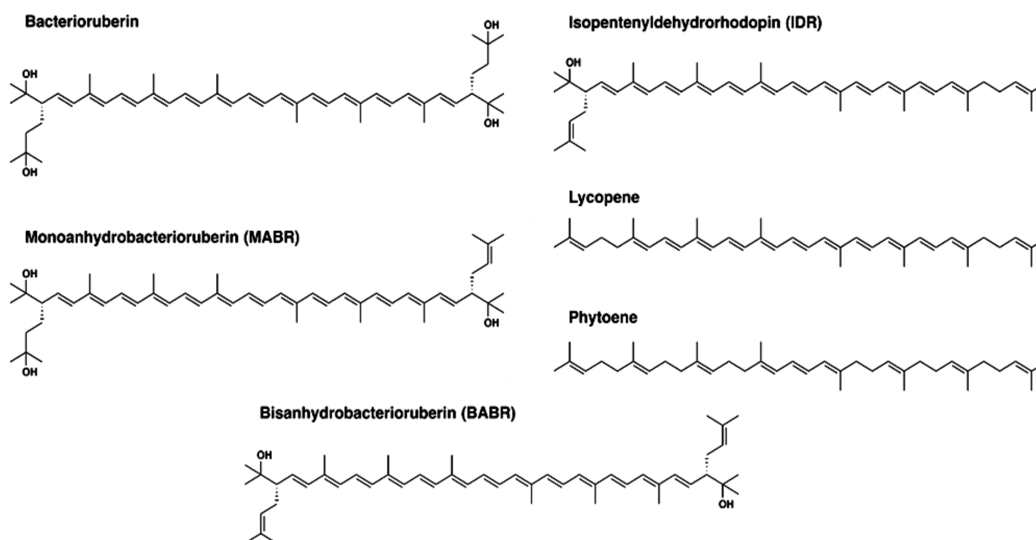

Fig. 1.1 Structure of carotenoids characteristic to the members of *Halobacteriaceae* (Yatsunami et al., 2014).

1.1.4 Mechanism of osmotic adaptation

As we know halophiles are salt loving organisms, inhabiting various extreme environments and solar salterns is one such habitat. In solar salterns, the salinity drastically varies along the salinity gradient from reservoir pond (3-5 %) to the evaporator pond (15-25 %) and finally the crystallizer pond (≥ 25 %) (Mani et al., 2012a). How does microorganisms inhabiting these solar salterns combat and survive such a drastic change in salinity? The cells of all three domains of life, residing in such environment are very dynamic and have a very important property of adapting to the changes in external salt (NaCl) concentration. Two types of strategies are employed by halophilic microorganisms to combat the high external solute concentrations; one strategy involves the accumulation of inorganic ions such as K^+ and is mostly used by members of the family *Halobacteriaceae* (domain *Archaea*) and order *Haloanaerobiales* (domain *Bacteria*). Another strategy involves the accumulation and / or *de novo* synthesis of certain organic solutes and is seen in a variety of halophilic organisms mostly belonging to the domain *bacteria* and *eukaryota* and few members of domain *archaea* (Roberts, 2005).

The type of organic molecules used for osmotic balance includes polyols, sugars, amino acids and their derivatives, betaine, ectoine, hydroxyectoine and peptides (Shivanand and Mugeraya, 2011). The organic solutes can be zwitterionic, noncharged, or anionic (along with an inorganic cation such as K^+). The diversity of these organic solutes is increasing day by day, as more novel organisms, especially polyextremophilic halophilic, thermophilic and hyperthermophilic *bacteria* and *archaea* are being studied. Normal cell in hypertonic solution tend to shrink due to loss of water along with intracellular ions to the environment. Interestingly, accumulation of such compatible solutes helps the cells in maintaining the turgor pressure, cell volume and electrolyte concentration (Roberts, 2005).

1.1.4.1 Spectrum of microbially accumulated osmolytes

HPLC and NMR are the two widely used methods for the characterization of osmolytes produced by halophilic microorganisms (Motta et al., 2004; Galinski and Trüper, 1994; Ventosa et al., 1998; Martins et al., 1997). The spectrum of compatible solutes used by microorganisms comprises of only a limited number of compounds: sugars (trehalose), polyols (glycerol and glucosylglycerol), free amino acids (proline and glutamate),

derivatives such as proline betaine and ectoine, quarternary amines and their sulfonium analogues (glycine betaine, carnitine and dimethylsulfoniopropionate), sulfate esters (choline-O-sulfate), N-acetylated diamino acids and small peptides (Nδ-acetylornithine and N-acetylglutaminylglutamine amide). In general, compatible solutes are highly soluble molecules and do not carry a net charge at physiological pH.

1.1.4.2 Biosynthesis of compatible solute (ectoine)

Numerous studies have been carried on ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) producing microorganisms, both Gram-negative and Gram-positive (Roberts, 2005). The biosynthesis of ectoine in halophile such as *Halomonas elongata* starts from aspartate semialdehyde, an intermediate in amino acid metabolism (Fig. 1.2). The aldehyde is converted to L-2,4-diaminobutyric acid, which is then acetylated to from Nγ-acetyldiaminobutyric acid (NADA). The final step is the cyclization of this product to form ectoine.

Ectoine synthesis is carried by a concerted effect of three genes: *ectABC*. The *ectA* gene codes for diaminobutyric acid acetyltransferase; *ectB* codes for the diaminobutyric acid aminotransferase and *ectC* codes for ectoine synthase (Canovas et al., 1998). Halophilic microorganisms able to synthesize ectoine are also able to convert this compound into *S,S*-1,4,5,6–tetrahydro-2-methyl-5-hydroxyl-pyrimidine-4-carboxylate, hydroxyectoine (Fig. 1.2), by means of a 2-oxoglutarate dependent non-heme-iron (II) containing dioxygenase (Bursy et al., 2007; Reuter et al., 2010; Widderich et al., 2014; Saum and Müller, 2008).

Characterization of the three recombinant Ect enzymes of *Halomonas elongata* is accomplished and the findings suggests that the osmolyte ectoine is partially regulated by intracellular cations (Roberts, 2005). The first enzyme diaminobutyrate aminotransferase (DABA), transaminates the aspartate semialdehyde with glutamate and generates the diaminobutyrate (Fig. 1.2). It is a 260 kDa complex (44 kDa subunits) and requires both pyridoxal 5'-phosphate and K^+ for its activity (Ono, 1999). The DABA aminotransferase of second step is activated by 0.5 M NaCl and / or KCl and also the third enzyme, ectoine synthase, requires NaCl for activation (Roberts, 2005).

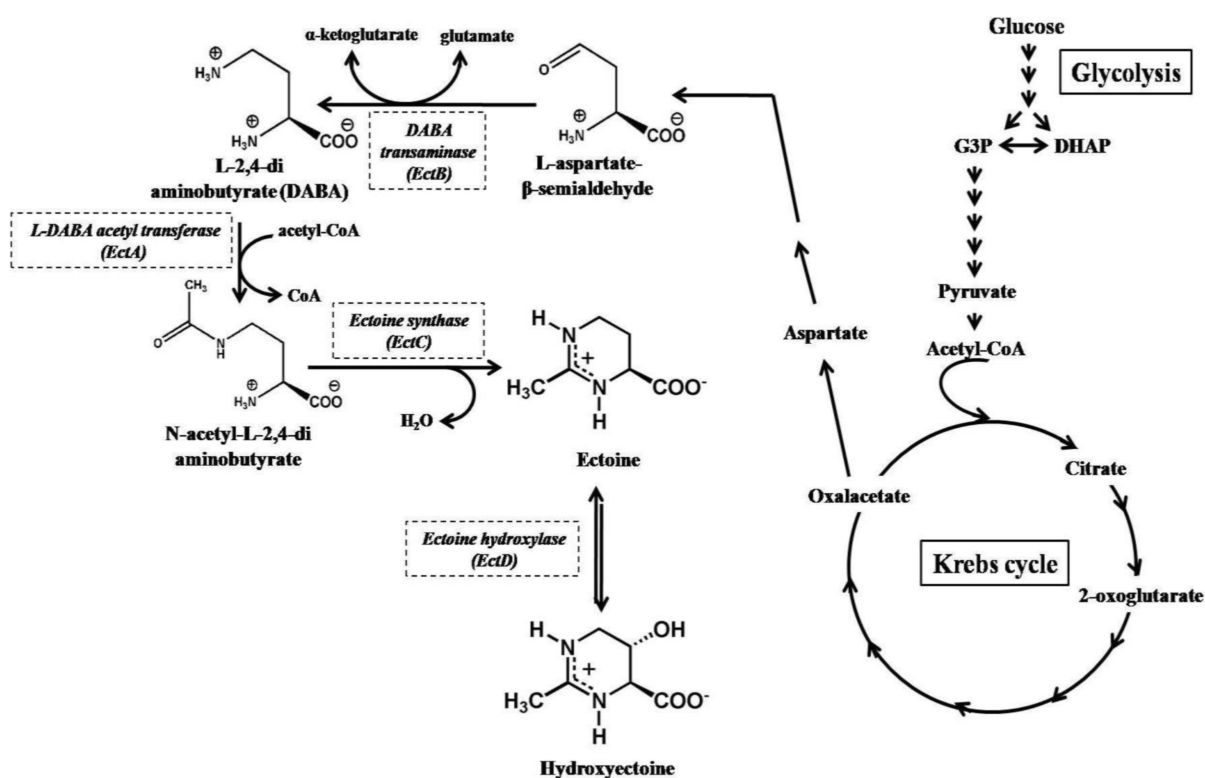

Fig. 1.2 Biosysnthesis of ectoine and hydroxyectoine in halophilic bacteria using glucose (Modified from Hänelt and Müller, 2013 and Garcıa-Estepa et al., 2006). The coding genes and enzymes involved in the biosysthesis are represented in dotted boxes.

1.2 Biodegradable plastics

For the past few years, anthropogenic activity such as increased use of both plastic and petroleum has resulted in environmental pollution. Conventional plastics such as polyethylene (PE), polypropylene (PP) and polyvinyl alcohol are petroleum based plastics obtained from non-renewable petro-chemical resources which are on verge of depletion and hence there is an increasing need to replace with the eco friendly bio-based green plastics obtained from renewable resources (Chen, 2012). Biodegradable plastics can be divided into three categories (Khanna and Srivastava, 2005);

1.2.1 Chemically synthesised polymers: Includes polyglycollic acid, PLA, poly(ε-caprolactone), polyvinyl alcohol, PE. These are susceptible to enzymatic or microbial activity on them. They do not match all the properties of plastics, hence are not commercially viable as substitute for plastics.

1.2.2 Starch-based biodegradable plastics: Includes starch–polyethylene which is a blend of starch and plastic wherein starch is added as filler and cross-linking agent. Most of the indigenous soil microorganisms are amylase producers and hence can degrade the starch easily, thus breaking down the polymer matrix. Only partial degradation is achieved and the leftover fragments (after starch removal) are recalcitrant and remain in the environment for a long time.

1.2.3 Microbially synthesized plastics: These are the only 100% biodegradable polymers. They are polyesters of various HAs which are synthesised by numerous microorganisms as energy reserve materials when nutrients such as nitrogen or phosphorus is available only in limiting concentrations, but in the presence of excess carbon source. They possess properties similar to various synthetic thermoplastics like polypropylene and hence can replace them. They are also completely degraded to water (H_2O) and carbon dioxide (CO_2) under aerobic conditions and to methane (CH_4) under anaerobic conditions by naturally accuring microorganisms (Chen, 2014; Chee et al., 2010).

1.3 Polyhydroxyalkanoates (PHA)

Polyhydroxybutyrate (PHB) was the first PHA to be discovered and is also the most widely studied biopolymer. It is accumulated as a membranous inclusion in many bacteria up to 80% of dry cell weight. It has mechanical properties very similar to conventional plastics like polypropylene or polyethylene and can be extruded, moulded, spun into fibres, made into films and used to make heteropolymers with other synthetic polymers. In spite of its numerous advantages, PHB has yet not been able to replace conventional plastics on a large scale because of its high production cost (Sudesh, 2013; Sudesh et al., 2000).

1.3.1 Structure and properties of PHA

The general structure of PHA is represented in Fig. 1.3. PHAs are polymers of carbon, oxygen and hydrogen. The polymer can be made from a single monomer or a combination of the monomers (Table 1.2).

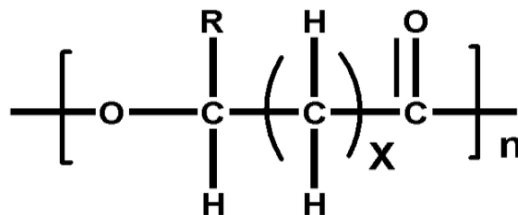

Fig. 1.3 General structure of polyhydroxyalkanoates (PHA).

Table 1.2 List of structural derivatives of PHA.

R Group			Polyhydroxyalkanoates (PHA)	
Group	**Formula**	**C Number**	**PHA Type**	**Abbrevation**
Methyl	$-CH_3$	C4	Poly(3-hydroxybutyrate)	PHB
Ethyl	$-C_2H_5$	C5	Poly(3-hydroxyvalerate)	PHV
Propyl	$-C_3H_7$	C6	Poly(3-hydroxyhexanoate)	PHHx
Butyl	$-C_4H_9$	C7	Poly(3-hydroxyheptanoate)	PHHp
Pentyl	$-C_5H_{11}$	C8	Poly(3-hydroxyoctanoate)	PHO
Hexyl	$-C_6H_{13}$	C9	Poly(3-hydroxynonanoate)	PHN
Heptyl	$-C_7H_{15}$	C10	Poly(3-hydroxydecanoate)	PHD
Octyl	$-C_8H_{17}$	C11	Poly(3-hydroxyundecanoate)	PHUD
Nonyl	$-C_9H_{19}$	C12	Poly(3-hydroxydodecanoate)	PHDD
decyl	$-C_{10}H_{21}$	C13	Poly(3-hydroxytridecanoate)	PHTD
Undecyl	$-C_{11}H_{23}$	C14	Poly(3-hydroxytetradecanoate)	PHTeD
Dodecyl	$-C_{12}H_{25}$	C15	Poly(3-hydroxypentadecanoate)	PHPD
Tridecyl	$-C_{13}H_{27}$	C16	Poly(3-hydroxyhexadecanoate)	PHHD

1.3.2 Classification of PHA

Microbial PHAs can be classified according to various criteria listed below (Loo and Sudesh, 2007).

1.3.2.1 Biosynthetic origin

(i) **Natural PHAs**: produced naturally by microorganisms from general substrates, eg synthesis of homopolymer P(3HB) by genus *Bacillus* (Valappil et al., 2007) and heteropolymer / copolymer P(3HB-*co*-3HV) by genus *Haloferax* (Bhattacharyya et al., 2014).

(ii) **Semisynthetic PHAs**: produced by addition of unusual precursors such as 3-mercaptopropionic acid to a general substrate, to promote the biosynthesis of poly(3-hydroxybutyrate-*co*-3-mercaptopropionate) [P(3HB-*co*-3MP)].

1.3.2.2 Monomer size

PHAs are classified into two major classes, depending on the number of carbon atoms in hydroxyalkanoate (HA) monomer,

(i) **Short chain-length PHAs (PHA_{scl})**: monomers containing 3-5 carbon atoms such as PHB and PHV.

(ii) **Medium chain-length PHAs (PHA_{mcl})**: monomers containing 6-14 carbon atoms such as PHHx, PHHp, PHO, PHN, etc (Table 1.2).

1.3.2.3 Number of different monomers in PHAs

(i) **Homopolymers**: are fromed by linkage of a single type of monomeric unit. The linkage is between ester bonds of one monomer to the carboxylic group of adjacent monomer to give polymer such as PHB, PHV, etc.

(ii) **Heteropolymers**: are formed by linkage of two or more different type of monomeric units such as, a copolymer of P(HB-*co*-HV).

1.3.2.4 Chemical nature of the monomers

(i) PHA containing aliphatic fatty acids such as PHB.

(ii) PHAs containing aromatic fatty acids such as Poly(3-hydroxy-5-phenylvalerate) PHPV (Shrivastav et al., 2013)

(iii) PHA heteropolymers containing both aliphatic and aromatic fatty acids. i.e P(3HB-*co*-3MP) (Ward and O'Connor, 2005).

1.3.3 Polymer characterization techniques

There are myriad of PHA monomeric units (≥ 150) available and different composition of these in PHA polymers results in their diverse chemical composition and material properties (Tan et al., 2014b). Characterization of the PHA is very important for its application. The physical and chemical property of a PHA polymer is determined by its monomeric composition (Strazzullo et al., 2008; Ward and O'Connor, 2005).

1.3.3.1Monomeric Composition

The monomeric composition of PHA polymer is determined by techniques such as gas chromatography (GC), liquid chromatography (LC), nuclear magnetic resonance (NMR) spectroscopy, fast-atom bombardment mass spectroscopy (FAB)-MS, and matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) (Ballistreri et al., 1989; Saeed et al., 2001). The PHA as an intact polymer is difficult to analyze. Therefore, the polymer is depolymerized first with chemical derivatization and then analyzed for its monomeric units using chromatography based methods (Tan et al., 2014a). The chemical composition of intact PHA polymers such as homopolymer, copolymer, terpolymer, etc can be studied using NMR, which provides details on the topology and functional group in molecules. Both, ^{1}H-NMR and ^{13}C-NMR can be employed for PHA characterization, however, the high proton NMR (^{1}H-NMR) is more sensitive and has shorter analytical time (≤ one hour) as compared to the carbon (^{13}C-NMR) which requires longer analysis time (up to 24 hour) and is less sensitive (Tan et al., 2014b). NMR is also used for analyzing the functional groups such as methane protons –CH_3, methylene protons, –CH=CH– and in the quantitative estimation of PHA monomers, by studying the intensity ratio of the signals (Sánchez et al., 2003). The MS technique such as MALDI-TOF-MS needs no chemical derivatization of the PHA therefore it can be used as a complementary to NMR. MALDI-TOF-MS is an extremely sensitive, accurate technique requiring picomole (10^{-12}) to femtomole (10^{-15}) quantity of sample.

1.3.3.2 Molecular Mass

Gel permeation chromatography (GPC) system, calibrated with polystyrene standard columns such as Styragel HMW 6E (5 to 10,000 kDa) and PLgel MIXED-C (0.2 to 2,000 kDa) is used to determine the polymer's average (a) molecular mass (Mw), (b) molecular mass distribution (Mn), and (c) polydispersity index (PDI; Mw/Mn) (Ashby et al., 2002). The Mw of PHA spans over a wide range from 50 kDa to 10,000 kDa and depending on Mn value, PDI could be between 1.1 and 6.0 (Tan et al., 2014b; Chen et al., 2010). Interestingly, two or more GPC columns, connected in series are employed for identifying polymer having varying Mw and Mn values. MALDI-TOF-MS, is also used for evaluating

the Mw and Mn of PHAs and their oligomers and offers accurate mass measurement of PHA (Li, 2013).

1.3.3.3 Thermal Properties

Polymer materials are usually characterized for their thermal properties such as glass transition temperature (T*g*), melting temperature (T*m*), and thermodegradation temperature (T*d*) using differential scanning calorimetry (DSC) and differential thermal analysis (DTA). Thermal properties determine the temperature conditions at which the polymer can be processed and utilized. The T*g*, T*m*, and T*d* values for PHA are listed in Table 1.3 and are usually in the range of −52 to 4 °C, non-observable to 177 °C, and 227 to 256 °C, respectively (Tan et al., 2014b; Chen, 2010). DTA measures the mass loss and provides qualitative thermal information. However, DSC employs a direct heat flow measurement, which provides qualitative as well as quantitative thermal information. Therefore, DSC is preferred over other thermal invstigative methods in PHA studies (Aoyagi et al., 2001; Hahn et al., 1995; Sánchez et al., 2003). Also, thermogravimetric analysis (TGA) is used to obtain the T*d* value of PHA, where a sample is heated in a controlled atmosphere at a defined rate while sample mass loss is measured. Interestingly, the development of the simultaneous thermal analysis (STA) combines TGA and DSC / DTA measurement techniques, enabling the determination of T*g*, T*m*, and T*d* on a single instrument (Tan et al., 2014b; Sánchez et al., 2003).

Table 1.3 Comparison of physical properties of various PHA and conventional plastics (Sudesh et al., 2000).

Samples	Tm (^{o}C)	Tg (^{o}C)	Tensile strength (Mpa)	Elongation at break (%)
PHB	177	4	43	5
P(HB-*co*-10%HV)	150	-	25	20
P(HB-*co*-20%HV)	135	-	20	100
P(HB-*co*-10%HHx)	127	-1	21	400
Polypropylene	170	-	34	400

Tm: melting temperature; Tg: glass transition temperature; Mpa: megapascal

1.3.3.4 Crystallinity

The crystallinity of PHA polymer could be measured by instruments such as FTIR, DSC and XRD. PHA polymers can be non-crystalline to highly crystalline with crystallinity values between 0% and 70% (Chanprateep, 2010).

(i) **The Fourier Transform Infra Red (FTIR) analysis** of PHA shows prominent characteristic absorption bands at certain wavenumbers which can be correlated to crystallinity (Tan et al., 2014b). Depending on the polymer composition, the band locations vary, for example scl-PHAs such as PHB and P(HB-*co*-HV) bands around 1279, 1228, and 1185 cm^{-1} indicates the degree of crystallinity (Porter and Yu, 2011). Band 1725 cm^{-1} and bands in the range of 1500 to 1300 cm^{-1}, 1300 to 1000 cm^{-1} and 1000 to 800 cm^{-1} indicates the conformational changes of mcl-PHA and scl-mcl PHA, in both the crystalline phase and amorphous phase (Chen et al., 2009).

(ii) **The DSC analysis** of the polymer, melting enthalpy (ΔHm) provides an estimated value for heat of fusion (ΔHf), which could be related to PHA's crystallinity. PHA polymers with very low crystallinity typically have low to non-observable ΔHf while highly-crystalline polymers such as P3HB can have ΔHf values up to 146 J g^{-1} (Barham et al., 1984). Both, FTIR and DSC are only adequate at measuring relative crystallinities within a given material and absolute crystallinity measurement can be done by XRD analysis (Simon-Colin et al., 2008).

(iii) **The X-ray diffraction (XRD) analysis** could be used to measure absolute crystallinity of the polymer. XRD analysis is able to shed light on the polymer's rate of crystallinity, as well as atomic structures such as chemical bonds and their disorder (Dufresne et al., 1999). Crystallinity percentage can be calculated according to semi-crystalline and amorphous polymer areas in the diffractogram using Lorentzian and Gauss functions (Sánchez et al., 2003).

1.3.3.5 Mechanical Properties

The mechanical properties that are commonly evaluated for PHA polymers are (a) Young's modulus, (b) elongation at break and (c) tensile strength and these can be performed with

tensile tester instrument by standardized test methods such as the ones recommended by American Society for Testing and Materials (ASTM) standards (Wu and Liao, 2014).

(i) **The Young's modulus / tensile modulus / elastic modulus** provide a measure of PHA's stiffness and ranges from the very ductile mcl-PHA (0.008 MPa) to the stiffer scl-PHA (3.5×103 MPa) (Rai et al., 2011).

(ii) **The elongation at break / fracture strain** measures the extent that a material will stretch before it breaks and is expressed as a percentage of the material's original length. PHA polymers can either be a soft elastomeric material or a hard rigid material, thereby displaying a wide elongation at break values between 2% and 1000% (Chen et al., 2010).

(iii) **The tensile strength** measures the amount of force required to pull a material until it breaks, and for PHA polymers it falls in the range of 8.8 to 104 MPa (Rai et al., 2011).

1.3.4 Metabolic pathways for PHA biosynthesis

Microorganisms employ various pathways for the metabolizing and / or transformation of carbohydrates and fatty acids into diverse range of PHAs with main intermediate being either acetyl-CoA and / or acyl-CoA and conclude with monomer polymerization by PHA synthases (Fig. 1.4) (Van-Thuoc et al., 2012; Philip et al., 2007; Van-Thuoc et al., 2008). The ability of microorganisms to synthesize a particular form of PHA is mainly due to the substrate specificity of PHA synthases and these enzymes may be divided into four classes (Van Thuoc et al., 2012; Rehm, 2003). Class I and II synthases are encoded by PhaC gene. Interestingly, class I PHA synthases utilize CoA thioesters of 3-hydroxyalkanoates (3-HAs) comprising 3‑5 carbon atoms, whereas, class II polymerases are also specific to CoA thioesters of 3-HAs comprising 6‑14 carbon atoms (Han et al., 2010). Class III and class IV synthases are composed of two genes (PhaC and PhaE) and (PhaC and PhaR), respectively. However, class III synthases possess substrate specificities similar to class I synthases and Class IV synthases and utilize 3-HA monomers with 3‑5 carbon atoms (Rehm, 2003).

The PhaCAB genes are localized on the chromosome of microorganisms and encodes for three cytoplasmic proteins / enzymes. The Pha A gene encodes for acetyl-CoA C-

acetyltransferase, the 393 amino acids protein. The Pha B gene encodes for acetoacetyl-CoA reductase, 246 amino acids protein. The Pha C gene encodes for poly-β-hydroxybutyrate polymerase / synthase, 589 amino acids protein. PHA synthase is the key enzyme for the PHA biosynthesis, which polymerizes monomers of (R)-3-hydroxyacyl-CoA thioester into polyester.

PHA synthesis begins with binding of PHA synthase (PhaC) to the periplasmic membrane of the cell (Stubbe and Tian, 2003; Fig. 1.4). Phasin is the most abundant granule-associated amphiphilic protein which is responsible for regulating PHA accumulation and forms proteinaceous barriers between surfaces of adjacent polymer cores thereby prevents blending of PHA granules (Banki et al., 2005). Along with phospholipids, there are number of other proteins associated with the surface of PHA granule and form monolayer of ~4 nm around the granule. It is hypothesized that these proteins protect the hydrophobic core from the cytoplasmic enzymes and also play a major role in regulating the size and number of PHA granules synthesised. PhaR is a regulatory protein bind to PHA granules. After attaining a critical granule concentration inside the cell cytoplasm and when no more binding sites are available for PhaR, it binds to gene promoters of phaP1 and phaR thereby downregulating transcription and hence reducing the rate of PHA synthesis. Pha depolymerase is granule associated and plays a role is depolymerising the PHA chains. However its proper function is yet to be understood in details (Hiraishi et al., 2010).

PHB homopolymer are mostly obtained if monosaccharide sugars such as glucose (hexose) and fructose (pentose) are processed via glycolysis / Embden–Meyerhof pathway. However, copolymers could be produced, if fatty acids or sugars are metabolized by other pathways such as fatty acid β-oxidation, fatty acid *de novo* biosynthesis, pentose phosphate pathway (Aldor and Keasling 2003; Steinbuchel and Lutke-Eversloh 2003). Interestingly, there are only a limited number of bacteria belonging to the genus *Pseudomonas* such as *P. putida* strain U, *P. putida* strain BM01 and *P. oleovorans*, reported to produce PHA containing aromatic monomers by utilizing aromatic hydrocarbons as a carbon and energy source (Ward and O’Connor, 2005; Abraham et al., 2001).

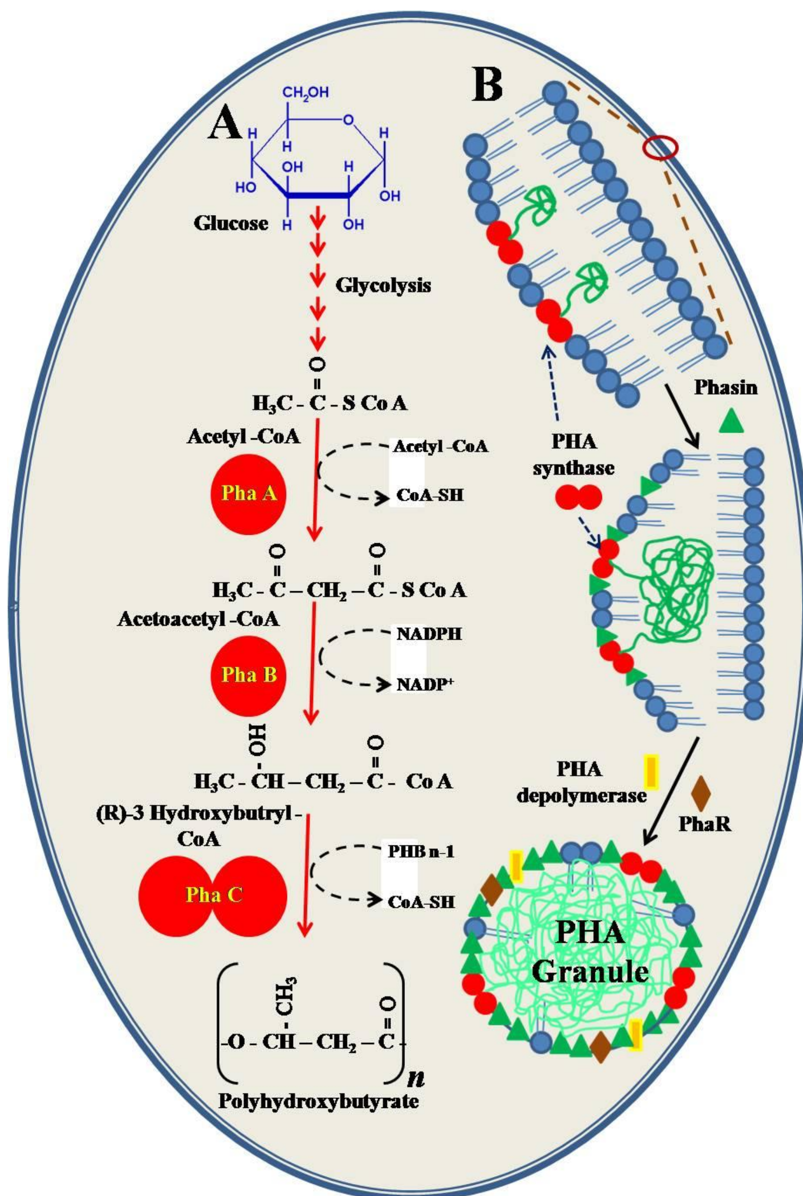

Fig. 1.4 General scheme of PHA synthesis in a microbial cell. (A) Biosynthesis pathways for synthesis of poly(3-hydroxybutyrate) (PHB) obtained from Glucose via Embden–Meyerhof pathway. (B) Mechanism of PHA synthesis in association with the cytoplasmic membrane by budding model.

1.3.5 Industrial agro-industrial wastes as substrates for PHA production

1.3.5.1 Sugarcane Bagasse

The sugarcane (genus *Saccharum*, family *Poaceae*, tribe *Andropogoneae*), world's largest cash crop, is a tall grass (2 – 6 meters), long with stout jointed fibrous stalks that are rich in sugar and used for sugar production. Sucrose is the main product of sugarcane, which is

accumulated in the internodes of stalks. Sucrose is extracted and purified in specialized mill factories and is mainly used in human food industries and / or is fermented to produce ethanol (Brazilian sugarcane industry). Apart from sugar, it is also used for the production of jaggery, ethanol, molasses, alcoholic beverages (rum), soda, etc, (Parameswaran, 2009).

The fibrous residue left after crushing and extraction of its juice is referred as bagasse, more specifically called as sugarcane bagasse (SCB). India is the world's second largest producer of sugarcane with Brazil being the world's leader thereby making SCB as one of the major cellulosic agro-industrial by-product in the world (Loh et al., 2013; Hernández-Salas et al., 2009). It is mostly used as source of energy, electricity / biogas production, pulp and paper production, fermentation products such as enzymes (cellulose, lipase, xylanase, inulinase, amylase), animal feed (single cell protein), amino acids, organic acids, bioethanol, bioplastics, etc. (Pippo and Luengo, 2013; Parameswaran, 2009).

SCB consists of approximately 46 % cellulose, 27 % hemicelluloses, 23 % lignin and 4 % ash (Pippo and Luengo, 2013). Lignocellulose material (LCM) is a complex carbohydrate polymer of cellulose, hemicellulose and lignin. Cellulose is a homopolymer of repeating glucose units linked by β-1,4 glycosidic bonds, forming cellobiose that is repeated several times in its chain and is linear and crystalline. Hemicellulose is a heteropolymer composed of hexoses (D-glucose, D-galactose, and D-mannose) and pentoses (D-xylose and L-arabinose) and is short and highly branched polymer. The lignin is tightly bound to these two carbohydrate polymers and is hydrophobic in nature, thus protecting the polymers from microbial attack (Sarkar et al., 2012). Lignin is formed by radical polymerization of three phenyl-propane alcohols such as p-coumarilic, coniferilic, and synapilic thereby making it a complex aromatic macromolecule (Canilha et al., 2012).

SCB can be converted to monomeric sugars at temperatures below 200°C and low acid concentrations. Such conditions have also been described as pretreatment methods for enzymatic hydrolysis of cellulose. While most industrial fermentation processes are based mainly on hexoses, the use of hemicellulose as a source of carbohydrates is gaining interest over the past few years. However, most of the microorganisms used in traditional processes are not able to ferment the pentose sugars, which are set free during hemicellulose

hydrolysis. To overcome this obstacle, a number of strains have been developed that are able to ferment pentoses to valuable chemical products such as ethanol, organic acids, solvents, and xylitol (Neureiter et al., 2002).

1.3.5.2 Coconut oil cake

Coconut oil cake (COC) is a byproduct obtained after oil extraction from dried copra (family *Arecaceae*; species *Cocos nucifera*). COC is generally fed to animals and finds no other application. It contains starch, soluble sugars, soluble proteins, lipids and trace amounts of nitrogen (Ramachandran et al., 2004). Solid state fermentation (SSF) has been tried using COC for different applications in bioconversion processes. Selvakumar et al., (1998) studied the production of glucoamylase by *Aspergillus niger* under SSF. Phytase, a feed enzyme, has been produced on COC by *Rhizopus oligosporus* (Sabu et al., 2002). *Candida rugosa* was cultivated on COC for lipase production by Benjamin and Pandey, (1996). COC served as a good substrate, which not only provided nutrients but also good surface area for proper growth and aeration (Benjamin and Pandey, 1997, 1998).

1.3.5.3 Sagostarch waste

Before 1940s, Starch from tapioca or cassava was primarily produced in countries like Brazil, Malaysia, Thailand and other tropical and subtropical regions (Bujang, 2008; Dufour et al., 2002). But the ban on import of starch during the Second World War prompted people of India to find alternatives to meet the demand. Salem, a district in Tamil Nadu spearheaded the problem by starting the first industry in 1943 for the production of sago starch from tapioca. Accompanied by the favourable climatic conditions for the production of tapioca in the surrounding areas, the starch industry grew steadily and now it has about 650 units constituting 89% of entire country's production. The increase in the industry also meant an increase in the cultivation of tapioca, making Salem as the highest tapioca productivity region in world with 25 ton/hectare against worldwide value of 10 ton/hectare (Thanuskodi, 2010). Starch and sago produced have found their use in cuisines, textile, laundry, detergent, adhesive manufacturing and other industries.

The important goal of tapioca processing is the release of the starch granules embedded within the cellular constituents (Oyewole and Obieze, 1995). The initial process involves

peeling of the tapioca skin, followed by immersion into the water tanks for washing and removing dirt adhering on the surface of tubers. Next step is the grating of washed tubers in which the tubers are crushed, releasing the starch granules. Then separation of starch from the pulp is done by mixing the grated tubers with water forming slurry followed by separation of crude pulp which is is dried and used as cattle fodder. The slurry left behind will be processed further for obtaining starch by allowing the crude slurry to sediment in a tank. More often the top layer of sediment will have a yellowish green tint and contain lot of impurities. This layer is removed as it degrades the starch quality. The remaining slurry is stirred again and allowed to settle. At the final step, the slurry mostly consisting of starch is recovered and dried to remove excess water and pulverised (Thanuskodi, 2010; Fish and Trim, 1993; Sanni et al., 2005).

Waste products are formed during various stages of sago starch manufacturing. The fibrous material left behind during starch production and the effluent from the setting tanks are the major pollutants originating from sago starch industries. Though it is being used as a cattle fodder supplement, high amount of silicaceous material hinders its use at a larger scale. Hence, large amount of this fibre remains unused contributing to a major environmental problem. Therefore treatment of the solid wastes become a major problem and needs immediate addressing (Thanuskodi, 2010).

The above mentionaed agro-industrial wastes could be used as substrates for the production of PHA by halophilic microorganisms. This can help one to manage the waste as well as to produce value added product.

1.3.6 Applications of industrially available PHAs

PHAs are potentially emerging as the next generation of environmentally friendly materials and have myriads of applications. Currently, microbial fermentations using recombinant *Escherichia coli* are being used for PHAs production for commercial applications (Zhou et al., 2011; Quillaguamán et al., 2010; Philip et al., 2007; Nikel et al., 2006; Margesin and Schinner, 2001).

1.3.6.1 BIOPOL

BIOPOL is a co-polymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) was initially manufactured by Imperial Chemical Industries (ICI) and currently produced by Metabolix (Cambridge,MA, USA). This polymer can be applied to coat paper / paperboards, blow moulding and film production. It is used for electric and electronic packaging as it has antistatic properties. It has been used for medical applications by companies like Fluka and Toray (Clarinval and Halleux, 2005). Studies have shown that, fishing nets, ropes are made from monofilaments and fibers of BIOPOL exhibit good strength and are naturally biodegradable in the sea.

1.3.6.2 Nodax

Nodax is a PHA copolymer consisting of 3-hydroxybutyrate (3-HB) and a comparatively small quantity of medium chain length PHA (mcl-PHA) monomers such as 3-hydroxyhexanoate (3-HHx), 3-hydroxyoctanoate (3-HO) and 3-hydroxydecanoate (3-HD). For commercial applications, the PHA should be tough and ductile, having low crystallinity and melting temperature, which is obtained by incorporating mcl-PHA monomers (Noda et al., 2005). This polymer has been developed by Procter and Gamble and promises anaerobic and aerobic degradability, hydrolytic stability and elastic and mechanical properties to suit specific needs. It is available as foams, fibres or nonwovens, films and latex among others. This polymer can be used to make hygienic wipes / tampon, medical surgical garments, carpet, packaging, compostable bags and lids (Philip et al., 2007).

Fifty thousand tonnes of the 50 million tonnes of European polymer market comprises of bioplastics (Philip et al., 2007). Biodegradable shopping bags are used in many chains of supermarkets such as Sainsburys and Tesco (Carmichael, 2006; Valappil et al., 2006; Chen and Wang, 2002). Using various animal models, many successful studies have clearly demonstrated that PHB, PHBV, P4HB, PHO, PHBHHx, etc possess the biodegradability, biocompatibility and thermoprocessibility for implant as well as controlled drug release applications (Chen and Wu, 2005). Depending on the applications, the mechanical properties or biocompatibility of PHA polymer can be modified either by blending or doing surface modification and / or making composits with other polymers, enzymes or even

inorganic materials (Wang et al., 2014). Recent study by Han et al., (2015) on haloarchaeon *Haloferax mediterranei* reported the biosynthesis of random / higher-order copolymers PHBV (R-PHBV / O-PHBV) produced by cofeeding valerate with glucose. The O-PHBV film had rough surface which exhibited increased platelet adhesion thereby accelerating blood clotting. Another report by Danis et al., (2015) employed *Natrinema pallidum* for P(HB-*co*-HV) produced which was used to prepare biocompatible films for drug delivery (rifampicin) by blending with polyethylene glycol.

1.4 Halophilic microorganisms accumulating PHA

So far, there have been relatively few studies on the PHA production from halophilic microorganisms as compared to their non halophilic counterparts (Yin et al., 2014; Cai et al., 2011).

1.4.1 Halophilic bacteria

Halophilic bacteria, mostly members of the family *Halomonadaceae* namely *Halomonas boliviensis*, *H. elongata* are known to accumulate PHAs. So far, *H. boliviensis* is the best strain to be reported for providing highest yields of PHA. Members of the genus *Halomonas* are also reported to co-produce PHA and osmolytes, like ectoines and hydroxyectoine in a combined process (Quillaguamán et al., 2010). Neverthless, other halophilic and halotolerant bacteria belonging to the Genus *Bacillus*, *Yangia*, *Alcalilimnicola*, etc. are also known to produce PHA (Salgaonkar et al., 2013; Van-Thuoc et al., 2012; Yakimov et al., 2001). Table 1.4 gives the details of the various halophilic bacterial strains reported to produce PHA using varying substrates.

Table 1.4 Accumulation of PHA by moderately and extremely halophilic bacteria using commercial substrates.

Halophilic bacteria	Substrate	PHA Type	Yield	Reference
Halomonas boliviensis LC1	Starch hydrolysate	PHB	56.0[a]	Quillaguamán et al., 2005
H. boliviensis LC1	Maltose	PHB	58.8[a]	Quillaguamán, et al., 2005
Halomonas sp. SK5	Glu	PHB	1.2 $\pm$ 0.3[b]	Rathi et al., 2013
Halomonas sp. SK5	OPTS	PHB	1.6 $\pm$ 0.2[b]	Rathi et al., 2013
H. nitroreducens	Glu	PHB	33.0[a] / 0.8[b]	Cervantes-Uc et al., 2014

Halophilic bacteria	Substrate	PHA Type	Yield	Reference
Bacillus megaterium	Glu	PHB	39.0[a]	Salgaonkar et al., 2013
Halomonas boliviensis	Glu	PHB	68.5[a] / 5.8[b]	Guzmán et al., 2009
Bacillus strain ND97	Glu / Glu + Pro	PHB/ PHBV	48[a] / 53[a]	Van-Thuoc et al., 2012
Bacillus strain ND153	Glu / Glu + Pro	PHB/ PHBV	65[a] / 71[a]	Van-Thuoc et al., 2012
Bacillus strain QN194	Glu / Glu + Pro	PHB	26[a] / 11[a]	Van-Thuoc et al., 2012
Yangia pacifica ND199	Glu / Glu + Pro	PHBV	34[a] / 12[a]	Van-Thuoc et al., 2012
Y. pacifica strain ND218	Glu / Glu + Pro	PHBV	24[a] / 11[a]	Van-Thuoc et al., 2012
Y. pacifica strain QN271	Glu / Glu + Pro	PHB/ PHBV	48[a] / 31[a]	Van-Thuoc et al., 2012
Y. pacifica strain QN187	Glu / Glu + Pro	PHB/ PHBV	44[a] / 27[a]	Van-Thuoc et al., 2012
Y. pacifica strain ND240	Glu / Glu + Pro	PHB/ PHBV	28[a] / 12[a]	Van-Thuoc et al., 2012
Alcalilimnicola halodurans	Glu	PHB	-	Yakimov et al., 2001
H. campisalis MCM B-1027	Maltose + YE	PHBV	45-81[a]	Kulkarni et al., 2010
H. maura		PHB		Bouchotroch et al., 2001
H. boliviensis	Sucrose + YE	PHB	54[a]	Quillaguaman et al., 2007
H. boliviensis	Glu + MSG	PHB	90[a]	Quillaguaman et al., 2008
H. salina	Glycerol	PHB	55.0[a]	Mothes et al. 2008
H. marina HMA 103	Glu	PHB	59.0[a]	Biswas et al. 2009
H. boliviensis LC1	Glu	PHB	56.0[a]	Quillaguamán, et al 2006
H. boliviensis	Glu	PHB	81.0[a]	Quillaguamán, et al 2008
H. boliviensis	WBH	PHB	34.0[a]	Van-Thuoc et al., 2008
H. boliviensis	Glu + Xylose	PHB	45.0[a]	Van-Thuoc et al., 2008
H. boliviensis	Glu	PHB	30.0[a]	Van-Thuoc et al., 2008
H. boliviensis	Glu + Butyric acid	PHB	50.0[a]	Van-Thuoc et al., 2008
B. sonorensis SM-P-1S	*Jatropha* biodiesel byproduct	PHB	71.8[a]	Shrivastav et al., 2010
H. hydrothermalis SM-P-3M	*Jatropha* biodiesel byproduct	PHB	75.0[a]	Shrivastav et al., 2010
Halomonas TD01	Glu	PHB	65-70[a]	Tan et al., 2011
H.campaniensis LS21	C/S/L/P	PHB	26.0[a]	Yue et al., 2014
Bacillus sp. ND153	Glu + Pro	PHBV	79[a]	Van Thuoc and Quillaguamán, 2014

a: % w/w of cell dry weight; b: g L^{-1}; CDW: cell dry weight; PHB: poly (3-hydroxybutyrate); PHBV: poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate); MSG : monosodium glutamate; YE : yeast extract; Glu : glucose; Pro : Propionate; OPTS: oil palm trunk sap; WBH : Wheat bran hydrolysate; C/S/L/P: cellulose/starch/lipids/proteins.

1.4.2 Halophilic archaea

As mentioned earlier, section 1.1.3, extremely halophilic archaea belonging to the family *Halobacteriaceae* contains 49 genera and 182 species which is the largest archaeal group (as of November 2014). Accumulation of PHA in halophilic archaea was first reported by Kirk and Ginzburg (1972) in *Haloarcula marismortui* much later after the discovery of PHA in *Bacillus subtilis* by Lemoigne (1923). Further screening of other halophiles has shown that halophilic organisms belonging to the eubacterial family *Halomonadaceae* and archaeal family *Halobacteriacea*, genera *Halococcus*, *Halorubrum*, *Natronobacterium*, *Natronococcus, Halopiger*, *Haloarcula*, *Halobiforma*, *Haloferax*, and *Halobacterium* are capable of accumulating PHA (Quillaguaman et al., 2008, 2010; Legat et al., 2010; Poli et al., 2011; Lynch et al., 2012). Some halophilic archaea such as *Haloterrigena hispanica*, *Haloquadratum walsbyi*, *Halorhabdus tiamatea*, *Halorhabdus utahensis*, and *Natrinema altinense* have also been reported to synthesize PHA. However, polymer has neither been quantified nor characterized (Antunes et al., 2008b; Burns et al., 2007; Romano et al., 2007; Waino et al., 2000; Xu et al., 2005). Among haloarchaea, *Haloferax mediterranei* so far is the best PHA producer (Poli et al., 2011). Early studies using batch and continous culture on *Hfx. mediterranei* revealed its ability to accumulate up to 65 wt % and ~ 46 wt.% PHA with respect to its cell dry weight (CDW) using starch / glucose as substrate (Rodriguez-Valera and Lillo, 1992).

Most of the microorganisms are reported to synthesise homopolymer of hydroxybutyrate (PHB) from carbohydrates and the natural synthesis of a copolymer is rare until provided with precursors (Van-Thuoc et al., 2015). However, there are few examples of bacteria like *Rhodococcus* sp. being able to synthesise copolymers [P(3HB-*co*-3HV)] in the absence of precursors like valerate (Valentin and Dennis, 1996). Recent studies have revealed the chemical structure of the PHA produced by *Hfx. mediterranei* to be co-polymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV) containing 10.7% of 3-HV when cultured by fed-batch fermentation with glucose as carbon source (Don et al., 2006). P(3HB-*co*-3HV) differs from PHB in being less crystalline, more elastic, tougher, and in having a lower melting point. Tables 1.5 and 1.6 gives the details of the various

haloarchaeal strains reported to produce PHA using commercial and agro-industrial wastes as substrates.

Table 1.5 Accumulation of PHA by extremely halophilic archaea using commercial substrates.

Halophilic archaea	**Substrate**	**PHA Type**	**Yield**	**Reference**
Haloferax mediterranei	YE & Glu	PHB	17.0[a]	Fernandez-Castillo et al., 1986
Hfx. gibbonsii	YE & Glu	PHB	1.2[a]	Fernandez-Castillo et al., 1986
Hfx. volcanii	YE & Glu	PHB	7.0[a]	Fernandez-Castillo et al., 1986
Hfx. mediterranei	Glu	PHBV	23.0[b]	Huang et al., 2006
Hfx. mediterranei	Starch	PHB	6.48[b]	Lillo & Rodriguez-Valera, 1990
Hfx. mediterranei	Cas aa & YE	PHBV	1.33 ± 0.05[b]	Lu et al., 2008
Hfx. mediterranei	YE & starch	PHBV	1.74 ± 0.04[b]	Lu et al., 2008
Haloarcula hispanica	YE & Glu	PHB	2.4[a]	Fernandez-Castillo et al., 1986
Har. hispanica	YE & Glu	PHBV	0.58 ± 0.03[b]	Lu et al., 2008
Har. marismortui	YE & Glu	PHB	21.0[a]	Han et al., 2007
Haloarcula sp. IRU1	Glu	PHB	63.0[a]	Taran and Amirkhani, 2010
Har. japonica strain T5	Glu	PHB	0.5[a]	Nicolaus et al., 1999
Halococcus dombrowskii	YE & HyCase	PHB/PHBV	0.15/0.01[a]	Legat et al., 2010
Hcc. salifodinae	YE & HyCase	PHB/PHBV	0.05/0.01[a]	Legat et al., 2010
Halobiforma haloterrestris strain 135T	YE & butyric acid	PHB	40.0[a]	Hezayen et al ., 2002
Hbf. haloterrestris strain 135T	YE, Cas aa, PP	PHB	15.0[a]	Hezayen et al., 2002
Haloterrigena hispanica	YE & Cas aa	PHB	0.14[a]	Romano et al., 2007
Halopiger aswanensis strain 56	YE/sodium acetate /BA	PHB	34.0[a]	Hezayen et al., 2010; Hezayen et al., 2000
Natronobacterium gregoryi	YE & Cas aa	PHB/PHBV	0.1/0.03[a]	Legat et al., 2010
Halobacterium noricense	YE / tryptone	PHB/PHBV	0.08/0.03[a]	Legat et al., 2010
Halogeometricum borinquense strain TN9	Glu	PHB	14.0[a]	Salgaonkar et al., 2013
Hgm. borinquense strain E3	Glu	PHBV	73.5[a]	Present study
Natrinema pallidum 1KYS1	Starch	PHBV	53.14 [a]	Danis et al., 2015

PHB: poly (3-hydroxybutyrate); PHBV: poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate); YE: yeast extract; Glu: glucose; Cas aa: casamino acids; PP: protease peptone; a: % w/w of cell dry weight; b: g L^{-1}

Table 1.6 Accumulation of PHA by extremely halophilic archaea using inexpensive agro-industrial wastes.

Halophilic archaea	Substrate	PHA Type	Yield	Reference
Haloferax mediterranei	Stillage using fresh salts	PHBV	16.42[b]	Bhattacharyya et al., 2014
Hfx. mediterranei	Stillage using recovered salts	PHBV	16.25[b]	Bhattacharyya et al., 2014
Hfx. mediterranei	25 % pre-treated vinasse	PHBV	19.7[b]	Bhattacharyya et al., 2012
Hfx. mediterranei	50 % pre-treated vinasse	PHBV	17.4[b]	Bhattacharyya et al., 2012
Hfx. mediterranei	ECS	PHBV	24.2[b]	Huang at al., 2006
Hfx. mediterranei	ERB/ECS	PHBV	77.8[b]	Huang at al., 2006
Hfx. mediterranei	EWB/ECS	PHBV	52.7[b]	Huang at al., 2006
Hfx. mediterranei	NWB/ECS	PHBV	28.0[b]	Huang at al., 2006
Hfx. mediterranei	Hydrolyzed whey	PHBV	5.5[b]	Koller et al., 2007
Hfx. mediterranei	Hydrolyzed whey	PHBV	12.2[b]	Koller et al., 2008
Hfx. mediterranei	Hydrolyzed whey	PHBVB	14.7[b]	Koller et al., 2008
Haloarcula sp. IRU1	Petrochemical waste water	PHB	46.0[a]	Taran, 2011
Har. marismortui	10% raw vinasse	PHB	2.8[b]	Pramanik et al., 2012
Har. marismortui	100% Pre-treated vinasse	PHB	4.5[b]	Pramanik et al., 2012
Har. japonica strain T5	Molasses	PHB	1.0[a]	Nicolaus et al., 1999
Haloterrigena hispanica	Carrot waste	PHB	0.13[a]	Di Donato et al., 2011

a: % w/w of cell dry weight; b: g L^{-1}; CDW: cell dry weight; PHB: poly (3-hydroxybutyrate); PHBV: poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate); PHBVB: poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate); ECS: extruded cornstarch; ERB: extruded rice bran; EWB: extruded wheat bran; NWB: native wheat bran.

1.5 Gaps in existing research

Halophiles are less explored group of extremophilesconstituting a huge untapped reserve of biotechnological metabolites. With majority of halophilic diversity in Indian solar salterns unexplored, it is the need of the hour to delineate the inherent microbial lineages and harness their biotechnological potential.

One of the major drawbacks of employing polyhydroxyalkanoates in wide range of applications is its high production and extraction costs. It is required to reduce the

production cost of PHA by improving the microbial strain, efficient fermentation strategies, recovery processes and use of low-cost raw materials. Screening of organisms from diverse eco-niches may lead to the detection of novel unidentified polymer producers. In this aspect, halophiles have an edge over their non-halophilic counterparts due to growth at high salinities.

From the existing literature, it is evident that not many microorganisms are known for producing or accumulating compatible solutes (Lentzen and Schwarz, 2006). Many of these compatible solutes like ectoine and hydroxyectoine have important biotechnological applications. Since halophiles, especially halophilic bacteria are known for accumulating compatible solutes, it is of importance to screen halophiles for novel compatible solutes.

Therefore it is proposed to isolate, identify and screen culturable halophilic bacteria and archaeafor their ability to accumulate polyhydroxyalkanoates and compatible solutes.

1.6 Aims and objectives of the research work

Research on PHA is gaining momentum, primarily because it is an eco friendly green material. Being completely biodegradable and biocompatible and properties similar to plastics could be a replacement for environmental non-friendly plastics obtained from non-renewable petrochemical sources. It has also been used in various applications like being used as a coating material on paper, cardboard, or food trays to form a water-resistant layer, an alternative to commercial plastics such as polyethylene (Anderson and Dawes 1990, Steinbüchel and Füchtenbush; 1998). Though, PHAs have been commercially produced for more than two decades, there are many roadblocks in manufacturing the product such as high fermentation costs, lack of industrial infrastructure, etc.

In PHA production processes using microorganisms, the carbon source / substrates employed affects the overall production costs significantly (Van-Thuoc et al., 2008). Therefore, to improve the overall economy of the process, one need to substantially reduce the fermentation costs and this can be achived, by making use of cheap and readily available agro-industrial wastes as carbon sources (Van-Thuoc et al., 2015; Verlinden et al., 2011; Ramadas et al., 2009).

In view of this, the following objectives were undertaken for study,

(i) Screening of the halophilic isolates for accumulation of polyhydroxyalkanoates (PHA) and compatible solutes.

(ii) Characterization of promising halophiles by phenotypic, chemotaxonomic and phylogenetic analysis.

(iii) Optimization, extraction and characterization of the PHA and compatible solutes.

Chapter II

Solar Salterns of India and Isolation of Halophilic Microorganisms

2.1 Introduction

Halophilic microorganisms are known to thrive in various low saline eco-niches such as sea water, marine sediments and predominantly in high-salt environments such as solar salterns, the saline lakes, soda lakes, deep-sea brines as well as other unusual environments like hides, fermented sea food, salted fish, etc (Fukushima et al., 2007; Purdy et al., 2004; Bragança and Furtado, 2009; Ma et al., 2010; Birbir et al., 2004; Roh et al., 2010). A solar saltern is a man-made ecosystem providing a salinity gradient for the production of solar salt (Javor, 2002). Salinity in the saltern varies from 0.5 – 0.86M (3-5%) NaCl in the reservoir pan to saturation ie above 4.28M (25%) NaCl in crystallizer pans (Mani et al., 2012b). Thus, microbes inhabiting these salt pans are divided into extreme halophiles (3.0–5.0M NaCl), borderline extreme halophiles (2.0–3.0M NaCl), moderate halophiles (0.5–2.0M NaCl) and halotolerant organisms (0.2M NaCl) (Oren, 2008). Solar salterns harbor organisms ranging from prokaryotes like bacteria (*Salinibacter ruber*) and archaea (family *Halobacteriaceae*) to eukaryotes like algae (*Dunaliella salina*) and fungi (*Hortaea* species, *Eurotium* species) (Gunde-Cimerman et al., 2009; Oren, 2002b). There are only preliminary studies on the isolation and characterization of halophilic archaea, bacteria and fungi from solar salterns of Goa and Tamil Nadu (Bragança and Furtado, 2009; Nayak et al., 2012; Mani et al., 2012a and Manikandan et al., 2009). Salterns of India are yet to be documented for microbial diversity and explored for halophilic microorganisms having potential biotechnological applications. In this chapter, the isolation, purification, phenotypic and chemotaxonomic characterization of moderately halophilic bacteria and extremely halophilic archaea has been reported.

2.2 Materials and methods

2.2.1 Sampling sites, sample collection and analysis

Solar salterns (referred to as Mitagars in Konkani and Uppalam in Tamil) along (a) the West coast of India: Ribandar and Siridao in Goa and (b) East coast of India: Marakkanam in Tamil Nadu, were sampled during the peak salt harvesting phase in the months of April to May in 2009-2011. Saline water samples (1L) were collected from respective salterns in clean plastic bottles by rinsing them with the same water prior to collection. Approximately

8-10 grams of sediment samples (approximately 5-10 cm from the surface) were collected in self sealing plastic bags using a clean spatula. The samples were stored at 4°C (cold room) if required prior to analysis or processed immediately. pH and salinity of the samples was determined at the sampling site using pH paper and Baumé hydrometer (Mani et al., 2012b).

2.2.2 Isolation, purification and maintenance

2.2.2.1 Moderately halophilic bacteria

Sediment samples were diluted (10^{-4}, 10^{-5} and 10^{-6}) in 5% NaCl solution whereas water/brine samples were directly surface spread plated, 100 µl of each on halophile medium (HM) and moderate halophilic medium (MHM) (Table 2.1) (Roeβler and Müller, 2002; Ventosa et al., 1982). The plates were incubated at room temperature (28°C) for 1-2 days and analysed for the total viable bacterial load. The colonies from the plates were picked, and surface streaked several times on the respective medium until a pure culture was obtained.

2.2.2.2 Extremely halophilic archaea

Four different media were used for selecting extremely halophilic archaea, all containing 25% (w/v) NaCl. They are (a) NaCl Tryptone Yeast Extract (NTYE) medium (b) NaCl Tri-Na-citrate (NT) medium, (c) Japan Collection of Microorganisms 168 (JCM168) and (d) Extremely Halophilic medium (EHM) (Bragança and Furtado, 2009; Elevi et al., 2004; Minegishi et al., 2008) (Table 2.1). Two methods namely (i) direct plating and (ii) enrichment techniques were employed for the isolation of haloarchaeal organisms from water and sediment samples. In direct plating method, 100 µl of water sample or a loopful of sediment sample (~ 0.1 gm) was directly surface spread plated on respective medium. In the enrichment technique method, one ml of water sample or one gram of sediment sample was aseptically transferred to 50 ml of respective liquid medium and incubated at 37°C for up to 5-10 days. Then, 10 µl aliquots were surface spread plated on media. Plates were incubated at room temperature (30°C) for 30 to 45 days in self sealing plastic bags until colonies appeared.

Table 2.1 Composition of maintenance media used in the study.

Ingredients (gL^{-1})	Maintenance media					
	Extreme halophilic archaea				**Moderate halophilic bacteria**	
	NTYE	**NT**	**EHM**	**JCM168**	**MHM**	**HM**
NaCl	250.0	250.0	250.0	200.0	178.0	62.6
$MgSO_4.7H_2O$	20.0	20.0	20.0	20.0	1.0	6.0
KCl	5.0	2.0	2.0	2.0	2.0	-
Tryptone	5.0	-	-	-	-	-
Yeast Extract	3.0	10.0	10.0	5.0	10.0	1.5
Beef Extract	-	-	-	-	-	1.5
Tri-Sodium citrate	-	3.0	-	3.0	-	-
$CaCl_2.2H_2O$	-	-	0.36	2.0	0.36	-
NaBr			0.23	-	0.23	-
$NaHCO_3$	-	-	0.06	-	0.06	-
Peptone	-	-	5.0	-	5.0	5.0
$FeCl_3.6H_2O$	-	-	Trace	0.036	Trace	-
Casamino acids	-	-	-	5.0	-	-
Sodium Glutamate	-	-	-	1.0	-	-
$MnCl_2.2H_2O$	-	-	-	Trace	-	-

pH was adjusted to 7.0-7.4 using 1M NaOH. 1.5-1.8% agar agar was use as solidifying agent. EHM medium is modification of MHM by increasing the concentration of NaCl and $MgSO_4$.

2.2.3 Phenotypic and chemotaxonomic characterization of the halophilic isolates

2.2.3.1 Morphological characterization

(i) Colony characteristics

Each of the halophilic isolate was surface streaked (quadrant streak) on the respective solid agar medium so as to get isolated colonies. Colony size, shape, margin, pigmentation, consistency, elevation and opacity were determined.

(ii) Phase contrast microscopy

Gram staining for moderate halophilic bacterial isolates was carried out (Appendix I). Smears of log phase cultures were prepared on clean grease free glass slide, air dried and heat-fixed. The smear was stained with primary stain, crystal violet for 1 min. The stain was discarded and smear covered with Gram's iodine for 30 sec. The smear was rinsed with water, then decolorized with 70% ethanol for 30 sec. Finally, counter stained with safranine for 1 min. Rinsed with water, dried and examined the slide under oil immersion objective (100 X) of the phase contrast microscope (Olympus BX41). Endospore staining was done according to Schaeffer and Fulton (1933). Briefly, smears were prepared as described above and were flooded with malachite green stain (Appendix I). This was steamed by placing the slide on glass beaker containing boiling water, so that the stain penetrates the endospores. The beaker containing the smear was continuously heated and maintained at 60 to 70 °C for 10 min. The stain was discarded and smear washed with water and stained with safranine stain for 3 - 4 min. The slides washed with water, dried and observed under oil immersion objective (100X). The extremely halophilic cell smears were prepared in a drop of 20% (w/v) NaCl solution and air-dried. The cells were desalted with 2% acetic acid followed by Gram staining described as above (Appendix I, Dussault, 1955).

(iii) Scanning electron microscopy (SEM)

Cell pellets of individual haloarchaeal isolates were dispensed in NaCl synthetic medium (NSM) to an absorbance of 0.8 at 600nm (Appendix I). 50 - 100 µl of suspension was mounted onto circular glass cover slips, and air dried. The salt crystals formed on drying, were removed by rinsing the smear with 2% (v/v) acetic acid (Dussault, 1955). The smears are then fixed with 2.0% glutaraldehyde fixative (prepared in NSM) and incubated at room temperature (28-30°C) overnight. The cover slips were then exposed, to a series of increasing gradient of acetone-water, corresponding to 30%, 50%, 70% and 90% for 10 min, respectively and finally to 100% acetone, for 30 min followed by air drying. The cover slips containing the samples was placed on a stub, followed by coating with thin gold film using sputter coating device and then viewed under scanning electron microscope

(JEOL-5800 LV SEM, Japan). For moderate halophilic bacterial isolates, SEM was done as above except for the desalting step.

2.2.3.2 Response of extremely halophilic isolates;

(i) Antibiotics

Log phase haloarchaeal cultures were spot inoculated on NaCl glucose synthetic medium (NGSM, Appendix I) individually containing 100 µg ml^{-1} of streptomycin, ampicillin, erythromycin, kanamycin, novobiocin, rifampicin and penicillin (100 IU ml^{-1}) respectively. Plates were incubated at 37°C for 5-8 days. Control plate without antibiotics was maintained to compare the growth of cultures.

(ii) Pigment of eubacterial *Pseudomonas aeruginosa* SB1

One ml of the log phase indicator organism consisting of the extremely halophilic cultures BK3, BK6, BK7, BK11, BK18, BK19, BK20, BBK1 and BBK2 respectively, were aseptically transferred and mixed with 20 ml of sterile NTYE medium containing 25% NaCl and 2% agar, and poured in sterile individual plates. After the seeded agar solidified, wells (7 mm in diameter) were punched aseptically with a sterile cork borer and 100 µl of fluorescent pigment was added to each well. The plates were incubated at room temperature for 15–20 days until the orange-red growth of the indicator culture was prominent (Salgaonkar et al., 2011).

(iii) Metals and metal oxide nanoparticles

Response of extremely halophilic archaea to two heavy metals, Cadmium chloride ($CdCl_2$) and Zinc chloride ($ZnCl_2$) and Zinc oxide nanoparticles (ZnO NPs with particle size ≤ 100 nm) was studied. Stock solutions of 0.5 M $CdCl_2$ (100.66 g L^{-1}), 0.5 M $ZnCl_2$ (68.145 gL^{-1}) and ZnO NPs (40.695 gL^{-1}) prepared in deionized water and filter sterilized using a 0.22 µm filter. The ZnO NPs solution was sonicated at pulse rate of 3–5 Hz for 15 min (MicrosonTM Sonicator). From the stocks, working concentrations were prepared in sterile deionized water. Minimum inhibitory concentration (MIC) of the heavy metal and metal nanoparticles was determined by growing the cultures in metal incorporated complex

medium such as NTYE / NT. The MIC was noted by gradually increasing the concentration of metal and metal NPs in the medium until the culture ceased to grow. The concentrations of $CdCl_2$, $ZnCl_2$ and ZnO NPs employed for MIC studies were 0.1, 0.5, 1.0, 2.0 and 4.0 mM. The growth was recorded after 5–15 days of incubation (Das et al., 2014; Salgaonkar et al., 2015).

2.2.3.3 Chemotaxonomic characterization

(i) Polar lipids analysis

Cell pellets from 50 ml stationary phase, 5 days old haloarchaeal isolates were suspended in 3.75 ml methanol : chloroform (2:1, v/v) and extracted for 4-6 hrs (Bligh and Dyer, 1959; Kates, 1978). The extract (supernatant) was collected by centrifugation at 10,000 rpm for 20 min, and the pellet was re-extracted with 4.75 ml methanol : chloroform : water (2:1:0.8, v/v). The extracts (supernatants) were combined and 2.5 ml of chloroform and 2.5 ml of water was added to it, for achieving phase separation. After centrifugation, the chloroform phase containing the lipids was collected in clean dry glass vial and dried by evaporation. Lipids were re-dissolved in 100 µl of chloroform and spotted on to silica gel plates (Silica gel 60 F254, Merck) using fine glass capillary tubes. The polar lipids were separated by single development using the solvent system, chloroform : methanol : acetic acid : water (85 : 22.5 : 10 : 4, v/v). Glycolipids were detected by spraying with 0.5% α – napthol in 50% methanol-water followed by 5% H_2SO_4 in ethanol and spots were visualized by heating the TLC plate at 100^oC. Phospholipids were detected by spraying separate set of plate with molybdenum blue spray reagent (Sigma-aldrich).

(ii) Pigments analysis

(a) Extraction

Haloarchaeal cells from stationary phase (6-8 days old) were harvested by centrifugation at 8000rpm for 10 minutes (Eppendorf Centrifuge). One set of the cell pellet was resuspended in acetone and the second set in methanol followed by chloroform so that the final ratio of chloroform : methanol is 2:1 (v/v). This mixture was placed on a gel rocker for 3-4 hrs until complete extraction of pigments was achieved. On centrifuging the mixture, the cell debris

appeared colourless, whereas the solvent appeared orange-red in colour. Cells of some isolates which failed to lyse and still appeared pigmented were sonicated in a glass beaker for 5-15 min using the medium probe of the sonicator, at a pulse rate of 0.5 s in acetone and/or chloroform: methanol 2:1 (v/v). The extracted pigments were scanned from 190–800 nm using a spectrophotometer (UV-2450 Shimadzu, Japan).

(b) HPLC analysis of pigment extracts

The pigment extracts were separated by high performance liquid chromatography (HPLC), using a Shimadzu UFLC and analyzed by SPDM 20A Prominence diode array detector. For this, the pigments extract was dried and redissolved in 500-µl methanol (HPLC grade), centrifuged at 10,000 rpm for 10 min for separation of cell debris and medium salts if any. 100-µl of supernatant was injected through a loop into a Phenomenex C18 HPLC column (250 mm × 4.6 mm, 5 µm) and eluted at a rate of 1ml min^{-1} by a gradient of methanol and water (Table 2.2). The elution of pigment was monitored at 450 nm. The solvent system used was Solvent A: Deionised distilled water with 0.1% Trifluoroacetic acid (TFA). pH maintained at 7.0 with ammonium acetate; Solvent B: methanol.

Table 2.2 Details of the gradient used for pigment separation and elution.

Time (min)	Flow rate (ml min^{-1})	A (%)	B (%)	Total time (min)
0-3	1	98	2	3
3-13	1	98-0	2-100	10
13-20	1	0	100	10
20-25	1	0-98	100-2	5
25-30	1	100	0	5

2.2.3.4 Biochemical characterization

(i) Moderately halophilic bacteria

Biochemical tests like utilization of various carbohydrates and other tests were conducted according to Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986). Catalase activity was determined by placing a drop of H_2O_2 (3% v/v) solution on actively grown bacterial culture. The formation of effervescence indicate positive test for catalase. A loopful of freshly grown culture was placed on a filter paper soaked with the oxidase

reagent (Appendix I). A purplish blue color change within 30 sec indicated oxidase activity. One day active culture was stab inoculated with the help of straight nichrome wire in semisolid sulfide indole motility (SIM) agar medium deep (HiMedia, Lab. Pvt. Ltd.). The SIM medium was stab inoculated to determine hydrogen sulfide (H_2S) production, indole formation, and motility. After incubation for 24-48 hrs at 37 °C, xylene was added to the tube and shaken vigorously, followed by few drops of Kovac's reagent. A magenta pink to red ring at the interface indicated a positive test. The cultures were streaked on citrate agar medium slants (Appendix I) and incubated for 24 to 48 hrs. Citrate positive test is indicated by growth on the slant surface converting the medium from original green to intense prussian blue. The nitrate reduction test was performed by inoculating the culture in nitrate broth (HiMedia, Lab. Pvt. Ltd.), followed by incubation at 37 °C for 24 hrs. After growth, a few drops of Griss-Illosways reagent A + B (sulfanilic acid + α-napthylamine) (Appendix I) was added to medium. Red coloration indicated as nitrate reductase positive test. For carbohydrate utilization, stock of various sugars (10% w/v) were made, sterilized separately at 121°C for 15 minutes and added to the sterilized basal medium (containing g L^{-1} tryptone 10; NaCl 5; phenol red 0.018) to a final concentration of 1%. The sugars used were D-glucose, D-galactose, sucrose, D-fructose, ribose, D-mannitol, D-maltose, D-raffinose, L-arabinose, D-mannose, D-sorbitol and D-lactose. The tubes were incubated for 24 to 48 h at 37°C. Growth was observed by turbidity and production of acid and gas was observed by change in the colour of the medium from original red to yellow and gas bubble in Durhams tube.

The isolates were also screened for various hydrolytic enzymes using commercial media (HiMedia, Lab. Pvt. Ltd.) such as, starch agar for amylase, skimmed milk agar for protease, tributyrin agar for lipase, pectin agar for pectinase, cellulose agar for cellulase and gelatin agar for gelatinase. The organisms were spot inoculated on various media and the plates were incubated at 37°C for 24-48 hrs. To determine extracellular amylase activity, after the culture growth, plates were flooded with iodine solution (Appendix I). Clearance around the growth, against a dark blue background was taken as an evidence of amylase activity. Protease, lipase and pectinase activities were determined by observing the formation of clear zones around culture growth. Gelatinase activity was determined by observing

liquefaction of the agar around the growth. Cellulase activity was determined by flooding the plate with methyl red for 5 min followed my washing it with 0.1N HCl. Transparent zones around the culture against red background indicated cellulose positive. Appropriate positive and negative controls were maintained for all of the above tests, and experiments were executed in triplicates.

(ii) Extremely halophilic archaea

Biochemical tests for extremely halophilic archaeal isolates tests were carried in accordance with proposed minimal standards (Tindall et al., 2010; Oren et al., 1997). The carbohydrate utilization tests were similar to halophilic bacteria except, that Norberg and Hofstein (NH) was used as the basal medium for growth (Appendix I) and the results were noted after 10-15 days of growth. Tests for catalase and oxidase were performed as mentioned before.

The haloarchaeal isolates were screened for various hydrolytic enzymes. To determine extracellular enzyme activity, the isolates were grown for 15-20 days by spot inoculating on NGSM with various substrates like starch (1% w/v) for amylase, gelatin (0.4% w/v) for gelatinase, skimmed milk (1% w/v) for protease, olive oil (0.1% w/v) for lipase, Tween 20 and 80 for esterase. Amylase and protease activity was determined as mentioned above. However, lipase activity was determined by checking for orange fluorescence after flooding the plate with rhodamine B (0.001% w/v) and exposing it to UV. Esterase activity was determined by observing the formation of opaque zones around culture growth. Gelatinase activity was determined as clearance around the growth after flooding the plates with a solution of 15 % (w/v) $HgCl_2$ in 20 % (w/v) HCl (Elevi et al., 2004).

Aerobic and anaerobic reduction of nitrate to nitrite was detected as described above. Indole production was determined by adding Kovac's reagent to the NTYE broth supplemented with 1% (w/v) tryptone. H_2S formation was determined by the black sulfide precipitate in the soft agar medium containing 0.5% (w/v) sodium thiosulfite. Anaerobic growth was tested with L-arginine, KNO_3 and DMSO in screw-topped sealed vials. Appropriate positive and negative controls were maintained for all the above tests, and experiments were carried in triplicates.

2.3 Results and discussion

2.3.1 Sampling sites and samples analysis

The solar salterns selected were (a) Ribandar and Siridao in Goa along the Arabian Sea (West coast) and (b) Marakkanam in Tamil Nadu along the Bay of Bengal (East coast) of India (Fig. 2.1). These salterns are of thalasohaline origin similar to other salterns worldwide with sodium and chloride ions dominating the water. Ribandar and Siridao salterns cover an area about 184690.44 m^2 and are located on the bank of the river Mandovi in Tiswadi taluka, North Goa, India (Mani et al., 2012a, b). The solar salterns in Goa are transient and experience three phases namely, the ceased phase during June to October (monsoon period), preparatory phase from December to January, and salt harvesting phase, from February to June. During the non salt production phase, the salinity of salt pans is 3-4%, whereas the salinity reaches up to 30% during salt production phase (Mani et al., 2012a). On the other hand, the solar salterns of Tamil Nadu ie Marakkanam cover an area of 466075.59 m^2 and are located along the coast of Bay of Bengal are operational throughout the year depending on the rain fall. The salt produced here varies annually from 65000 to 75000 tonnes (Ministry of Commerce and Industry, 2012).

Fig. 2.1 Sampling sites of solar salterns of (a) Ribandar and Siridao located in Goa and (b) Marakkanam in Tamil Nadu.

Sampling was carried out in the crystallizer pond prior to and during the salt harvesting phase in April 2010 (Fig. 2.2). Sample sites with locations, climatic data like temperature, physico-chemical parameters like pH and conductivity, total cell counts on varying media, and the results are summarized in Table 2.1. The pH of the samples was neutral between 7.0-7.5 and the salinity ranged from 22 % to salt saturation (> 34% w/v). The salterns were found to be inhabited by halophilic microorganisms able to grow in wide range of NaCl and $MgSO_4$. The number of colonies varied from 60 to 4.0 x 10^4 cfu/ml for water / brine samples whereas ranged from 2.8 x 10^2 to 5.1 x 10^4 cfu/ml for sediment samples.

Fig. 2.2 Sampling sites (A-D): Salt pans of Ribandar and Siridao, Goa during the salt harvesting phase, (E, F): Tools used during the process of salt harvesting (G): Heap of consumption grade solar salt at the bank of the salt pan. The arrows indicates the dried algal mass harvested from the reservoir pan which is used as fertilizers.

2.3.2 Isolation, purification and designation of moderately and / or extremely halophilic microorganisms

Sediments and water / brine samples were spread on varying media agar plates such as NTYE, NT, EHM, JCM168, MHM and HM. On incubation for 6-8 days white, cream to yellow colonies were observed. Colonies of the moderate halophilic isolates appeared within 1-2 days, whereas the extremely halophilic isolates colonies took 10-20 days to appear. Faster growth (within 24 hrs) was seen on MHM and HM which turned denser within 2 days with white / cream / yellow colonies (Fig. 2.3). Low NaCl content of the HM and MHM medium of 6.2% and 17.8%, respectively, supports the growth of moderately halophilic microorganisms which grow faster than their extremely halophilic counterparts. Orange colonies were obtained on NTYE and JCM168 media, whereas brick red colonies along with some colourless colonies appeared on NT, EHM and MHM, respectively. Prominent light pink colonies were observed on samples from Tamil Nadu salterns. White, cream and yellow colonies were selected from MHM and HM medium whereas pink, orange, red and brick red pigmented colonies were selected from NTYE, NT and EHM. Cultures were purified through repeated streaking on respective agar media to obtain pure culture (Fig. 2.3).

The isolates were labelled as halophilic bacterial series and moderate halophile bacterial series and were thereafter maintained on HM and MHM slopes/plates at room temperature (28°C) or 4°C.

(i) H (Halophiles) series cultures (H1-H26)

(ii) M (Moderate) series cultures M2, M4, SP17, RP26 and SI3

(iii) E (Extreme) series cultures (E1-5), J1

(iv) BK (Bragança; Kabilan) series cultures (BK3, BK6, BK7, BK11, BK18, BK19 and BK20)

(v) BBK (Bragança; Bhakti; Kabilan) series cultures (BBK1 and BBK2)

(vi) BS (Bragança; Salgaonkar) series cultures (BS1, BS2, BS3, BS4, BS5, BS6, BS7, BS8, BS11, BS13, BS15, BS16, BS17 and BS19)

(vii) TN (Tamil Nadu) series cultures (TN1, TN2, TN3, TN4, TN5, TN6, TN7, TN8, TN9, TN10, TN13, TN15, TN16, TN17, TN18, TN19, TN20 and TN21).

Table 2.3 The physico-chemical and microbiological analysis of the sediments and brine samples from various saltern sampling sites.

Sample site	pH	Salinity %	Viable cell count (CFU/ml) or (CFU/gm)					
			MHM	HM	NTYE	NT	EHM	JCM
Ribandar sample								
Brine	6.2	28	2.08×10^3	9.8×10^3	1.2×10^2	2.6×10^3	60	1.4×10^4
Sediment	6.0	24.4	TNTC	5.4×10^4	2.8×10^2	1.4×10^3	2.0×10^3	2.5×10^3
Siridao sample								
Brine	7.0	28	1.4×10^3	ND	NG	NG	1.6×10^2	2.3×10^4
Sediment	6.8	19.3	TNTC	ND	1.1×10^3	6.0×10^2	4.7×10^4	5.2×10^2
Marrakanam sample								
Brine	7.2	25	3.2×10^2	ND	NG	NG	TNTC	4.0×10^4
Sediment	6.5	17	TNTC	ND	4.2×10^3	TNTC	5.1×10^4	4.6×10^2

TNTC: too numerous to count; ND: Not done; NG: No growth.

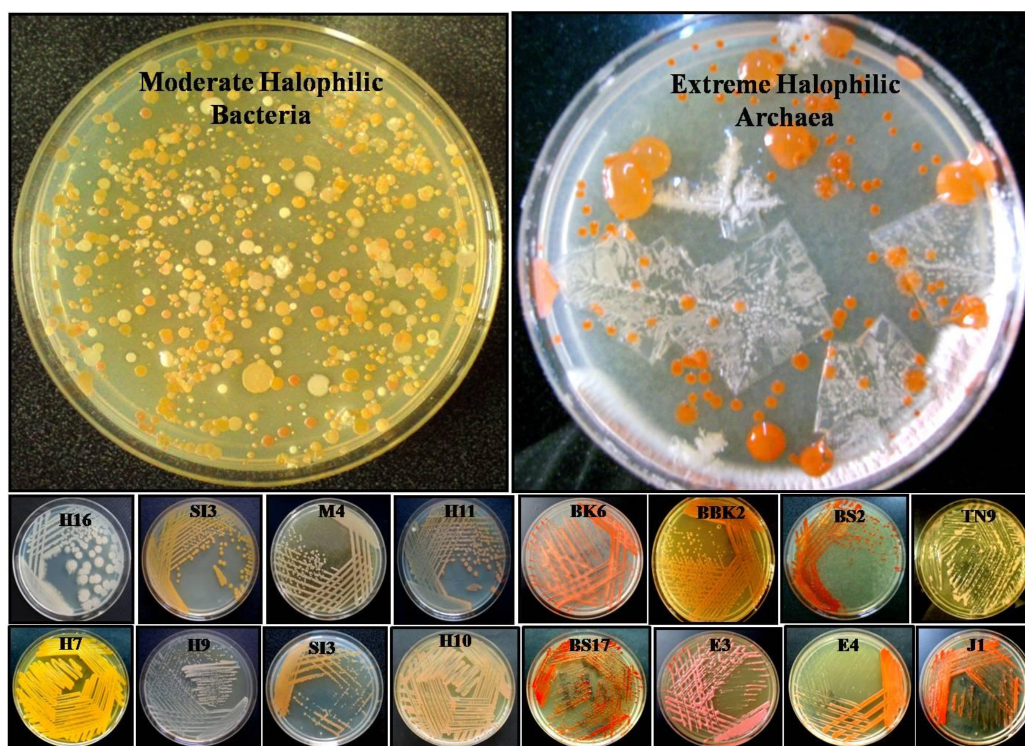

Fig. 2.3 Colony appearance and pure cultures of the moderately halophilic bacterial isolates and extremely halophilic archaeal isolates obtained from solar salterns of Goa and Tamil Nadu.

2.3.3 Morphological characterization of the isolates

Table 2.4 – 2.7 describes the colony and cell morphology of the halophilic bacterial and archaeal isolates. Among the 31 halophilic bacterial isolates obtained, 20 isolates appeared entire margin whereas 11 isolates had irregular colony morphology. The isolates showed varying pigmentation such as cream, yellow, brown and orange. Gram staining and phase contrast microscopic (100X objective) observation revealed that the cell stained uniformly Gram positive and / or Gram negative depicting its morphology as rods, short rods and cocci. The cells were mostly single and / or arranged in pairs, chains or groups.

All the forty four extremely halophilic isolates had circular colony morphology and showed varying shades of pigment such as light orange, orange, bright orange, orange red, brick red and light pink. Some of the isolates were cream (TN16, TN17 and TN18) or colourless (TN19, N20 and TN21). The isolates BBK2, BS16, TN1–7 and TN10–11 phenotypically appeared similar with light orange pigmentation and produced extracellular slimy / mucoid secretion. Interestingly, the isolates TN9 and E3 showed unique light pink pigmentation and also appeared slimy.The cells of all the 44 isolates stained uniformly Gram negative. Most of the cells were cocci with few being short rods and rods. Interestingly, some of the isolates, BS5, BS7 and BS11 showed both coccoid and rod morphology depicting their pleomorphic nature.

The SEM micrographs of some of the halophilic isolates are presented in Fig. 2.4. The cells of the moderately halophilic bacterial isolate M4 appeared as long filaments. Cells of the isolate H16 appeared as rods in chain with the cell size being ~ 4.12 x 1.72 µm in diameter. This cell morphology is characteristic of the genus *Bacillus*. Bunk et al., (2010) reported the cell size of *Bacillus megaterium* to be 4.0 µm in length with 1.5 µm diameter. Extremely halophilic archaeal isolates showed varying cell morphology and cell size. Cells of BK6 isolate appeared as cocci in pairs of ~ 1.0 µm in diameter which compared well with the cell size (0.8-1.2 µm) reported by Denner at al., (1994) for the halophilic archaeon *Halococcus salifodinae*. The isolate E3 and TN9 had similar cell morphology and appeared as irregular cocci in groups of ~ 1.21 ± 0.05 µm and 1.2 ± 0.05 µm in diameter, respectively. Interestingly, cells of the isolate BBK2 appeared involuted oval discs of ~

1.17 ± 0.05 x 0.79 ± 0.05 µm in diameter. Oren et al, (2009) described the key characteristics of various genera of the family *Halobacteriaceae.* On the basis of cell morphology of the isolates, BK6 and BBK2 were tentatively identified as *Halococcus* and *Haloferax*, respectively. Pigmentation and cell morphology of isolates E3 and TN9 correlated well with the genus *Halogeometricum* (Malfatti et al., 2009).

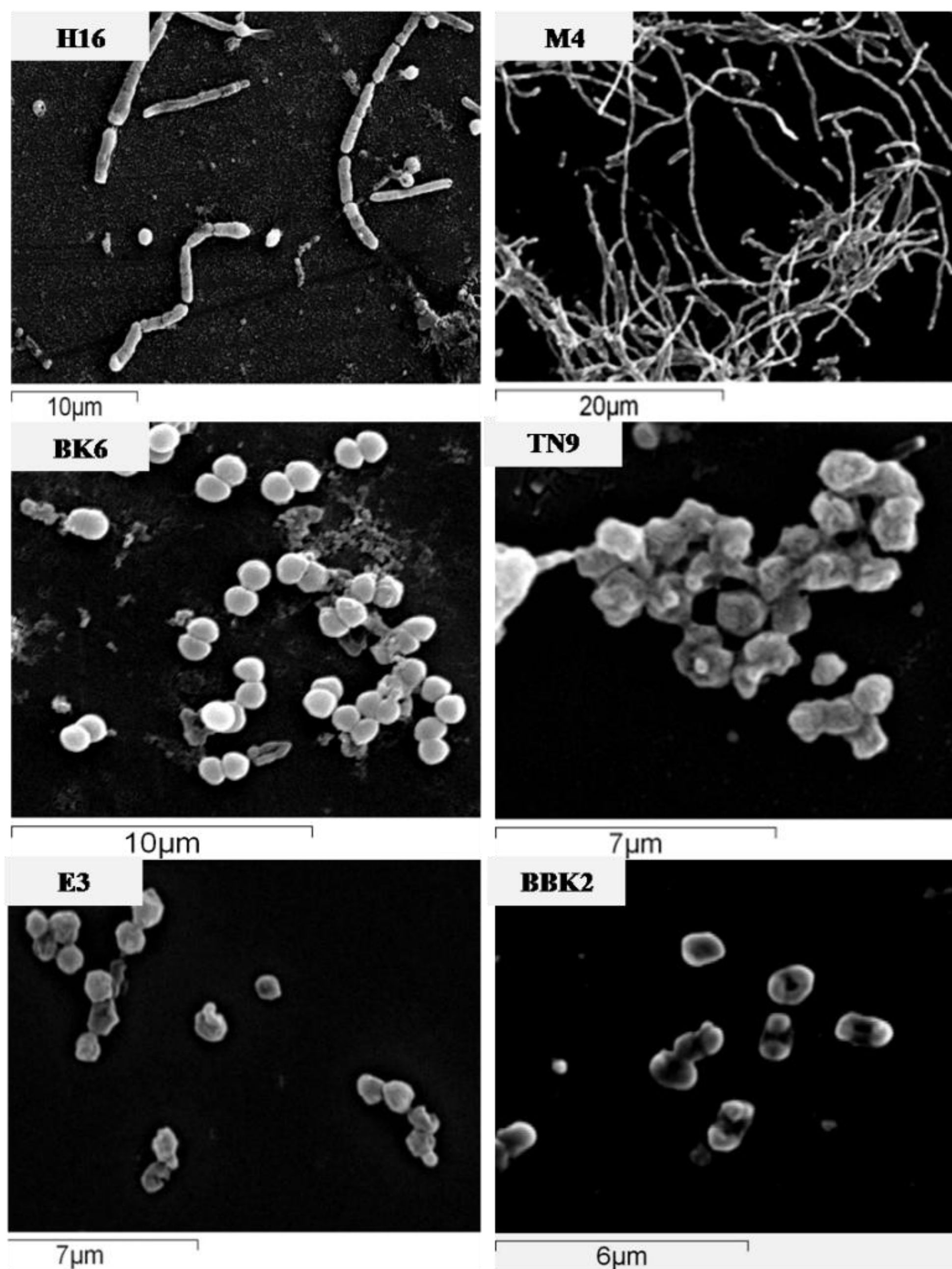

Fig. 2.4 Scanning electron micrographs of halophilic isolates grown in complex liquid medium.

Table 2.4 Characteristics of the moderately halophilic bacterial isolates.

Culture designation	Eco-niches	Colony morphology	Pigmentation	Cell Morphology	Cell arrangement	Gram Character
H1	Ribandar, Goa	Circular	Yellow	Rods	Singles / pairs	negative
H2	Ribandar, Goa	Circular	Cream	ND	ND	ND
H3	Ribandar, Goa	Circular	Cream	Cocci	Pairs / groups	negative
H4	Ribandar, Goa	Circular	White	Cocci	Pairs / groups	negative
H5	Ribandar, Goa	Circular	Brown	ND	ND	ND
H6	Ribandar, Goa	Circular	Yellow	Rod	Singles / pairs	negative
H7	Ribandar, Goa	Circular	Yellow	Short rods	Singles / pairs	negative
H8	Ribandar, Goa	Irregular	Orange	Rods	Singles	negative
H9	Ribandar, Goa	Circular	Yellow	Short rods	Singles / pairs	negative
H10	Ribandar, Goa	Irregular	Orange	Rods	Singles / pairs	negative
H11	Ribandar, Goa	Circular	Yellow	Rods	Singles / pairs	negative
H12	Ribandar, Goa	Circular	Cream	ND	ND	ND
H13	Ribandar, Goa	Circular	Cream	ND	ND	ND
H14	Ribandar, Goa	Circular	Brown	ND	ND	ND
H15	Ribandar, Goa	Irregular	Cream	Rods	Singles / chains	Positive
H16	Ribandar, Goa	Irregular	Cream	Rods	Singles / chains	Positive
H17	Ribandar, Goa	Irregular	Yellow	Short rods	Singles / pairs	negative
H18	Ribandar, Goa	Irregular	Orange	Rods	Singles / Pairs	Negative
H19	Ribandar, Goa	Irregular	Orange	Rods	Pairs / chains	Negative
H20	Ribandar, Goa	Circular	Cream	Rods	Singles / pairs	negative
H21	Ribandar, Goa	Irregular	Light orange	Rods (thick)	Singles / pairs	negative
H22	Ribandar, Goa	Irregular	Cream	Short rods	Singles	Negative
H23	Ribandar, Goa	Circular	Yellow	Short rods	Singles	Negative
H24	Ribandar, Goa	Irregular	White	ND	ND	ND
H25	Ribandar, Goa	Circular	Cream	ND	ND	ND
H26	Ribandar, Goa	Irregular	Cream	Rods	Singles / chains	Positive
M2	Ribandar, Goa	Circular	Orange	Cocci	Singles	Negative
M4	Marakkanam, TN	Circular	Cream	Rods	Singles / chains	Negative
RP26	Ribandar, Goa	Circular	Cream	Rods	Singles	Positive
SI3	Siridao, Goa	Circular	Cream	Cocci	Singles / pairs	Positive
SP17	Siridao, Goa	Circular	Cream	Cocci	Singles	Positive

ND Not done; TN Tamil Nadu

Table 2.5 Characteristics of the extremely halophilic archaeal isolates.

Culture designation	Eco-niches	Colony morphology	Pigmentation	Cell Morphology	Cell arrangement	Gram Character
E1	Marakkanam, TN	Circular	Orange Red	Discs	Singles	Negative
E2	Siridao, Goa	Circular	Red	Discs	Singles	Negative
E3	Marakkanam, TN	Circular	Light Pink	Irregular cocci	Singles/groups	Negative
E4	Ribandar, Goa	Circular	Orange	Cocci	Pairs /groups	Negative
E5	Ribandar, Goa	Circular	Orange Mucoid	Involuted	Singles	Negative
J1	Marakkanam, TN	Circular	Bright orange	Cocci	Singles	Negative
BK3	Ribandar, Goa	Circular	Orange	Cocci	Pairs/groups	Negative
BK6	Ribandar, Goa	Circular	Orange	Cocci	Pairs/groups	Negative
BK7	Ribandar, Goa	Circular	Orange	Cocci	Pairs/groups	Negative
BK11	Ribandar, Goa	Circular	Orange	Cocci	Pairs/groups	Negative
BK18	Ribandar, Goa	Circular	Orange	Cocci	Pairs/groups	Negative
BK19	Ribandar, Goa	Circular	Orange	Cocci	Pairs/groups	Negative
BK20	Ribandar, Goa	Circular	Orange	Cocci	Pairs/groups	Negative
BBK1	Ribandar, Goa	Circular	Orange	Cocci	Pairs/groups	Negative
BBK2	Ribandar, Goa	Circular	Orange	Involuted	Singles	Negative
BS1	Ribandar, Goa	Circular	Orange	Cocci	Singles	Negative
BS2	Ribandar, Goa	Circular	Orange	Short rods	Singles/pairs	Negative
BS3	Ribandar, Goa	Circular	Orange	Short rods	Singles/pairs	Negative
BS4	Ribandar, Goa	Circular	Orange red	Short rods	Singles/pairs	Negative
BS5	Ribandar, Goa	Circular	Bright orange	Cocci/Short rods	Singles/pairs	Negative
BS6	Ribandar, Goa	Circular	Orange	Short rods	Singles/pairs	Negative
BS7	Ribandar, Goa	Circular	Orange	Cocci / Short rod	Singles/pairs	Negative
BS8	Ribandar, Goa	Circular	Red	Cocci	Singles	Negative
BS11	Ribandar, Goa	Circular	Orange	Cocci / Short rods	Singles/pairs	Negative
BS13	Ribandar, Goa	Circular	Red	Rods	Pairs	Negative
BS15	Ribandar, Goa	Circular	Orange red	ND	ND	ND
BS16	Ribandar, Goa	Circular	Light orange	Involuted	Singles	Negative
BS17	Ribandar, Goa	Circular	Orange	Cocci	Singles	Negative
BS19	Ribandar, Goa	Circular	Orange	Cocci	Singles	Negative
TN1	Marakkanam, TN	Circular	Bright orange	Involuted	Singles	Negative
TN2	Marakkanam, TN	Circular	Bright orange	Involuted	Singles	Negative

ND Not done; TN Tamil Nadu

Culture designation	Eco-niches	Colony morphology	Pigmentation	Cell Morphology	Cell arrangement	Gram Character
TN3	Marakkanam, TN	Circular	Bright orange	Involuted	Single	Negative
TN 4	Marakkanam, TN	Circular	Bright orange	Involuted	Single	Negative
TN5	Marakkanam, TN	Circular	Bright orange	Involuted	Single	Negative
TN6	Marakkanam, TN	Circular	Bright orange	Involuted	Single	Negative
TN7	Marakkanam, TN	Circular	Bright orange	Involuted	Single	Negative
TN8	Marakkanam, TN	Circular	Orange red	Discs	Single	Negative
TN9	Marakkanam, TN	Circular	Light pink	Irregular cocci	Single / Group	Negative
TN10	Marakkanam, TN	Circular	Bright orange	Cocci	Single	Negative
TN13	Marakkanam, TN	Circular	Bright orange	Cocci	Single	Negative
TN15	Marakkanam, TN	Circular	Bright orange	Cocci	Single	Negative
TN16	Marakkanam, TN	Circular	Cream	Rod	Single	Negative
TN17	Marakkanam, TN	Circular	Cream	Cocci	Single	Negative
TN18	Marakkanam, TN	Circular	Cream	Cocci	Single	Negative
TN19	Marakkanam, TN	Circular	Colourless	Rod	Single	Negative
TN20	Marakkanam, TN	Circular	Colourless	Rod	Single	Negative
TN21	Marakkanam, TN	Circular	Colourless	Rod	Single	Negative

ND Not done; TN Tamil Nadu

2.3.4 Response of extremely halophilic archaea

2.3.4.1 Antibiotics

Most of the antibiotics that inhibit bacteria are non effective against archaea (Oren, 2013a). Therefore, antibiotics can be used to distinguish members of the domain archaea from that of bacteria due to unique structural / biochemical characteristics, altered antibiotic binding proteins, synthesis of antibiotic degrading enzymes and / or efflux pumps (Levy and Marshall, 2004). Most halophilic archaeal species are sensitive to bacitracin, rifampicin and novobiocin (Bonelo et al., 1984; Hilpert et al., 1981; Pecher and Bock, 1981; Gadelle and Forterre, 1994). The extremely halophilic isolates screened for their susceptibility to various antibiotics were found to be resistant to penicillin (100 IU ml^{-1}), ampicillin (100 µg ml^{-1}), kanamycin (100 µg ml^{-1}), erythromycin (100 µg ml^{-1}) and streptomycin (100 µg ml^{-1}). However, growth of the isolates was inhibited by novobiocin (100 µg ml^{-1}).

Interestingly, rifampicin (100 µg ml^{-1}) inhibited the growth of isolate BK6, BS17, TN9 and E3, whereas growth of isolates BBK2, BS2, E4 and J1 was unaltered (Fig. 2.5).

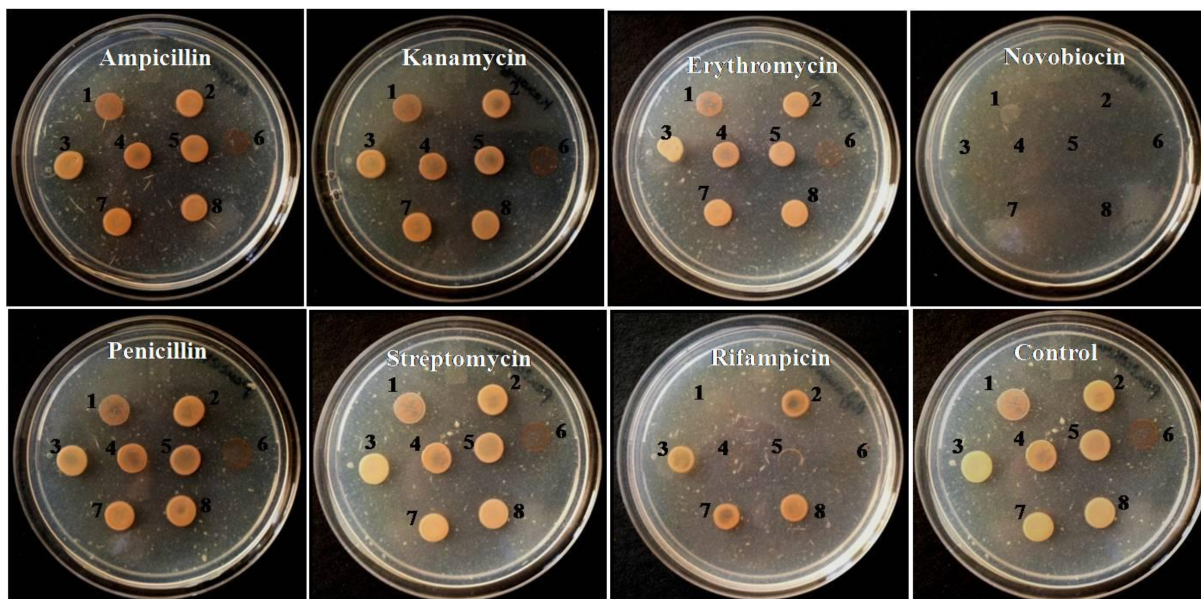

Fig. 2.5 Response of haloarchaeal isolates, (1) BK6, (2) BBK2, (3) BS2, (4) BS17, (5) TN9, (6) E3, (7) E4 and (8) J1, to various antibiotics incorporated in NGSM agar.

Penicillin and ampicillin belong to *β*-lactam class antibiotics. They interfere with cell wall synthesis in the final transpeptidation process of the cell wall synthesis in eubacteria by acting as a structural analog of acyl-D-alanyl-D-alanine (the substrate of the enzyme) and acylating the transpeptidase enzyme. By preventing cross-linking of the cell wall, they effectively lead to cell death. Archaea is resistant to *β*-lactams because of lack of peptidoglycan. Similarly, erythromycin, streptomycin and kanamycin are protein synthesis inhibitor acts by binding to the 70S ribosome of eubacteria and belonging to class macrolide and aminoglycosides, respectively. It has no effect on archaeal growth because of the resemblance of archaeal rRNA structure to eukaroytes.

The antibiotic rifampicin belongs to the group rifamycin, which is a subclass of the DNA-interfering antimicrobials, family ansamycins. It is a bactericidal drug which acts by inhibiting bacterial DNA-dependent RNA polymerase thereby inhibiting DNA-dependent RNA synthesis in bacteria. It binds to RNA polymerase, at an area near to its active site,

hence blocks the formation of phosphodiester bond in the RNA backbone, thereby blocking the RNA synthesis (Campbell et al., 2001). Since, the archaeal RNA polymerase is structurally more closely related to the eukaryotic one, this antibiotic may inhibit archaea too (Kelman and Kelman, 2004; Dridi et al., 2011). Bonelo et al., (1984) reported the *Hfx. mediterranei* DSM 1411, an extremely halophilic archaeon was sensitive to rifampicin, bacitracin and novobiocin. Novobiocin belongs to the DNA interfering antimicrobials of family aminocoumarin and is one of the few compounds active against both the domains bacteria and archaea. It is an inhibitor of *gyrB* subunit of DNA gyrase (type II DNA topoisomerase).

2.3.4.2 Pigment of *Pseudomonas aeruginosa* SB1

The fluorescent green pigment from *Pseudomonas aeruginosa* SB1 (292.5 mg/277 mg dry weight of cells) showed anti-haloarchaeal activity, indicated by zone of clearance around the wells containing pigment. Extremely halophilic archaea are largely non-pathogenic organisms and differ significantly in their cell membrane and posess diether type of lipids in contrast to the diester lipids of their eubacterial counterpart. However they are mainly associated with the spoilage of salted food such as fish, meat and most of the fermented food products like pickles etc (Graikoski, 1973). They also damage hides which are brine-cured (Bailey and Birbir, 1993; Birbir and Ilgaz, 1996). *Pseudomonas* belonging to the domain eubacteria has been known to inhibit *Salmonella* and other bacteria usually associated with poultry (Oblinger and Kraft, 2006) as well as other Gram positive and Gram negative bacteria of dairy interest (Gobbetti et al., 1997). The phenazine groups of compounds produced by Pseudomonas are known to posses a broad spectrum of antibiotic activity towards fungi and animal tissue (Laursen and Nielsen, 2004; Mavrodi et al., 2006; Dwivedi and Johri, 2003).

This is the first report describing anti-haloarchaeal activity of the fluorescent pigment of *Pseudomonas aeruginosa*. Rinsing and pretreatment of food with high salt content with the phenazine compounds could be used to prevent the growth of haloarchaea and thus retain the aesthetic appearance of the salted products. This is the first report on the cross domain interaction of *Pseudomonas aeruginosa* and members of haloarchaea.

2.3.4.3 Metal and metal oxide nanoparticles

The extremely halophilic isolates could resist and grow in presence of metals and metal nanoparticles. The $CdCl_2$ and $ZnCl_2$ resistance was seen as BK6 (4.0/2.0mM) > BBK2 (4.0/2.0 mM) > BS17 (0.5/0.5 mM) = BS2 (0.5/0.5 mM) whereas for ZnO NPs resistance was BK6 (2.0mM) > BBK2 (2.0mM) > BS17 (0.5mM) > BS2 (0.1mM).

Nieto et al. (1987) in his study on haloarchaeal susceptibility to different heavy metals found that the MIC of Cd and Zn was 0.05-2.5 and 0.05–0.5mM, respectively. Williams et al. (2013) reported that haloarchaeal strains *Natronobacterium gregoryi* and *Halobacterium saccharovorum* can tolerate only up to 0.001 and 0.01 mM of Cd and Zn, respectively. Cd resistance and bioaccumulation in Gram negative, *Brevundimonas* sp. ZF12 and Gram positive, *Streptomyces pimprina* have been reported (Puranik et al., 1995; Masoudzadeh et al., 2011). The best MIC of Cd and Zn for the extremely halophilic isolates obtained from solar salterns of Goa and Tamil Nadu was 4.0 mM and 2.0 mM which were much higher as compared to the literature (Das et al., 2014; Salgaonkar et al., 2015). Reports on the MIC values of ZnO NPs for Gram positive *Staphylococcus aureus* was 0.8–1.5 mM whereas that of Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* was 6.1 mM (Gunalan et al., 2012; Premanathan et al., 2011). Till date, there are no reports on resistance of extremely halophilic archaea to metal nanoparticles.

2.3.5 Chemotaxonomic characterization

2.3.5.1 Polar lipids analysis

One of the characteristic features of the members of the family *Halobacteriaceae,* domain archaea is the unique lipids with ether bonds and isoprenoid hydrophobic side chains (Kamekura, 1993, Kates, 1978; Oren, 2012; Oren, 2006b). These polar lipids are used to discriminate between different genera, and certain lipid components are used as chemotaxonomic signatures for certain genera (Oren et al., 2009). Considering this, polar lipids were extracted from the extremely halophilic isolates, separated and examined using silica gel thin layer chromatography (TLC) (Fig. 2.6).

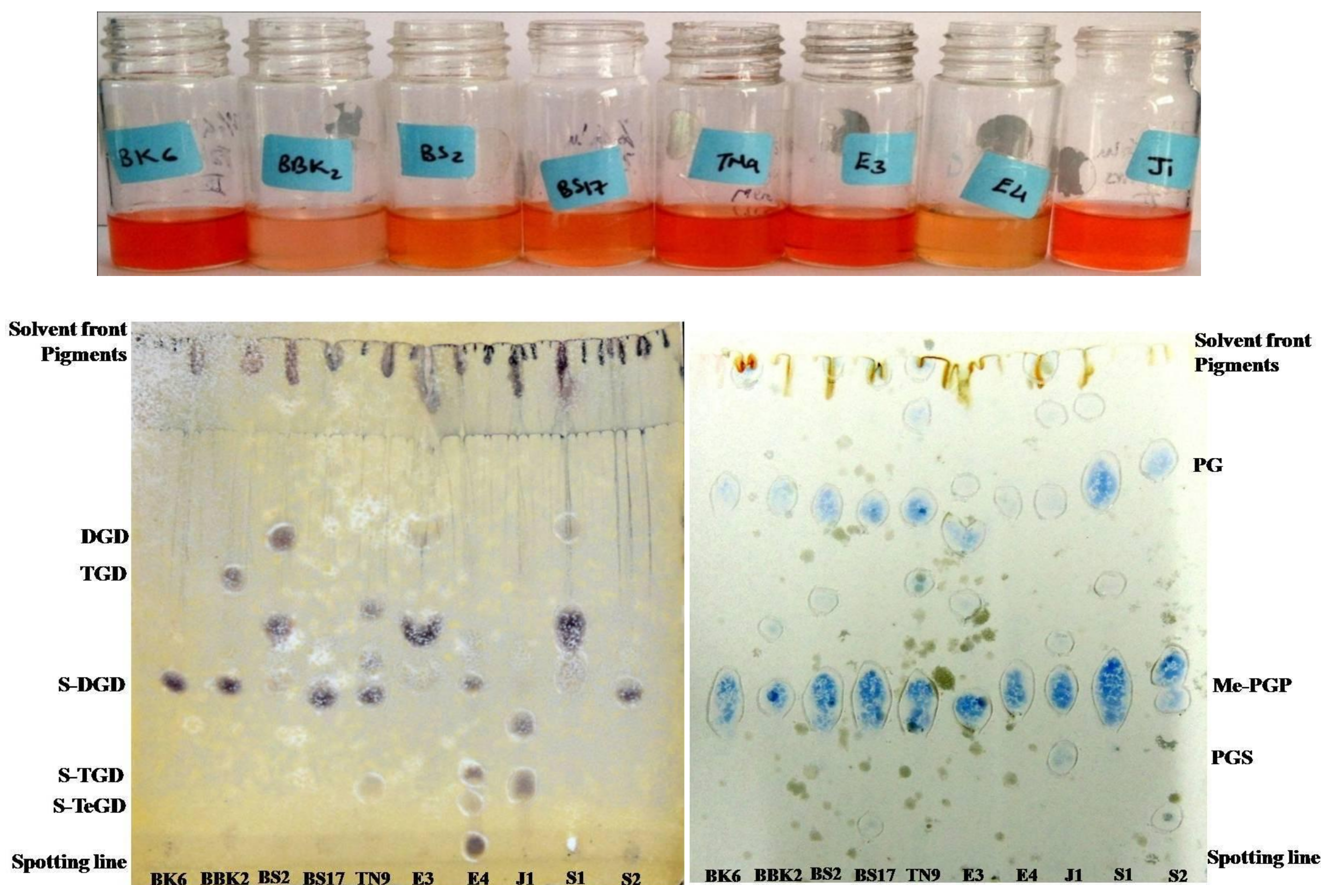

Fig. 2.6 Vials containing the polar lipid extracts from extremely halophilic isolates and thin layer chromatography of polar lipids (Glycolipids – left panel; phospholipids – right panel) of haloarchaeal isolates, BK6, BBK2, BS2, BS17, TN9, E3, E4, J1, S1 – *Haloarcula hispanica* JCM8911, S2 – *Halorubrum* s*accharovorum* JCM 8865.

The phospholipids appeared as distinctly resolved blue spots for all the isolates (Fig. 2.6 right panel). There were two distinct spots one slightly elongated with Rf ranged from 0.26 - 0.3 characteristic of methyl ester of phosphatidylglycerophosphate (Me-PGP) and another one with Rf range of 0.62 – 0.68 characteristic of phosphatidylglycerol (PG). Apart from these, another less intense spot of Rf range corresponding to 0.24 - 0.26 was seen, characteristic of phosphatidylglycerosulfate (PGS). These phospholipids were well in comparison to that of the standard haloarchaeal strains used *Haloarcula hispanica* JCM 8911 and *Halorubrum saccharovorum* JCM 8865. The pigments of the haloarchaeal

isolates run along the solvent front with an Rf of 0.98-0.99 and are visualized as orange or yellow without any visualizing agent.

The TLC chromatogram developed for glycolipids showed distinctly resolved purple to dark blue spots which varied for each isolate. The purple spots having an Rf range of 0.25-0.29 were of the sulfated diglycosyl diether (S-DGD) derivative of PG and were present in the isolates BK6, BBK2, BS17, TN9, E4 and S2. The S-DGD was absent in the isolates BS2, E3, J1 and S1. Rf values at around 0.09-0.15 are characteristics of sulfated triglycosyl / tetraglycosyl diether (S-TGD; S-TeGD) and were detected in the strain TN9, E4 and J1 (Oren et al., 2001). Interestingly, strain E4 showed some minor glycolipid spots which were retained along the spotting line with Rf of 0.01. Similar glycolipid was reported by Goh et al., (2006) for the strain *Halococcus hamelinensis* which was named as glycolipid X or unknown glycolipid. Also, spots in the Rf range of 0.5-0.57 were seen for the isolates BBK2, BS2, E3 and S1 which may be due to the presence of triglycosyl diglycosyl diether and diglycosyl diether (TGD and DGD), respectively. The glycolipid profile of the isolates BS2 and BS17 matched well with the standard cultures *Haloarcula hispanica* JCM 8911 (S1) and *Halorubrum saccharovorum* JCM 8865 (S2), respectively. TLC profiles of the total lipids (glycolipids and phospholipids) of extremely halophilic isolates from solar salterns of India were characteristic of halophilic archaea of the family *Halobacteriaceae* (Oren and Rodriguez-Valera, 2001). Oren et al., (2009), describes the major polar lipids of various genera of the family *Halobacteriaceae* with PG and Me-PGP being the major phospholipids of the genus *Halococcus, Haloferax, haloarcula, Halorubrum, Haloterrigena, Halobacterium, Halobiforma, Natrialba* and *Natronorubrum*. Also, phosphatidylglycerosulfate (PGS) is present in most of the genus except *Haloferax,* some alkaliphilic species of *Halorubrum*, *Natronorubrum*, *Halobiforma*, *Natrialba* and *Haloterrigena*.

The major glycolipids might vary depending on the genus as well as species. For example the major glycolipid for the genus *Halococcus* are S-DGD, genus *Haloferax* are S-DGD-1 (1-*O*-[α-D-mannose-(6'-SO_3H)-(1'→2')-α-D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol) and DGD-1 (1-*O*-[α-D-mannose-(1'→2')-α-D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol), the

genus *Haloarcula* are TGD-2 (1-*O*-[β-D-glucose-(1'→6')-*α*-D-mannose-(1'→2')-*α*-D-glucose]-2,3-di-*O*-phytanyl-sn-glycerol), the genus *Halorubrum* are S-DGD-3 (1-O- [*α*-D-mannose-(2'-SO_3H)-*α*-D-(1'→4')-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol), the genus *Haloterrigena*, specifically neutrophilic species is S2-DGD. The nature of the glycolipid belonging to *Halogeometricum borinquense* was PG, PGP and a single glycolipid (Montalvo-Rodriguez et al., 1998).

2.3.5.2 Pigments analysis

The extremely halophilic isolates when grown in NTYE/ NT/ MHM/ EHM media showed pink / mauve / orange to brick red pigment (Fig. 2.2). These pigments occurring in extremely halophilic strains were extracted and identified based on their spectroscopic characteristics (UV-Vis spectrometry) and High-Performance Liquid Chromatography (HPLC) profiles.

The typical UV-visible spectrophotometric scans for the pigment composition extracted using (A) chloroform: methanol (2:1 v/v) and (B) acetone are shown in Fig. 2.7 (Salgaonkar et al., 2015). The spectra of the pigment extracted in acetone showed characteristic absorption maxima at 496 nm with two shoulder peaks at 471 nm and 528 nm, respectively (Fig. 2.7). Apart from these main peaks, another peak at 389 nm was seen. Interestingly, spectra of the pigment extracted in chloroform: methanol (2:1 v/v) showed similar profile but there were shift in the peaks of the main absorption maxima was at 503 nm with two shoulder peaks at 477 nm and 536 nm, respectively (Fig. 2.7). Apart from these main peaks, two more peaks were observed at 323 nm and 394 nm, respectively. The spectrophotometric scans were well comparable with the archaeal C-50 bacterioruberin, which have absorption maxima at 496 and 530 nm, and a shoulder peak at 470 nm (Oren, 2002a).

The HPLC elution pattern of the pigments extracted from cells of the extremely halophilic strains BBK2, BS17 and E3 revealed five peaks (a-e) with various proportions (Fig. 2.8A-C). Interestingly, the BS2 strain showed a unique elution profile with seven peaks (a-g) (Fig. 2.8D). The pigment obtained from BBK2, BS17 and E3 had similar elution profile, elution time and absorption spectrum of each peak representing each compound. These

compared well with halophilic archaeal (*Haloarcula vallismortis* ATCC 29715T and *Haloarcula japonica* strain TR-1) C_{50} carotenoids such as the α-bacterioruberin (BR) and their derivatives like monoanhydrobacterioruberin (MABR), bisanhydrobacterioruberin (BABR) and isopentenyldehydrorhodopin (IDR) (Yatsunami et al., 2014; Oren and Rodriguez-Valera, 2001). The members of the family *Halobacteriaceae* (Kamekura and Kates, 1988) are known to harbor C_{20}–C_{50} isoprenoids and isoprenoid-derived compounds.

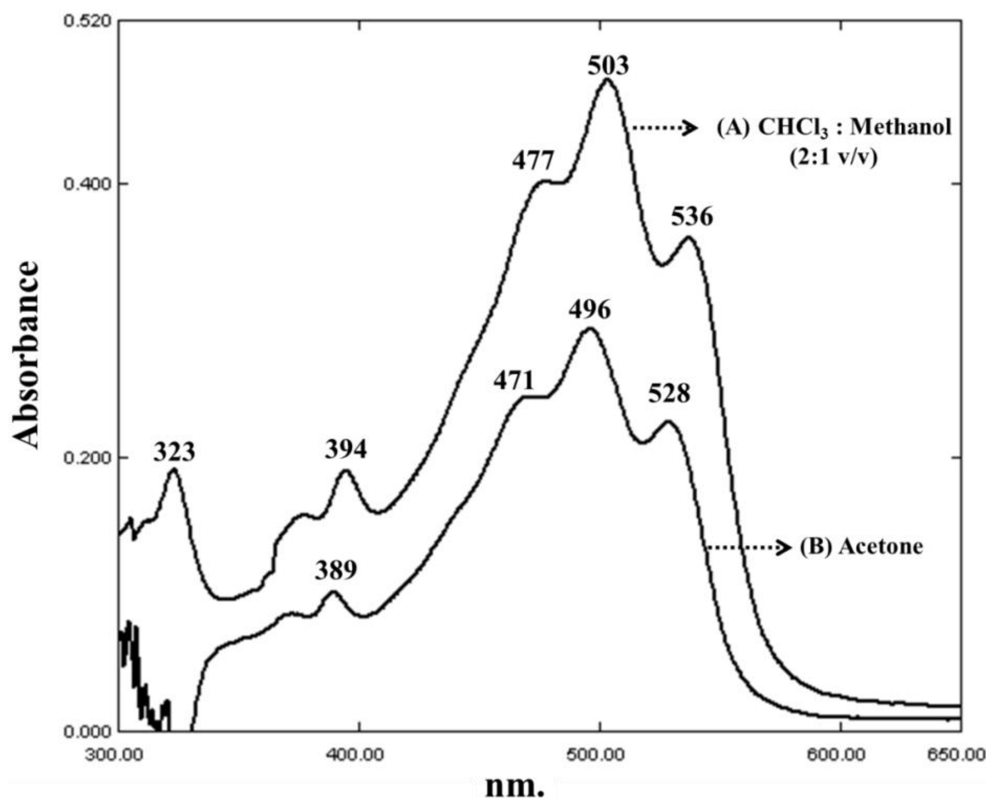

Fig. 2.7 Spectrophotometric scans of pigments from haloarchaeal strain BS17 extracted using (A) chloroform : methanol (2:1 v/v) and (B) acetone.

The halophilic microorganism *Salinibacter ruber*, though phylogenetically belongs to the domain bacteria shares many properties with members of the domain archaea, family *Halobacteriaceae* (Oren, 2013b). Similar to most of the haloarchaea, the colonies of *Salinibacter* appears orange red due to the production of salinixanthin (an unusual carotenoid). This forms a light antenna and transfers energy to the retinal group of xanthorhodopsin, a light-driven proton pump (Oren, 2013b). The HPLC elution profile of pigments from *Salinibacter ruber* has only one elution peak which indicate single

compound whose spectrum has maximum absorption at 482 nm with one shoulder peak at about 510 nm (Oren and Rodriguez-Valera 2001).

Carotenoids are yellow to red pigments, synthesized by some species of plants, algae, fungi, bacteria, and archaea. They are derived from the terpenoid biosynthetic pathway and play role in photosynthesis as accessory pigments, also functioning as antioxidants, lethal UV light protection pigments, and membrane stabilizers (Yatsunami et al., 2014; DasSarma, et al., 2001). They are efficient scavengers of reactive nitrogen / oxygen species (RNS / ROS), especially singlet oxygen species, and non-biological radicals (Chisté et al., 2011).

Oren (2009) studied the microbial abundance in salt saturated brines and concluded that the red colouration of salt saturated brines (North Arm Lake, Dead Sea) is mainly due to three types of microorganisms (i) the alga (*Dunaliella salina*) rich in β-carotene, (ii) halophilic archaea (family *Halobacteriaceae*) containing bacterioruberin (C_{50} carotenoids) and its precursors, such as IDR, BABR, and MABR and bacteriorhodopsin, halorhodopsin (retinal proteins), and (iii) the *Bacteroidetes* (*Salinibacter*) containing salinixanthin (acylated C_{40} carotenoid glucoside) and some retinal pigments.

There are number of reports on whole community pigments extracted from crystallizer ponds of solar salterns (Oren and Rodriguez-Valera 2001; Litchfield et al., 2000). Litchfield et al., (2000), examined the pigments from the whole microbial community in two commercial salterns located in United States (California saltern) and Israel (Eilat saltern). In their study, the HPLC analysis of pigment extracts from the whole community biomass obtained from saltern crystallizers brine showed the presence of bacterioruberins, carotenoids, chlorophylls *a* and *b* and β-carotene. The spectra of pigments showing absorption maximum around 450 nm and 495 nm was due to the β-carotene from *Dunaliella* sp. and C_{50} bacterioruberins from the halophilic Archaea. Nevertheless, Oren and Rodriguez-Valera (2001) studied the microbial community of crystallizer pans of salterns in Spain (Santa Pola and Mallorca salterns) and investigated that the red colour of the crystallizer pan is not only due to the algal β-carotene and / or archaeal bacterioruberins

but is also due to bacteria (*Salinibacter*) which is an important member of the microbial community (Oren, 2013c).

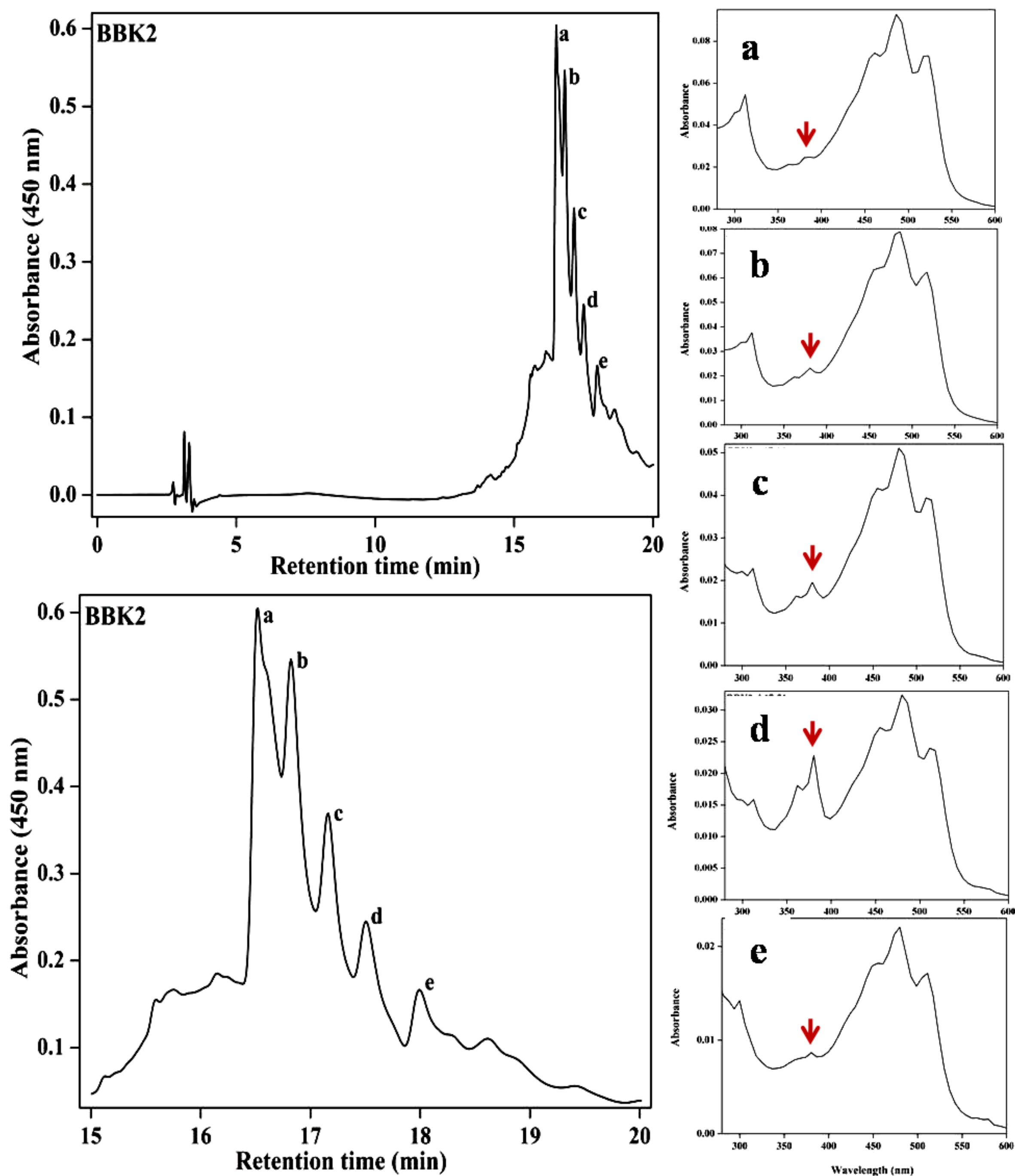

Fig. 2.8(A) HPLC separation of pigments of extremely halophilic archaeon BBK2 along with absorption spectrum.

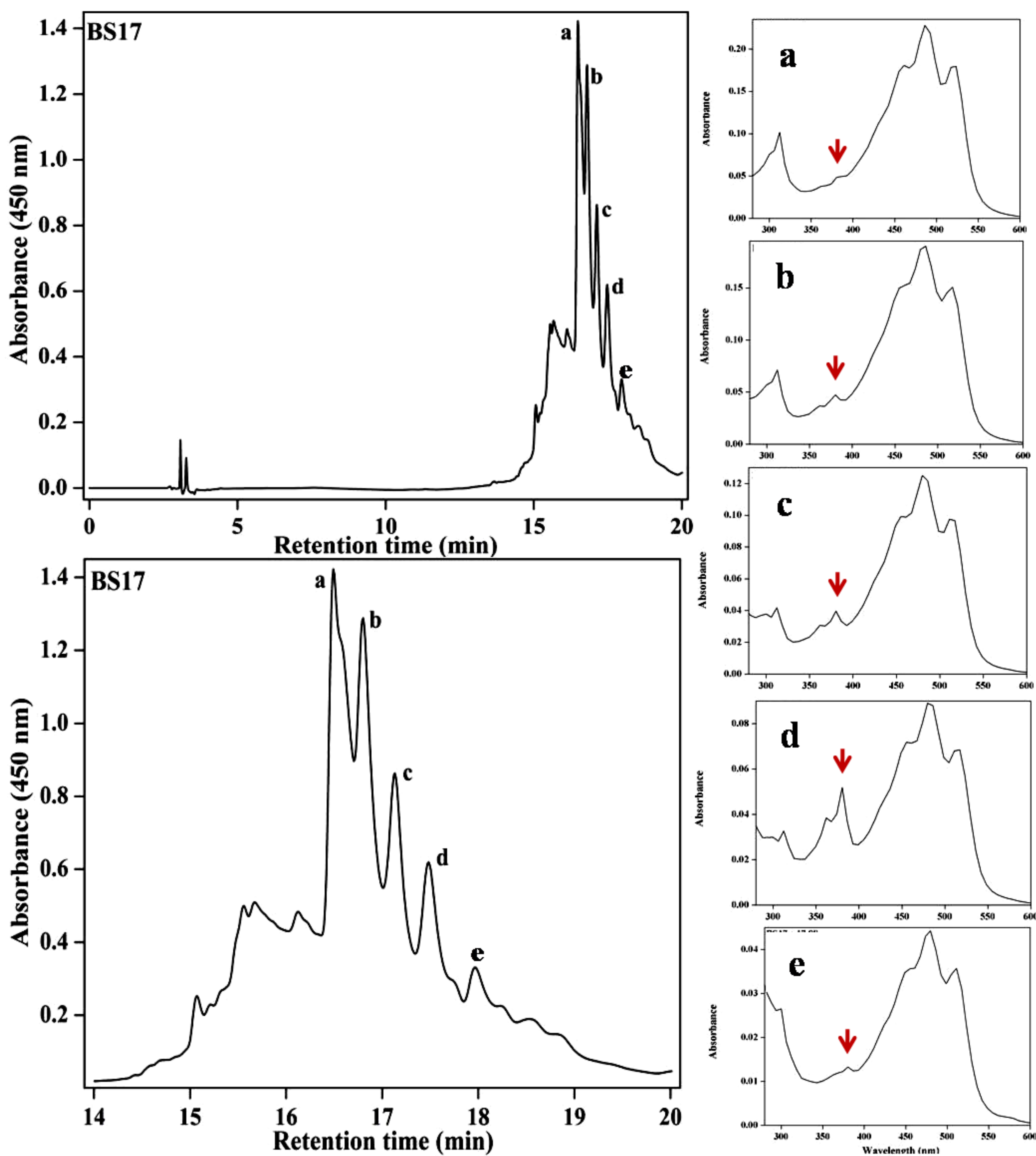

Fig. 2.8(B) HPLC separation of pigments of extremely halophilic archaeon BS17 along with absorption spectrum.

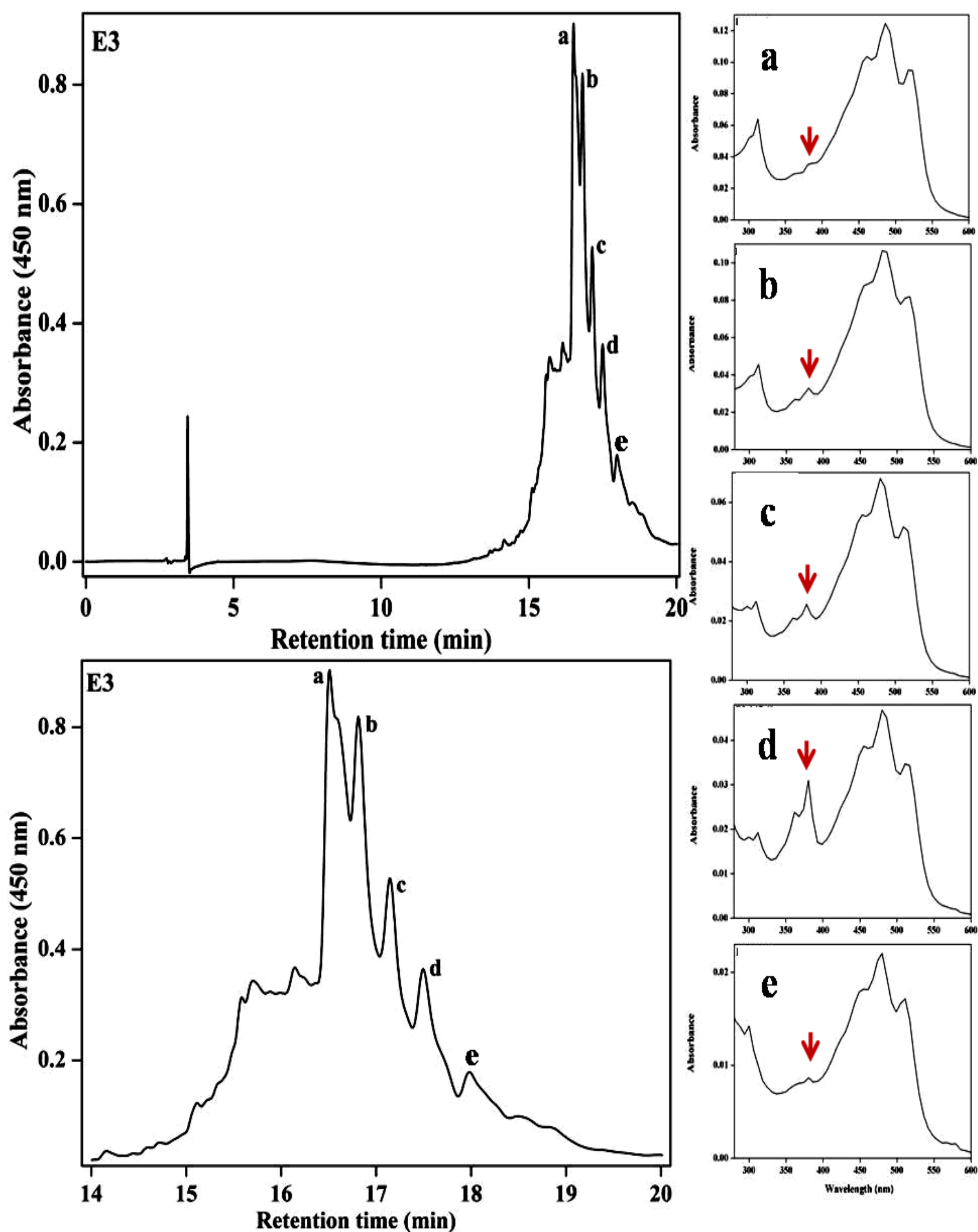

Fig. 2.8(C) HPLC separation of pigments of extremely halophilic archaeon E3 along with absorption spectrum.

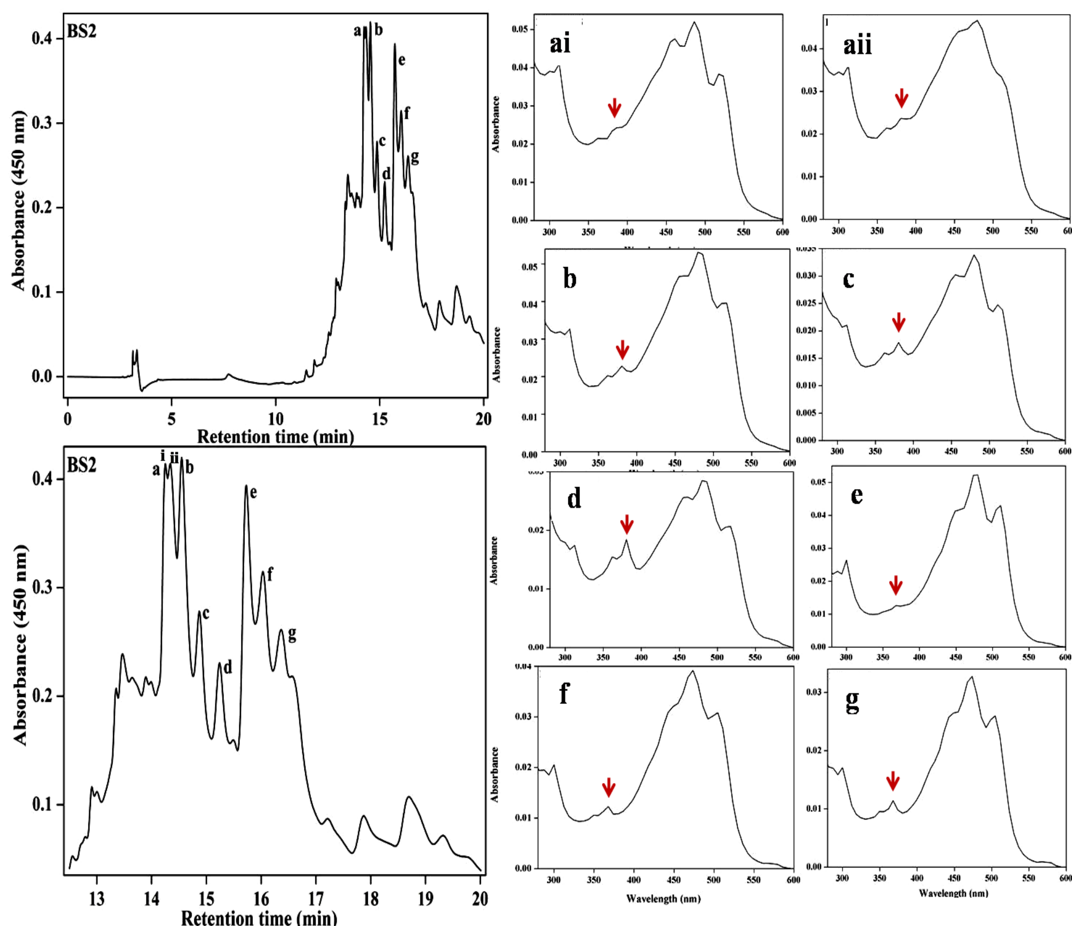

Fig. 2.8(D) HPLC separation of pigments of extremely halophilic archaeon BS2 along with absorption spectrum.

2.3.6 Biochemical characterization

The biochemical characteristics of the moderately and extremely halophilic isolates are represented in Tables 2.6 and 2.7. All the halophilic bacterial isolates were able to utilize and produced acid from D-glucose, sucrose, D-fructose, D-mannitol, D-maltose, glycerol and produced catalase and oxidase. Therefore they were chemoorganotrophic, possessing aerobic metabolism with O_2 as terminal electron acceptor and did not grow anaerobically. Apart from this, the strains H15, H16 and H26 could also utilize D-sorbitol. Strain M4 and SI3 showed nitrate reductase activity whereas isolates H15, H16 and H26 did not. The isolates were indole and citrate negative. The isolates H15, H16, H26 and SI3 were methyl red (MR) positive except for H15 which was MR negative. The isolate H15 and M4 were Voges–Proskauer (VP) positive.

Table 2.6 Morphological and biochemical characterization of the moderately halophilic isolates.

Characteristics	Cultures				
	H15	**H16**	**H26**	**M4**	**SI3**
Pigmentation	Cream	Cream	Cream	Cream / White	Cream
Colony size (mm)	2-3	2-3	2-3	1	0.5-1
Elevation	Flat	Flat	Flat	Convex	Convex
Consistency	Stringy	Stringy	Stringy	Butyrous	Butyrous
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Margin	Irregular	Irregular	Irregular	Entire	Entire
Cell morphology	Rod	Rod	Rod	Rod	Coccus
Arrangement	Pairs / Chain	Pairs / Chain	Pairs / Chain	Single / Chain	Single
Gram character	Positive	Positive	Positive	Negative	Positive
Motility	+	+	+	+	+
H_2S Production	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	-
Nitrate reduction	-	-	-	+	+
Carbohydrate Utilization					
D-Glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Lactose	-	-	-	+	+
Ribose	-	-	-	-	-
D-Mannose	-	-	-	ND	-
D-Mannitol	+	+	+	+	+
D-Maltose	+	+	+	+	+
L-Arabinose	-	-	-	ND	-
D-Galactose	-	-	-	ND	-
D-Sorbitol	+	+	+	-	-
Glycerol	+	+	+	+	+
Substrate hydrolysis					
Starch	+	+	+	-	-
Skimmed milk	+	+	+	-	+
Gelatin	+	+	+	-	-
Pectin	-	-	-	-	-
Cellulose	+	+	+	-	-
Olive oil	+	+	+	+	+
IMViC					
Indole	-	-	-	-	-
Methyl red	+	+	+	-	+
Vogues-Proskauer	+	-	-	+	-
Citrate	-	-	-	-	-
Growth Optima					
Temperature (15-45°C)	+	+	+	+	+
pH	6.0 - 10	6.0 - 10	6.0 - 10	5 – 9	5 - 9
NaCl (%)	0-7.5	0-7.5	0-7.5	5-25	0.5-25

"+" positive/growth/utilization "-" negative/no growth/ non utilization

Isolates H15, H16 and H26 were found to hydrolyse starch, skimmed milk, gelatin, cellulose and olive oil confirming their ability for production of amylase, protease, gelatinase, cellulase and lipase activity. The substrate pectin was not hydrolysed by any of the isolates. Overall, the isolates M4 and SI3 showed no hydrolytic enzyme activity except for lipase. All the halophilic isolates showed growth at varying temperature range from 15^oC to 45^oC. H15, H16 and H26 grew at pH range from 6.0 to 10.0, whereas the M4 and SI3 could tolerate and grow from acidic to neutral pH of 5.0-9.0. The isolates H15, H16 and H26 could tolerate and grow over a range of 0.5-7.5% NaCl, whereas the M4 and SI3 could grow over a vast range of NaCl from 5-25% and 0.5-25%, respectively. Therefore, all the isolates H15, H16 and H25 were categories as moderately halophilic.

All the extremely halophilic isolates were oxidase and catalase positive (Table 2.7). BK6, BBK2 and E4 were non motile whereas BS2, BS11, TN9, E3, BS17 and BS19 were motile and showed diffused rhizoidal growth along the stabbed NGSM agar deep. H_2S was not produced from sodium thiosulfite by most of the isolates except for the strain TN9. Indole was produced from tryptone by the isolates BS2, BS11, TN9 and E3. Aerobically nitrate was reduced to nitrite by all the extremely halophilic isolates. Ability for anaerobic growth and nitrate reduction was indicated by most of the isolates except strain BBK2. The isolates BS11, BS17 and BS19 showed anaerobic growth with L-arginine, DMSO and KNO_3. The isolate BS2 grew in presence of L-arginine and KNO_3. Hydrolysis of various substrates was seen by the extremely halophilic isolates and the pattern of hydrolysis varied according to the isolates (Table 2.7). All the isolates showed lipase activity which was detected by bright orange fluorescence of rhodamine on exposure to UV light. BK6, E4, TN9 and E3 could grow on NSM supplemented with starch, skimmed milk, olive oil and gelatin indicating the ability of these isolates to produce amylase, protease, lipase and geletinase activity. Clearance zone was seen around the growth of the culture BBK2 when Tween 80 was supplied as the substrate, indicating the presence of esterase activity. BBK2 also showed amylase, lipase and gelatinase activity. None of the isolates could hydrolyse Tween 20. BS2, BS11, BS17 and BS19 were unable to hydrolyse most of the substrates indicating that these isolates lack the ability to produce hydrolytic enzymes.

Table 2.7 Morphological and biochemical characterization of the extremely halophilic isolates.

Characteristics	Haloarchaeal isolates								
	BK6	**BBK2**	**BS2**	**BS11**	**TN9**	**E3**	**E4**	**BS17**	**BS19**
Colony Morphology	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Consistency	Butyrous	Mucoid	Butyrous	Butyrous	Mucoid	Mucoid	Butyrous	Butyrous	Butyrous
Colony Size (mm)	1	2	1	1	1	1	1	1	1
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex
Opacity	Opaque	Translucent	Translucent	Translucent	Opaque	Opaque	Opaque	Translucent	Translucent
Cell Morphology	Coccus	Cup / Involuted	Disk	Disk	Irregular Coccus	Irregular Coccus	Coccus	Disk	Disk
Arrangement	Pair	Single	Single	Single	Single / groups	Single / groups	single	Single, pair	Single, pair
Pigmentation	Bright Orange	Light Orange	Bright Orange red	Bright Orange red	Baby Pink	Baby Pink	Bright Orange	Bright Red	Orange Red
Gram Stain	Gram Negative	Gram Negative	Gram Negative	Gram Negative	Gram Negative	Gram Negative	Gram Negative	Gram Negative	Gram Negative
Motility	-	-	+	+	+	+	-	+	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
Indole	-	-	+	+	+	+	-	-	-
H_2S formation	-	-	-	-	+	-	-	-	-
Nitrate reduction (aerobic)	+	+	+	+	+	+	+	+	+
Anaerobic growth									
L-arginine	ND	-	+	+	ND	ND	ND	+	+
KNO_3	ND	-	-	+	+	ND	ND	+	+
DMSO	ND	-	-	+	-	ND	ND	+	+
Nitrate reduction (anaerobic)	ND	-	+	+	+	ND	ND	+	+
Substrate hydrolysis									
Starch (0.2%)	+	+	-	-	+	+	+	-	-
Tween 20 (0.1%)	-	-	-	-	-	-	-	-	-
Tween 80 (0.1%)	-	+	-	-	-	-	-	-	-
Skimmed milk (1.0%)	+	-	-	-	+	+	+	-	-
Olive oil (1.0%)	+	+	+	+	+	+	+	+	+
Gelatin (0.4%)	+	+	-	-	+	+	+	-	-
Carbohydrate utilization									
D-Glucose	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	-	+
D-Fructose	+	ND	+	+	+	+	+	-	-
D-Mannitol	ND	ND	+	+	-	-	ND	-	-

Characteristics	Haloarchaeal isolates								
	BK6	**BBK2**	**BS2**	**BS11**	**TN9**	**E3**	**E4**	**BS17**	**BS19**
Carbohydrate utilization									
D-Maltose	ND	ND	ND	ND	+	+	ND	+	+
D-Raffinose	+	ND	ND	ND	+	+	+	-	-
L-Arabinose	+	+	-	-	+	+	+	-	-
D-Sorbitol	ND	-	+	+	-	-	-	-	-
D-Lactose	ND	-	ND	ND	+	+	-	-	-
D-Galactose	+	+	ND	ND	+	+	+	-	+
Glycerol	+	+	+	+	+	+	+	-	-
Ribose	+	+	+	+	+	+	+	-	-
D-Mannose	ND	-	-	-	+	+	-	-	+/-
D-Xylose	+	-	ND	ND	+	+	+	+	+
D-Trehalose	+	-	ND	ND			+	ND	ND
Control (without sugar)	+	+	+	+	+	+	+	+	+

"+" positive/growth/utilization "-" negative/no growth/ non utilization "ND" not done

BK6 and E4, were able to utilize D-glucose, sucrose, D-fructose, D-raffinose, L-arabinose, D-trehalose, xylose, D-galactose, ribose and glycerol with slight acidification of the medium. TN9 and E3 were able to utilize many sugars like D-glucose, sucrose, D-fructose, D-maltose, D-raffinose, L-arabinose, D-trehalose, xylose, D-mannose, D-lactose and glycerol. The isolates BS17 and BS19 were unable to utilize many sugars except D-glucose and D-maltose. Whereas, BS2 and BS11 could utilize sugars like D-glucose, sucrose, D-fructose, D-mannitol, D-sorbitol, ribose and glycerol. Halophilic archaea are either aerobic or facultatively anaerobic heterotrophs, utilizing both amino acids and/or carbohydrates as carbon and energy sources. Nutrient transport in haloarchaea occurs via various sugars, amino acids, peptides, nucleotides / bases symporters and ATP-binding cassette (ABC) transporters (Anderson et al., 2011). A study by Anderson et al., (2011) identified three secreted proteases in the genomes of halophiles suggesting that all of the halophiles may be capable of degrading and utilizing extracellular proteins as nutrient source. Once there is uptake of amino acids, several amino acid degradation pathways are found in most of the halophiles genomes. Interestingly, the ability of haloarchaea to utilize amino acids is higher than that of the carbohydtrates. Members of family *Halobacteriaceae* are known to use the tricarboxylic acid cycle (TCA) in the process of aerobic degradation of carbon and, through

a combination of the glyoxylate cycle and the respiratory electron transport if necessary (Andrei et al., 2012). Haloarchaeal genomes encode the complete set of enzymes of the TCA cycle (Falb et al., 2008). Activity of all enzymes of the cycle was detected in *Halobacterium salinarum* (Aitken and Brown, 1969).

2.4 Conclusion

Halophilic microorganisms were isolated from sediment and water samples from solar salterns of Goa and Tamil Nadu, India. The microorganisms were able to grow over a wide range of NaCl concentrations (0.5-30 %, w/v), and were segregated into moderately and extremely halophilic bacteria / archaea. All these isolates were characterized morphologically by Gram staining and Scanning Electron Microscopy (SEM) and showed varying cell morphologies such as rods in chain, cocci in groups, irregular cocci in groups, involuted oval discs. The halophilic bacterial and archaeal isolates were characterized chemotaxonomically by studying their response to antibiotics, metals, presence of specific polar lipids and carotenoid pigments. The bacterial isolates were susceptible to penicillin, ampicillin, kanamycin, erythromycin and streptomycin whereas archaea were susceptible to novobiocin and rifampicin. Extremely halophilic isolates showed the presence of characteristic haloarchaeal signature glycolipids and phospholipids such as sulfated diglycosyl diether (S-DGD), phosphatidylglycerol (PG), sulfated triglycosyl/tetraglycosyl diether (S-T/TeGD), phosphatidylglycerosulfate (PGS) and phosphatidylglycerophosphate (Me-PGP) when separated by thin layer chromatography (TLC). They harboured pink to orange to brick red pigment having characteristic absorption maxima at 496 nm and two shoulder peaks at 471 nm and 528 nm, characteristics of archaeal C-50 α-bacterioruberins. Most of the halophilic bacterial and archaeal isolates were able to utilize carbohydrates such as D-glucose, sucrose, D-fructose, D-mannitol, D-maltose, glycerol and produced catalase and oxidase whereas some were able to hydrolyse substrates such as starch, skimmed milk, gelatin, cellulose and olive oil confirming their ability to produce extracellular enzymes such as amylase, protease, gelatinase, cellulase and lipase.

Chapter III

Accumulation of Polyhydroxyalkanoates by Halophilic Bacteria

3.1 Introduction

Polyhydroxyalkanoates (PHAs) are a family of polyhydroxyesters synthesized by numerous microorganisms from various carbon sources as intracellular carbon and energy storage compounds under nutrient limiting conditions (Steinbuchel and Schlegel, 1991; Tian et al., 2009). Research on PHA is gaining momentum due to its myriad applications. It is an eco-friendly green material, completely biodegradable and biocompatible thermopolyester with material properties similar to plastics, which are obtained from non-renewable petrochemical sources. PHAs from diverse sources with various chemical structures find attractive applications in medical and surgical fields such as bone plates, sutures etc. (Pachence and Kohn, 2000; Sudesh et al., 2000; Anderson and Wynn, 2001; Chen and Wang, 2002). Other applications include water resistant coatings on cardboard or paper, food processing industries and as additives in cosmetics (Poli et al., 2011; Anderson and Dawes, 1990; Steinbuchel and Fuchtenbush, 1998) PHAs are synthesized by several bacteria and few members of archaea (family *Halobacteriaceae*). Halophilic organisms such as genus *Halomonas*, *Salinibacter*, *Bacillus*, belong to the eubacterial domain have been isolated from saline environments (Quillaguamán et al., 2004; Lim et al., 2006; Wang et al., 2007; Xue et al., 2008; Echigo et al., 2012). Members of the genus *Bacillus* have also been known to accumulate polyhydroxybutyrate (PHB), which is the basic biopolyester (homopolymer) of the PHA family (Labuzek and Radecka, 2001; Shamala et al., 2003; Singh et al., 2009; Mizuno et al., 2010). One of the major challenges faced by PHA industry is to reduce its production cost, which includes medium sterilization and polymer extraction from cells. Halophilic microorganisms belonging to domain bacteria (*Halomonas boliviensis*) and archaea (*Haloferax mediterranei*) are considered as attractive organisms for PHA production than their non halophilic counterparts. As these organisms are salt loving, their production medium contains high salt, which reduces the cost required for medium sterilization (Lu et al., 2008; Quillaguaman et al., 2008, 2010; Ibrahim and Steinbuchel, 2010; Legat et al., 2010). This chapter mainly focuses on the screening of potential PHA-accumulating halophilic isolates from salt pans of Goa and Tamil Nadu, characterization of the promising isolates by morphological, biochemical and 16S rRNA analysis as well as characterization of the polymer obtained.

3.2 Materials and methods

3.2.1 Halophilic bacterial isolates

Moderately halophilic bacterial isolates belonging to H and M series (Chapter II) obtained from sediment and brine samples from solar salterns of Ribandar in Goa and Marakkanam in Tamil Nadu, India were screened for the production of PHA.

3.2.2 Screening of halophilic isolates for PHA production

3.2.2.1 Colony staining by Nile Red dye

Thirty one moderately halophilic isolates, H1-26, M2, M4, SP17, RP26 and SI3 were screened for the accumulation of PHA. The ability of the halophilic isolates to accumulate PHA was checked on E2 medium (Lageveen et al., 1988) containing 2% (w/v) glucose as substrate with or without 5% (w/v) NaCl. The moderately halophilic isolates were spot-inoculated and incubated at room temperature (28°C). Accumulation of PHA was monitored for every 12 hrs for 2 days. This was carried out by either incorporating 50 µl of Nile Red stain [0.01% (w/v) stock in DMSO] prior into 100 ml of medium or flooding the fully grown culture plates with Nile Blue A [0.05% (w/v) in absolute ethanol] and incubating in the dark for 20 min. The stain was decanted and plates were exposed to UV light (Bio-Rad Laboratories, Segrate, Milan, Italy). Bright orange fluorescence was graded and recorded (Kitamura and Doi, 1994; Spiekermann et al., 1999).

3.2.2.2 Cell staining

(i) Sudan black B staining

Sudan black B staining was done as described by Murray et al., (1994) (Appendix I). Briefly, smears of log phase cultures were prepared on clean grease free glass slides, air dried and heat-fixed. The smears were flood with primary stain, Sudan black B for 15 min. the excess stain was discarded, followed by flooding the smear with xylol for 15-30 sec. Excess xylol was discarded and the slides was dried. Finally, the glass slides were countered stained with safranin for 1 min, and rinsed with water, dried and examined under the oil immersion objective (100X) of the phase contrast microscope (Olympus BX41, Tokyo, Japan).

(ii) Nile Red staining

For Nile Red staining, cell smear was made from 42 hrs old H16 culture grown in E2 medium with 2% glucose. The smear was washed 2–3 times with sterile distilled water, dried and stained for 15–20 min with 0.01% Nile Red in DMSO. The excess stain was drained and the stained smear was washed 3–4 times with distilled water and air-dried. The cells were examined using propidium iodide (PI) filter lens of fluorescence microscope (Nikon Eclipse TS100, Tokyo, Japan). The entire procedure was carried out in the dark.

3.2.3 Genotypic characterization of the PHA positive halophilic bacterial isolates

3.2.3.1 Genomic DNA extraction

Genomic DNA was extracted according to Pospiech and Numann. (1995). Briefly, 2 ml of overnight bacterial culture grown in the respective maintenance medium (Chapter II) was centrifuged at 12000 rpm for 3 min. Then the pellet was re-suspended in 400 µl SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). 50 µl lysozyme (10 mg/ml) and 20 µl proteinase K (15 mg/ml) was added and incubated at 37°C for 30 min. Then, 50 µl of 10% SDS was added and further incubated at 37°C for 30 min. To this mixture, 500 µl of PCI (Appendix II) was added and centrifuged at 13000 rpm for 10 min. Following this 300 µl of CI (Appendix II) was added to the supernatant and centrifuged at 13000 rpm for 10 min. The aqueous phase was removed and the DNA was precipitated by adding 1/3rd volume of isopropanol and incubating at 4°C overnight. The precipitated DNA was pelleted by spinning at 13000 rpm for 10 min. The DNA pellet was washed with 70% ethanol and re-suspended in TE buffer.

3.2.3.2 Amplification of 16S rRNA gene fragment using PCR

16S rRNA gene fragment was amplified using 27(F) 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492(R) 5′-GGTTACCTTGTTACGACTT-3′ (Frank et al., 2008) under the following conditions: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min and 15 s. The final extension was at 72°C for 10 min. Amplified product was subjected to electrophoresis on a 1.5% agarose gel and was found to be approximately 1.4 kb in size.

The purified product was sequenced bidirectionally, using an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

3.2.3.3 Sequencing and phylogenetic tree construction

The amplified PCR products were purified and sequenced using an automated DNA sequencer (Applied Biosystems, USA). The 16S rRNA sequences obtained from halophilic isolates H15, H16, H26, M4, SI3, RP26 and SP17 were analysed for taxonomic affiliation using BLAST search tool and Multiple Sequence Alignment was performed using MUSCLE. MEGA 5.0 was used for the construction of phylogenetic tree by neighbour-joining method with bootstrapping analysis for 1000 replicates and displayed for 100 (Tamura et al., 2011).

3.2.4 PHA production and its quantitative estimation by various halophilic isolates

PHA positive halophilic isolates, H15, H16, H26, M4, SI3, RP26 and SP17 were grown in liquid E2 medium with 2% glucose as sole carbon source and with 5% NaCl (w/v). The biomass content and intracellular PHA accumulation was determined for every 24 hrs.

3.2.4.1 Determination of biomass

Cells from two ml of the liquid culture was harvested in regular intervals by centrifugation at 10,000 rpm for 10 min and washed twice with distilled water. The cell pellet obtained was dried at 60 °C till constant weight was obtained.

3.2.4.2 Determination of PHA

Two ml of concentrated sulphuric acid was added to the dried cell pellet containing the polymer. The mixture was hydrolyzed by heating in water bath at $100^{o}C$ for 20 min to obtain crotonic acid. The amount of accumulated polymer was quantified by recording the absorbance at 235 nm using concentrated sulphuric acid as blank with UV-visible spectrophotometer (Shimadzu UV-2450, Japan). The values obtained were quantified by comparing with a standard curve with commercial Poly[(R)-3-hydroxybutyric acid] natural

origin (Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India) (Appendix III; Law and Slepecky, 1961).

3.2.5 Growth kinetics of halophilic isolate H16 and PHA accumulation study

Growth kinetics and PHA content was determined as follows: Starter culture was obtained by growing the culture in 50 ml liquid E2 medium with 0.2% glucose as sole carbon source contained in 150-ml Erlenmeyer flasks. Two percentage of this starter culture was used to inoculate 500 ml of E2 medium supplemented with 2% (w/v) glucose contained in 1000 ml Erlenmeyer flasks. After every 6 hrs interval, following parameters were determined (i) absorbance at 600 nm, (ii) biomass as cell dry weight (CDW) and (iii) PHA content. OD600 nm was determined using UV-visible spectrophotometer (Shimadzu UV-2450, Japan). The CDW and amount of accumulated polymer was quantified as described in section 3.2.4.1 and 3.2.4.2., respectively

3.2.6 Morphological studies of the halophilic isolate H16

Promising polymer producing halophilic isolate H16 was selected and scanning electron microscopy (SEM) was done as described previously in Chapter II, section 2.2.3.1(iii).

3.2.7 Production of polymer and extraction

Isolate H16 was grown in 2 L of E2 medium with 2% (w/v) glucose. Cells were harvested at 36 hrs of growth by centrifugation at 12,000 rpm, 15 min and 4°C. The PHA was extracted from the cell pellet by the sodium hypochlorite method of Rawte and Mavinkurve, (2002) with one modification. The cell pellet harbouring the polymer was extracted in two times its volume with sodium hypochlorite containing 2% (v/v) chlorine.

3.2.8 Characterization of polymer (PHA)

3.2.8.1 Spectrophotometric identification (crotonic acid assay)

Purity of the polymer was determined by crotonic acid assay. The polymer (PHA) was dissolved in warm $CHCl_3$ such that the final concentration is 1.0 mg ml^{-1}. Five hundred micro liters of this suspension was added to 4.5 ml of concentrated H_2SO_4, mixed well and

heated at 100°C in boiling water bath for 10 min. PHA content was determined as crotonic acid and quantified by spectrophotometry at 235 nm.

3.2.8.2 Fourier transform infra red (FT-IR) spectroscopy

The IR spectrum of the obtained PHA was recorded on Shimadzu FTIR-8201 PC. The polymer was dissolved in warm $CHCl_3$ and the solution was directly applied on the window and scans were recorded in the range of 500–4000 cm^{-1} and at a resolution of 4 cm^{-1}.

3.2.8.3 X-ray diffraction (XRD) analysis

X-ray diffraction analysis of the polymer film obtained from isolate H16 and standard PHB (Sigma-Aldrich) was done with Rigaku diffractometer (Mini Flex II, Japan), operated with CuKα wavelength of 15.42 nm and data was acquired in the angular region (2θ) of 10-60° with scanning speed of 2°min^{-1}. The crystallite size L (nm) was determined for the highest peaks using the Scherrer equation which is defined as: L (nm) = 0.94 λ / B Cos θ, where "λ" is the wavelength of the X-ray radiation which is 1.542 Å (wavelength of the Cu), "*B*" is the full width at half maximum (FWHM) in radians, "*θ*" is the Bragg angle (Vidhate et al., 2012; Oliveira et al., 2006).

3.2.8.4 Thermogravimetric analysis (TGA)

To evaluate the melting temperature (Tm), Thermogravimetric analysis (TGA) was performed with a DTG-60 (Shimadzu, Kyoto, Japan) Differential Scanning Calorimeter. Approximately 1.3 mg of the polymer was subjected to thermal analysis with the following thermal cycle: heating from 30 to 180°C at a heating rate of 10°C min^{-1}, isothermally held at 180°C for 5 min, then cooled to 100°C at a rate of 50°C min^{-1} and re-heated to 500°C at 20°C min^{-1}.

3.2.8.5 Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H NMR scans of the PHA polymer were recorded after suspending the PHA in high purity deuterochloroform ($CDCl_3$). The ^{1}H NMR spectra of the samples were obtained at 400 MHz using a model Bruker Avance 300 NMR spectrometer (Rheinstetten, Germany).

3.3 Results and discussions

3.3.1 Screening of PHA producing isolates

Thirty one halophilic isolates obtained from halophilic and moderately halophilic media (Chapter II) were screened for PHA accumulation. Halophilic isolates H15, H16, H26, M4, SI3, RP26 and SP17 were positive for PHA when screened by the Nile Red / Nile Blue plate assay. Among the seven, halophilic isolates H15, H16 and H26 showed high fluorescence intensity with Nile Red when exposed to ultra violet (UV). The cells of these halophilic cultures also showed bright red fluorescence when observed under PI filter of fluorescence microscope (Fig. 3.1). All the isolates showed presence of brown black granules when stained for lipids with Sudan black B.

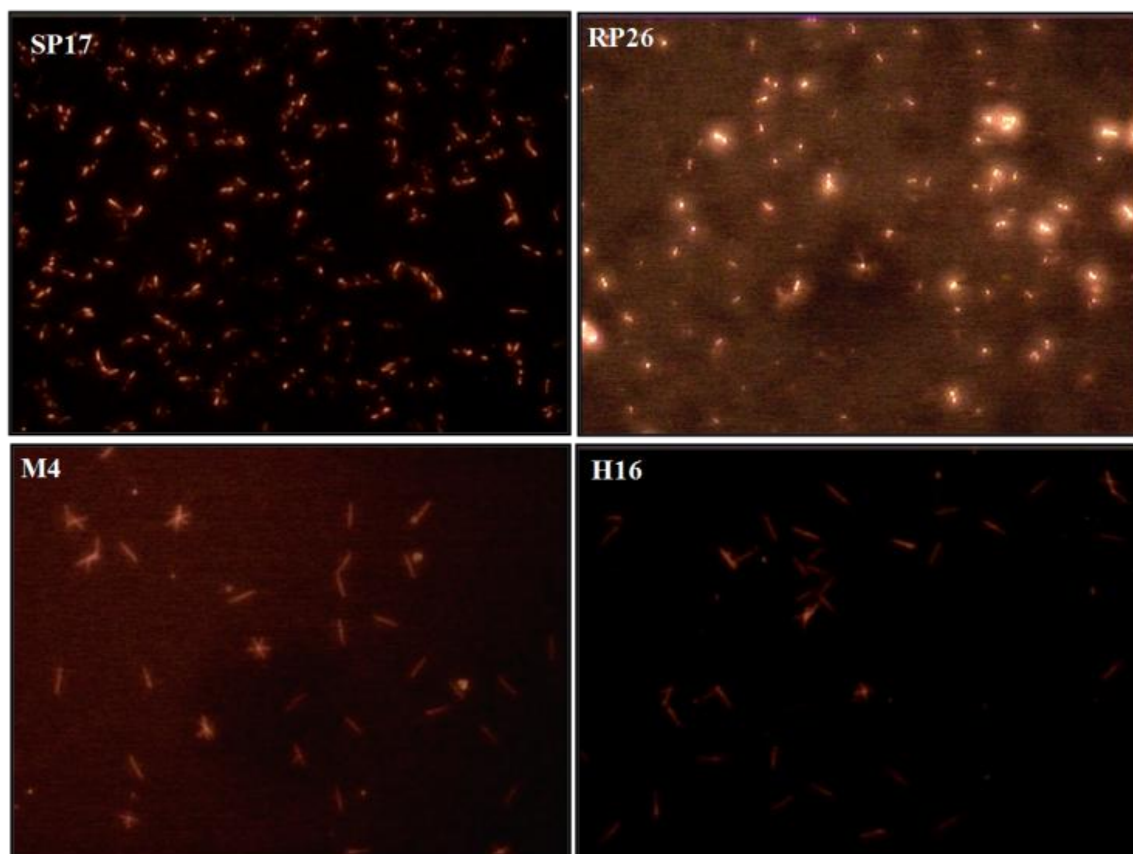

Fig 3.1 Fluorescence exhibited by cells of halophilic bacterial isolates on stained with Nile Red.

3.3.2 Genetic characterization of promising isolates

The BLAST analysis of the 16S rRNA gene fragments of the halophilic isolates H15 and H26 showed 100% similarity to *Bacillus megaterium* MS5 and 3S7, respectively. The isolate isolate H16 showed 96% similarity to *Bacillus megaterium* Y-5. 99.45% similarity was shown by isolate M4 to *Halomonas elongata* DSM2581. Isolates SI3, RP26 and SP17 showed 98-99% similarity to *Salinicoccus hispanicus*, *Alkalibacillus salilacus* and *Halobacillus alkaliphilus*, respectively (Fig. 3.2; Table 3.1). The sequences were deposited in EMBL Nucleotide Sequence Database with accession numbers H15 (HF564606), H16 (HF564607), H26 (HF564608) and M4 (AB904839). Halophilic microorganisms belonging to domain bacteria and archaea are known to inhabit solar salterns. Although research on solar salterns has been extensively focussed on the halophilic archaea, species belonging to genus *Bacillus* such as *Bacillus aidingensis*, *Bacillus qingdaonensis*, *Bacillus salaries*, *Halomonas elongata*, *Halomonas boliviensis*, etc. have also been recovered from these econiches (Lim et al., 2006; Wang et al., 2007; Xue et al., 2008, Quillaguaman, et al., 2004).

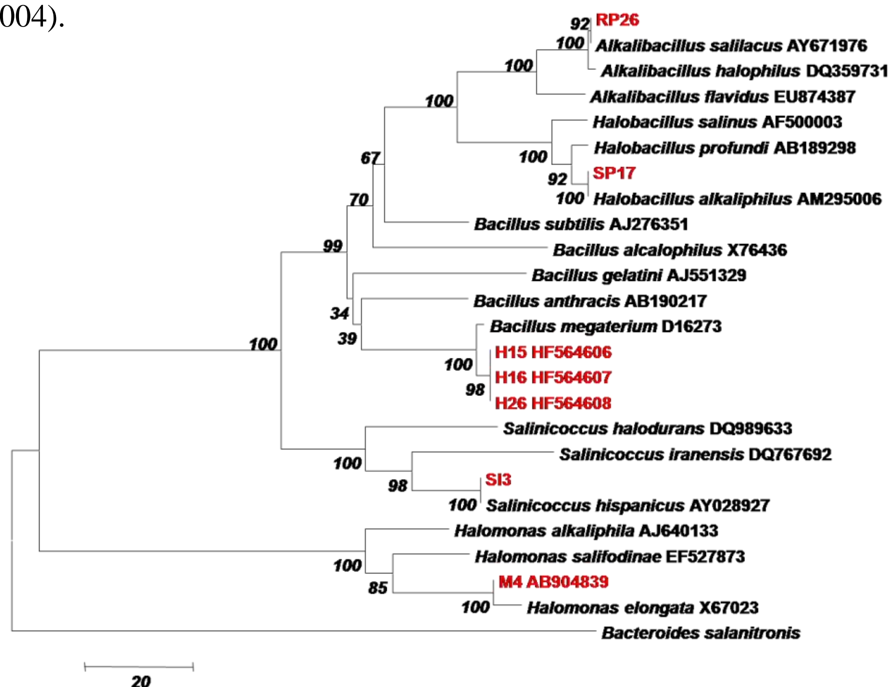

Fig. 3.2 Phylogenetic tree showing the position of polymer accumulating moderately halophilic salt pan isolates based on the 16S rRNA. Neighbor-Joining tree was constructed with MEGA 5.0 with bootstrap values for 1000 replicates. *Bacteroides salanitronis* was used as an outgroup.

Table 3.1 Identification of the moderately halophilic bacterial isolates obtained from solar salterns of India.

Culture designation	Identification	Gene bank / EMBL / DDBJ accession number
H15	*Bacillus megaterium*	HF564606
H16	*Bacillus megaterium*	HF564607
H26	*Bacillus megaterium*	HF564608
SI3	*Salinicoccus sp.*	ND
M4	*Halomonas elongata*	AB904839
RP26	*Alkalibacillus salilacus*	ND
SP17	*Halobacillus alkaliphilus*	ND

"ND" not done

3.3.3 PHA production by various halophilic isolates

Halophilic isolates H15, H16, H26, M4, SI3, RP26 and SP17 were grown in liquid E2 medium with 2% (w/v) glucose as sole carbon source and with 5% NaCl (w/v). The PHA yield of these isolates is calculated as the ratio between the polymer content and the cell biomass in terms of cell dry weight (CDW). Among the seven halophilic isolates grown in liquid E2 medium, *Salinicoccus* strain SI3 failed to grow. Isolates H15, H16 and H26 showed the best growth and PHA yield (%) as compared to isolate M4, RP26 and SP17 (Fig. 3.3). Upon comparison of the biomass content and PHA yield for three consecutive days (24 hrs, 48 hrs and 72 hrs) it was observed that the time of incubation plays an important and critical role influencing the polymer accumulation. The growth and polymer accumulation (% w/w CDW) was faster in E2 medium without NaCl [H16 (27%) > H15 (25%) > H26 (23.2%)] as compared to with 5% NaCl [H16 (3.0%) > H26 (2.8%) > H15 (2.5%)]. However, upon incubation for 48 hrs, the growth and polymer yield increased in E2 medium without NaCl and was H16 (35.5%) > H15 (32.5%) > H26 (31.5%)] as compared to with 5% NaCl [H16 (27.1%) > H26 (25.1%) > H15 (24.1%)]. On further incubation of the isolates for 72 hrs there was a decrease in the PHA content in the isolates which was H16 (17.29%) > H26 (17.09%) > H15 (15.9%)] in E2 medium without NaCl

and H16 (23.9%) > H15 (22.7%) > H26 (21.9%)] in E2 medium with 5% NaCl. Recent study by Chen et al (2009) identified an intracellular PHB depolymerase in *Bacillus megaterium* ATCC 11561 which could rapidly degrade intracellular native PHB into its monomeric hydroxybutyrate (3HB) units. *Bacillus* spp. are used widely in various biotechnological applications such as for the production of surfactants, antibiotics, flavour enhancers, etc. (Ruiz-Garcia et al., 2005).

Research on PHAs has gained momentum worldwide because of its biocompatible and completely biodegradable nature. Hence, PHAs could reduce the pollution problems associated with non degradable synthetic plastics obtained from nonrenewable petrochemical resources. PHAs are accumulated in response to stress by many genera of bacteria such as the Gram positive genera *Bacillus*, *Caryophanon*, *Clostridium*, *Corynebacterium*, *Micrococcus*, *Microlunatus*, *Microcystis*, *Nocardia*, *Rhodococcus*, *Staphylococcus*, *Streptomyces* and Gram negative genera *Pseudomonas, Halomonas, Azohydromonas*, *Burkholderia*, and *Cupriavidus* and a few members of halophilic archaea (Tan et al., 2014). Use of Gram-negative bacteria has limitations due to the co-extraction of lipopolysaccharide (LPS) endotoxins along with PHA polymer. This is highly unsuitable for biomedical applications as LPS endotoxin being a pyrogen, can elicit a strong inflammatory response in individuals. In spite of the existing methods on the removal of LPS, the treatment process itself changes the polymer properties and an overall increase in production cost. Lack of LPS in Gram-positive bacteria gives them an advantage over their Gram-negative counterparts but the relative production of PHAs is lower (Valappil et al., 2007). These issues make archaea interesting candidates to look for biodegradable polymers.

Extreme and unexplored eco-niches are a hub for novel microorganisms with versatile biotechnological potential. In this chapter, halophilic bacterial isolates were screened for the production of PHA and the *B. megaterium* strains H15, H16 and H26 were found to be the best potential producers of PHA. These three isolates were able to grow in NaCl concentration up to 7.5% (w/v) but showed optimum growth at 5% NaCl (chapter II). As

all three isolates were identified as *B. megaterium*, and among them the isolate H16 accumulated PHA slightly higher as compared to isolate H15 and H26 and therefore further studies were carried only with isolate H16.

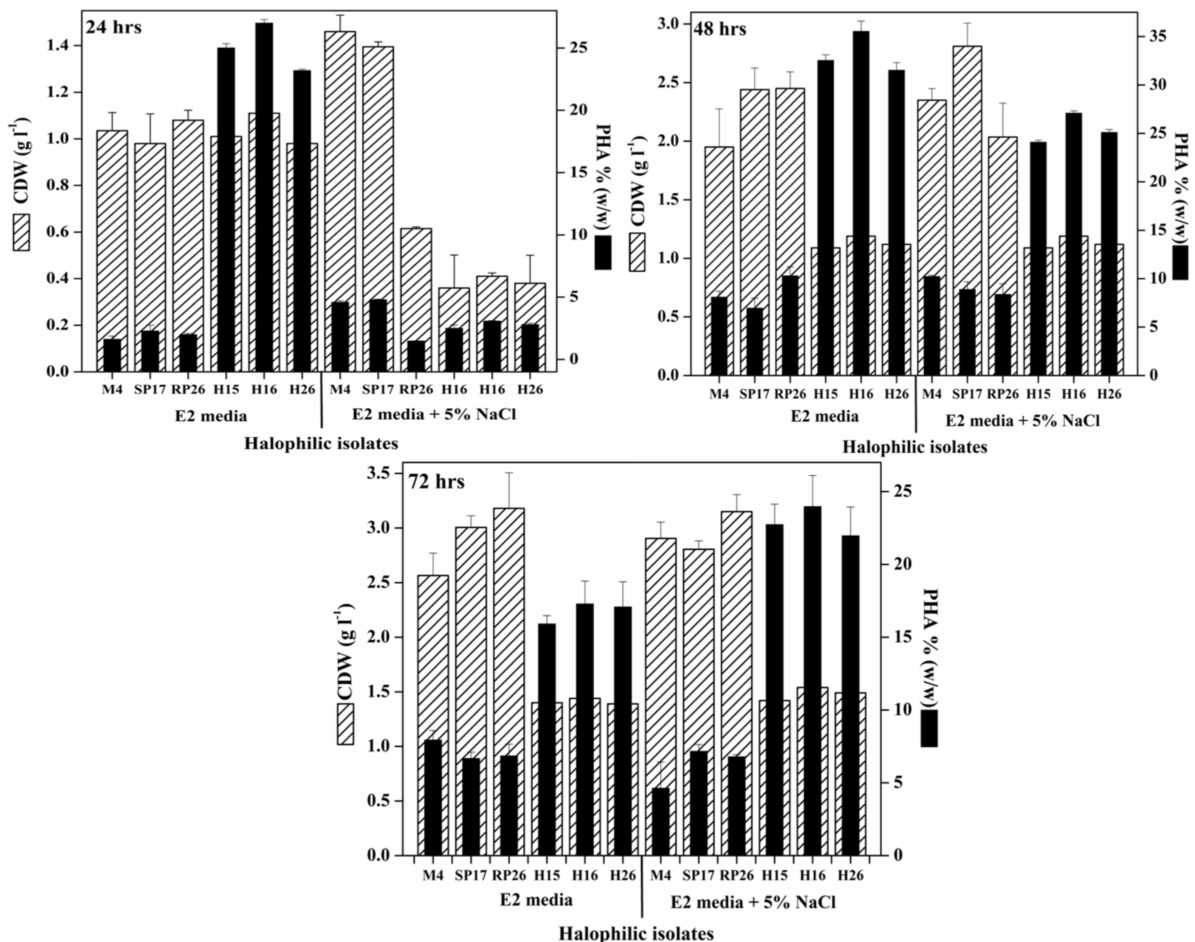

Fig. 3.3 Comparison of CDW and PHA production in moderately halophilic isolates with varying time period.

3.3.4 Growth kinetics and polymer accumulation study of halophilic isolate *Bacillus megaterium* strain H16

3.3.4.1 E2 medium without NaCl

As all the three isolates were phenotypically and genotypically identical, further studies were carried out using isolate H16. The time course of growth and accumulation of polymer by H16 culture is presented in Fig. 3.4. Growth of the organism increased steadily with a lag phase of 12 hrs followed by the logarithmic phase till the 36th hour and finally

the stationary phase which lasted for 78 hrs. Accumulation of polymer started at the 6th hour (lag phase) of growth and maximum accumulation was observed in the early stationary phase, 42nd hour (40.0% w/w of polymer of CDW), after which a decline in the polymer level was observed (Fig. 3.4A).

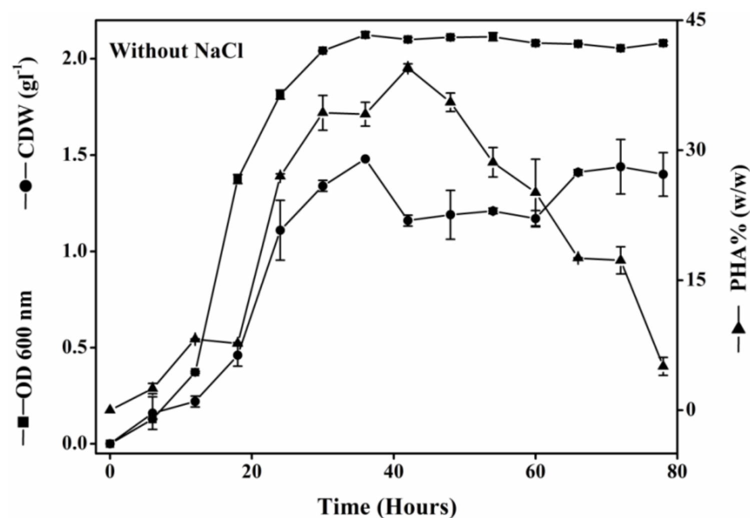

Fig. 3.4 (A) Growth kinetics and PHA accumulation studies for isolate H16 in E2 medium supplemented with 2% (w/v) glucose.

3.3.4.2 E2 medium with NaCl

When the culture was subjected to growth in same medium supplemented with 2% glucose and 5% (w/v) NaCl, it showed a lag of 24 hrs followed by logarithmic phase at 30th hour and attained stationary phase at 54th hour. Polymer was accumulated maximally at 60th hour (early stationary phase), which was 39% (w/w) of its CDW (Fig. 3.4B).

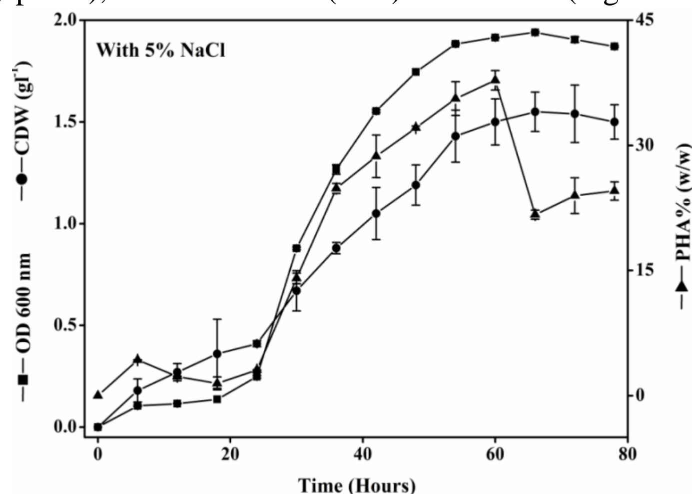

Fig. 3.4(B) Growth kinetics and PHA accumulation studies for isolate H16 in E2 medium supplemented with 2% (w/v) glucose and 5% (w/v) NaCl.

The growth kinetics and polymer accumulation studies revealed that the isolate H16 showed PHA accumulation in E2 medium in presence and absence of NaCl. Isolate H16 accumulated PHB in presence and absence of NaCl up to 40% of its CDW. This compares well with other reports with *Bacillus* sp., which accumulated 30‑46% of PHB per CDW (Gouda et al., 2001; Shamala et al., 2003; Vazquez et al., 2003; Valappil et al. 2008).

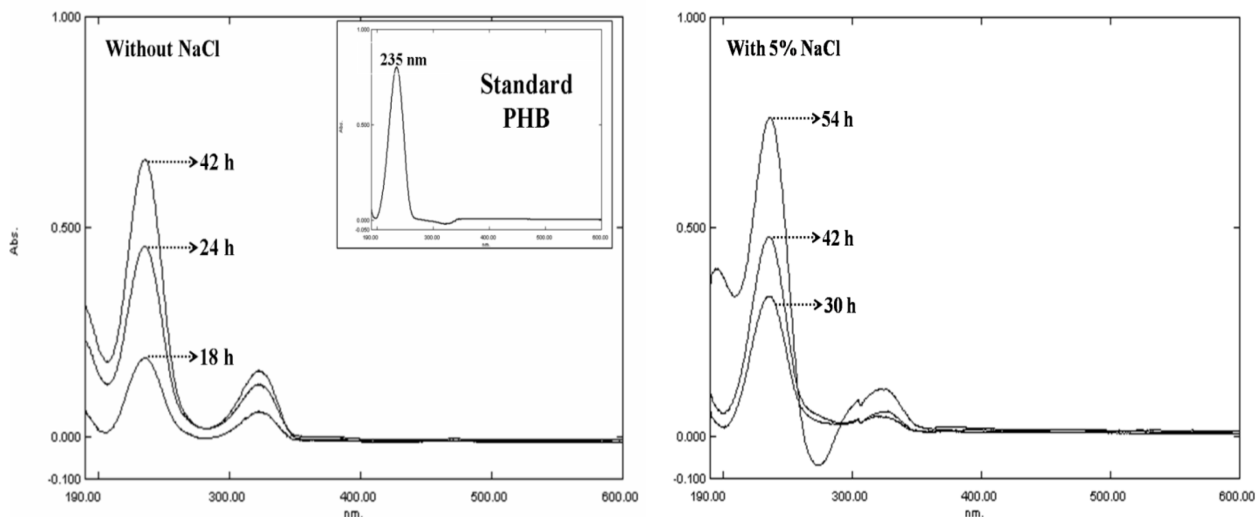

Fig. 3.5 Spectrophotometric scans of crotonic acid of PHA obtained during different phases of growth from culture H16 grown in E2 medium with 2% glucose (a) without NaCl and (b) with 5% NaCl.

3.3.5 Cell morphology study of the isolate H16

The size of the isolate H16 in nutrient medium (NA) is 1.5 x 3.0 µm, whereas in production medium, (E2 with 2% glucose) the size was reduced to 0.9 x 2.1 µm (Fig. 3.6A, B). The cell changed its morphology drastically in production medium with 5% NaCl, from rods to spheres, which are approximately 2.4 µm in diameter (Fig. 3.6C). Cells were also seen to be clumping together when the culture was exposed to the double stress of excess glucose and NaCl (Fig. 3.6C). Similar observation was made by Gouda et al., (2001) in PHA positive *Bacillus megaterium*, wherein the formation of PHB granules was reported to change the cell shape from rod to oval and spherical so as to accommodate the growing PHA granules.

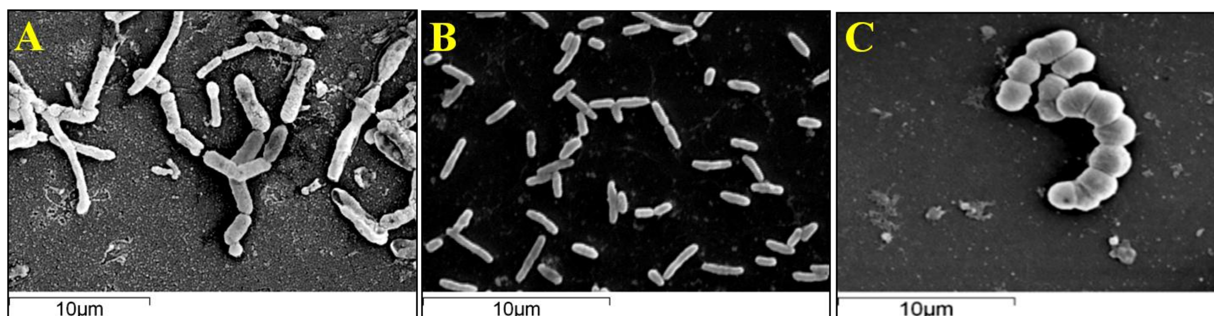

Fig 3.6 Morphological changes observed in H16 isolate when grown in (A) nutrient broth, (B) E2 medium with 2% glucose and (C) 2% glucose plus 5% NaCl.

3.3.6 Polymer characterization

The accumulated polymer was extracted from the cell pellet using sodium hypochlorite and a white precipitate was obtained. This precipitate was washed with diethyl ether, dissolved in hot $CHCl_3$. This was poured in a clean dry glass Petri dish and allowed to evaporate overnight at 4°C to obtain a polymer film (Fig. 3.7)

3.3.6.1 UV-visible spectrophotometric analysis

On addition of concentrated H_2SO_4, the polymer was hydrolysed to crotonic acid, which gave a distinct peak at 235 nm similar to that of the standard PHB (Fig. 3.5; Sharma and Mallick, 2005). The peak consistently increased with the growth of the culture during the growth cycle till it reached the late stationary phase (Fig. 3.5).

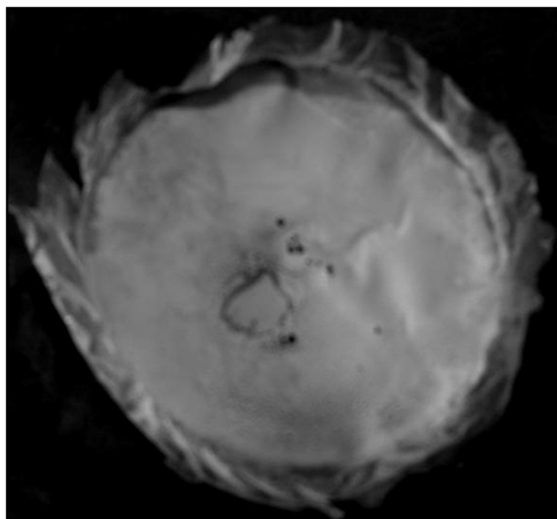

Fig. 3.7 Polymer film obtained from moderately halophilic bacteria *Bacillus megaterium* strain H16.

3.3.6.2 FT-IR analysis

The infrared spectra of all polymers exhibited prominent peaks at 1733.9 cm^{-1} which represents (Fig. 3.8) band of carbonyl (C=O) stretch and is reported to appear between 1744 - 1722 cm^{-1} (Arcos et al., 2010). The C-O-C and C-H stretching is represented by band at 1278 - 1281 cm^{-1} and 2900 cm^{-1}, respectively (Padermshoke et al., 2004). Other small bands were also seen as a result of stretching shifts due to interactions between the -OH and C=O groups (da Silva Pinto and Wypych, 2009).

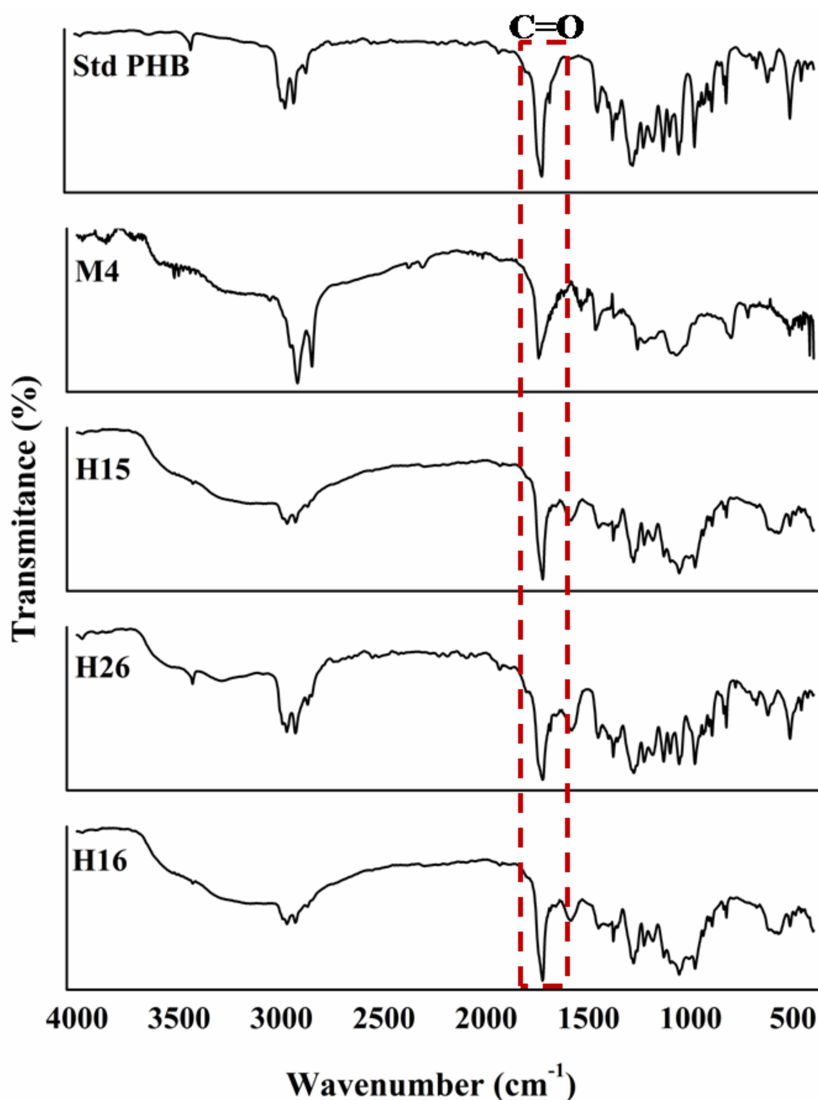

Fig. 3.8 Comparison of FT-IR spectra of standard PHB (Sigma) with polymers obtained from various halophilic isolates.

3.3.6.3 XRD analysis

Fig.3.9 shows the X-ray diffraction (XRD) patterns of polymer obtained in comparison with standard PHB (Sigma-Aldrich). Overall, the diffraction pattern was similar to that of the standard PHB. The standard PHB profile exhibits prominent peaks at 2θ = 13.4°, 17.15°, 20.1°, 22.7°, 25.5° and 30.3° corresponding to (020), (110), (101), (111), (121) and (002), reflections of the orthorhombic crystalline lattice in accordance with the study by da Silva Pinto and Wypych, (2009) and Thire et al., (2011).

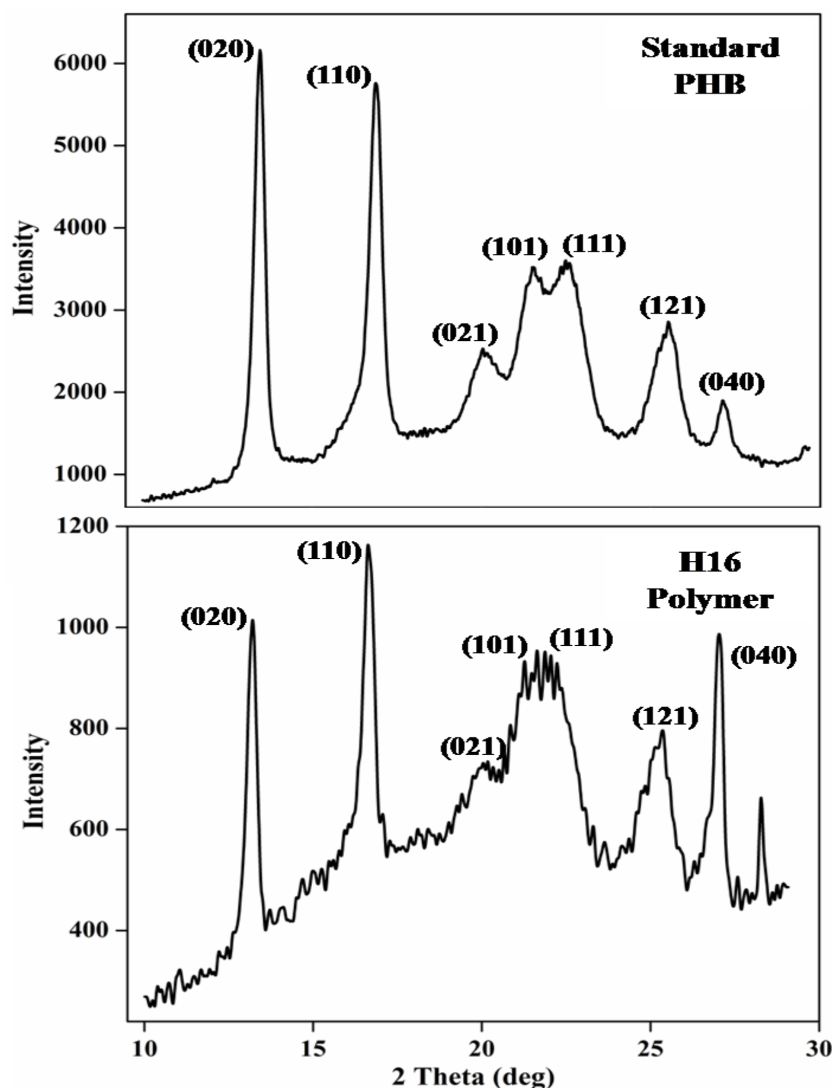

Fig. 3.9 Comparison of XRD patterns of polymers obtained from halophilic *Bacillus megaterium* strain H16 with standard PHB.

3.3.6.4 TGA-DTA analysis

The TGA–DTA thermogram of polymer is shown in Fig. 3.10. The TGA plot shows approximately 67% weight loss in the temperature range of 160–250°C. Corresponding to this weight loss, exothermic peaks in the range of 175–280°C were observed. No weight loss or heat change was observed in temperature range of 35–160°C which confirms the stability of the polymer up to 160°C.

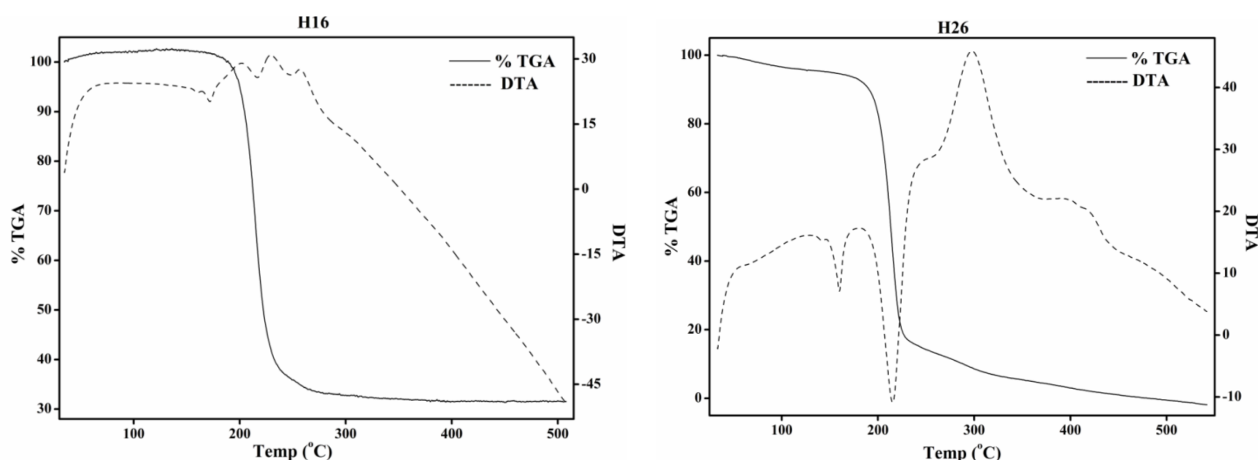

Fig. 3.10 TGA-DTA analysis of polymers obtained from moderately halophilic *Bacillus* strains H16 and H26.

3.3.6.5 ^{1}H NMR analysis

Scans of ^{1}H NMR showed a doublet at 2.5 ppm corresponding to methylene group ($-CH_2$), the signal at 5.3 ppm corresponded to methine group (-CH-) and another signal at 1.3 ppm corresponded to methyl group ($-CH_3$) (Fig. 3.11) indicating the polymer to be homopolymer of 3-hydroxybutyrates (Fig. 3.11) (Bonthrone et al., 1992; Chaijamrus and Udpuay, 2008).

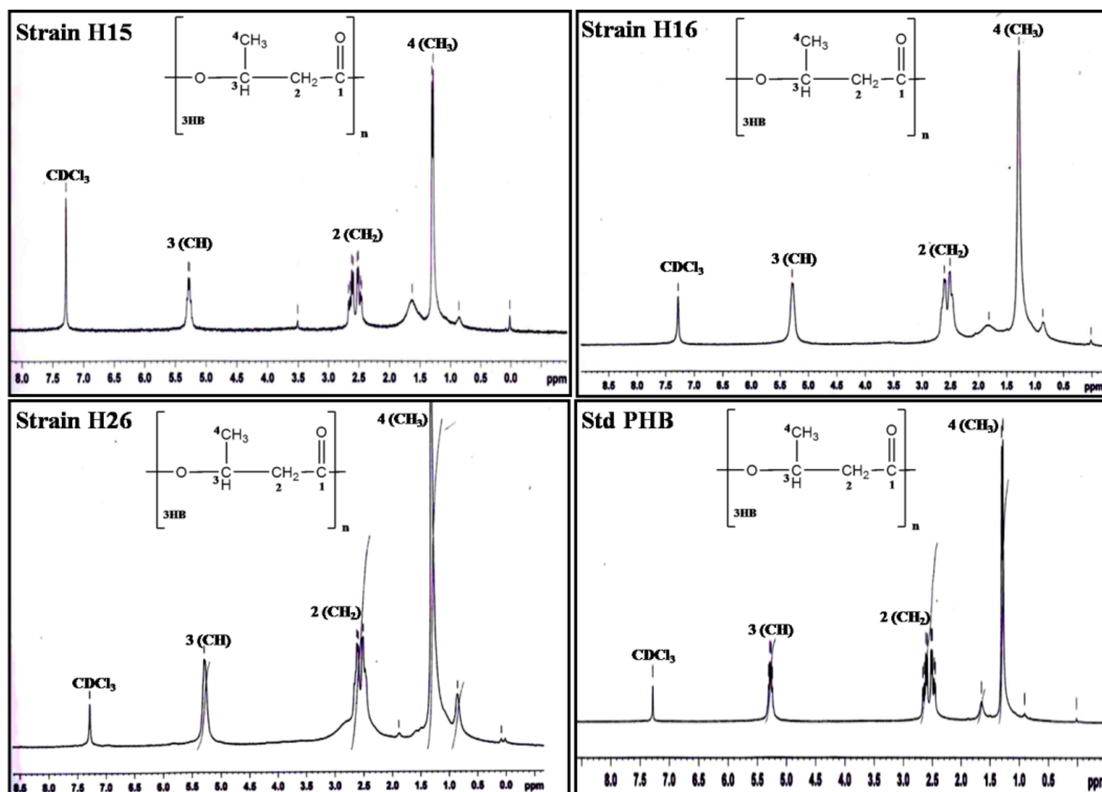

Fig. 3.11 ^{1}H NMR Spectra of standard PHA and polymers obtained from moderately halophilic isolates H15, H16 and H26.

3.4 Conclusion

Among the thirty one moderately halophilic isolates screened for PHA, three isolates H15, H16 and H26 showed the best PHA accumulation and were identified as *Bacillus megaterium* based on phenotypic and genotypic characterization. H16 was able to accumulate PHA in the presence and absence of NaCl. In the absence of NaCl, PHA of 40.0% (w/w) of CDW was accumulated at 42 hrs of growth, whereas in presence of 5% w/v NaCl, the culture showed longer lag phase of up to 24 hrs and accumulated a maximum PHA of 39.0 % (w/w) of CDW at 54 h of growth. The FT-IR, XRD and ^{1}H NMR confirmed the polymer to be homopolymer of 3-hydroxybutyrates (PHB). The TGA-DTA confirmed the polymer to be stable up to temperature of 160°C. This isolate could be exploited further for the production of PHA at an industrial scale due to its tolerance and growth in presence of up to 5% (w/v) of NaCl.

Chapter IV

Accumulation of Polyhydroxyalkanoates by Extremely Halophilic Archaea

4.1 Introduction

Microorganisms cope stress by various responses like accumulation of osmolytes, volutin granules, spore formation, etc. (Roberts, 2005; Seufferheld et al., 2011; Osman et al., 2008). Accumulation of polyhyroxyalkanotes (PHA) is also a stress response employed by many bacteria and few members of halophilic archaea. The nutrient limiting stress conditions will usually be an environment with excess carbon substrate and deficient in other elements like nitrogen and phosphorus. PHAs are potential renewable and biodegradable polyesters synthesized by numerous bacteria as an intracellular carbon and energy storage granules (Rehm, 2007; Tian et al., 2009).

Haloarchaea thrive in saline and hypersaline environments like solar salterns, salt lakes, salt deposits, fermented sea foods, etc. with NaCl concentration ranging from 3 % to saturation (Mani et al., 2012b). Microorganisms growing in extreme environments usually have versatile and well developed survival mechanisms for combating stress. These microorganisms have been the prime focus in the past decade for their far superior biotechnological applications when compared with their counterparts (Oren, 2010). Haloarchaea have been screened for many potential applications as sources of hydrolytic enzymes, pigments etc. (Moreno et al., 2013; Quillaguaman et al., 2010).

Accumulation of PHA in halophilic archaea was first reported by Kirk and Ginzburg (1972) in *Haloarcula marismortui* much later after the discovery of PHA in *Bacillus subtilis* by Lemoigne in 1923. Further screening of other halophiles has shown that halophilic organisms belonging to the eubacterial family *Halomonadaceae* and archaeal *Halobacteriacea*, genera *Halococcus*, *Halorubrum*, *Natronobacterium*, *Natronococcus, Halopiger*, *Haloarcula*, *Halobiforma*, *Haloferax*, and *Halobacterium* are capable of accumulating PHA (Quillaguaman et al., 2008, 2010; Legat et al., 2010; Poli et al., 2011; Lynch et al., 2012). In this chapter, extremely halophilic archaeal isolates from solar salterns of Goa and Tamil Nadu India (Chapter II) were screened for PHA accumulation and their subsequent extraction and characterization was carried out. Promising haloarchaeal isolates were also characterized using molecular analysis.

4.2 Materials and methods

4.2.1 Screening of haloarchaeal isolates for PHA production

Haloarchaeal isolates belonging to BK, BBK, BS, E and TN series, (Chapter II) were screened for the production of PHA. This was tested using NGSM containing 2 % (w/v) glucose as substrate (Appendix I). 50 µl of Nile Red (Hi-media) was added to 100 ml of NGSM agar medium such that the final concentration is 0.5µg ml^{-1} medium [stock of 0.001% (w/v) Nile Red in DMSO]. The isolates were streak/spot inoculated in triplicates on agar medium plates and incubated at room temperature (37°C). Accumulation of PHA by haloarchaeal isolates was determined on exposure of the grown culture plates (3-8 days old) to UV light using Gel documentation system (BIO-RAD Laboratories CA, USA) (Spiekermann et al., 1999). The fluorescence was quantified using TotalLab Quant software. Staining of cells for lipid granules and PHA was done using Sudan black B and Nile Red stain, as described previously in Chapter III; section 3.2.2.2 (Murray et al., 1994; Salgaonkar et al., 2013a). The staining for the haloarchaeal isolates was done only after the air dried cell smears were desalted with 2 % acetic acid (Dussault, 1955).

4.2.2 Molecular characterization of the promising haloarchaeal isolates

4.2.2.1 Genomic DNA extraction

Molecular characterization of the isolates was performed according to the protocol described in Halohand book (Dyall-Smith, 2008). In brief, 1 ml of culture broth (5–6 days old) were centrifuged at 5,000 rpm for 5 min and treated with 200 µl of sterile distilled water. To this, 200 µl of buffer-saturated phenol was added and incubated at 60°C for 60 min. After centrifugation at 8,000 rpm for 5 min, 400 µl of cold ethanol was added to the aqueous phase for DNA precipitation. The precipitated DNA pellet was washed with 70 % ethanol and resuspended in nuclease free water.

4.2.2.2 Amplification of 16S rRNA gene using PCR

The extracted genomic DNA was used as template for the amplification of 16S rRNA gene fragment with primers A109 (F) AC(G/T)GCTCAGTAACACGT and 1510(R) GGTTACCTTGTTACGACTT (Mani et al., 2012a; Birbir et al., 2007). Each PCR reaction contained 10X Taq buffer, 2 mM $MgCl_2$, 10 mM of dNTPs (Sigma), 10 µM of each primer

(IDT technologies, Singapore), 2 U Taq Polymerase and 1 µl of template DNA. The reaction was carried out with initial denaturation for 5 min at 94°C, denaturation for 30 s at 94°C, annealing for 40 s at 53.5^{o}C, elongation for 60 s at 68°C (35 cycles) and final elongation at 68°C for 5 min.

4.2.2.3 Sequencing and phylogenetic tree construction

The amplified PCR products were purified and sequenced using an automated DNA sequencer (Applied Biosystems, USA). The obtained results were subjected to similarity search using EzTaxon server (Kim et al., 2012). Multiple sequence alignment was performed with MUSCLE and the Phylogenetic tree was constructed by the neighborjoining method of MEGA 5.0 (Tamura et al., 2011). Bootstrap analysis was carried for 1000 replicates, which was performed to assess the confidence limits of the branching. The obtained sequences were deposited in GenBank and DDBJ database.

4.2.3 Detection of intracellular PHA by transmission electron microscopy analysis

Halophilic archaeal isolates, *Haloarcula* strain BS2, *Haloterrigena* strain J1 and *Halogeometricum* strain E3 were grown for 4 days in NGSM supplemented with 2% (w/v) glucose. The cells were harvested from the liquid culture by centrifugation, lyophilized using lyophilizer (Christ, Alpha 1-2 LD plus). Transmission electron microscopy (TEM) analysis of the cells was done as described by Galán et al., (2011) with slight modification. Briefly, the lyophilized cells were fixed in 4 % (v/v) glutaraldehyde and 1 % osmium tetroxide, followed by gradual dehydration in a series of increasing acetone concentrations (20, 40, 60, 80 and 100 % acetone in water). The samples were then embedded in epoxy resin and ultrathin sections (50-70 nm) were cut with microtome using a diamond knife, followed by staining with uranyl acetate and lead citrate. The sections were examined under a transmission electron microscope (HRTEM, JEOL JEM 1400, Japan).

4.2.4 PHA production and its quantitative estimation by various haloarchaeal isolates

PHA positive halophilic isolates BK6, BBK2, BS2, J1, TN9 and E3 were grown in NGSM with 2% (w/v) glucose as sole carbon source. The biomass content and intracellular PHA

accumulation was determined after 4 days as described previously in Chapter III; section 3.2.4.

4.2.5 Growth kinetics and polymer accumulation by haloarchaeal isolate TN9 and E3

Growth rate and intracellular PHA content in *Hgm. borinquense* strains TN9 and E3 was determined as follows. Isolates TN9 and E3 grown in NT and EHM broth respectively for 2-3 days (mid-log phase) was used as starter culture. Two percent inoculum was used to inoculate the NGSM supplemented with 2% (w/v) glucose. The flasks were incubated at 37°C on a rotary shaker at 110 rpm. After regular intervals of 24 hrs the following parameters were analyzed, (i) absorbance at 600nm, (ii) cell dry weight (CDW), (iii) pH of medium, (iv) reducing sugar in medium and (v) PHA content in the cells. Growth was determined by recording the optical density (OD 600nm) of the liquid culture using UV-visible spectrophotometer (Shimadzu UV-2450, Japan). Reducing sugar was estimated by DNSA method (Miller, 1959) and compared to the standard curve for reducing sugar (Appendix III). The CDW and polymer content in the cells was determined as described in Chapter III, sections 3.2.4.1 and 3.2.4.2, respectively.

4.2.6 Polymer extraction

Haloarchaeal cultures *Hgm. borinquense* strains TN9 and E3 were grown for 4–5 days at 37 ^{o}C, 110 rpm in 2 L of NGSM with 2 % (w/v) glucose. After 5 days, cells were harvested by centrifugation at 10000 rpm, 15 min and 4°C. The cells were briefly rinsed with distilled water and the intracellular polymer was extracted using sodium hypochlorite method as described in Chapter III, section 3.2.7. The polymer pellet obtained was washed twice with diethyl ether and dried at 70–80°C. The dried PHA pellet was dissolved in warm $CHCl_3$ (60–65°C) poured in glass petri dish and allowed to evaporate at 4°C.

4.2.7 Polymer characterization

Polymer characterization was done using crotonic acid assay, FT-IR, TGA and ^{1}H NMR analysis as described previously in chapter III, section 3.2.8.

4.3 Results and discussion

4.3.1 Haloarchaeal isolates and screening for PHA

Forty four isolates belonging to BK, E, BBK, BS and TN series obtained from salterns of Ribandar and Siridao in Goa (Chapter II) were screened for the accumulation of the PHA. Among these isolates screened, all the isolates of BK and BBK series showed fluorescence on plates stained with Nile Red, whereas only seven isolates (BS1, BS2, BS4, BS5, BS7, BS13 and BS16) of the BS series, 11 isolates (TN1–TN11) of the TN series and isolate E3, E4, J1 exhibited fluorescence. The intensity of the fluorescence varied with time from slight to bright fluorescence (Table 4.1). BS series cultures showed very less growth as well as polymer accumulation (even after 10–15 days of incubation). Among all the isolates screened, isolates TN9 and E3 grew fast with accumulation of polymer in 3–5 days, followed by BBK2, TN4–7 and TN10 in 5–8 days (Fig. 4.1AB). Most of the isolates also showed presence of brown black granules when stained for lipids with Sudan black B (Fig. 4.2). The isolates fluoresced at an emission wavelength of 590 nm revealing bright orange-red granules when stained with Nile Red and observed under fluorescence microscope (propidium iodide filter) (Fig. 4.3).

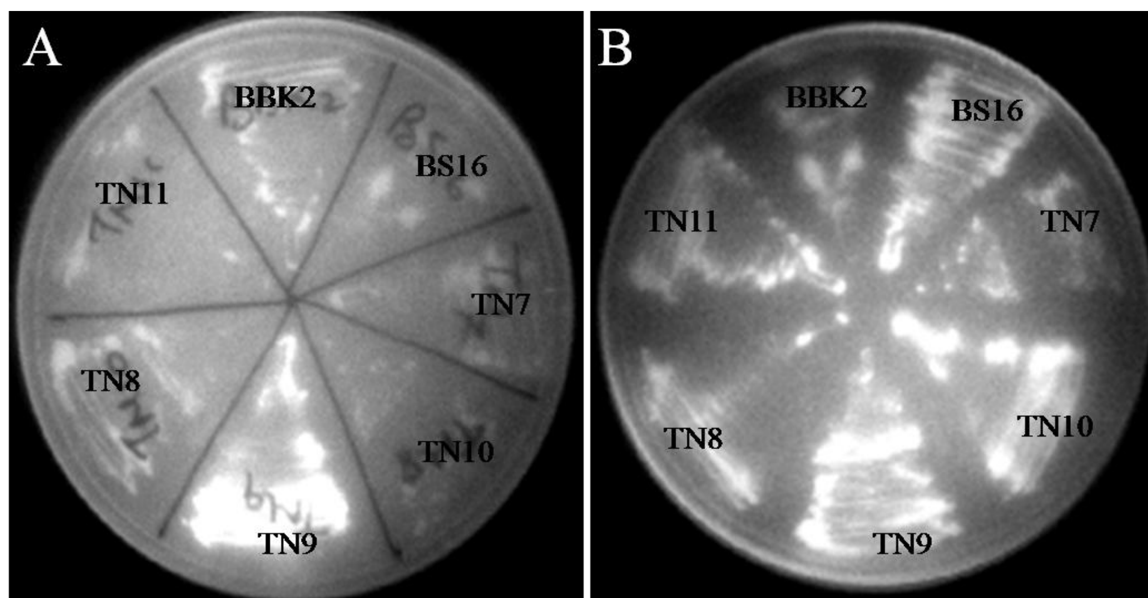

Fig. 4.1 Fluorescence exhibited by the haloarchaeal isolates grown in NGSM with Nile Red stain (A) after 3 days, (B) after 8 days of growth.

Table 4.1 Comparison and quantification of fluorescence exhibited on exposure to UV light by some of the extremely halophilic archaeal cultures grown on NGSM with prior incorporated of Nile Red dye.

Haloarchaeal Isolates	Average intensity of fluorescence	
	After 3rd day (72 hours)	After 8th day (192 hours)
TN7	157.53	214.52
TN8	168.41	167.77
TN9	236.76	212.94
TN10	181.36	212.05
TN11	191.08	195.63
BBK2	175.85	218.18
BS16	153.40	215.06

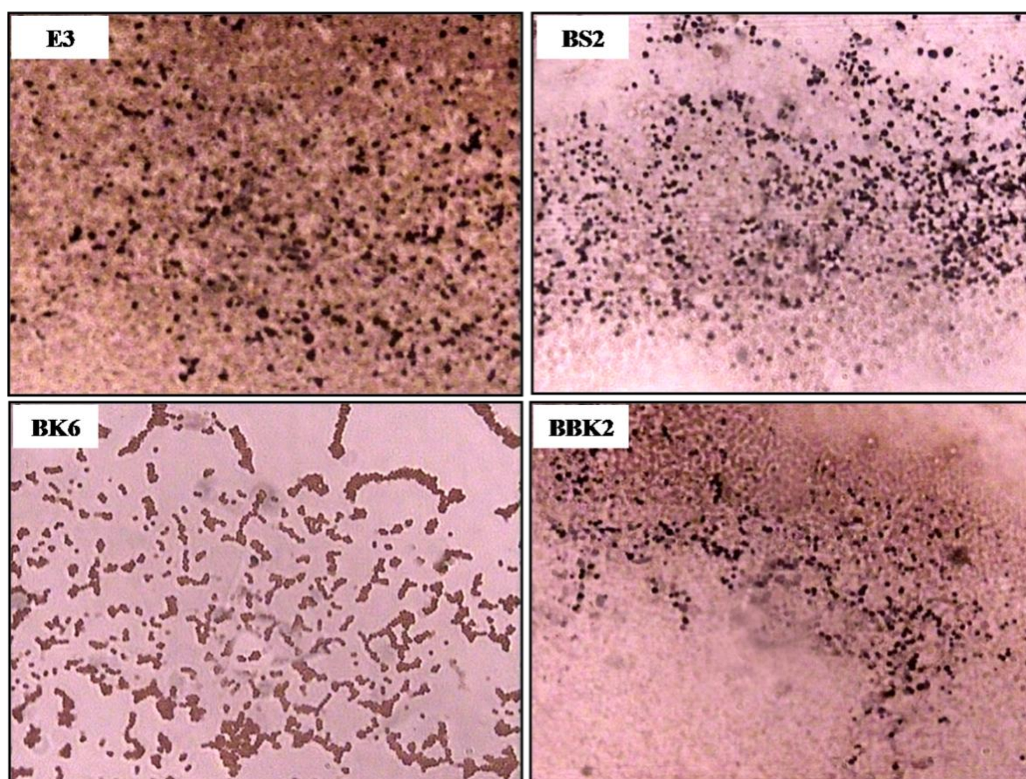

Fig. 4.2 Staining of the haloarchaeal isolates grown in NGSM by Sudan black B.

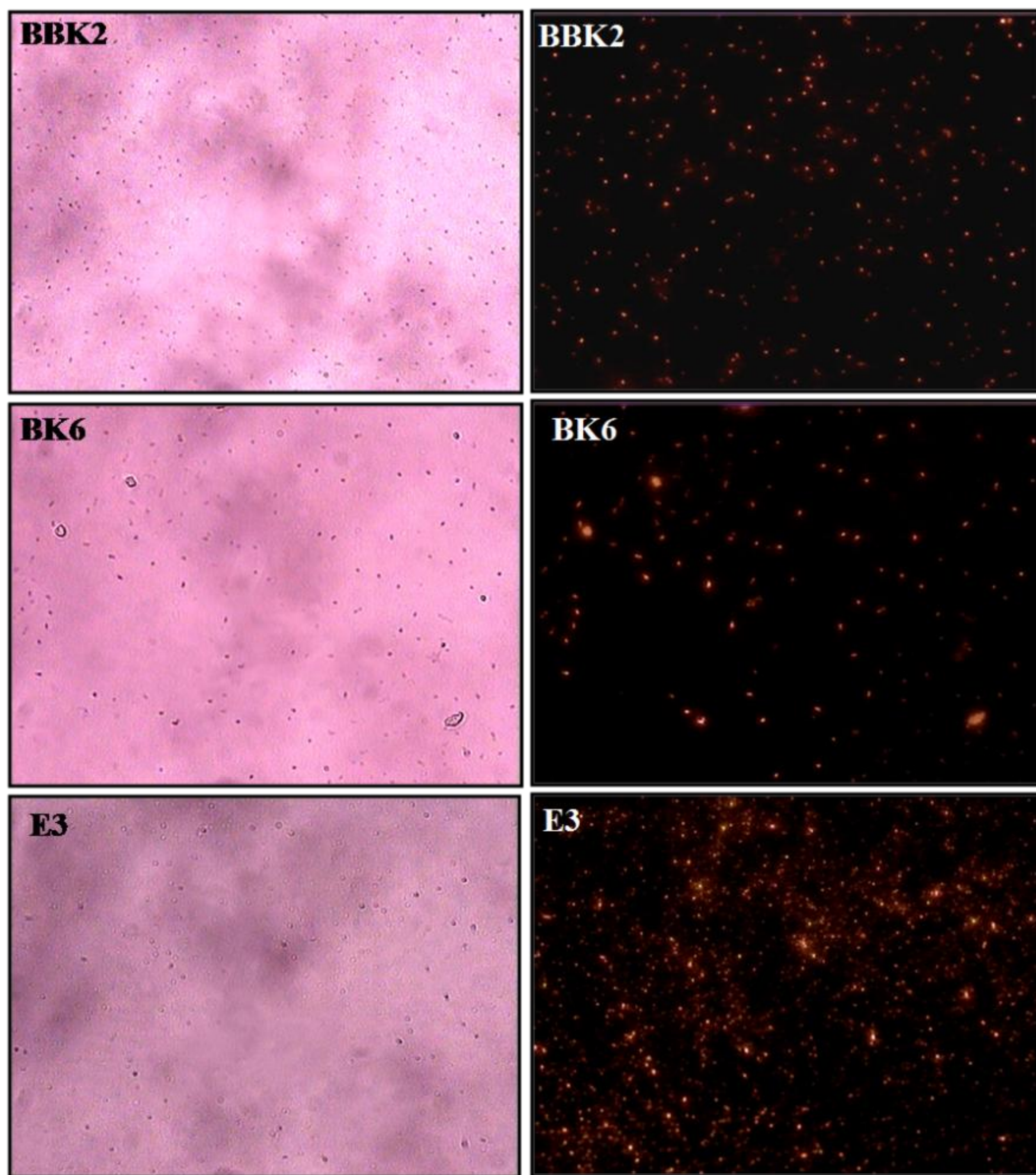

Fig. 4.3 Comparison of haloarchaeal cells under phase contrast and fluorescence microscope, when grown in NGSM and stained with Nile Red stain.

4.3.2 Molecular characterization of the haloarchaeal isolates

The PHA producing isolates from the BK, BBK, BS, E and TN series were characterized at molecular level. Phylogenetic analysis of the 16S rRNA gene sequence data revealed that

the E4, BBK1 and all BK series isolates belonged to genus *Halococcus*. Isolates BK3 and E4 were most closely related to *Hcc. saccharoliticus* ATCC49257 (99.52% and 99.14%) whereas the isolate BBK1 was most closely related to *Hcc. salifodinae* DSM8989 (99.39%). The isolates BBK2, BS16, TN4, TN5, TN6 and TN10 belonged to genus *Haloferax*, wherein the isolates TN4, TN5, TN6 and TN10 showed 100% similarity to *Hfx. prahovense* TL6. The isolates BBK2 and BS16 were most closely related to *Hfx. volcanii* DS2 (99.79%) and *Hfx. lucentense* JCM9276 (99.69%), respectively. Isolates BS1, BS2, BS5, BS7, BS8, BS13 and TN8 belonged to genus *Haloarcula*, wherein isolates BS1, BS5, BS7, and TN8 were most closely related to *Har. quadrata* 801030/1 (99.25%), *Har. marismortui* ATCC43049 (99.52%), *Har. salaria* HST01-2R (99.47%) and *Har. hispanica* ATCC33960 (99.56%), respectively. Isolates BS4 and J1 belonged to genus *Halorubrum* and *Haloterrigena* and were most closely related to *Hrr. trapanicum* NRC34021 (98.3%) and *Htg. thermotolerance* PR5 (98.91%), respectively. The isolates TN9 and E3 were most closely related to *Halogeometricum borinquense* DSM11551 (100% and 99.69%) (Fig. 4.4). Organisms from five different genera namely *Halococcus*, *Haloferax*, *Haloarcula, Haloterrigena* and *Halogeometricum* screened were found positive for PHA production. Four of these isolates were deposited at Microbial Culture Collection (MCC), National Centre for Cell Science, Pune, India (Table 4.2). They are *Hfx. volcanii* strain BBK2 (GenBank/EMBL/DDBJ accession number AB588756; MCC2589), *Hcc. salifodinae* strain BK6 (GenBank/EMBL/DDBJ accession number AB588757; MCC2602), *Har. japonica* strain BS2 (GenBank/EMBL/DDBJ accession number HQ455798; MCC2588), and *Hrr.* spp. strain BS17 (GenBank/EMBL/DDBJ accession number AB971753; MCC2603).

The haloarchaeal isolates belonging to genus *Haloferax* and *Halogeometricum* showed good PHA accumulation which was quantified by Nile Red fluorescence (Table 4.1). The haloarchaeal culture *Hgm. borinquense* strains TN9 and E3 grew faster with high cell density and therefore cell pelleting was simpler and polymer quantification was easier (Fig. 4.2). Further studies were conducted on *Hgm. borinquense* strains TN9 and E3.

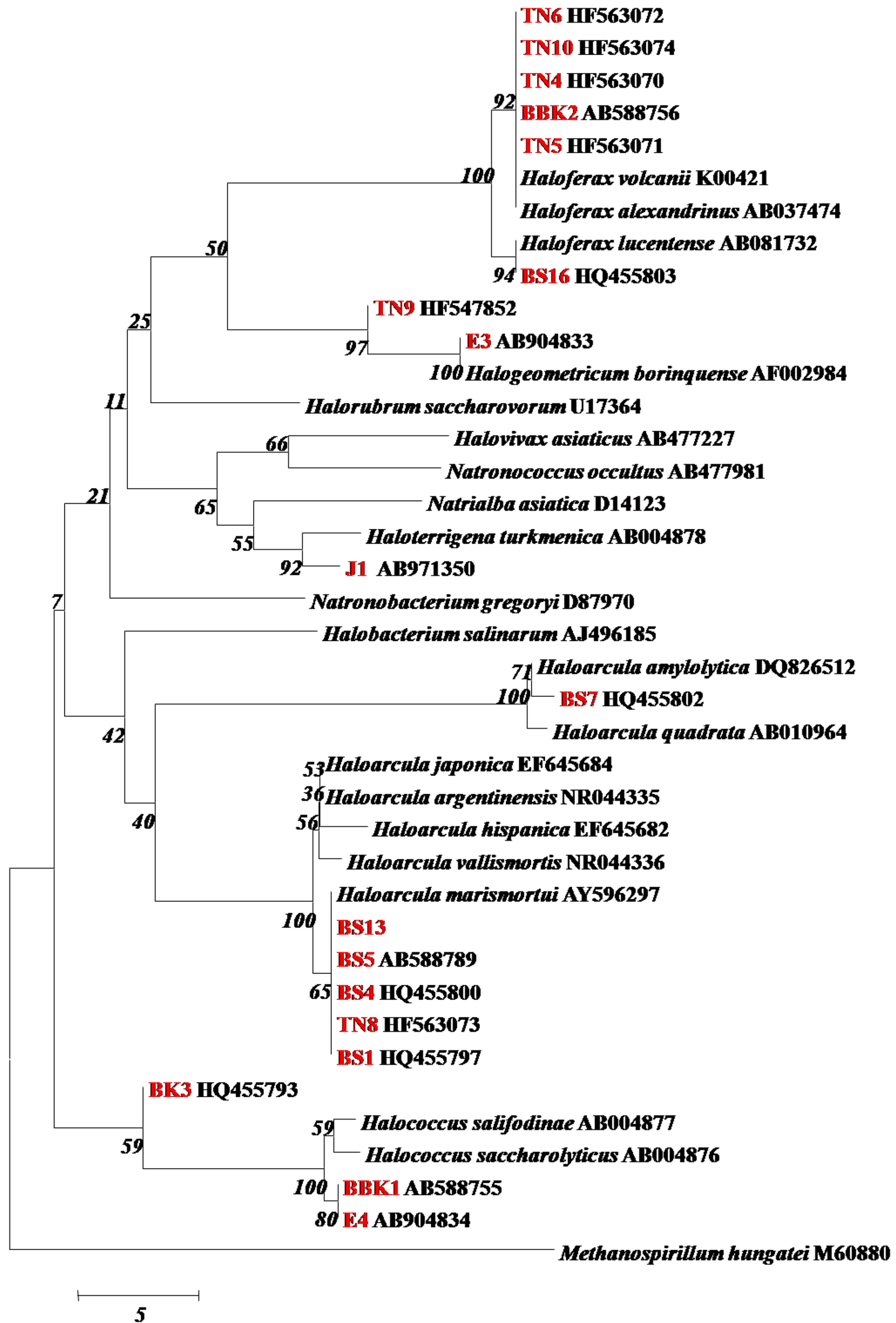

Fig. 4.4 Phylogenetic tree showing the positions of PHA accumulating haloarchaeal isolates based on 16S rRNA gene sequencing. Neighbor-joining tree was constructed with MEGA 5.0 with bootstrap values for 1000 replicates and displaying for 100. *Methanospirillium hungatei* is represented as an outgroup of the tree.

Table 4.2 Identification of the extremely halophilic archaeal isolates obtained from solar salterns of India.

Culture designation	Identification	Gene bank / EMBL / DDBJ accession number
BBK2	*Haloferax volcanii*	AB588756
TN4	*Haloferax volcanii*	HF563070
TN5	*Haloferax volcanii*	HF563071
TN6	*Haloferax volcanii*	HF563072
TN10	*Haloferax volcanii*	HF563074
BS16	*Haloferax lucentense*	HQ455803
TN9	*Halogeometricum borinquense*	HF547852
E3	*Halogeometricum borinquense*	AB904833
J1	*Haloterrigena turkmenica*	AB971350
BS17	*Halorubrum* spp.	AB971753
BS2	*Haloarcula japonica*	HQ455798
BS1	*Haloarcula marismortui*	HQ455797
BS4	*Haloarcula marismortui*	HQ455800
BS5	*Haloarcula marismortui*	HQ455789
BS7	*Haloarcula amylolytica*	HQ455802
TN8	*Haloarcula marismortui*	HF563073
BK3	*Halococcus salifodinae*	HQ455793
BK6	*Halococcus salifodinae*	AB588757
BBK1	*Halococcus salifodinae*	AB588755
E4	*Halococcus salifodinae*	AB904834

4.3.3 TEM analysis

Three halophilic archaeal isolates, *Haloarcula* strain BS2, *Haloterrigena* strain J1 and *Halogeometricum* strain E3 grown in NGSM supplemented with 2% glucose as sole source of carbon was analyzed for presence of intracellular PHA using TEM (Fig. 4.5). The cells of haloarchaeal isolates were oval shaped, with an average size ranging from 1.38 ± 0.05 x 0.89 ± 0.08 μm for BS2, 1.24 ± 0.2 x 0.56 ± 0.1 μm for E3 and 0.98 ± 0.05 x 0.717 μm for J1 (Fig. 4.5). Furthermore, all the cells contained PHA inclusions with various shapes from oval to irregular with average diameters ranging from 0.39 ± 0.01 x 0.27 ± 0.004 μm for BS2, 0.42 ± 0.05 μm for E3 and 0.64 μm for J1, respectively. The oval cells of isolate BS2

contained one, two, or three PHA inclusions, E3 contained single circular inclusion whereas J1 showed irregular inclusions.

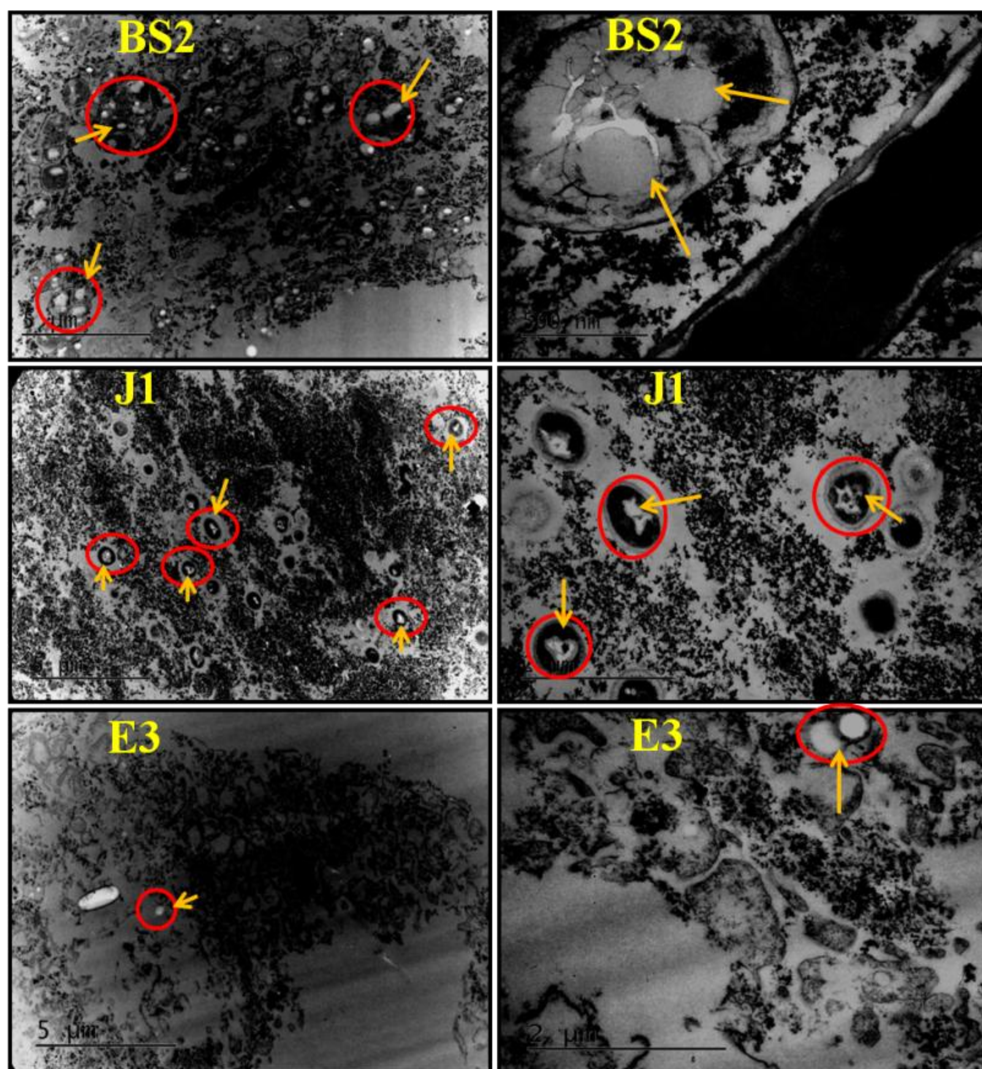

Fig. 4.5 TEM images of cells of *Haloarcula* strain BS2, *Haloterrigena* strain J1 and *Halogeometricum* strain E3 grown in NGSM supplemented with 2% (w/v) glucose.

4.3.4 PHA production by various haloarchaeal isolates

The halophilic archaeal isolates, BK6, BBK2, BS2, J1, TN9 and E3 grew in NGSM with 2% (w/v) glucose as sole carbon source. The growth as cell dry weight (CDW) (g l^{-1}) and PHA content (g l^{-1}) for all the haloarchaeal isolates is represented in Fig. 4.6. Among the six halophilic archaeal isolates grown in liquid NGSM, the isolates TN9 and E3 grew faster

(within 2 days) and luxuriously compared to others. The isolates with the highest biomass content as CDW (g l^{-1}) was E3 (5.8 ± 0.4) > TN9 (2.38 ± 0.04) > BK6 (2.145 ± 0.33) > BBK2 (1.915 ± 0.43) > BS2 (1.91 ± 0.09) > J1 (1.89 ± 0.48). The intracellularly accumulated PHA content (g l^{-1}) was highest in isolate E3 (4.0 ± 0.045) > TN9 (0.872 ± 0.045) > BS2 (0.87 ± 0.02) > BBK2 (0.606 ± 0.05) > J1 (0.12 ± 0.04) > BK6 (0.063 ± 0.001).

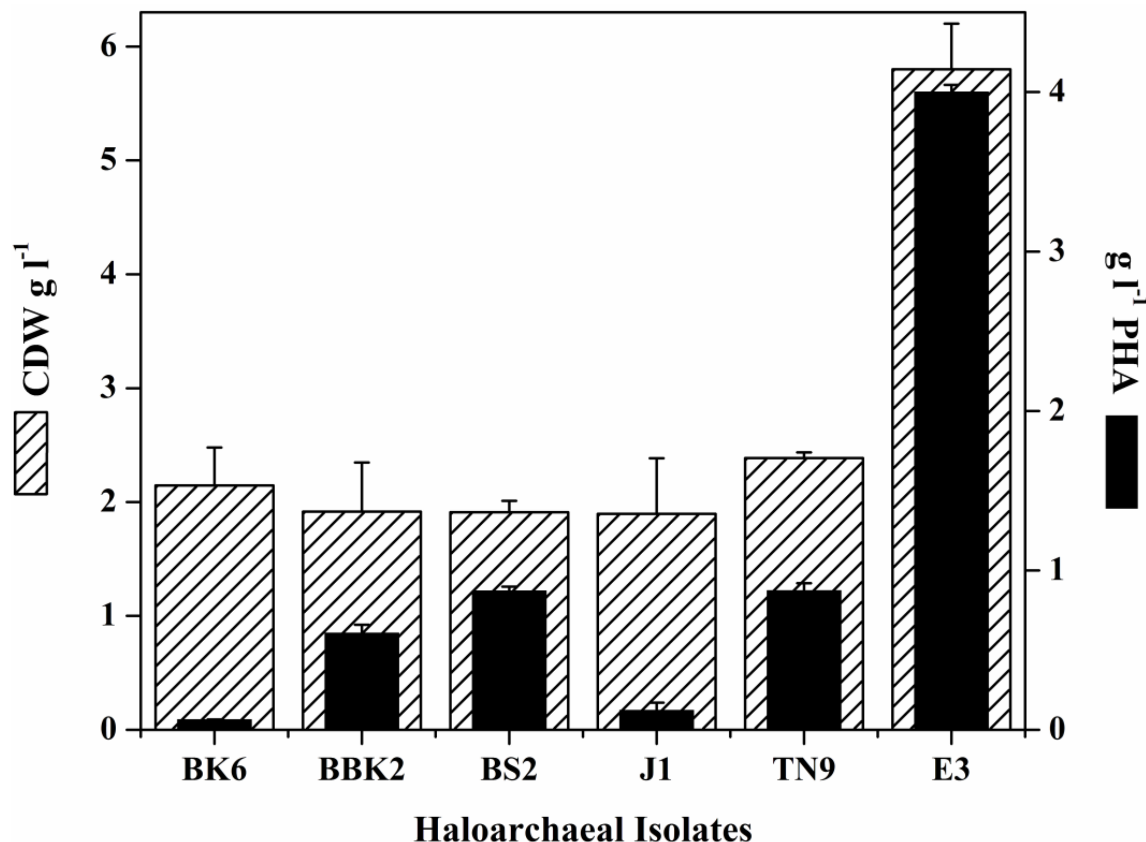

Fig. 4.6 Comparison of CDW and PHA (gl^{-1}) produced by extremely halophilic archaeal isolates grown in NGSM with 2% (w/v) glucose.

Extremely halophilic microorganisms are known to be the dominant microflora of the crystallizer ponds of solar salterns and are gaining attention for their versatile biotechnological potential (Quillaguaman et al., 2010; Anton et al., 2000). Among the halophilic archaeal isolates screened for the PHA production, *Halogeometricum borinquense* strains TN9 and E3 were found to be the best producers of PHA. These two isolates were able to grow lavishly at high salinity (20% NaCl) and showed better

accumulation of PHA than the rest of the isolates. Hence, further studies were carried with *Hgm. borinquense* strains TN9 and E3.

Table 4.3 Comparison of cell dry weights (CDW) and PHA yields of *Hgm. borinquense* strain E3 with various haloarchaeal isolates.

Haloarchaeal strain	Media	Carbon source	CDW (gL^{-1})	Maximum PHA (gL^{-1})	PHA Yield (% w/w)	PHA type	Reference
Hgm. borinquense strain E3	NSM	Glucose	4.06 ± 0.04	5.8 ± 0.4	70.0 ± 1.7	PHBV	Present study
Hgm. borinquense strain TN9	*NSM	Glucose	4.6	0.65	14	PHB	
	NSM	Glucose	2.38	0.88	36.9	ND	
Har. japonica strain BS2	NSM	Glucose	1.91	0.87	45.5	ND	
Htg. thermotolerance strain J1			1.89	0.12	6.34	ND	
Hfx. volcanii strain BBK2			1.19	0.606	50.92	ND	
Hcc. salifodinae strain BK6			2.14	0.063	2.94	ND	
Haloarcula sp. IRU1	BM	Glocose	1.58	0.98	62.03	PHB	Taran, 2015
		Starch	1.43	0.81	56.64		
Haloarcula hispanica	MST	Glocose	4.7	0.58	12.26	PHBV	Lu et al., 2008
Haloferax mediterranei	MG	Starch	7.01	1.74	24.88		
Haloarcula hispanica AS2049	MG	Glucose	10.2	0.9	8.6	PHBV	Han et al., 2009
Haloferax volcanii (pWLG1EC)			4.2	0.5	3.1		
Hfx. mediterranei ATCC 33500	MM	Glucose	85.8	23	27	PHBV	Huang et al., 2006

CDW: cell dry weight; PHB: poly (3-hydroxybutyrate); PHBV: poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate); NSM: NaCl synthetic medium; BM: basal media; MM: mineral medium; *NSM: NSM devoid of yeast extract.

4.3.5 Growth profile and polymer accumulation study of haloarchaeal isolate

4.3.5.1 *Hgm. borinquense* strain TN9

The time-course of growth and polymer accumulation by TN9 culture in NGSM with 2 % (w/v) glucose is presented in Fig. 4.7. Growth of the culture increased steadily with a lag phase of 2 days (48 hrs) followed by logarithmic phase of 4 days (96 hrs) and attained the stationary phase on 7th day (168 hrs) which lasted till 11th day (264 hrs). Accumulation of polymer, though started at the early log phase [~ 0.9–1.6 % (w/w) of CDW], maximum accumulation was at the mid-log phase, 5th day (~ 14 % (w/w) of CDW), after which a decline in the polymer level was observed till the 8th day, ~ 8.3–7.7 % (w/w) of CDW. Interestingly, after 8th day there was aggregation or clumping of the cells in the culture medium due to which it was difficult to estimate the concentration of the polymer, ~ 10–12 % (w/w) of CDW.

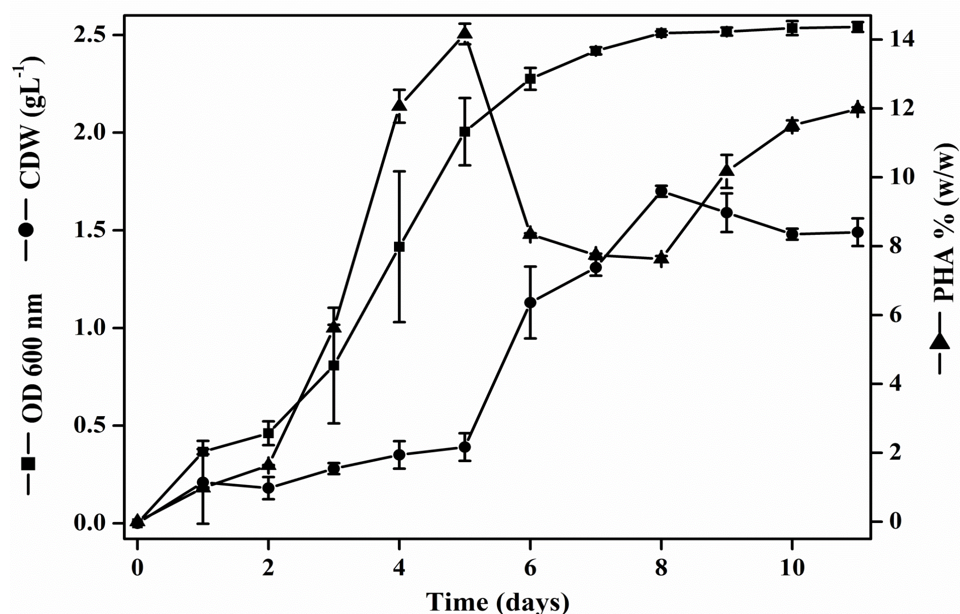

Fig. 4.7 Growth profile of extremely halophilic archaeon, *Hgm. borinquense* strain TN9 in NGSM supplemented with 2% (w/v) glucose.

4.3.5.2 *Hgm. borinquense* strain E3

The time-course of growth of *Hgm. borinquense* strain E3 in NGSM with 2% (w/w) glucose is presented in Fig. 4.8. The isolate E3 in presence of 2% glucose grew steadily

with an initial optical density (OD at 600 nm) 0.09 ± 0.04 which increased to 1.17 ± 0.006 on 3rd day and reached its maximum of 1.89 ± 0.08061 on 7th day. Similarly, the CDW of the culture increased steadily with time from 0.7 ± 0.007 on 1st day to 1.3 ± 0.007 on 4th day with maximum of 2.1 ± 0.08 on 7th day. The culture showed a growth rate (μ) of 0.235 ± 0.01. Active intracellular polymer synthesis of 6.5 ± 0.76% (w/w) of CDW was observed on the 1st day, which increased gradually and reached a maximum of 73.5 ± 1.7% (w/w) of the CDW on the 7th day. Rapid consumption of glucose by the isolate was observed within the first 3 days from the start of study and slowed down till the 7th day. A steady drop in the pH of the medium from 7.3 to 4.5 was observed.

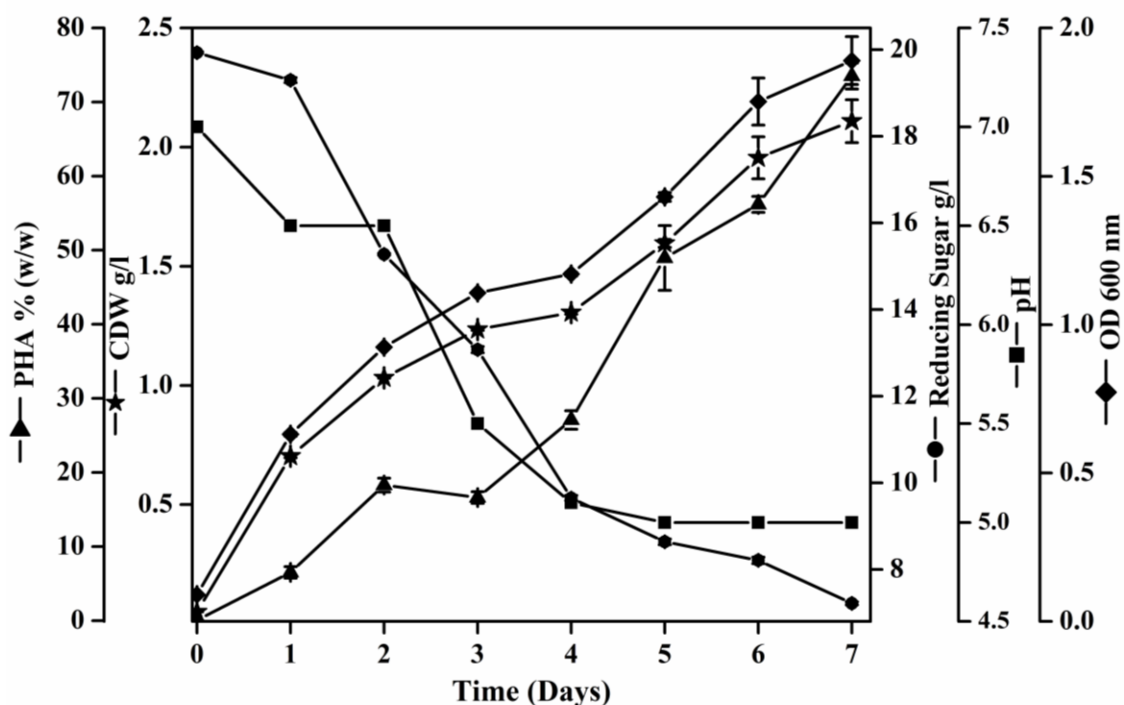

Fig. 4.8 Growth profile of extremely halophilic archaeon, *Hgm. borinquense* strain E3 in NGSM supplemented with 2% (w/v) glucose.

As compared to bacteria, there are few reports on PHA synthesis and accumulation using glucose as substrate by haloarchaeal generas mainly *Haloferax* and *Haloarcula* of family *Halobacteriaceae* (Table 4.3; Han et al., 2010). Fernandez-Castillo et al, 1986, reported PHB accumulation using yeast extract and glucose by *Hfx. mediterranei*, *Hfx .volcanii*, *Har. hispanica* and *Hfx. gibbonsii* 17.0 > 7.0 > 2.4 > 1.2 % (w/w) of CDW, respectively. Lillo and Rodriguez-Valera, (1990), reported PHB accumulation by *Hfx. mediterranei*

using glucose and starch as substrate was stable over a three month period. Han et al., 2007 reported the synthesis of 21.0 % (w/w) of CDW of PHB using yeast extract/glucose by *Hfx. mediterranei* and *Har. marismortui*. Moreover, there are reports on PHBV synthesis, 23.0 g L^{-1} and 0.58 ± 0.03 g L^{-1} by *Hfx. mediterranei* and *Har. hispanica* using glucose and yeast extract and glucose as substrate (Huang et al., 2006; Lu et al., 2008). 0.5 and 63 % (w/w) of CDW of PHB was synthesized by *Har. japonica* strain T5 and *Haloarcula* sp. IRU1 using glucose (Nicolaus et al., 1999; Taran and Amirkhani, 2010). In our present study, we have reported for the first time, the ability of *Hgm. borinquense* strains TN9 and E3 to synthesize PHA, 14.0 and 73.5% (w/w) of CDW, respectively using glucose as sole source of carbon.

4.3.6 Polymer characterization

Intracellularly accumulated polymer of *Hgm. borinquense* strains TN9 and E3 was extracted using sodium hypochlorite as described in Chapter III, section 3.2.7. The polymer was characterized using crotonic acid assay, FT-IR, XRD, TGA–DTA and NMR analysis.

4.3.6.1 Crotonic acid assay

Acid hydrolysis of the polymer to crotonic acid gave a characteristic peak at 235 nm comparable to that of the standard PHB (Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India) (Fig. 4.9) (Law and Slepecky, 1961; Sharma and Mallick, 2005).

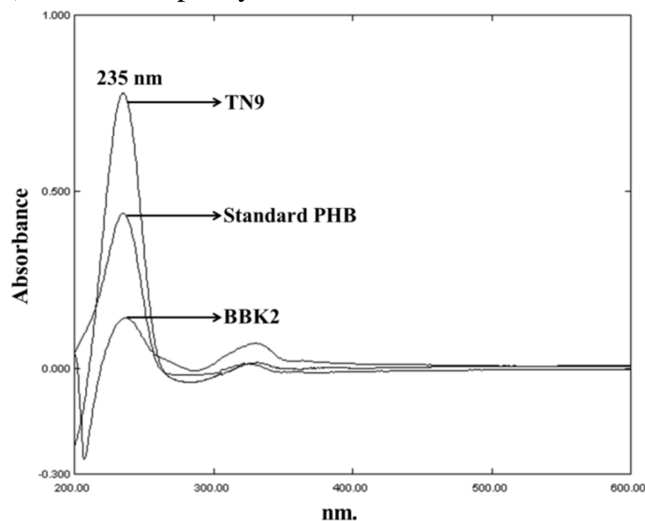

Fig. 4.9 Spectrophotometric scans of crotonic acid of PHA obtained from haloarchaeal isolate TN9, BBK2 and standard PHB.

4.3.6.2 FT-IR analysis

The FT-IR spectra of the polymers showed a prominent absorbtion band at 1724 cm^{-1} characteristic of ester carbonyl group (C=O) stretching. Band of carbonyl stretching appearing between 1744 - 1722 cm^{-1} provides information on the degree of crystallinity of the polymer (Arcos et al., 2010). The band at 1278 cm^{-1} and 1281 cm^{-1} represented C-O-C stretching whereas in the region around 2900 cm^{-1} represents C-H stretching (Padermshoke et al., 2004) (Fig. 4.10). There was a distinct band at 980 cm^{-1} in the polymer. Bands around 977 cm^{-1} corresponds to copolymer of P(HB-*co*-HV) (Bloembergen,\ et al., 1986). Apart from these absorption bands, other minor bands observed between 750-1500 cm^{-1} may be as a result of stretching shifts due to interactions between the -OH and C=O groups (da Silva Pinto and Wypych, 2009).

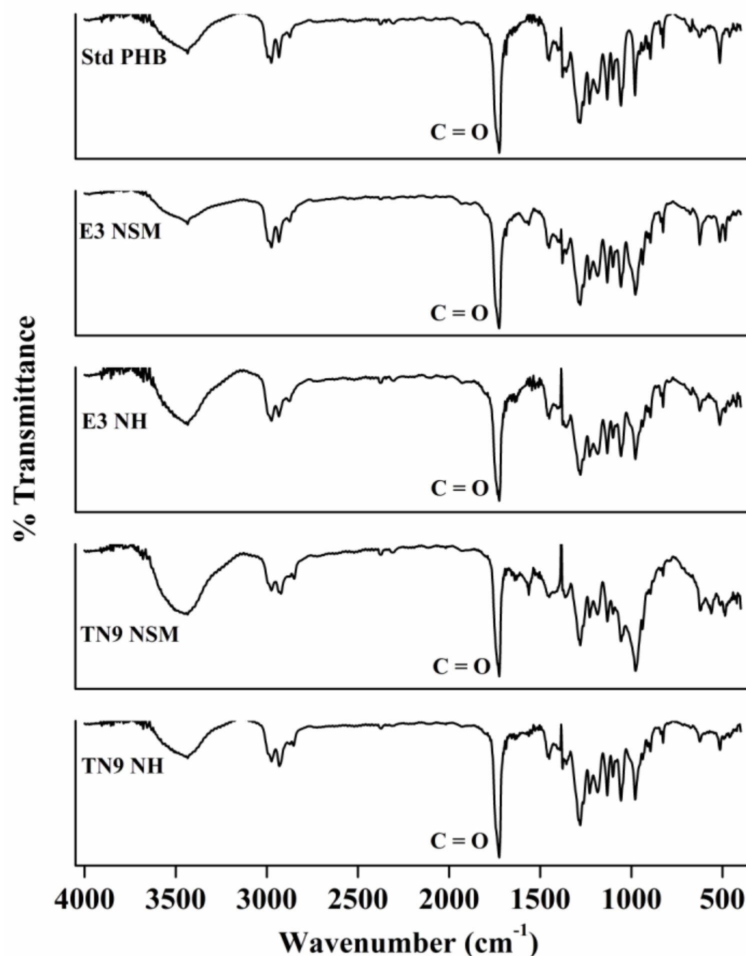

Fig. 4.10 Comparison of the FT-IR spectrum of the standard PHB (Sigma-Aldrich) with the polymers obtained from haloarchaeal isolate TN9 and E3 grown in NH and NGSM.

4.3.6.3 TGA analysis

The TGA thermogram of the polymer obtained from *Hgm. borinquense* strains E3 and standard PHB is represented in Fig. 4.11. A steady weight loss of ~ 14% up to temperature 186°C was obtained in the E3 polymer which further increased ~ 36% (weight loss) up to temperature 241°C. Total weight loss (~99.4%) was seen at temperature of 305°C. For standard PHB, the TGA showed only ~ 3.9% weight loss up to temperature 205°C which increased further to ~ 10% (weight loss) up to temperature 228°C with total weight loss (100%) at 260°C.

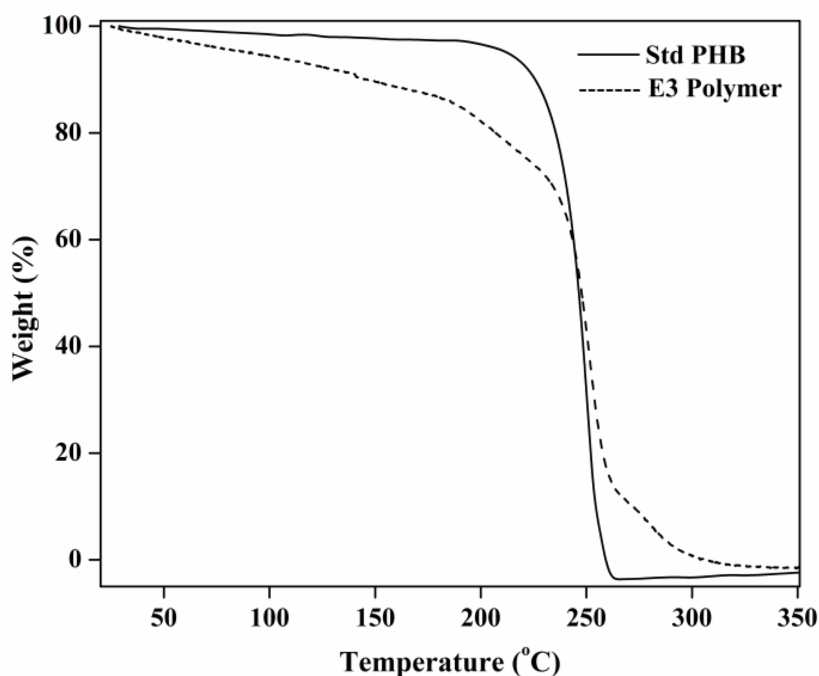

Fig. 4.11 Comparison of the TGA curve of polymers obtained from *Hgm. borinquense* strain E3 with standard PHB.

4.3.6.4 XRD analysis

The X-ray diffraction (XRD) patterns of the E3 polymer in comparison with standard PHB is depicted in Fig. 4.12. The E3 polymer exhibited prominent peaks at 2θ = 14.5°, 17.17°, 26.08° and 32.28° corresponding to (020), (110), (121) and (002) reflections. The standard PHB profile exhibited prominent peaks at 2θ = 13.4°, 17.15°, 20.1°, 22.7°, 25.5° and 30.3° corresponding to (020), (110), (101), (111), (121) and (002) reflections (Their et al., 2011). Overall, the diffraction pattern of the polymer was similar to that of the standard PHB except for a decrease in peak intensity and shifts. The three diffraction peaks between 2θ =

20–25° (ie. 20.1^{o}, 21.6^{o}, 22.6^{o}) observed in case of PHB were absent in E3 polymer. Broadening of the peaks clearly indicates decrease in crystallinity indicating amorphous nature of the polymer. The crystallite size was calculated from the Scherrer equation as described in Chapter III, section 3.2.8.3, using the (020) and (110) peak and was found to be 22.3 nm and 12.17 nm in case of standard PHB and E3 polymer, respectively. The XRD profile of polymer obtained using glucose as substrate correlated with the XRD spectra of pure PHBV reported by Farago et al, (2008).

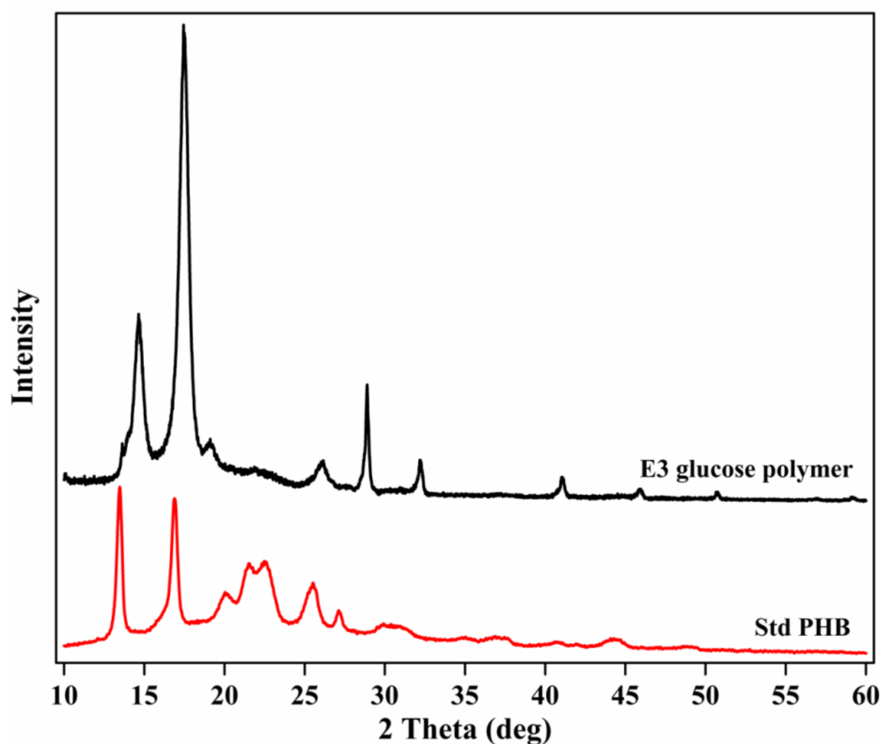

Fig. 4.12 Comparison of the X-ray diffraction patterns of polymer from *Hgm. borinquense* strain E3 grown in NGSM with 2% glucose with the standard PHB.

4.3.6.5 ^{1}H NMR analysis

The monomeric composition of the polymer was determined by ^{1}H NMR spectrum (Fig. 4.13) which provided the details of the functional groups in the polymer. ^{1}H NMR scan of the polymer obtained from *Hgm. borinquense* strain TN9 showed a doublet at 2.06–2.65 ppm corresponding to methylene group (–CH_2) as compared to signals obtained at 2.45–2.66 ppm of standard PHB from Sigma-Aldrich (Chapter III, section 3.3.6.5). The signal at 5.25–5.27 ppm corresponded to methine group (–CH–), while standard PHB gave signal at

5.24–5.30 ppm. Another signal at 1.3 ppm corresponded to the methyl group (–CH_3) was also observed. Signals were also obtained between 1.24 and 1.29 ppm for methyl protons as compared to 1.27–1.29 ppm of the standard PHB. The signal at 7.27 ppm corresponds to that of $CDCl_3$ (Fig. 4.13A). From this data, one can confirm that the polymer obtained from strain TN9 is a homopolymer of 3-hydroxybutyrate (HB).

The ^1H NMR scan of the polymer obtained from *Hgm. borinquense* strain E3 showed a signal at 0.85 - 0.91 ppm and 1.26-1.28 ppm corresponding to methyl (–CH_3) group of hydroxyvalerate (HV) and hydroxybutyrate (HB), respectively. Another signal at 1.6 ppm represented the methylene (–CH_2) group of HV. A doublet at 2.44-2.49 and 2.52 - 2.63 ppm corresponded to methylene group (–CH_2) of both HV and HB, respectively. Signals at 5.22 and 5.26 ppm corresponded to methine group (–CH–) of both HV and HB, respectively. The signal at 7.26 ppm corresponds to that of $CDCl_3$ (Fig. 4.13 B). All these analysis confirmed that the polymer obtained from *Hgm. borinquense* strain E3 is a co-polymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)]. The intensity ratios of the signals were used for the quantative estimation of the monomeric units of the polymer.

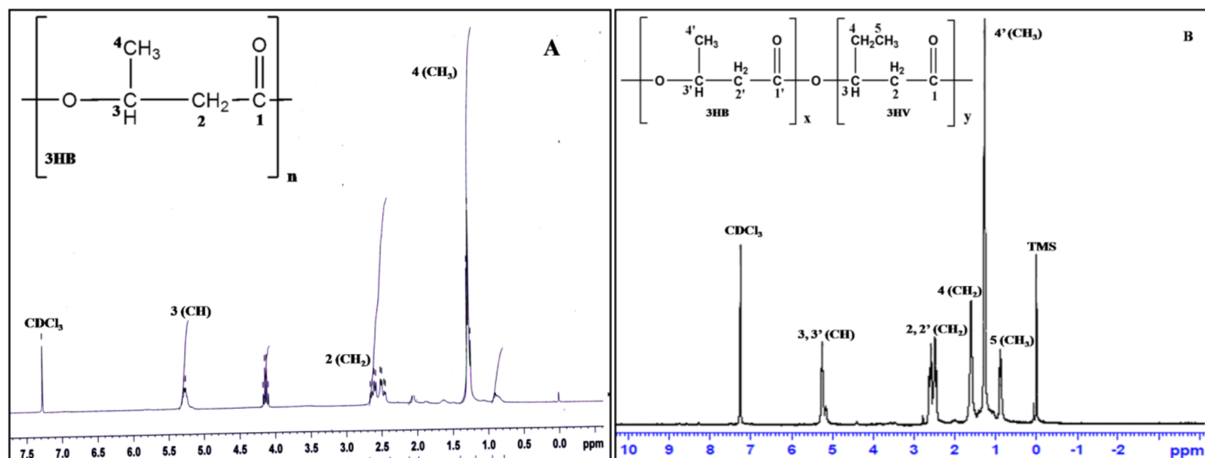

Fig. 4.13 ^1H NMR spectrum of the polymers obtained from *Hgm. borinquense* strains TN9 and E3 grown in NGSM with 2% Glucose.

Composition of HV (%) in the co-polymer of P(HB-*co*-HV) was calculated by substituting the area values of HV (0.9 ppm) and HB (1.25 ppm) in the following equation as described by Liu et al., (2010).

$$\text{HV composition (\%)} = \frac{\text{Area } CH_3 \text{ (HV)}}{\text{Area } CH_3 \text{ (HV)} + \text{Area } CH_3 \text{ (HB)}} \times 100\ \%$$

Using the above equation, it was calculated that the co-polymer [P(HB-*co*-HV)] produced by the extremely halophilic archaeon *Hgm. borinquense* strain E3 by utilization of glucose comprises 21.47% HV units (Fig. 4.13B). PHAs with varying monomeric units can be synthesized by feeding the microorganism with various substrates. Recent study by Wang et al., (2013) investigated the effect of levulinic acid as co-carbon sources along with different carbon substrates like fructose, glucose, sucrose, lactose, etc on copolymer P(HB-*co*-HV) production using bacterial strain *Ralstonia eutropha* H16. When glucose was used as the sole source of carbon a 2.44 ± 0.59 gL^{-1} of homopolymer of PHB was obtained. Interestingly, when glucose along with levulinic acid as co-carbon source was used 3.18 ± 0.25 gL^{-1} copolymer of P(HB-*co*-HV) with 21.4 ± 1.7% HV content was obtained. A recent detailed review by Quillaguaman, et al., (2010) however stated that haloarchaea such as *Hfx. mediterranei*, *Har. hispanica* are able to synthesize P(HB-*co*-HV) even in the absence of additional co-carbon substrates such as propionic and / valeric acid.

Polyhydroxybutyrate (PHB) was the first polymer of microbial origin, to be identified and characterized. However, its crystalline, stiff and brittle nature limits its application. Moreover, its temperature of degradation (T_d = ~273°C) is just a few degrees above its melting temperature (T_m = ~170°C), due to which it shows poor stability at temperatures above the melting. Therefore research is presently focused on modification of the PHB with improved properties and this can be achieved by incorporation of other monomers like HV in the polymer chain of HB to obtain copolymers. Though both PHB and P(HB-*co*-HV) are short-chain-length PHAs (scl-PHAs) consists of 4 and 5 carbon atoms in length 3-hydroxyalkanoate (3HA) monomers, the copolymer has better physical properties like toughness, flexibility and impact resistance than its homopolymer counterpart. Moreover, different proportions of HV can greatly influence the performance of the copolymer for

instance; increase in the HV content decreases the T_m of the polymer (Wang et al., 2013). Importantly, P(HB-*co*-HV) has attracted widespread attention because of its biocompatible and biodegradable nature due to which it has been used as material for medical applications such as designing scaffolds for various tissue engineering approaches (Sultana and Khan, 2012). Study by Liu et al, compared *in vitro* degradation, bone cell (osteoblast cell) attachment and proliferation of PHBV matrices with various concentrations of HV and found that HV concentration drastically affects the cell attachment and proliferation, with a concentration of 12% HV being the best.

4.4 Conclusion

Among the forty four extremely halophilic isolates screened, TN9 and E3 isolates grew fast with the accumulation of PHA within 3–5 days, followed by strains BBK2, TN4–7 and TN10 within 5–8 days. Based on phenotypic, chemotaxonomic and genotypic characterization, the organisms were grouped in five different genera namely *Halococcus*, *Haloferax*, *Haloarcula, Haloterrigena* and *Halogeometricum* of the family *Halobacteriaceae*. The isolates *Hgm. borinquense* strains TN9 and E3 showed maximal PHA accumulation of 14.0 and 73.5% (w/w) of CDW in NGSM supplemented with 0.2% nitrogen source and 2% carbon source. The crotonic acid assay, FT-IR, XRD, TGA-DTA and ^{1}H NMR analysis indicated the polymer produced by *Hgm. borinquense* strain TN9 to be a homopolymer of 3-hydroxybutyrates (PHB) while E3 accumulated a co-polymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)]. Industrial processes require organisms withstanding harsh physicochemical conditions like high salt concentration analogous to high ionic strength and low water activity (aw). *Hgm. borinquense* strain E3 could be engineered further for industrial production of PHA due to its robust growth at varying pH, temperature and NaCl concentration.

Chapter V

Optimization of Culture Conditions and Polymer Extraction Procedures

5.1 Introduction

PHAs are accumulated by microorganisms when exposed to excess carbon substrates and low amounts of essential nutrients such as nitrogen and oxygen. Therefore it is important to determine the nature and amount of carbon substrate for optimal PHA production. PHA's are accumulated intracellularly as insoluble granules. To recover the intracellular PHA, one needs to lyse the cell wall and cell membranes, followed by solubilization and purification of the PHA polymer. The routinely employed methods for bacteria are (i) solvent based extraction employing various solvents like $CHCl_3$, acetone, methanol, etc, (ii) chemical digestion method employing sodium hypochlorite (NaClO) and (iii) physical disruption of cells by sonication (Kunasundari and Sudesh 2011; Tan et al., 2014b).

Extremely halophilic archaea thrive in econiches having high NaCl concentrations of 25 % (w/v) (4.3 M). Such high salt concentrations in growth/production media lead to low risks of contamination by eubacteria and yeasts. The haloarchaeal cells are also known to lyse in water/low osmolarity solutions thus aiding in the easy extraction of the intracellular PHA (Quillaguaman et al., 2008, 2010). In this chapter parameters such as various substrate concentrations, NaCl, pH, temperature and different methods of extractions were employed to give an optimal PHA yield.

5.2 Materials and methods

5.2.1 PHA production in *Halogeometricum borinquense* strain E3 under varying growth conditions

Extremely halophilic archaeon, *Halogeometricum borinquense* strain E3 was selected for optimization of culture conditions and polymer extraction procedures. To evaluate the optimum culture conditions for PHA production, mid log phase (10^6 cells/ml) of E3 grown in EHM was used as starter culture.

E3 was grown in NSM containing 1% (w/v) of two different substrates, glucose and starch.

The effect of varying concentrations of substrate and salinity on cell growth and PHA accumulation was determined by varying glucose (0.5%, 1%, 2%, 3%, and 4%) and sodium

chloride (5%, 10%, 15%, 20%, 25% and 30%) in NGSM (Appendix I) at pH 7.0. Cultures were incubated at 37 °C at 100 rpm.

The optimum temperature for cell growth and PHA accumulation was determined by incubating E3 at temperatures such as 27°C, 37°C and 47°C, respectively.

The pH optimum for cell growth and PHA accumulation was determined in NGSM at different pH values (pH range 4-11, with 1 pH unit increase). Determination of cell mass was done gravimetrically as cell dry weight (CDW) and quantification of the polymer production was done by crotonic acid assay, as described in Chapter III, section 3.2.4.

5.2.2 Various polymer extraction methods

E3 was grown in batches of one liter of NGSM with 2% (w/v) glucose as carbon source. The flasks were incubated for 7 days at 37°C on a rotary shaker at 100 rpm after which the polymer was extracted. Various methods for optimal extraction of the polymer were carried out and are summarized in Fig. 5.1.

5.2.2.1 Physical disruption

To cell pellet from 20 ml of the liquid culture, 20 ml of distilled water was added. The cells were uniformly suspended in water by vortexing for 5 min. This suspension was kept at room temperature (27°C) on a gel rocker for 5-6 hrs. The suspension was centrifuged 8000 rpm for 20 min. The supernatant was discarded and the polymer pellet was dried in oven at 60°C.

5.2.2.2 Non-solvent based extraction

(i) Sodium hypochlorite (NaClO)

The cell pellet obtained from 20 ml of the liquid culture was extracted using sodium hypochlorite method (Rawte and Mavinkurve, 2002). To the cell pellet, 40 ml NaClO was added with 2% and / or 4% active chlorine. The cells were completely suspended in the NaClO and kept at 37°C for 20-30 min on shaker. The suspension was centrifuged 8000 rpm for 20 min and the supernatant was discarded. Polymer pellet obtained was washed twice with diethyl ether and dried at 60°C.

(ii) Sodium dodecyl sulfate (SDS)

The cell pellet obtained from 20 ml of the liquid culture was extracted using 20 ml of 0.1% (w/v) Sodium Dodecyl Sulphate (SDS) in distilled water. The cell suspension was kept at 37°C for 24hrs on an orbital shaker at 110 rpm. The suspension was centrifuged at 8000 rpm for 20 min and the polymer pellet obtained was dried at 60°C.

5.2.2.3 Solvent based extraction

The solvent based extraction methods employed chloroform ($CHCl_3$). The cell pellet obtained from 20 ml of the liquid culture was lyophilized (Christ, Alpha 1-2 LD plus) for 12 hrs and then extracted using chloroform (a) at 37°C for 24 hrs on shaking condition at 100 rpm and (b) at 60°C for 8-10 hrs using soxhlet apparatus. Up to 95% of the chloroform was collected by distillation using a rotary evaporator under vacuum at 60°C and the remaining 5% of the chloroform containing polymer was poured in a clean glass Petri dish and kept undisturbed in the cold (4°C) overnight for total evaporation to obtain the polymer film.

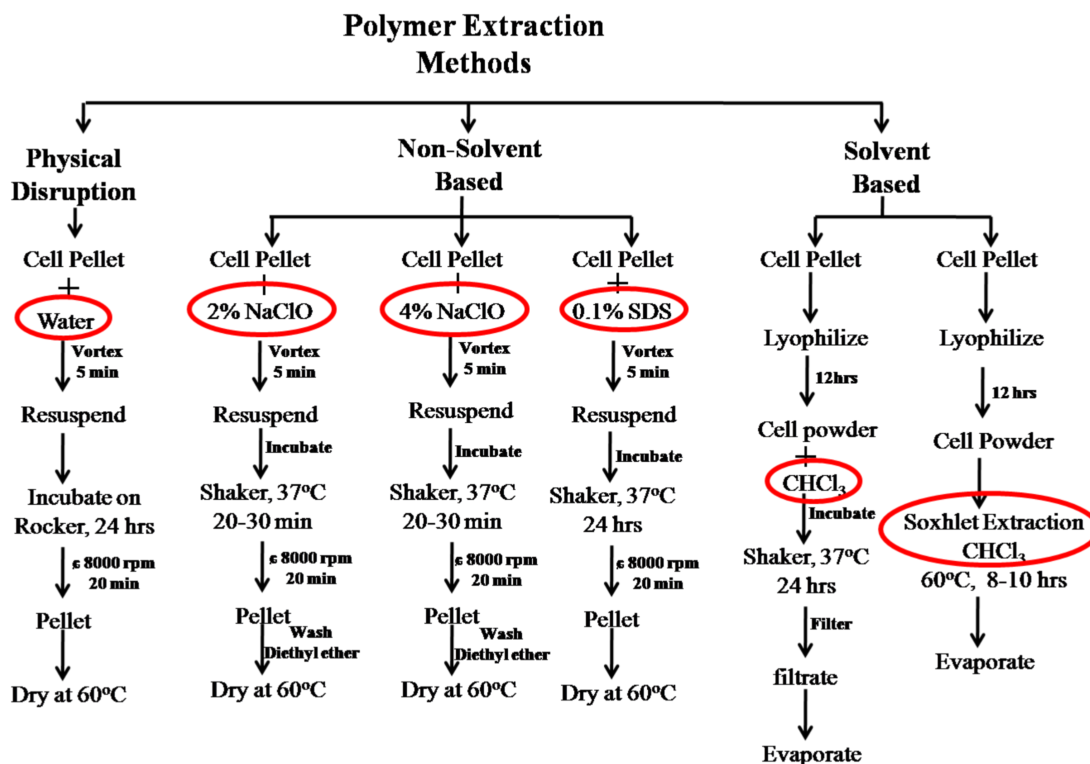

Fig. 5.1 Methods for extraction of PHA from extremely halophilic archaeon *Hgm. borinquense* strain E3 using various methods.

Quantification of the polymer obtained using various methods were done gravimetrically by drying the final product at 60°C until constant weight was obtained. The cell dry weight (CDW) of the culture was determined as described in Chapter III, section 3.2.4. The amount of PHA was calculated as PHA % (w/w) of CDW.

5.2.3 Characterization of the polymer

5.2.3.1 FT-IR analysis

The FT-IR spectra of the polymer pellet extracted using various methods were recorded on Shimadzu IRAffinity-1 spectrometer (Kyoto, Japan). Polymer was pulverized to fine powder using mortar and pestle mixed with moisture free KBr (IR grade). The polymer-KBr mixture was ground to fine powder and thin transparent pellets were made with the help of pellet maker. The pellet scans were recorded in the range of 500 cm^{-1} to 4000 cm^{-1} and a resolution of 4 cm^{-1} for 40 scans.

5.2.3.2 Differential scanning calorimeter (DSC) analysis

Thermal property of the polymer was tested by a differential scanning calorimeter (Shimadzu DSC 60). The polymer samples of approx. 2 mg weight were weighed and sealed in the aluminum DSC pans and placed in the DSC cell and heated under air, from 30°C to 500°C at a heating rate of 10°C/min.

The polymer obtained by soxhlet extraction was pigmented orange in colour. Therefore the polymer film was decolourised by immersing it in NaClO and acetone separately for 10 min (Fig. 5.5). Characterization of both the original pigmented polymer and decolourised polymer was done using ^{1}H NMR analysis as described in Chapter III, section 3.2.8.

5.3 Results and discussion

5.3.1 Optimization of culture conditions and PHA production

E3 was able to grow and accumulate PHA in NSM with 1% (w/v) glucose / starch with cell mass as CDW (g l^{-1}) of 3.19 ± 0.76 / 6.2 ± 0.3 and PHA content of (g l^{-1}) 2.49 ± 0.028 / 4.6 ± 0.23, respectively. In presence of starch, the residual starch interfered in the final PHA product, hence further studies were done using glucose. E3 grew over a range of glucose

concentrations, pH, temperature and salinity. E3 was able to grow over a wide range of pH from 4 - 11 and temperatures 27°C, 37°C and 47°C. PHA production at pH 8 - 10 was 3.0 ± 0.23 to 3.16 ± 0.1 (g l^{-1}) which decreased drastically at pH 11 to 0.8 ± 0.16 (g l^{-1}) (Fig. 5.2). At pH 4 and 5 very less amount of biomass was produced. Though optimum temperature for biomass production was at 47°C, 9.6 ± 0.8 (g l^{-1}) but low PHA yield of 1.3 ± 0.02 (g l^{-1}) was obtained. Negligible growth was observed at 0.5% and 4% glucose, and 5% NaCl concentration respectively. The best cell mass of 5.8 ± 0.4 (g l^{-1}) and PHA yield of 4.0 ± 0.045 (g l^{-1}) was obtained at 2.0% glucose, pH 7.0, 37°C and 20% NaCl (Fig. 5.2).

5.3.2 Methods of polymer extraction and quantification

The best method for obtaining PHA [% (w/w) of CDW] was, 4% NaClO (77.85 ± 0.3) > soxhlet (72.4 ± 3) > $CHCl_3$ 37°C (49.0 ± 1) > 2% NaClO (40.89 ± 1.4) > 0.1% SDS (19.05 ± 2.1) and water (14.1 ± 1.4) (Fig. 5.3).

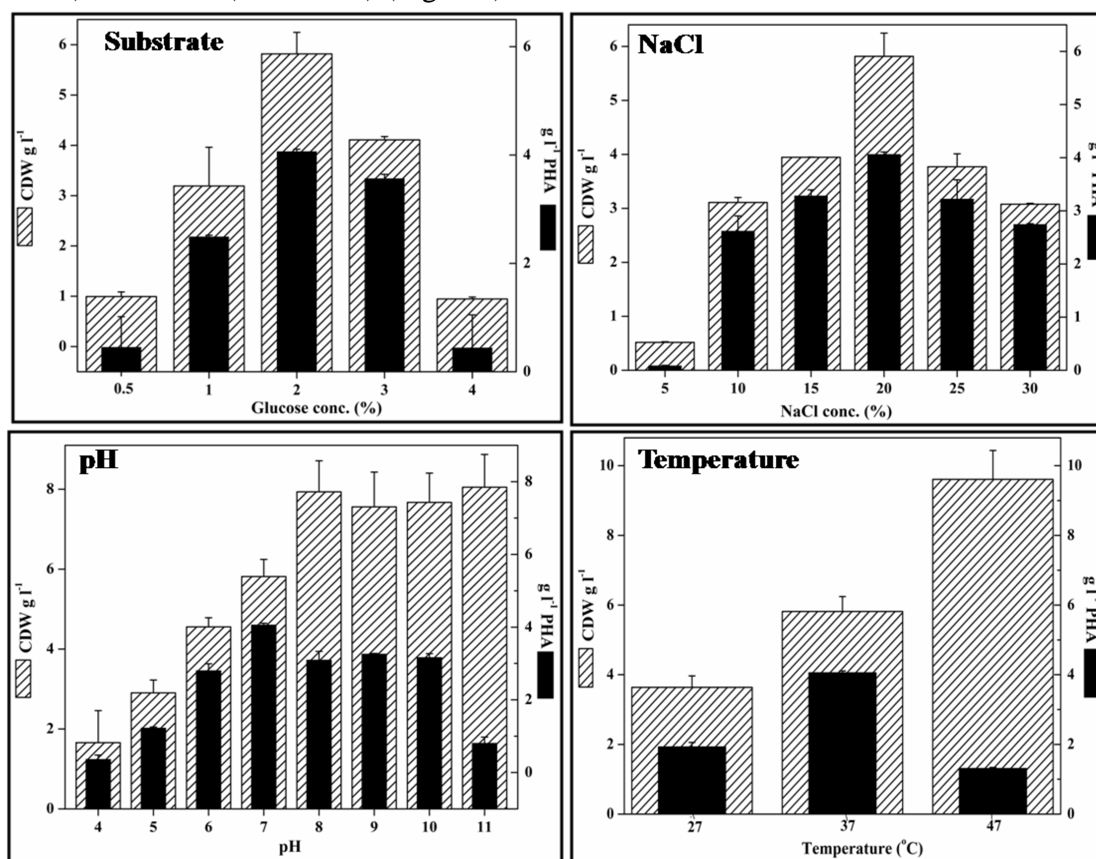

Fig. 5.2 Effect of varying substrate concentrations, salinity, pH and temperature on the cell growth and PHA production by isolate E3.

PHAs in microorganism are stored intracellularly as insoluble granules and their extraction from the cells is a very crucial process. For recovery of PHA, one needs to lyse the cell wall/cell membrane, followed by solubilization and purification of PHA polymer. Common methods for PHA polymer recovery from microbial biomass are solvent extraction methods, the non-solvent extraction methods are mostly chemical and / or enzyme based digestions methods (Kunasundari and Sudesh, 2011; Tan et al., 2014b).

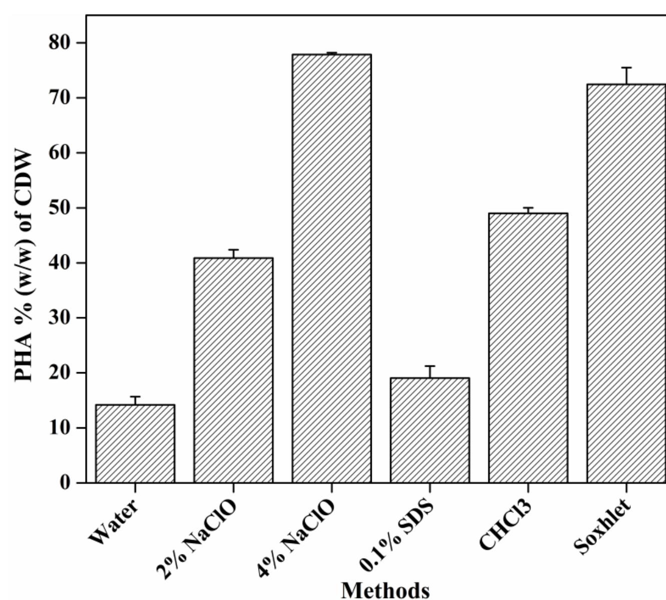

Fig. 5.3 Comparison of PHA % (w/w) of CDW obtained using various polymer extraction methods.

Hgm. borinquense strain E3 is an extremely halophilic archaeon which grows at high salinity. Suspending the cells in low osmolarity solution like distilled water resulted in physical disintegration of the S-layer containing cell wall, thereby releasing the intracellular PHA. However, PHA yield obtained was 14.1 ± 1.4 % (w/w) of CDW which was far less as compared to the other methods employed (Fig. 5.3). This may be due to the loss of polymer during harvesting, as the cell suspension in water tends to become viscous, thereby making the pellet very loose. Disruption of the cells using 0.1% SDS also gave low yield of PHA 19.05 ± 2.1% (w/w) of CDW.

Chemical methods routinely employed use compounds like sodium hypochlorite, (NaClO) which degrades the non PHA cellular material. Due to the simplicity and effectiveness,

NaClO, is widely employed for PHA extraction. However, there could be polymer degradation due to NaClO resulting in lower molecular mass polymers (Tan et al., 2014b). Polymer from E3 extracted using 4% NaClO gave the best PHA yield of 77.85 ± 0.3 % (w/w) of CDW as compared to 2% NaClO which gave yield of 40.89 ± 1.4 % (w/w) of CDW, respectively. The PHA obtained was also brittle due to the effect of NaClO on the polymer.

On the other hand, PHA recovery using enzymes leads to overall increase in the process cost. Hence, we employed solvent like $CHCl_3$ for the polymer recovery. Polymer film was obtained on evaporating $CHCl_3$ containing the polymer. The PHA film extracted using soxhlet apparatus at 60°C gave the best yield of 72.4 ± 3 % (w/w) of CDW as compared to 37°C which gave yield of 49.0 ± 1 % (w/w) of CDW, respectively. *Hgm. borinquense* strain E3 produces pink to orange carotenoid pigment which is an important chemotaxonomic marker of the family *Halobacteriaceae*. The pigments got co-extracted with the polymer, thereby giving the polymer a bright orange appearance. Treatment with NaClO and acetone turned the orange polymer film to creamish (Fig. 5.5).

5.3.3 FT-IR analysis of polymer

To confirm the final products obtained from E3, using various extraction methods were PHA, FT-IR analysis was done. Comparison of the FT-IR spectra of polymer obtained using starch and glucose by various extraction methods with standard PHB is represented in Fig. 5.4. Presence of prominent spectrum peak in the range of 1728 - 1738 cm^{-1} is characteristic of ester carbonyl group (C=O), is the diagnostic signal for PHAs and compared well with that of standard PHB (Sigma) (Kansiz et al., 2000). Additional peaks at around 1658 $cm^{-1}$and 1527 cm^{-1} were seen in polymer extracted using distilled water, SDS and soxhlet and represents the bacterial cellular protein amide (–CO–N–) I and II respectively (Gumel et al., 2012). Stretching of the ester group C‑O bond results in series of bands between 1,000 and 1,300 cm^{-1} (Ramezani et al., 2014). The polymer film obtained using $CHCl_3$ at 37°C was plastic in nature and could not be ground well with the KBr, hence did not show prominent peaks (Fig. 5.4).

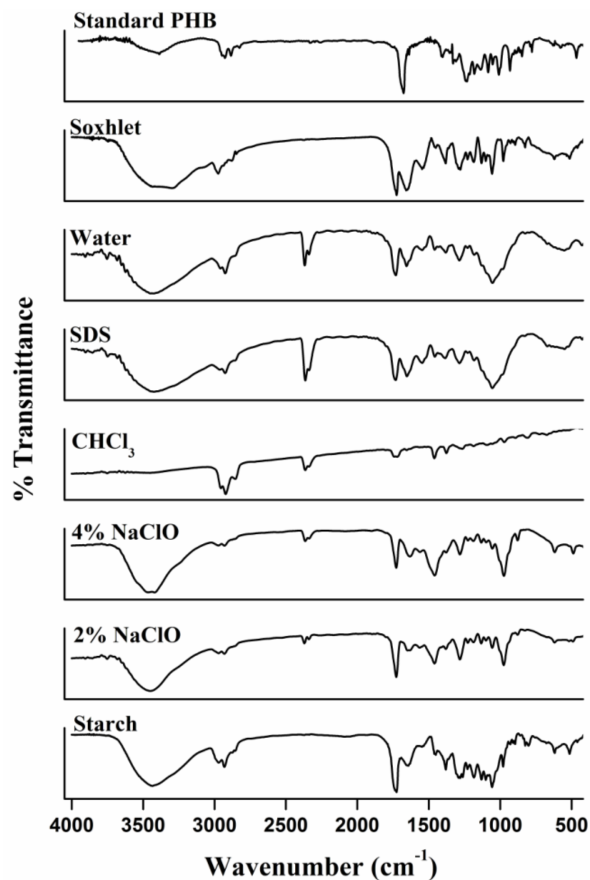

Fig. 5.4 Comparison of the FT-IR spectra of polymers from *Hgm. borinquense* strain E3 obtained using various extraction methods.

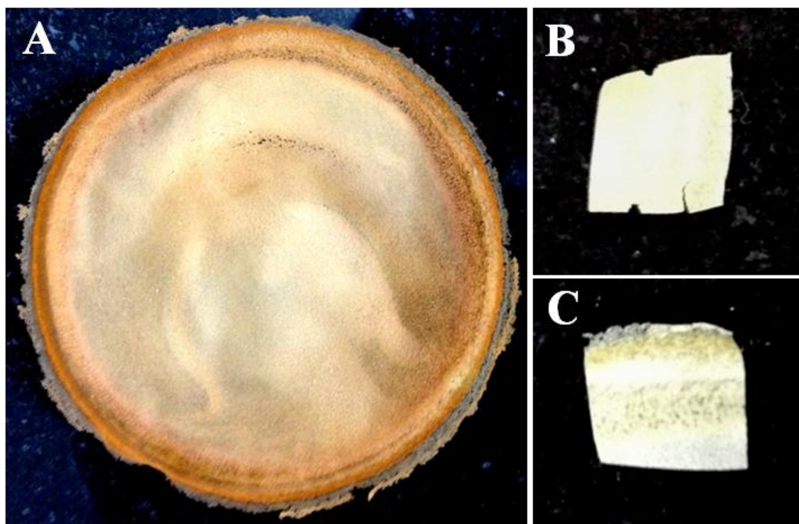

Fig. 5.5 Polymer film from *Hgm. borinquense* strain E3 grown in NGSM with 2% glucose (A) after soxhlet extraction, and decolourised polymer using (B) sodium hypochlorite (NaClO) and (C) acetone.

5.3.4 Comparison of polymers using DSC

The thermal properties were studied using DSC analysis (Fig. 5.6). No difference was observed in the polymer before and after decolourisation with acetone or NaClO. The thermograms displayed two melting endotherms at around T_{m1} = ~138°C and T_{m2} = ~154.5°C and a single thermal degradation (T_d) endotherm at T_d = ~231°C. The two T_m seen in case of the E3 polymer were much lower as compared to standard PHB which showed a single melting endotherm at T_m = 172 °C (Wang et al., 2013). According to Mitomo et al., (1999), multiple melting peaks may indicate the coexistence of two monomers in a polymer ie. a copolymers with varying monomer units like P(3HB-*co*-3HV). Hermann-Krauss et al., (2013) employed halophilic archaeon *Hfx. mediterranei* for production of 3HV-containing copolyesters and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate-co-4-hydroxybutyrate] (PHBHV4HB) terpolyesters from inexpensive crude glycerol phase from biodiesel production. Two melting endotherms at 122 °C and 137 °C were seen for the terpolyester. Recent study by Zakaria et al. (2013), have shown the production of P(3HB-*co*-3HV) copolymer using volatile fatty acids derived from palm oil mill effluent by a bacterial isolate *Comamonas* sp. EB172. Reports by Sudesh et al. (2000), suggested that incorporation of hydroxyvalerate (HV) units in a homopolymer (PHB) reduces its melting point thereby increasing polymer flexibility and decreasing its brittleness. This improves the overall thermal and mechanical properties of the polymer.

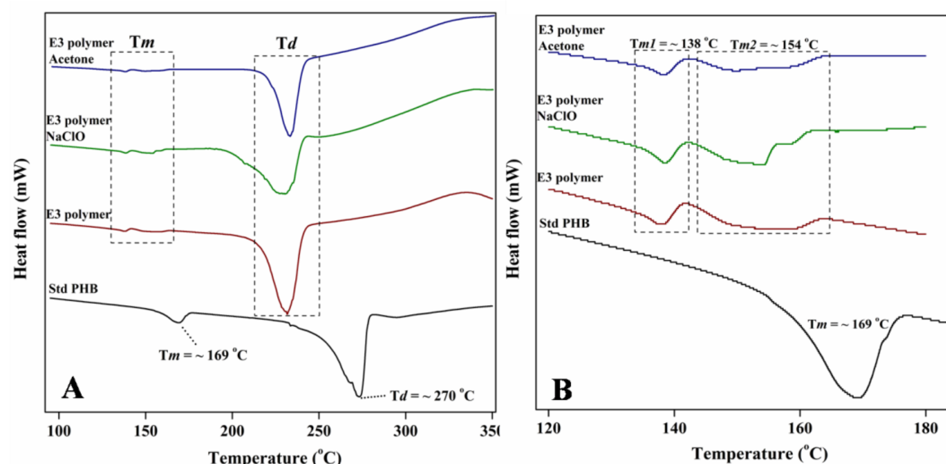

Fig. 5.6 (A) Comparison of DSC curves of E3 polymer before and after decolourisation with NaClO / acetone with standard PHB. **(B)** The highlighted area (dotted line) is depicting the melting profile (T*m*) of the polymer.

5.3.5 Comparison of polymers using FT-IR analysis

The FT-IR spectra of the polymer before and after decolourization (Fig. 5.7) showed a prominent absorption band at 1724 cm^{-1} characteristic of ester carbonyl group (C=O) stretching. Band of carbonyl stretch appearing in the range of 1744 - 1722 cm^{-1} provides information on the degree of crystallinity of the polymer (Arcos et al., 2010). In this study after decolourisation of the polymer, there was no shift in the band at 1724 cm^{-1}, therefore one can say that the polymer crystallinity was unaffected. As mentioned above, the vibration bands at 1658 $cm^{-1}$and 1527 cm^{-1} present in the original polymer represents the bacterial cellular protein amide (–CO–N–) I and II respectively (Gumel et al., 2012). Interestingly, acetone treatment of the polymer shifted these bands to 1629 cm^{-1} and 1548 cm^{-1}whereas NaClO treatment of the polymer resulted in disappearance of these bands altogether (Fig. 5.7). NaClO probably removes any protein moieties from the polymer, thereby resulting in pure polymer (Fig. 5.7).

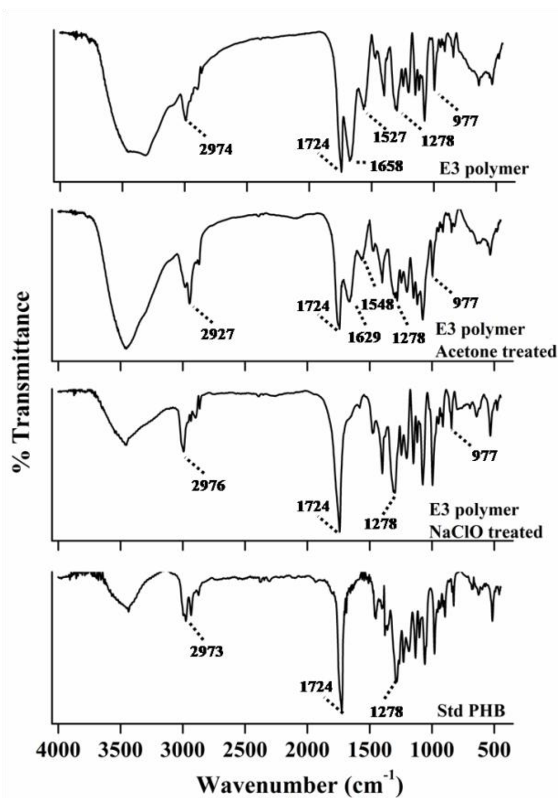

Fig. 5.7 Comparison of the FT-IR spectra of E3 polymer before and after treatment with NaClO / acetone.

The band at 1278 cm^{-1} and 1281 cm^{-1} represents C-O-C stretching whereas in the region around 2900 cm^{-1} ie. 2974 cm^{-1}, 2976 $cm^{-1}$and 2927 cm^{-1} represents C-H stretching (Padermshoke et al., 2004) (Fig. 5.7). There was a distinct band at 980 cm^{-1} in the original polymer and was retained in decolorized polymer too. Bands around 977 cm^{-1} are indicative of copolymer of P(HB-*co*-HV) (Bloembergen et al., 1986). Apart from these absorption bands, other bands observed may be the result in stretching shifts due to interactions between the -OH and C=O groups (da Silva Pinto and Wypych, 2009).

5.3.6 Comparison of polymers using ^{1}H NMR analysis

Comparison of the monomeric composition of the polymer was determined by ^{1}H NMR spectrum (Fig. 5.8) which provided the details of the functional groups in the polymer.

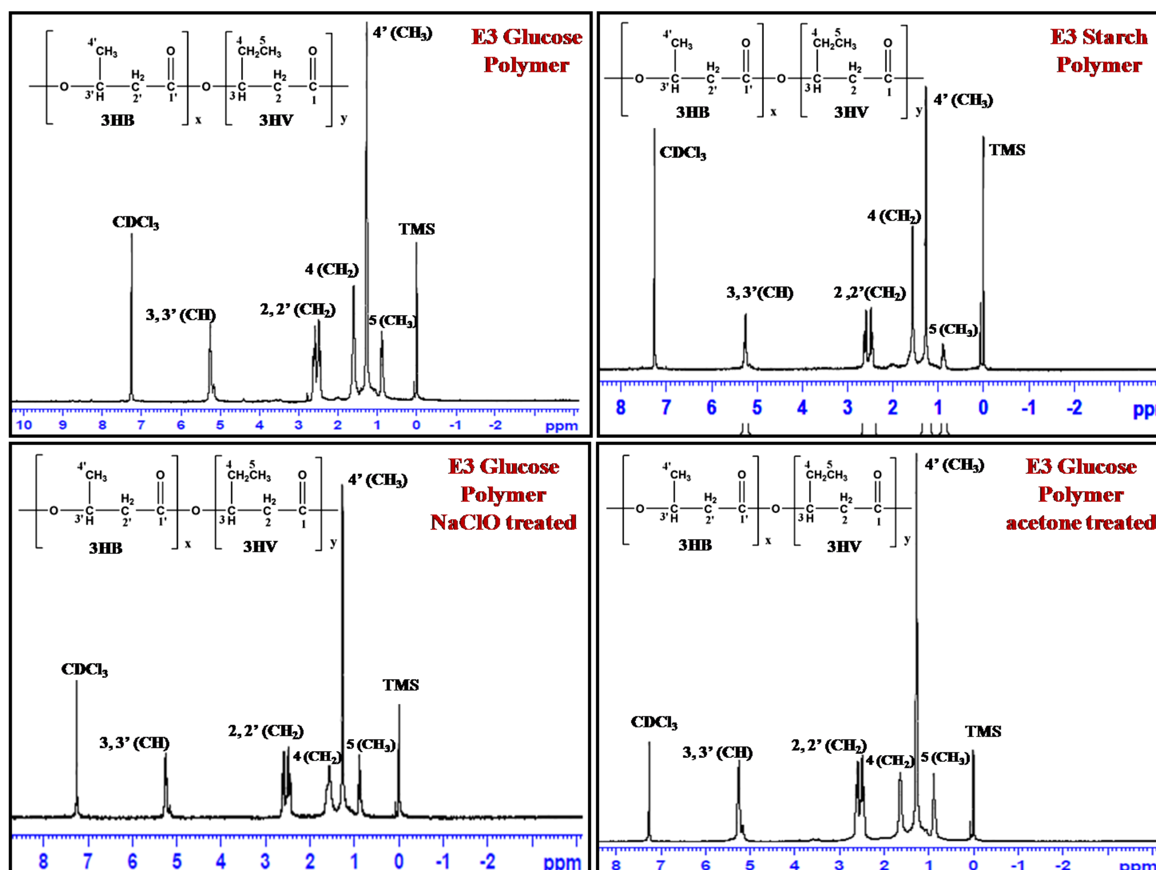

Fig. 5.8 ^{1}H NMR spectra of E3 polymer from glucose, starch and after decolourisation with NaClO / acetone.

Composition of HV (%) in the co-polymer of P(HB-*co*-HV) was calculated as described in Chapter IV, section 4.3.6.5. The co-polymer [P(HB-*co*-HV)] produced by the extremely halophilic archaeon *Hgm. borinquense* strain E3 by utilization of glucose and starch comprises 21.47 and 13.11 % HV units, respectively (Fig. 5.8). However, a minor reduction in the HV units to 19.51% and 19.39% was observed in the E3 glucose polymer after decolourisation with NaClO / acetone.

5.4 Conclusion

Optimization of culture conditions for *Hgm. borinquense* strain E3 showed that it could grow over a range of glucose concentrations, pH, temperature and salinity but the best cell mass to PHA yield was 5.8 ± 0.4 (g l^{-1}) and 4.0 ± 0.045 (g l^{-1}) respectively was obtained at 2.0% glucose, pH 7.0, 37°C and 20% NaCl. Chemical extraction using sodium hypochlorite (NaClO) with 4 % (w/v) chlorine could extract maximum PHA from the cell mass of 77.85 ± 0.3% (w/w) of CDW and soxhlet extraction using $CHCl_3$ gave PHA yield of 72.4 ± 3% (w/w) of CDW, respectively. The soxhlet extraction for polymer recovery is advantageous as upto 95% of $CHCl_3$ employed can be recovered by rotary evaporator. ^{1}H NMR analysis of the [P(HB-*co*-HV)] revealed it to have 21.4% hydroxylvalerate (HV) units which on decolourisation with NaClO/acetone slightly reduced to 19.39 / 19.51% HV units.

Chapter VI

Utilization of Agro-Industrial Wastes for PHA Production by Extremely Halophilic Archaea

6.1 Introduction

Conventional plastics obtained from non-renewable petro-chemical resources have created environmental havoc due to their non-degradable and recalcitrant nature (Thompson et al., 2009). To tackle this problem various bio-based materials derived from renewable resources have been explored. These materials can be (i) directly extracted from biomass as polysaccharides, lignocelluloses, proteins and lipids (ii) chemically synthesized by *in vitro* polymerization of bio-derived monomers such as lactate to produce poly(lactic acid) (PLA) (iii) biologically synthesized by microorganisms, *in vivo* polymerization of hydroxylalkanoic acid (HA) units to give polyhydroxyalkanoates (PHAs). (Chen and Patel, 2012; Chee et al., 2010; Steinbüchel, 2001).

PHAs produced from renewable sources are an alternative to conventional plastics (Chee et al., 2010). But, the cost of production and other downstream processing hampers its use in commercial applications. PHA accumulation by many Gram positive and Gram negative bacteria and few members of archaea especially halophilic archaea have been investigated previously (Valappil et al., 2007; Shrivastav et al., 2013; Han et al., 2010).

Carbon sources / substrates represent half of the PHA fermentation cost (Silva et al., 2004). High production cost and low yields are the major hurdle for commercial production and application of PHA, making microbially synthesized PHA 5–10 times more expensive than the petroleum-derived polymers. However, this can be taken care by various strategies like microbial strain improvements and / or finding novel PHA accumulating microorganisms. Replacing commercial substrates with inexpensive renewable agro-industrial wastes for PHA production and reducing the cost of PHA recovery/downstream process may also make the fermentation process more cost-effective (Kahar et al., 2005, Tsuge, 2002).

Sugarcane (*Saccharum officinarum*) is the world's largest cash crop (Parameswaran, 2009), India being the world's second largest producer of sugarcane, leaded by Brazil. The sugarcane bagasse (SCB) is the left over fibrous residue of sugar cane stalk after the extraction of juice and is one of the major cellulosic agro-industrial by-product of the sugar

cane industry (Pippo and Luengo, 2013). Microbial degradation and utilization of untreated SCB is slow due to its fibrous nature. To overcome this, pre-treatment of SCB is needed for improved substrate utilization and to speed up the fermentation process.

There are few reports on PHA production from agro-industrial wastes / cheap substrates by halophilic microorganisms both bacteria and archaea. Van-Thuoc et al., (2008) reported the production of homopolymer of poly(3-hydroxybutyrate) (PHB) by moderately halophilic *Halomonas boliviensis* using agro-industrial byproducts like wheat bran and potato wastes. Among extremely haloarchaeal, *Hfx. mediterranei* is the most widely studied and is reported to produce copolymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)] using various renewable agro-industrial wastes like the extruded corn starch, rice bran, wheat bran, hydrolysed whey, waste stillage from rice based ethanol industry and vinasse (Huang et al., 2006; Koller et al., 2007a; Bhattacharyya et al., 2014; Bhattacharyya et al., 2012).

In this chapter, the ability of extremely halophilic archaeal isolates to accumulate PHA using agro-industrial waste hydrolysates of sugarcane bagasse (SCB), coconut oil cake (COC) and sago starch waste (SSW) was checked. Characterization of the polymer accumulated by extremely halophilic archaeon *Halogeometricum borinquense* strain E3 using the above substrates was done.

6.2 Materials and Methods

6.2.1 Haloarchaeal strains and media used

Eight haloarchaeal isolates, (a) *Halococcus salifodinae* strain BK6, (b) *Haloferax volcanii* strain BBK2, (c) *Haloarcula japonica* strain BS2, (d) *Halogeometricum borinquense* strain E3, (e) *Halogeometricum borinquense* strain TN9, (f) *Halorubrum sp.*strain BS17, (g) *Halococcus sp.* strain E4 and (h) *Haloterrigena jeotgali* strain J1 were used in this study (Salgaonkar et al., 2013b; Mani et al., 2012a). The cultures were isolated and maintained on complex media (NTYE, NT, EHM and JCM168) whereas the NaCl synthetic medium

(NSM) (Chapter II; Appendix I) supplemented with various substrates as per the requirement was used as production medium.

6.2.2 Agro-industrial wastes

6.2.2.1 Procurement, processing and hydrolysis

Three agro-industrial wastes were used of (i) sugarcane bagasse (SCB), collected from a local sugarcane juice extractor, Vasco-da-Gama, Goa, India (ii) Coconut oil cake (COC), obtained from local oil extracting mill, Bardez taluka, North Goa and (iii) Sago starch waste (SSW), which was procured from discharge of sago starch industry in Salem, Tamil Nadu. The SCB was dried in sunlight for 3-5 days, cut into small pieces (5 – 10 cm). The COC were dried in oven at 60°C for 12 hrs where as SSW was dried for 5-7 days under sunlight for complete removal of moisture. All three substrates were further pulverized to fine powder using a blender. The powdered form of the wastes was subjected to dilute acid hydrolysis. Briefly, 5gm of the agro-industrial waste powder was put in 100 ml of 0.75% (v/v) sulfuric acid in water. The mixture was heated at 100°C for 1 hr with constant stirring at regular intervals. This was filtered using non absorbent cotton to separate the solid residue from the liquid hydrolysate. The liquid hydrolysate was neutralized (pH 7.0 - 7.4) using NaOH, followed by sterilization at 121°C for 10 min and stored at 4°C.

6.2.2.2 Characterization of the agro-industrial wastes

The powdered agro-industrial wastes were characterized for various physical and chemical parameters. Total solids (TS) and volatile solids (VS) were estimated according to American Public Health Association (APHA 1998). The chemical oxygen demand (COD) was determined as described by Raposo et al., (2008). The carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) content of the agro-industrial wastes was determined using Elementar CHNS Analyzer (Germany). Total carbohydrates were estimated by phenol sulphuric acid method (Dubois et al., 1956) and Total Kjeldahl Nitrogen (TKN) was determined as described by Labconco, 1998.

6.2.3 Screening of the haloarchaeal isolates for PHA accumulation using agro-industrial wastes hydrolysate

Haloarchaeal isolates were screened for the production of PHA on NSM by Nile Red stain as described in chapter IV. Briefly, for screening, the NSM plates were prepared by adding 2% agar-agar (w/v) to the medium followed by autoclaving and while still molten the medium was supplemented with varying concentrations (0.5 – 30 % v/v) of SCB hydrolysate along with 50 µl of Nile Red stain [0.01% (w/v) stock in DMSO]. Log phase haloarchaeal cultures were spot inoculated on the agar plates and incubated at 37°C for 6 - 7 days. The plates were exposed to ultra violet (UV) light using Gel documentation system (BIO-RAD Laboratories CA, USA) and emitted fluorescence from the culture was quantified using TotalLab Quant software (Chapter IV, section 4.2.1).

6.2.4 Selection of the agro-industrial waste and the best PHA accumulator strain for further study

Based on the accumulation of PHA using various agro-industrial waste hydrolysates, SCB was selected for further studies. Among all the haloarchaeal strains screened, *Hgm. borinquense* strain E3 showed the best growth and fluorescence when grown with all the three agro-industrial wastes as substrates. Hence, *Hgm. borinquense* strain E3 was used for further studies. Further, growth of this culture on solid agar plates of NSM with higher SCB hydrolysate concentrations (v/v) of 25%, 50%, 75% and 100% was studied. For 100 % SCB the NSM medium components (Appendix I) were directly dissolved in the SCB hydrolysate. Based on the growth on the plate, the culture was further grown in liquid broth with that particular concentration of SCB hydrolysate.

6.2.5 Growth kinetics and polymer quantification

Growth and intracellular PHA content for *Hgm. borinquense* strain E3 was determined as discussed previously in Chapter IV. Briefly, mid log phase culture (3 - 4 days) was used as inoculum and one percent of this was noculated into flasks containing NSM (Appendix I) with 25 % and 50 % (v/v) SCB hydrolysate, respectively. The flasks were maintained at 37°C on a rotary shaker at 110 rpm. At regular intervals of 24 hrs, the following parameters

were monitored (i) absorbance at 600nm, (ii) pH of the medium (iii) cell dry weight (CDW), (iv) Polymer quantification and (iv) the total carbohydrates was determined according to Dubios et al., 1956 (Appendix III). All the parameters were monitored as described in Chapter IV, section 4.2.5. Since the SCB hydrolysate had some particle precipitate, the CDW of the medium was taken and nullified from the culture CDW so as to avoid the error.

6.2.6 Extraction of the PHA

Hgm. borinquense strain E3 was grown in NSM with 25 % (v/v) containing SCB hydrolysate for 6 days. The cells were harvested by centrifuging at 10,000 rpm for 10 minutes and were dried for 12 hrs in oven. The dried cells were ground using mortar and pestle and the polymer was recovered by soxhlet extraction using chloroform as described in Chapter V, section 5.2.2.3(b).

6.2.7 Characterization of the PHA

The polymers produced by *Hgm. borinquense* strain E3 using various agro-industrial wastes of SCB, COC and SSW were characterized using UV-visible spectrophotometry, XRD analysis, DSC analysis, FT-IR spectroscopy, NMR spectroscopy as described in Chapter III/V, section 3.2.8/5.2.3.2.

6.3 Results and discussion

6.3.1 Agro-industrial wastes characterization

Sugarcane bagasse (SCB) used in this study appeared greenish brown with a sweet odour, the coconut oil cake (COC) appeared grey and had coconut oil odour, however the sago starch waste (SSW) also appeared grey with foul odour. On pulverization, the long fibrous SCB, granular COC and semi sticky SSW was converted to fine powder (Fig. 6.1). The total solids (TS) of the agro-industrial wastes followed the following order 94.3 ± 0.14% (SCB) > 92.5 ± 0.21% (SSW) > 91.0 ± 0.07% (COC), whereas the volatile solids (VS) were 92.7 ± 0.14% (SCB) > 84.7 ± 0.01% (COC) > 78.8 ± 0.14% (SSW). CHNS analysis revealed the C/N ratio to be 165.83 ± 8.0% (SCB) > 47.55 ± 0.21% (SSW) > 14.6 ± 0.13%

(COC). The SCB is the major by-product of the sugarcane industry and comprises of cellulose (46%), hemicelluloses (27%), lignin (23%) and ash (4%) (Pippo and Luengo, 2013). Study by Lavarack et al., (2002) revealed that xylose, arabinose, glucose along with other products can be obtained from dilute acid hydrolysis of SCB hemicelluloses. Therefore, the agro-industrial wastes were hydrolyzed by dilute H_2SO_4 acid (0.75% v/v) at 100 ^oC for 1 hr and on hydrolysis, the total carbohydrates (g l^{-1}) obtained was 24.78 ± 1.7 (SSW) > 12.64 ± 0.7 (SCB) > 5.35 ± 0.5 (COC). Total Kjeldahl nitrogen (TKN) (g l^{-1}) was 4.79 ± 0.014 (COC) > 0.7 (SCB) > 0.56 (SSW). Hence, the C/N ratio for the agro-industrial wastes hydrolysate was 44.25 (SSW) > 18.05 (SCB) > 1.11 (COC). The physico-chemical characterization of the agro-industrial wastes are represented in Table 6.1.

Table 6.1 Physico-chemical characterization of the agro-industrial wastes.

Sr. no.	Characteristic	Agro-industrial wastes		
		SCB	**COC**	**SSW**
A	**Physical characteristics**			
1.	Colour	Greenish Brown	Grey	Grey
2	Odour	Sweet	Coconut oil	Bad
3.	Texure			
	(a) Procured form	Long fibrous	Granular cake	Semi sticky
	(b) Pulverized form	Fine fibrous powder	Fine coarse powder	Fine powder
4.	TS (%)	94.3 ± 0.14	91.0 ± 0.07	92.3 ± 0.21
5.	VS (%)	92.7 ± 0.14	84.7 ± 0.01	78.8 ± 0.14
B	**Chemical characteristics**			
1.	CHNS analysis			
	(a) Nitrogen (%)	0.265 ± 0.02	2.985 ± 0.09	0.755 ± 0.007
	(b) Carbon (%)	43.455 ± 1.2	43.615 ±0.9	35.775 ± 0.4
	(c) Hydrogen (%)	6.095 ± 0.27	7.035 ± 0.16	6.12 ± 0.08
	(d) Sulphur (%)	0.3225 ± 0.12	0.319 ± 0.02	0.158 ± 0.008
	(e) C/N ratio (%)	165.83 ± 8.0	14.615 ± 0.13	47.55 ± 0.21
2.	COD (g kg^{-1})	1.18 ± 0.05	1.712 ± 0.1	1.33 ± 0.32
3.	Total carbohydrates (g L^{-1})	12.64 ± 0.7	5.35 ± 0.5	24.78 ± 1.7
4.	TKN (g L^{-1})	0.7	4.79 ± 0.014	0.56
5.	C/N ratio (%)	18.05	1.11	44.25

gL^{-1}: gram per litre; %: percentage

Sugarcane being the world's largest cash crop is used for the production of sugar, jaggery, ethanol, molasses, alcoholic beverages (rum), soda, etc. (Parameswaran, 2009). The sugarcane bagasse (SCB) is probably the largest, renewable, inexpensive agro-industrial waste generated and needs special attention for its management. Currently, SCB is primarily used as source of energy such as electricity / biogas production (Pippo and Luengo, 2013) apart for being used as raw material for obtaining various fermentation products such as enzymes (cellulose, lipase, xylanase, inulinase, amylase), animal feed (single cell protein), amino acids, organic acids, bioethanol, bioplastics, etc (Mazutti et al., 2006, Wahono et al., 2014, Parameswaran, 2009).

Fig. 6.1 Agro-industrial wastes, SSW, SCB, COC (A) procured form and (B) pulverized form.

Dilute acid (H_2SO_4 / HCl) hydrolysis though is a fast and simple method, it releases various byproducts such as phenolics, furans (furfural and hydroxymethylfurfural) and aliphatic acids (formic / acetic / levulinic acid) along with total carbohydrates, which acts as inhibitors of the fermentation process (Gray et al., 2006). Therefore, treatment of the acid

hydrolysate with anion exchange resin / activated charcoal / laccase can effectively reduce the furans and total phenolics (Chandel et al., 2007).

6.3.2 Haloarchaeal isolates and screening for PHA using agro-industrial wastes hydrolysate

Eight haloarchaeal isolates representing six genera namely *Halococcus*, *Haloferax*, *Haloarcula*, *Haloterrigena*, *Halorubrum* and *Halogeomertricum* of the family *Halobacteriaceae* were used in the study. All the eight cultures were able to grow on NSM plates supplemented with various agro-industrial wastes hydrolysate as substrate and Nile Red dye. Interestingly, when the plates were exposed to UV light, only six cultures, *Hfx. volcanii* strain BBK2, *Har. japonica* strain BS2, *Hgm. borinquense* strain TN9, *Hgm. borinquense* strain E3, *Halococcus sp.* strain E4, and *Htg. jeotgali* strain J1 showed bright orange fluorescence indicating the growth and accumulation of PHA (Fig. 6.2). *Hcc. salifodinae* strain BK6 and *Halorubrum* sp. strain BS17 showed weak growth but failed to show any fluorescence. Microbial degradation and utilization of untreated agro-industrial wastes is slow due to its fibrous / coarse nature. To overcome this, pre-treatment of agro-industrial wastes is needed for improved substrate utilization and to speed up the fermentation process. Many reports have focussed on the pre-treatment of SCB using alkali acid or a combination of varying parameters. Yu and Stahl (2008) reported dilute acid hydrolysis of SCB at moderate temperatures is an efficient treatment for the production of poly (3-hydroxybutrate) (P3HB) as well as copolymer poly (3-hydroxybutyrate-co-3hydroxyvelerate) (P3HB3HV) by aerobic bacterium *Ralstonia eutropha*.

Among the agro-industrial wastes used in this study, SCB was selected for further study as it was best tolerated and the cultures grew well upto 30% (v/v). Interestingly, among the six strains that accumulated PHA, *Hgm. borinquense* strain E3 grew faster, lavishly and showed better fluorescence over a range of substrate concentrations as compared to the other starins (Fig. 6.2). Hence, *Hgm. borinquense* strain E3 was selected for further studies.

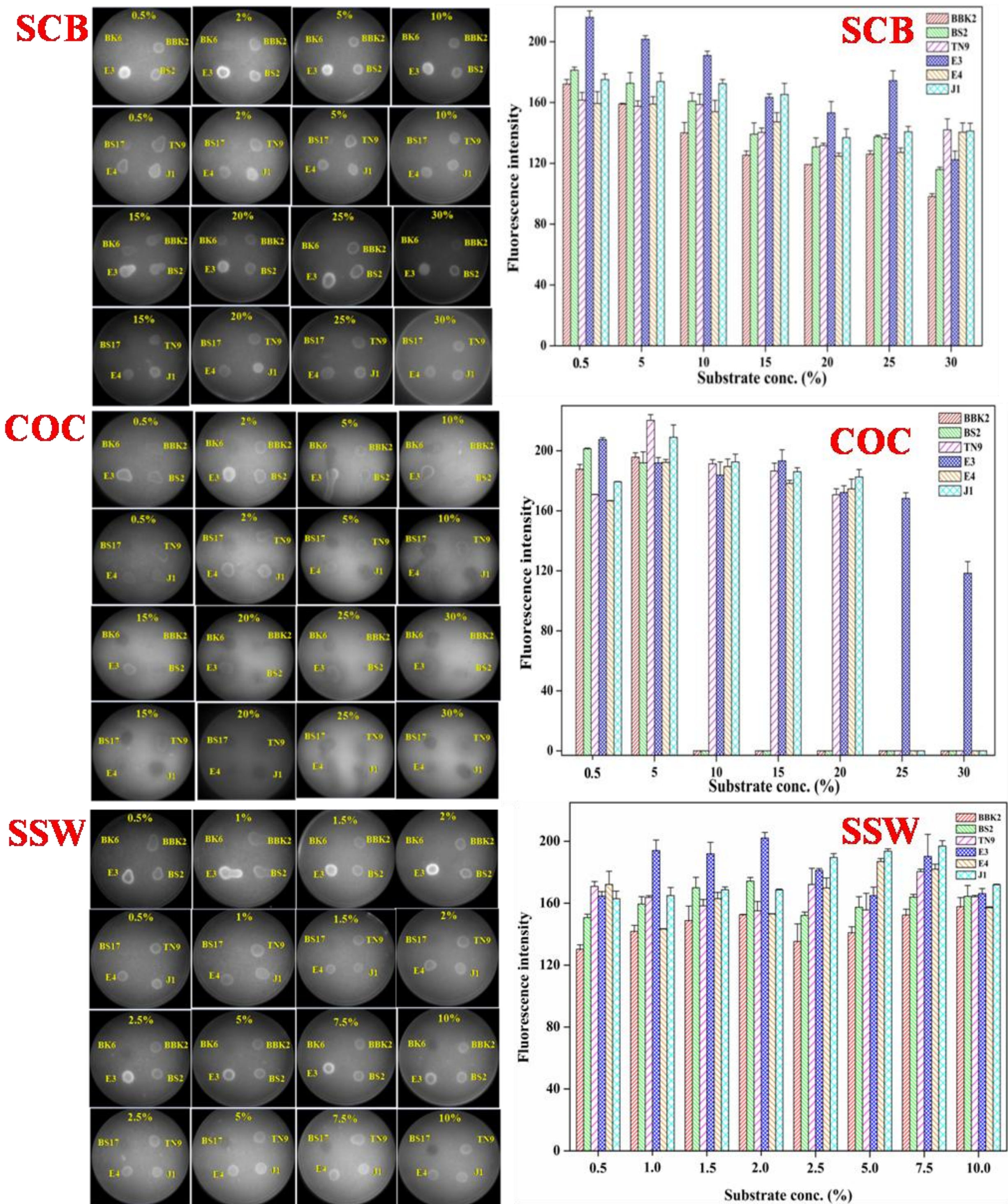

Fig. 6.2 Fluorescence exhibited by the PHA accumulating haloarchaeal culture when grown on NSM supplemented with Nile Red dye (left) and various concentrations of SCB, COC and SSW hydrolysates. The emitted fluorescence was quantified and compared using TotalLab Quant software (right).

6.3.3 Optimization of SCB hydrolysate

The *Hgm. borinquense* strain E3 when grown on NSM agar plates supplemented with 25%, 50%, 75% and 100% SCB hydrolysate, showed tolerance and growth upto a concentration of 75% (Fig. 6.3, inset). Interestingly, in liquid NSM, the culture grew with 25% (OD 600 nm = 1.2) and 50% (OD 600 nm = 0.6) SCB hydrolysate. The culture failed to grow at 75% and above (Fig. 6.3). Therefore, further growth pattern of the *Hgm. borinquense* strain E3 was studied in liquid NSM supplemented with 25% and 50% SCB hydrolysate, respectively.

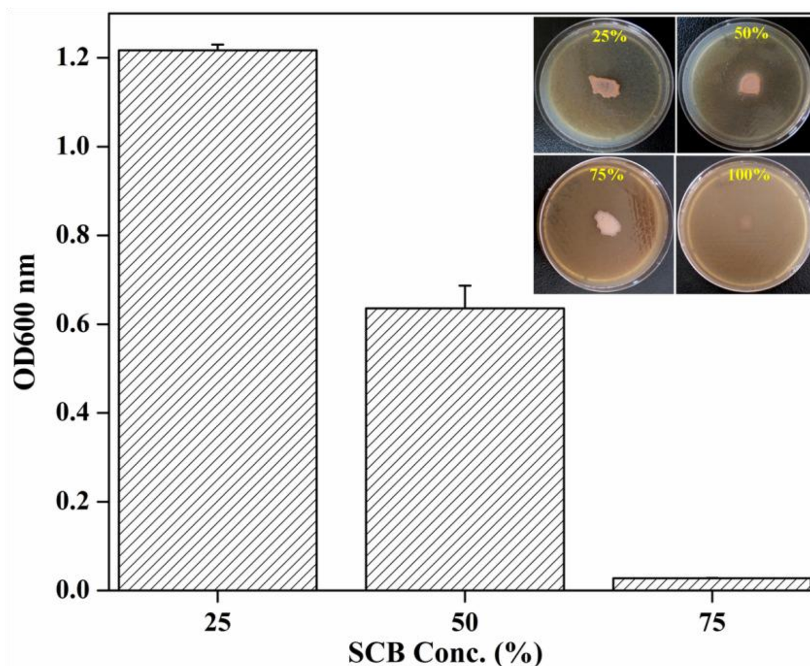

Fig. 6.3 Tolerance and growth of *Hgm. borinquense* strain E3 on solid (inset) and in liquid NSM with varying concentrations of SCB hydrolysate.

6.3.4 Growth profile of *Hgm. borinquense* strain E3 and polymer quantification study

The time-course of growth of *Hgm. borinquense* strain E3 in NSM with 25% and 50% SCB hydrolysate, respectively is presented in Fig. 6.4. The strain E3 in presence of 25% SCB hydrolysate grew steadily with an initial optical density OD600nm of 0.025 ± 0.003 which increased to 1.0 ± 0.03 on 4th day and reached its maximum of 1.24 ± 0.02 on 7th day. Interestingly, in presence of 50% SCB hydrolysate the strain E3 showed a longer lag phase of 4 days with an OD600nm of 0.045 ± 0.06 which started increasing gradually to 0.2 ± 0.1 on 5th day and reached its maximum of 1.4 ± 0.01 on 9th day. Similarly, the CDW of the

culture, in presence of 25% SCB hydrolysate increased steadily with time from 0.3 ± 0.02 on 1st day to 2.56 ± 0.18 on 4th day with maximum of 3.0 ± 0.19 on 9th day. Active intracellular polymer accumulation of 10.6 ± 0.008 % (w/w) of CDW was observed on the 1st day, which increased gradually and reached a maximum of 50.4 ± 0.1% on the 7th day. The CDW of the culture, in presence of 50% SCB hydrolysate was stable ~ 0.02 ± 0.01 for the first 4 days but increased steadily with 0.9 ± 0.5 on 5th day with maximum of 6.9 ± 0.7 on 9th day. Intracellular polymer accumulation of 4.6 ± 0.008% of CDW was observed on the 5th day, which increased gradually and reached a maximum of 45.7 ± 0.19% on the 7th day and reduced drastically up to 6.5 ± 0.04% on 9th day. The rapid consumption of the total carbohydrates was observed as the growth progressed in 25% SCB where as in 50% SCB there was negligible amount of total carbohydrate utilization for the first 4 days as the culture was in its lag phase but gradual utilization was observed only after the 5th day. A steady drop in the pH of the medium was observed from 7.2 to 5.0.

PHA accumulation by extremely halophilic archaeal and moderately halophilic and / or halotolerant bacteria, inhabiting marine and hypersaline regions of countries such as China, Turkey, Vietnam, Bolivia, India, etc, have been reported (Han et al., 2010; Danis et al., 2015; Van-Thuoc et al., 2012; Quillaguaman et al., 2006; Salgaonkar et al., 2013a,b). Moderately halophilic bacteria, belonging to the genus *Halomonas* such as *H. boliviensis* LC1, *H. nitroreducens*, *H. salina* have been reported to accumulate 56.0, 33.0 and 55.0 wt/wt % cell dry weight of homopolymer of hydroxybutyrate (HB) ie PHB by utilizing versatile substrates such as starch hydrolysate, glucose and glycerol, respectively (Quillaguaman et al., 2005; Cervantes-Uc et al., 2014; Mothes et al., 2008). Similarly, Van-Thuoc et al., (2012) reported the ability of halophilic and halotolerant bacteria, *Bacillus* sp. ND153 and *Yangia pacifica* QN271 to accumulate PHB of 65.0 and 48.0 % (w/w) of CDW or PHBV of 71.0 and 31.0 % wt/wt of CDW when glucose and / or glucose along with propionate was provided as the carbon source. However, there are very few reports on halophilic bacteria such as *H. campisalis* MCM B-1027, *Yangia pacifica* ND199 / ND218 synthesizing copolymer PHBV, irrespective of precursors like propionic / valeric acid in the culture medium (Kulkarni et al., 2010, Van-Thuoc et al., 2012). Shrivastav et al., (2010) reported the utilization of *Jatropha* biodiesel byproduct as substrate by *Bacillus*

sonorensis strain SM-P-1S and *Halomonas hydrothermalis* strain SM-P-3M for PHB production of 71.8 and 75.0 % (w/w) of CDW, respectively.

Extremely halophilic archaea, belonging to the family *Halobacteriaceae*, such as *Haloarcula* sp. IRU1, *Halobiforma haloterrestris* strain 135T, *Halopiger aswanensis* strain 56, *Hgm. borinquense* strain TN9 have been reported for PHB accumulation of 63.0, 40.0, 34.0 and 14.0 % (w/w) of CDW by utilizing versatile substrates such as glucose, yeast extract, butyric acid, sodium acetate (Taran and Amirkhani, 2010; Hezayen et al., 2002; Hezayen et al., 2010; Salgaonkar et al., 2013a). Interestingly, *Hfx. mediterranei* is known for copolymer PHBV accumulation of 23.0 % (w/w) of CDW from glucose, naturally without any addition of precursor (Huang et al., 2006).

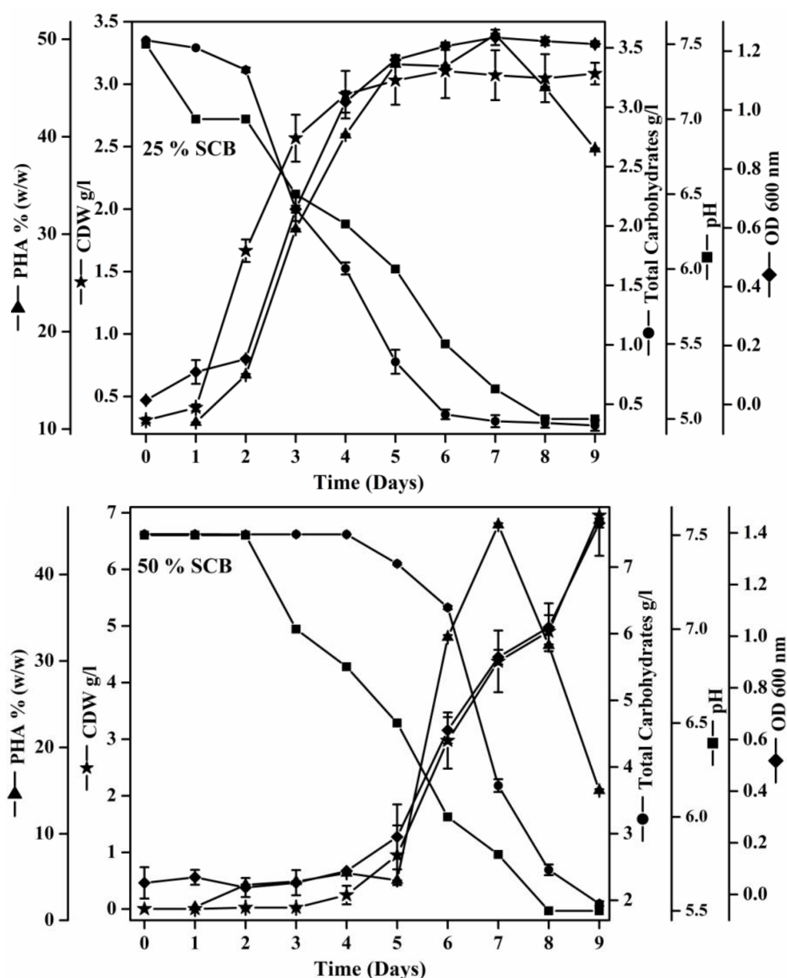

Fig. 6.4 Growth profile and PHA accumulation by *Hgm. borinquense* strain E3 in 25% and 50% SCB hydrolysate.

6.3.5 Bench scale PHA production and extraction by *Hgm. borinquense* strain E3

PHA was extracted from the cell biomass as schematically described in the Fig. 6.5. The dried cells of *Hgm. borinquense* strain E3 (before soxhlet extraction) when subjected to concentrated H_2SO_4 hydrolysis showed clear peak at 235 nm which is of the crotonic acid indicating the presence of PHA (Fig. 6.6A). After soxhlet extraction no peak at 235 nm was observed in the cell debris, thus confirming complete extraction of the polymer from the cell mass (Fig. 6.6B).

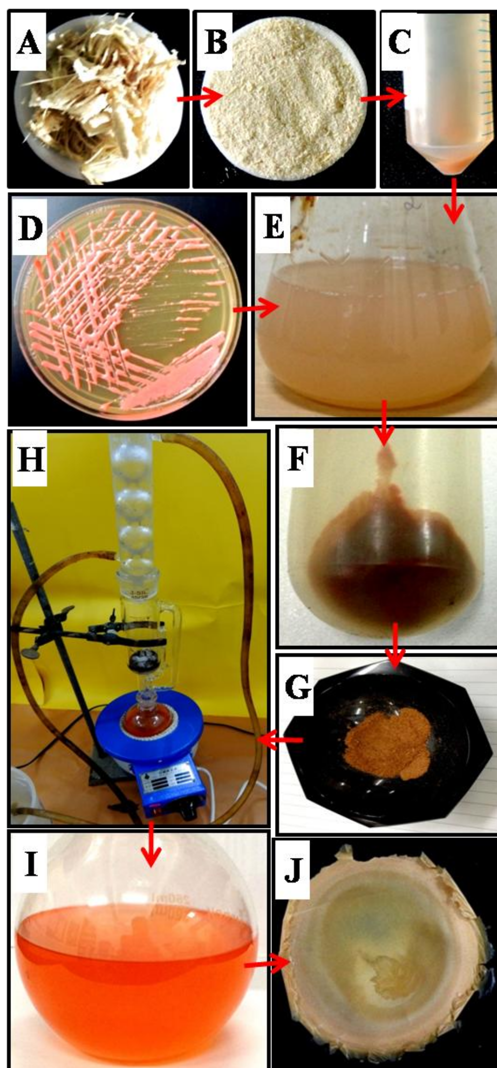

Fig. 6.5 Production and extraction of PHA from *Hgm. borinquense* strain E3. (A) SCB fibers, (B) pulverized SCB, (C) dilute acid hydrolysate of SCB (D) pure culture of *Hgm. borinquense* strain E3, (E) liquid culture grown in NSM supplemented with SCB hydrolysate, (F) culture pellet containing PHA, (G) cells containing PHA dried at 70°C and ground using motor and pestle, (H) chloroform extraction by soxhlet apparatus, (I) chloroform containing the extracted polymer and (J) polymer film obtained after solvent evaporation.

Table 6.2 Comparison of cell dry weights (CDW), maximum PHA concentrations and PHA yields of *Hgm. borinquense* strain E3 with various haloarchaeal isolates.

Haloarchaeal strain	Media	Carbon source	CDW (gL^{-1})	Maximum PHA (gL^{-1})	PHA Yield % (w/w)	PHA type	Reference
Hgm. borinquense strain E3	NSM	25% SCB	3.17 ± 0.19	1.6 ± 0.09	50.4 ± 0.1	PHBV	Present study
		50% SCB	3.07 ± 0.7	1.9 ± 0.3	45.7 ± 0.19		
		5% COC	8.2	0.9	10.9		
		2% SSW	3.4	1.52	44.7		
		Glucose	4.06 ± 0.04	5.8 ± 0.4	70.0 ± 0.04		
Natrinema pallidum 1KYS1	BMM	2% CS	0.174	0.075	53.14	PHBV	Danis et al., 2015
		2% Sucrose	2.219	0.055	2.48		
		2% Whey	0.457	0.091	19.92		
		2% Melon	0.371	0.039	10.50		
		2% Apple	2.550	0.077	3.02		
		2% Tomato	3.858	0.464	12.03		
		Starch	1.43	0.81	56.64		
Hfx. mediterranei ATCC 33500	MM	ECS	62.6	24.2	38.7	PHBV	Huang et al., 2006
		ERB:ECS	140.0	77.8	55.6		
		EWB:ECS	131.0	52.7	40.2		
		NWB:ECS	68.4	28.0	40.9		
Har. marismortui MTCC 1596	NDM	10% RV	12.0 ± 0.2	2.8 ± 0.2	23.0 ± 1.0	PHB	Pramanik et al., 2012
		100% PTV	15.0 ± 0.3	4.5 ± 0.2	30.0 ± 0.3		
Hfx. mediterranei DSM 1411	MST	25% PTV	28.14	19.7	70	PHBV	Bhattacharyya et al., 2012
		50% PTV	29	17.4	60		
		Stillage FS	23.12	16.42 ± 0.02	71.0 ± 2.0	PHBV	Bhattacharyya et al., 2014
		Stillage RS	23.55	16.25 ± 0.01	69.0 ± 3.0		
	salt medium	Whey lactose	16.71	12.2	73	PHBV	Koller et al., 2008
		Whey lactose	11.0	5.5	50	PHBV	Koller et al., 2007b

CDW: cell dry weight; PHB: poly (3-hydroxybutyrate); PHBV: poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate); BMM: Basal minimal media, MM: Mineral medium CS: corn starch; ECS: extruded cornstarch; ERB: extruded rice bran; EWB: extruded wheat bran; NWB: native wheat bran; RV: raw vinasse; PTV: pre-treated vinasse, FS: fresh salts; RS: recovered salts; NSM: NaCl synthetic medium; NDM: nutrient deficient medium

Table 6.3 Comparison of the bioprocess parameters during growth and PHA production by *Hgm. borinquense* strain E3 in NSM with substrates such as glucose, 25 and 50 % (v/v) SCB hydrolysate.

Culture	Media	Lag (h)	CDW (g/l)	PHA (g/l)	PHA % (w/w) CDW	μmax (1/h)	qp (g/g ×10⁻³ / h)	$Y_{P/S}$	Vol productivity (g/L/h)	Reference
Hgm. borinquense strain E3	NGSM 2% Glucose	-	5.8 ± 0.4	4.06 ± 0.045	70.0 ± 0.045	0.235±0.01	4.166	0.562	0.024	Present study
	NSM 25% SCB	48	3.17 ± 0.19	1.6 ± 0.09	50.4 ±0.1	0.529	2.9	4.32	0.0091	
	NSM 50% SCB	120	3.07 ± 0.7	1.9 ± 0.3	45.7 ± 0.19	0.508	2.57	0.5	0.011	
Haloferax mediterranei	NDM	96 ± 12	11.5 ± 0.25	2.6 ± 0.1	22.5 ± 0.5	0.105±0.001	1.18±0.01	0.23±0.01	0.014±0.001	Han et al., 2007
Haloferax mediterranei	10% Raw vinasse	96 ± 12	12.0 ± 0.20	2.8 ± 0.2	23 ± 1.0	0.086±0.001	1.21±0.01	2.17±0.03	0.015±0.001	Pramanik et al., 2012
Haloferax mediterranei	100% pre-treated vinasse	144 ± 12	15.0 ± 0.35	4.5 ± 0.2	30 ± 0.3	0.128±0.001	1.39±0.02	0.77±0.02	0.020±0.001	

Duration of growth in NSM with various substrates was 7 days (168 hrs); NDM : Nutrient deficient medium; μmax: maximum specific growth rate; Specific productivity of PHA (qp) = PHA (g l^{-1}) / time (h) x CDW (g l^{-1}) [$g.g^{-1}.h^{-1}$] (Folloniar et al., 2011); Volume productivity of PHA = PHA (g l^{-1}) / time (h) [$g.l^{-1}.h^{-1}$] (Castilho et al., 2009); Yield coefficient of PHA ($Y_{P/S}$) = PHA (g l^{-1}) / TOC (g l^{-1}) (Albuquerque et al., 2007).

In this study, 1.6 ± 0.09 and 1.9 ± 0.3 g L^{-1} of PHA polymer was obtained on consumption of 25% and 50% of SCB hydrolysate, respectively. Attempts have been made in reducing the fermentation cost of PHA, by employing various haloarchaeal strains and checking their ability to utilise cheap substrates (Table 6.2, 6.3). Danis et al., (2015), showed the ability of *Natrinema pallidum* 1KYS1 to produce 0.075, 0.055, 0.091, 0.039, 0.077 and 0.464 g L^{-1} of polymer by utilising various wastes such as corn starch, sucrose, whey, melon, apple, tomato as carbon substrates. Pramanik et al., (2012) studied the ability of *Haloarcula marismortui* to utilize 10% raw vinasse and 100% pre-treated vinasse to produce 2.8 g L^{-1} and 4.5 g L^{-1} of PHB. Recent study on *Hfx. mediterranei* DSM 1411 reported the synthesis of 7.2 g L^{-1} of polymer from whey permeate (Koller, 2015). Koller et al., (2008) showed the ability of *Hfx. mediterranei* to utilize hydrolyzed whey and produce 12.2 g L^{-1} of PHBV. Similarly, Bhattacharyya et al., (2012) employed *Hfx. mediterranei* to produce 19.7 g L^{-1} and 17.4 g L^{-1} from 25% and 50% pre-treated vinasse, respectively. Also, 24.2 g L^{-1} PHBV biosynthesis was noted in *Hfx. mediterranei* with extruded cornstarch (Huang et al., 2006).

Yu and Stahl, (2008), investigated the ability of Gram negative bacterium *Ralstonia eutropha* to produce 57.0 % (w/w) of CDW of PHA by utilising organic inhibitors such as formic / acetic acid, furfural, etc, from SCB hydrolysate. The bacterium, *Ralstonia eutropha* showed good growth when grown on acids such as acetic / lactic / propionic / butyric / valeric as well as monosaccharides such as glucose (hexose) and fructose (pentose). However, poor growth was reported when xylose and arabinose was provided as carbon source. Study by Lavarack et al., (2002) revealed that xylose, arabinose, glucose along with other products can be obtained from dilute acid hydrolysis of SCB hemicelluloses. The present study employed extremely halophilic archaeon, *Hgm. borinquense* strain E3, which utilises and grows lavishly when xylose, arabinose and glucose are provided as sole source of carbon. *Ralstonia eutropha* grew and accumulated 30.4 % (w/w) of CDW of PHA, when high cell density biomass was used as inoculums in SCB hydrolysate supplemented with glucose. However, no PHA was produced / accumulated by the bacterial cells when grown exclusively in the SCB hydrolysate solution without glucose supplement (Yu and Stahl, 2008). Interestingly, *Hgm. borinquense* strain

E3, was able to grow and produce PHA when grown in liquid SCB hydrolysate without treatment and or any additional carbon supplement.

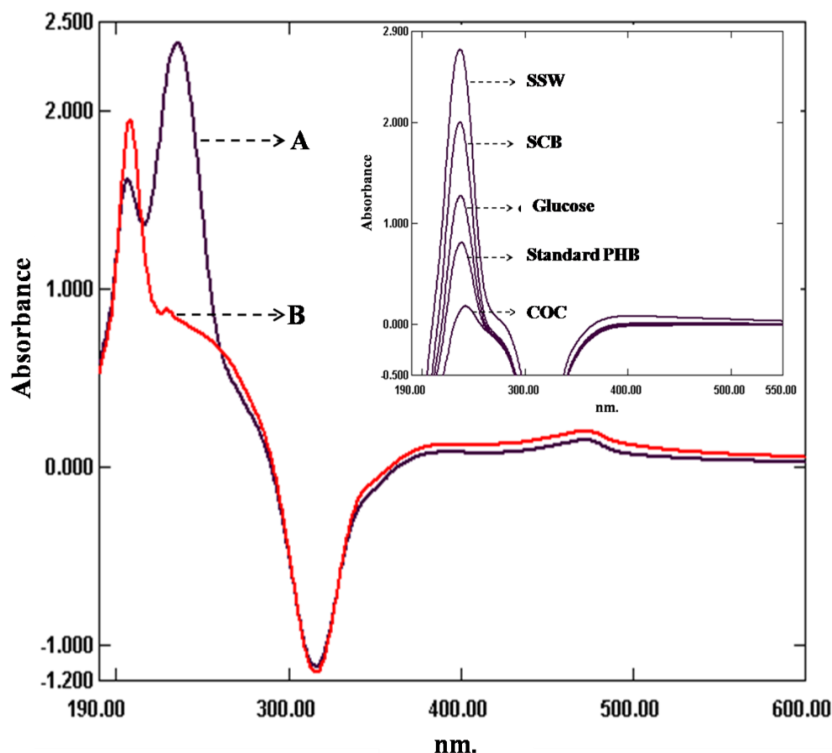

Fig. 6.6 Crotonic acid assay of cells of *Hgm. borinquense* strain E3 grown in NSM containing SCB hydrolysate: (A) before soxhlet extraction and (B) after soxhlet extraction. Inset showing comparison of crotonic acid spectrum of pure polymers obtained from glucose, SCB, SSW, COC hydrolysates and standard PHB.

6.3.6 Polymer characterization

The polymer obtained using various agro-industrial wastes is presented in Fig. 6.7 and was characterized using UV–visible spectrophotometer, XRD, DSC, FT-IR and NMR as described in Chapter III/V, section 3.2.8/5.2.6.

6.3.6.1 UV-visible spectrophotometric analysis

Concentrated H_2SO_4 hydrolysis of the polymers to crotonic acid gave a characteristic peak at 235 nm comparable to that of the polymer obtained from glucose and standard PHB (Sigma-Aldrich) (Inset of Fig. 6.6) (Law and Slepecky, 1961; Salgaonkar and Bragança, 2015).

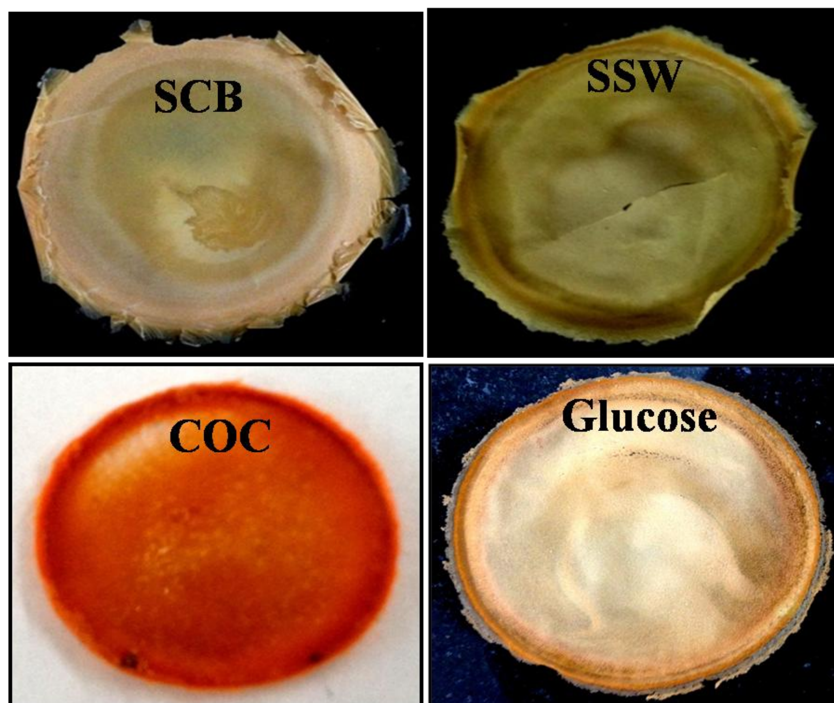

Fig. 6.7 PHA polymer films produced by *Hgm. borinquense* strain E3 by utilising substrates such as SCB, SSW, COC and glucose.

6.3.6.2 XRD analysis

Fig. 6.8 shows the X-ray diffraction (XRD) patterns in the range $2\theta = 10 - 60^{o}$ of polymer obtained from SCB, COC and SSW hydrolysate in comparison with standard PHB (Sigma-Aldrich). The XRD diffractogram of standard PHB revealed its semicrystalline nature showing characteristic peaks at 2θ = 13.4°, 17.0°, 20.1°, 21.2°, 22.2°, 25.5°, 27.1°, 30.1° and 44.4° corresponding to (020), (110), (021), (101), (111), (121), (040), (002) and (222) reflections of the orthorhombic crystalline lattice (Oliverira et al., 2006). The polymers obtained from agro-industrial wastes hydrolysate were more amorphous in nature and showed 2θ peaks almost at the same values as for the PHB (Fig. 6.8). The agro-industrial wastes polymer profile exhibited prominent peaks at 2θ = 13.4-13.9°, 17.3-17.9°, 20-20.2°, 21.2-21.9°, 22-22.3°, 25.4-25.9°, 27.2-27.5°, 30.4-31.7° and 44.4-45.8° corresponding to (020), (110), (021), (101), (111), (121), (040), (002) and (222) reflections of the orthorhombic crystalline lattice. Peak shifts as well as a decrease in peak intensity was observed in polymer when compared with standard PHB. It is clearly observed that the three diffraction peaks between $2\theta = 20 - 25^{o}$ in case of PHB has broadened and are

drastically decreased in intensity in case of SCB polymer. In case of COC polymer, there was a broad hump with drastic decrease in the peak intensity. This may be due to the greasy nature of the polymer due to the partial retention of coconut oil from the hydrolysate. The SSW polymer showed a prominent sharp peak at 2θ =31.7° corresponding to (002) reflection, along with the other peaks (Fig. 6.8). Broadening of the peaks clearly indicates a decrease in crystallinity, therefore an amorphous nature of the polymer (da Silva Pinto and Wypych, 2009; Da Silva Moreira Thire et al., 2011). The XRD profile of SCB, COC and SSW hydrolysate polymers were well comparable with the XRD spectra of pure PHBV reported by Farago et al., (2008). The crystallite size L (nm) was determined for the highest peaks using the Scherrer equation as described in Chapter III, section 3.2.8.3. The crystallite size for the (020), (110), (111) and (002) reflections was found to be 22.3 nm, 10.4 nm, 3.4 nm and 33.9 nm in case of standard PHB, SCB, COC and SSW polymers, respectively.

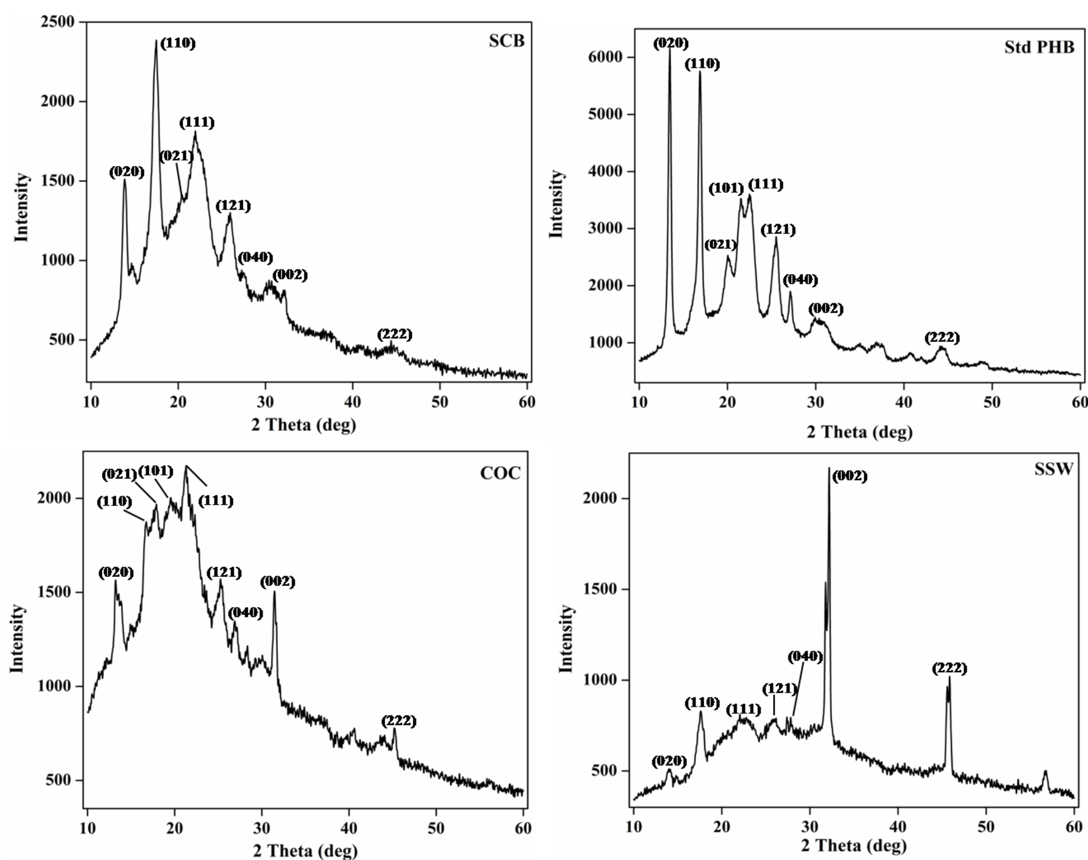

Fig. 6.8 Comparison of the X-ray diffraction patterns of standard PHB and polymer from *Hgm. borinquense* strain E3 grown in NSM with SCB, COC and SSW hydrolysate.

6.3.6.3 DSC analysis

The thermograms derived from differential scanning calorimetry (DSC) analysis for the polymer obtained using SCB hydrolysate and standard PHB (Sigma) are represented in Fig. 6.9. There were two melting endotherms Tm_1 = 136.59-142.69 ^oC and Tm_2 = 149.4-155.27 ^oC (Table 6.4) whereas in case of standard PHB a single melting endotherm at Tm = 169.2 ^oC was observed. The two Tm observed for SCB polymer were much lower as compared to PHB. The polymer synthesized by *Hgm. borinquense* strain E3 using glucose as substrate also showed two melting peaks at 138.15^oC (Tm_1) and 154.74^oC (Tm_2) (Salgaonkar and Bragança, 2015).

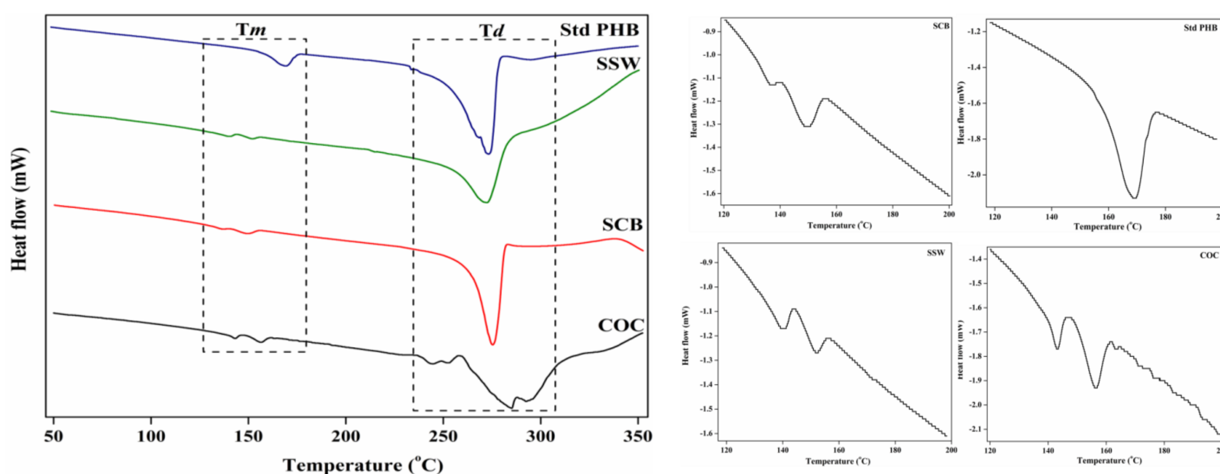

Fig. 6.9 (A) Comparison of DSC curves showing the melting temperatures (T*m*) and the degradation temperatures (T*d*) of standard PHB (sigma) with that of the polymers obtained from *Hgm. borinquense* strain E3 grown in NSM with SCB, COC and SSW hydrolysate. (B) Details of the melting temperature (T*m*) of the individual polymers.

Recent study by Buzarovska et al., (2009) reported, pure copolymer PHBV containing 13% hydroxyvalerate (HV) to have two melting endotherms. The lower melting peak (Tm_1) at 138^oC and could be due to the melting of the primary formed crystallites, whereas the upper one (Tm_2) at 152^oC is mostly due to the recrystallization of species during the scan (Buzarovska et al., 2009). The existence of multiple melting peak in a polymer indicate the polymers to have varying monomer units such as HB and HV units (Sudesh, 2013). *Haloferax mediterranei* is known to produce PHA with multiple melting endotherms, by utilising various carbon substrates (Hermann-Krauss et al., 2013). Chen et al., (2006) and

Koller et al., (2007b) showed the ability of *Haloferax mediterranei* ATCC 33500 / DSM 1411 to utilize extruded cornstarch / whey sugars as carbon substrates for the production of copolymer P(3HB-*co*-3HV) containing 10.4 and 6 mol% of hydroxyvalerate (HV), respectively. The P(3HB-*co*-3HV) produced by strain DSM 1411 showed two melting peaks at 150.8°C (Tm_1) and 158.9°C (Tm_2) whereas, the melting endotherms for strain ATCC 33500 were at 129.1°C (Tm_1) and 144.0°C (Tm_2) (Koller et al., 2007b, Chen et al., 2006). The degradation temperature (Td) peak for the SCB polymer and PHB was at 275.4°C and 273.2°C, respectively.

Table 6.4 Comparison of the DSC data of polymers synthesized by *Hgm. borinquense* strain E3 using various agro-industrial wastes with the literature.

PHA from various substrates	**Haloarchaeal isolate**	**DSC characterization (°C)**			**Reference**
		T*m1*	T*m2*	T*d*	
SCB	*Halogeometricum borinquense* strain E3	136.59	149.4	275.4	Present study
SSW		140.09	151.14	271.9	
COC		142.69	155.27	284.7	
Glucose		138.15	154.74	231.08	
Cornstarch	*Haloferax mediterranei* ATCC 33500	129.1	144.0	NR	Chen et al., 2006
Whey	*Haloferax mediterranei* DSM 1411	150.8	158.9	241	Koller et al., 2007b

T*m*: melting temperature; T*d:* degradation temperature

6.3.6.4 FT-IR analysis

The FT-IR spectra of polymer obtained using SCB, COC and SSW hydrolysate was compared with that of the standard PHB (Sigma) (Fig. 6.10). The IR spectra of polymer obtained from agro-industrial wastes and standard PHB showed one intense absorption band at 1724 cm^{-1} and 1731 cm^{-1} characteristic of ester carbonyl group (C=O) stretching. Also, band at 1281 cm^{-1} representing C-O-C stretching whereas that in the region 3100-2800 cm^{-1} such as 2983 cm^{-1} and 2981 cm^{-1} representing C-H stretching was detected (Padermshoke et al., 2004) (Fig. 6.10). Apart from these, other prominent bands were also

observed, which may be due to interactions between the OH and C=O groups resulting in shift of the stretching (da Silva Pinto and Wypych, 2009).

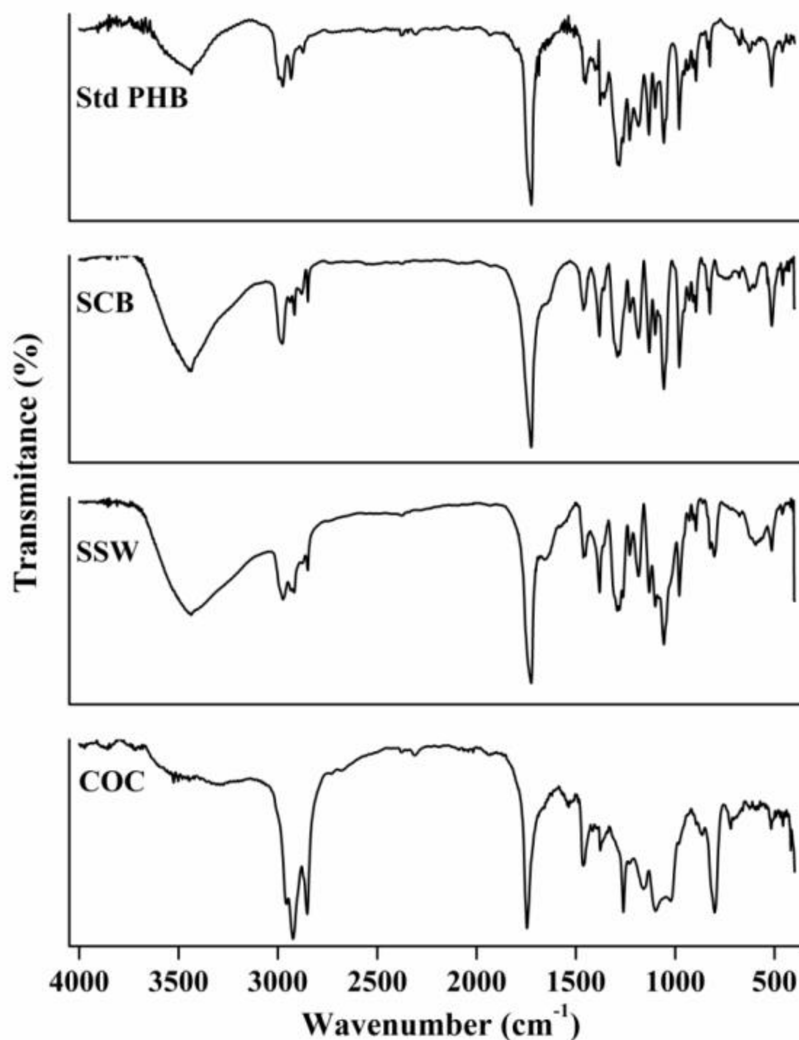

Fig. 6.10 Comparison of FT-IR spectrum of the standard PHB with that of the polymers obtained from *Hgm. borinquense* strain E3 grown in NSM with SCB, COC and SSW hydrolysate.

6.3.6.5 ^{1}H NMR analysis

Fig. 6.11 shows the comparison of ^{1}H NMR spectrum of polymer obtained using SCB, COC and SSW hydrolysate with that of the standard PHB (sigma). The chemical shift of peak and their chemical structure is represented in Table 6.5. characteristic peaks at 0.9 ppm is of methyl (CH_3) from hydroxyvalerate (HV) unit and at 1.25 ppm is of methyl (CH_3) from hydroxybutyrate (HB) unit, respectively. ^{1}H NMR spectrum of standard PHB

showed only one prominent peak at 1.25 ppm of methyl (CH_3) from HB unit (Chapter III, section 3.3.6.5). Therefore, it can be confirmed that the polymer obtained using SCB, COC and SSW hydrolysate is a co-polymer of [P(HB-*co*-HV)]. Composition of HV (%) in the co-polymer of P(HB-*co*-HV) was calculated as described in Chapter IV, section 4.3.6.5. It was found that the co-polymer produced by the extremely halophilic archaeon *Hgm. borinquense* strain E3 by utilization of SCB and SSW hydrolysates comprises 13.29% and 19.65 % HV units. ^{1}H NMR spectrum of COC polymer did not give proper signal peak area due to the retention of residual oil in the polymer.

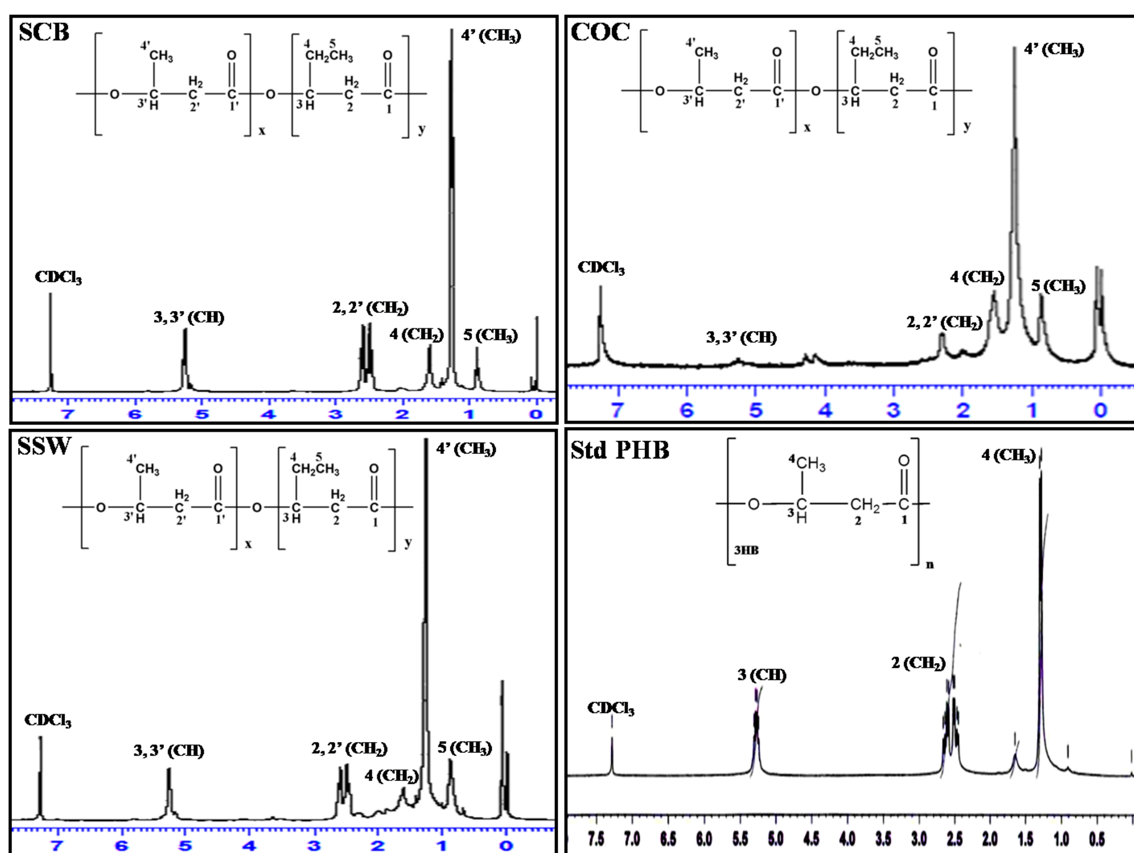

Fig. 6.11 Comparison of ^{1}H NMR spectrum of standard PHB with that of the polymers obtained from *Hgm. borinquense* strain E3 grown in NSM with SCB, COC and SSW hydrolysate.

Yu and Stahl (2008) reported the capability of aerobic bacterium *Ralstonia eutropha* to produce homopolymer of hydroxybutrate (PHB) as well as copolymer of P(HB-*co*-HV) by utilising SCB hydrolysate along with glucose as substrate. However, the bacterium failed to synthesize polymer when grown in hydrolysate solution devoid of glucose. Moreover,

Ralstonia eutropha strain was unable to utilise pentose sugars like xylose and arabinose as a sole source of carbon. Nevertheless, the strain could efficiently mineralize compounds like furfurals, formic / acetic acid and phenolic compounds which are the major hydrolysis inhibitors (Yu and Stahl 2008). Interestingly, the present isolate *Hgm. borinquense* strain E3 was able to grow and synthesise PHA [P(HB-*co*-HV)] from crude SCB hydrolysate without any additional supplement of glucose and pre-treatment for removal of inhibitors. The strain was also able to utilise arabinose and xylose when supplied as sole source of carbon (Chapter II, Table 2.7). Further investigation is needed, to study the cell and polymer yield, after treatment of the SCB hydrolysate and also the effect of glucose as an supplement to SCB hydrolysate could be done.

Table 6.5 Comparison of the chemical shift of peak and their chemical structure obtained from ^{1}H NMR data of polymers synthesized by *Hgm. borinquense* strain E3 using various agro-industrial wastes with the literature.

PHA from various substrates	**Haloarchaeal isolate**	**Relative chemical structure**					**Reference**
		CH_3 (HB)	**CH_2 (HV/ HB)**	**CH (HV/ HB)**	**CH_3 (HV)**	**CH_2 (HV)**	
		Chemical shifts of each peak (ppm)					
SCB	*Halogeometricum borinquense* strain E3	1.26-1.27	2.44-2.63	5.22-5.27	0.889	1.618-1.635	Present study
SSW		1.26-1.27	2.44-2.63	5.24-5.26	0.9	1.59-1.61	
COC		1.258	2.43-2.62	5.256	0.878	1.556	
Glucose		1.26-1.28	2.44-2.63	5.26	0.85-0.91	1.6	
Cornstarch	*Haloferax mediterranei* ATCC 33500	1.2	2.5	5.2	0.9	1.6	Chen et al., 2006
Vinasse	*Haloferax mediterranei* DSM 1411	1.26-1.28	2.43-2.645	5.22-5.28	0.86-0.95	1.586	Bhattacharyya et al., 2012

HB: hydroxybutyrate; HV: hydroxyvalerate; CH_3: methyl; CH_2: methylene; CH: methane

Study by Han et al., (2015) on haloarchaeon *Hfx. mediterranei* reported the biosynthesis of random / higher-order copolymers PHBV (R-PHBV / O-PHBV) which exhibited increased platelet adhesion thereby accelerating blood clotting. Report by Danis et al., (2015) employed *Natrinema pallidum* for P(HB-*co*-HV) produced which was used to prepare biocompatible films for drug delivery (rifampicin). The above studies confirms the potential of polymers synthesized by haloarchaea in various biomedical applications.

6.4 Conclusion

Ability of extremely halophilic archaeon *Hgm. borinquense* strain E3 to use the agro-industrial wastes for the production of PHA was investigated. *Hgm. borinquense* strain E3, was able to grow well on renewable agro-industrial wastes such as sugarcane bagasse (SCB), coconut oil cake (COC) and sago starch waste (SSW). SCB was selected for further studies as the culture showed best tolerance and grew well in NSM medium with 25% and 50% (v/v) SCB hydrolysate. The time-course of growth of *Hgm. borinquense* strain E3 in minimal medium with 25% and 50% (v/v) SCB hydrolysate showed maximum PHA of 50.4 ± 0.1% and 45.7 ± 0.19% (w/w) of CDW on the 7th day, respectively. The DSC thermogram showed the two melting endotherms in range of Tm_1 = 136.59-142.69 ^{o}C and Tm_2 = 149.4-155.27 ^{o}C. ^{1}H NMR analysis of the polymer obtained from SCB and SSW hydrolysates revealed it to be a co-polymer of [P(HB-*co*-HV)] comprising 13.29 and 19.65 % HV units. Various agro-industrial wastes that can be degraded by such halophilic microbes should be explored, for the production of biopolymers as this may help us in both, managing the agro-industrial wastes and cutting down the costs of commercial substrates. Since *Hgm. borinquense* strain E3 is an extremely halophilic archaeon requiring ≥ 25% NaCl for its growth, media sterilization cost can be cut down at industrial scale production.

Chapter VII

Osmoadaptation in Halophilic Microorganisms

7.1 Introduction

Solar salterns are unique eco-niches wherein the inherent microflora encounters a salinity gradient from 3.5 % to saturation. Microorganisms inhabiting these eco-niches have developed versatile osmoadaptation strategies to resist these salinity changes (Hänelt and Müller, 2013; Kempf and Bremer, 1998; Galinski, 1993; Roberts, 2005). Most halophilic and halotolerant microorganisms that grow at elevated salt concentrations synthesize and/or accumulate organic osmotic solutes known as compatible solutes (CS) to adjust intracellular water activity so as to provide osmotic balance between the cytoplasm and the surrounding medium (Jehlicka et al., 2012). CS are polar, low molecular weight, highly water soluble molecules and uncharged at physiological pH. They include heterogeneous group of organic compounds such as amino acids: proline and glutamate, amino acid derivatives: betaines, ectoines, N-acetylated diamino acids and N-derivatized carboxamides of glutamine (Galinski, 1993), carbohydrates or their derivatives: trehalose and sucrose, and polyols (glycerol and *Myo*-inositol) (Shivanand and Mugeraya, 2011). Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is one of the most widely found compatible solutes in the domain bacteria (Kuhlmann et al., 2008; Reshetnikov et al., 2006; Kuhlmann and Bremer, 2002). These CS have various applications in biotechnology, as they protect enzymes, membranes, and whole cells against stress caused by exposure to salt, heat, freezing, and desiccation.

Archaea surviving in saline environments follow a different strategy with intracellular accumulation of K^+ ions to counteract the external Na^+ ions (Oren, 2006a; Welsh, 2000; Oren, 2008). If the ionic balance is not achieved, cells will be subjected to osmosis followed by lysis. Active transport is required to maintain the ionic distribution because of the passive permeability of the cell membrane to K^+ and Na^+ ions, which is low. The K^+ gradient is maintained by a combination of an electrogenic Na^+/H^+ antiporter and two K^+ uniporters. Light driven rhodopsins provide energy for achieving the active energy for functioning of the pumps.

Only one report exists on the accumulation / synthesis of CS such as glycine betaine, trehalose and glutamate by *Halococcus hamelinensis* (Goh et al., 2011). Whole genome sequencing of *Haloarcula* sp., CBA1115 and *Halococcus hamelinensis* have indicated the presence of genes for the accumulation of CS such as (Gudhka et al., 2015; Yun et al., 2015; Goh et al., 2011). In this chapter, the osmoadaptation strategies in moderately halophilic bacteria and extremely halophilic archaeal isolates, from solar salterns of Goa and Tamil Nadu India (Chapter II) were studied along with characterization of the osmolytes obtained.

7.2Materials and methods

7.2.1 Halophilic bacterial and archaeal isolates

Three halophilic bacteria, (i) *Halomonas elongata* strain M4, (ii) *Alkalibacillus salilacus* strain RP26 and (iii) *Halobacillus alkaliphilus* strain SP17 and four halophilic archaea, (i) *Halococcus salifodinae* strain BK20, (ii) *Haloferax volcanii* strain BBK2, (iii) *Haloarcula japonica* strain BS2 and (iv) *Halogeometricum borinquense* strain TN9 were used in this study (Chapter III/IV).

7.2.2 Growth of microorganism in varying concentration of NaCl

Bacteria were grown in E2 medium and archaea were grown in Norberg and Hoefsteins (NH) or NaCl glucose synthetic medium (NGSM) medium (Appendix I) without NaCl or containing (w/v) 5%, 10%, 15%, 20%, 25%and 30% NaCl. The flasks were incubated on shaker at 37°C, 100 rpm. Absorbance of the culture broth was monitored at 600nm using UV-visible spectrophotometer (Shimadzu UV-2450, Japan). For bacteria the absorbance was taken after 24 hrs and for archaea after 4 days. Changes in the cell morphology of *H. elongata* strain M4 was studied using Scanning electron microscopy (SEM).

7.2.3 Screening for compatible solutes in halophilic bacteria

7.2.3.1 Extraction

Halophilic bacteria *H. elongata* strain M4, *A. salilacus* strain RP26 and *H. alkaliphilus* strain SP17 were grown for 2 days in 100 ml of MHM and E2 medium supplemented with

1% glucose (w/v) and 20% and 5% NaCl concentration. The cells from respective liquid medium were pelleted by centrifuging at 8,000rpm, 4°C for 12 min. The cell pellets obtained were freeze-dried using a lyophilizer (Christ, Alpha 1-2 LD plus). Cell extracts were prepared as described by Kunte et al. (1993). Briefly, to 0.5 gm of the lyophilized cells, 9 ml of an extraction mixture (methanol/chloroform/water 10 : 4 : 4, v/v/v) was added. The suspension was vigorously shaken for 5-10 min, at room temperature (27-29°C) followed by the addition of equal volumes of chloroform (2.5 ml) and water (2.5 ml). This mixture was shaken for 10-20 min on a gel rocker. The mixture was centrifuged at 8,000 rpm for 10 min for phase separation. The lower hydrophobic phase was discarded along with the cell debris which occurred at the interphase. The top hydrophilic layer was recovered and transferred to clean dry glass vials. The vials containing the cell extracts were kept at -80°C overnight and lyophilized.

7.2.3.2 Determination and analysis using HPLC

Freeze dried cell extracts were redissolved in 200µl acetonitrile/water (80: 20 v/v) (HPLC grade, Sigma), centrifuged at 10,000 rpm for 10 min for removal of cell debris and media salts if any. 100µl of this supernatant was injected through a loop into a Phenomenex C18 HPLC column (250 mm × 4.6 mm, 5 µm) and eluted at a rate of 1ml min^{-1} by a gradient of acetonitrile and water (Table 7.1). The elution profile of the extract was monitored at 210 nm. This was then separated on a HPLC (Shimadzu UFLC) and analyzed by SPDM 20A Prominence diode array detector. The solvent system used was (i) Solvent A,water : acetonitrile (9:1 v/v) and (ii) Solvent B, acetonitrile : water (9:1 v/v). Both the solvents were acidified with 0.1% formic acid. Commercial ectoine and betaine were used as standards.

Table 7.1 Gradient used for halophilic bacterial cell extracts separation and elution.

Time (min)	Flow rate (ml min^{-1})	A (%)	B (%)	Total time (min)
0-1	1	99	1	1
1-10	1	99-0	1-100	11
10-20	1	0	100	21

7.2.4 Screening for compatible solutes in halophilic archaea

7.2.4.1 Extraction

Halogeometricum strain TN9, *Haloarcula* strain BS2 and *Halorubrum sp.* strain BS17 were grown for 6-7 days in 100 ml of NH medium supplemented with 1% glucose (w/v) and 20% (w/v) NaCl. The cells were pelleted by centrifugation at 8,000 rpm, 4°C, for 15 min and freeze-dried using a lyophilizer (Christ, Alpha 1-2 LD plus). The cell extract was prepared as described by Kunte et al. (1993) with minor modification. Briefly, to 10 mg of the lyophilized cells, 570µl of extraction mixture (methanol/chloroform/water 10: 4: 4 v/v/v) and 30 glass beads were added. The archaeal cells were ruptured by placing the suspension in bead mill for 5 min, 30 Hz / sec. The cell lysate was centrifuged at 5,000rpm, 4°C, for2 min. The pellet was discarded and supernatant was lyophilized, resuspended in 500 µl of milli-Q water and filtered using Vivaspin 500 membrane with 3000 molecular weight cut-offs (MWCO).

7.2.4.2 FMOC derivatization

The filtered cell extract and standards (ectoine, citrulline and alanine) were derivatized using 9-fluorenylmethoxycarbonyl (FMOC) and identification of CS which was carried out using an isocratic HPLC with an NH_2 column using a UV detector (220 nm) as described by Kunte et al., 1993 and Saum et al., 2006. To 150 µl of sodium borate buffer (0.5 M, pH 7.7), 150 µl of cell extract or standard (20 µM) was added, followed by addition of 150 µl FMOC reagent (15 mM in acetone). The vial was vortexed for 45 sec and the mixture was extracted with 600µl pentane. This extraction step was repeated twice. The aqueous phase containing the FMOC derivatives of the cell extracts were analyzed using HPLC.

7.2.4.3 HPLC analysis

FMOC-derivatized cell extracts / standards were separated by reversed-phase chromatography (4-µm Supersphere 60 RP-8 end capped column; Merck, Germany). The solvent system used comprised of solvent A: 20% acetonitrile and 0.5% tetrahydrofuran (THF) and solvent B: 80% acetonitrile. Both were prepared in 50 mM, pH 4.2, and sodium acetate buffer. The details of the gradient used are given in Table 7.2. The derivatives were

monitored with an RF 2000 fluorescencedetector (Dionex, Germany) with an excitation wavelength of 254 nmand an emission wavelength of 315 nm (Saum et al., 2006).

Table 7.2 Gradient used for halophilic archaeal cell extract separation and elution.

Time (min)	Flow rate (ml min^{-1})	A (%)	B (%)	Total time (min)
0-2	1	100	0	2
2-8	1	100-80	0-20	6
8-16	1	80-73	20-27	8
16-29	1	73-46	27-54	13
29-31	1	46-0	54-100	2

7.2.5 Determination of intracellular K^+

Haloferax volcanii strain BBK2, *Halococcus salifodinae* strain BK20, *Halorubrum* sp. strain BS17 and *Halomonas elongata* strain M4 was grown in media with optimal NaCl concentrations as described in section 7.2.2. Cells were harvested by centrifugation and cell pellet was resuspended in 10-20% NaCl as per the NaCl requirenment of the isolates. Protein content of the cell pellets was assessed using the Lowry procedure (Lowry et al., 1951). One milliliter of liquid cell cultures was taken and filtered through cellulose nitrate membrane (0.45µm). To this, 1 ml of acid mix was added containing 1.5M $HClO_4$ + 15% TCA, mixed well and incubated for 24 hrs. K^+ concentration in the haloarchaeal cells was estimated by atomic absorption spectrometry (AAS) (PerkinElmer, USA).

7.2.6 FT-IR analysis of the cell extract

The FT-IR spectra of lyophilized cell extract of *H. elongata* strain M4 grown in MHM and E2 medium were recorded on Shimadzu IRAffinity-1 spectrometer (Kyoto, Japan) as described previously in Chapter V, section 5.2.5.

7.2.7 Nuclear magnetic resonance (NMR) spectroscopy

The chemical structure of the cell extract of *H. elongata* strain M4 was determined by proton nuclear magnetic resonance (^{1}H-NMR) analysis. For this the cell extracts were suspended in high purity heavy water / deuterium oxide (D_2O) and the ^{1}H NMR spectra

was obtained at 400 MHz using a model Bruker Avance 300 NMR spectrometer (Rheinstetten, Germany).

7.3 Results and discussion

7.3.1 Growth of halophilic bacteria and archaea at various NaCl concentrations

The growth of halophilic bacteria and archaea in E2 and NGSM/NH with varying NaCl concentrations is represented in Figs. 7.1 and 7.2, respectively. Among all the halophilic bacteria, *Halomonas elongata* strain M4 showed the best resistance and growth in E2 media over a range of NaCl concentrations, from 5% to 25% (w/v). On the other hand, *A. salilacus* strain RP26 and *H. alkaliphilus* strain SP17 showed NaCl tolerance and growth from 0% up to 15% (w/v) with optimum growth at 5% NaCl (Fig. 7.1). *H. elongata*, *A. salilacus* and *H. alkaliphilus* belong to the group of moderately halophilic bacteria (Mothes, et al., 2008; Jeon et al., 2005; Romano et al., 2008).

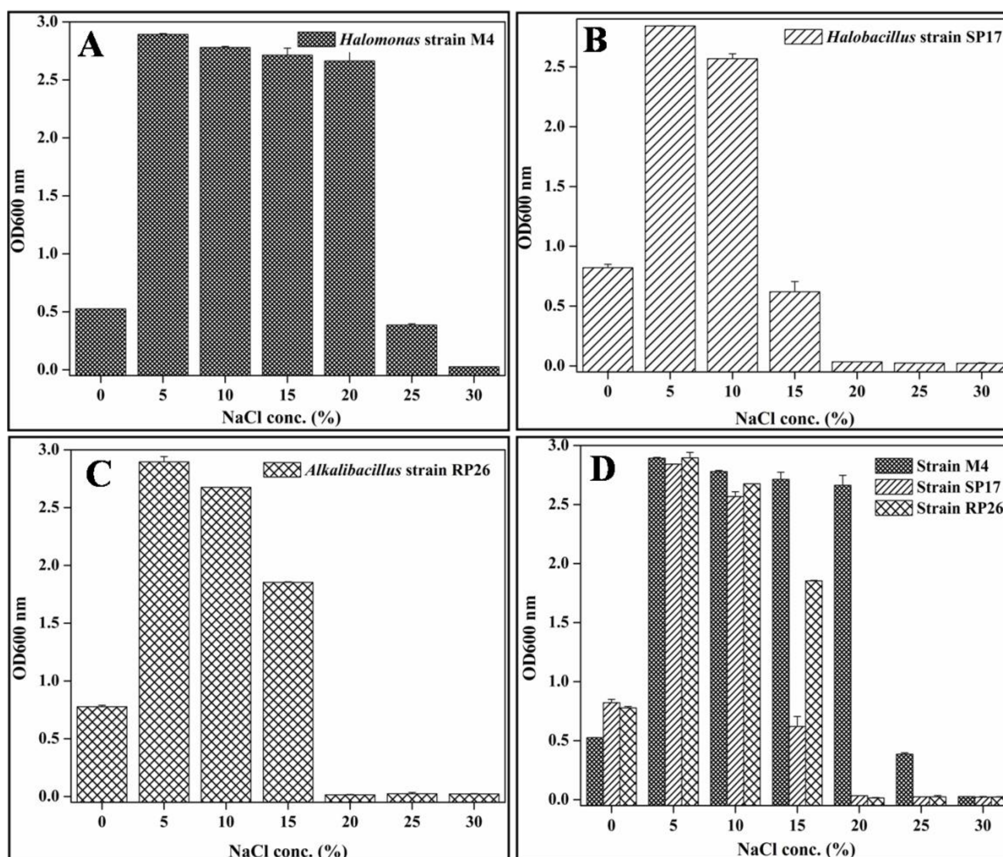

Fig. 7.1 Growth of halophilic bacteria in liquid E2 medium with varying NaCl concentrations.

Halophilic archaea grew well in both NGSM and NH media and showed minimum NaCl requirement of 5% (w/v). *Halococcus* strain BK20 showed optimum NaCl requirement of 20% (w/v) in both NH and NGSM media. *Haloferax* strain BBK2 showed optimum NaCl requirement of 15-20% (w/v) in NH medium and 15% in NGSM medium. *Haloarcula* strain BS2 overall grew well in NGSM medium with the optimum NaCl requirement of 20% (w/v) whereas weak growth was observed in NH medium with 15% NaCl being the optimum . *Halogeometricum* strain TN9 showed optimum NaCl requirement of 15% (w/v) in NH medium and 20% (w/v) in NGSM medium (Fig. 7.2).Studies on osmoadaptation in bacteria and archaea can be achieved by examining their growth in media with varying salt concentrations and based on the salt requirement, these microorganisms can be further classified as moderate halophiles and extreme halophiles (da Costa et al., 1998).

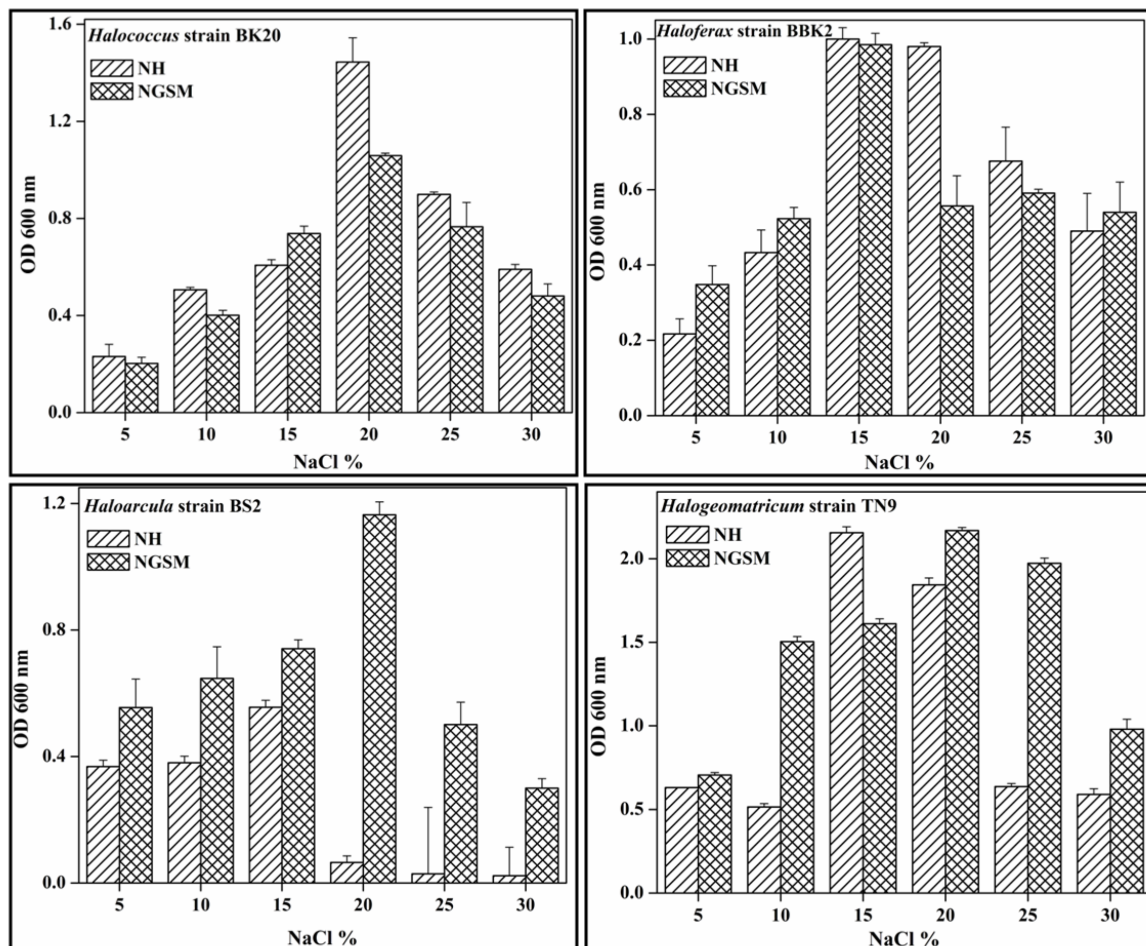

Fig. 7.2 Growth of halophilic archaea in liquid NGSM and NH media with varying NaCl concentrations.

7.3.2 Cell morphology study using SEM

The SEM of *H. elongata* strain M4 grown in the E2 medium containing varying concentrations of NaCl (%) is shown in Fig. 7.3. The cells of *H. elongata* strain M4 at lower NaCl concentrations of 5-15% w/v NaCl, appear as long rods arranged in chains. As the NaCl concentration increases from 20-25% w/v NaCl, the cell size reduces and the cells become shorter, however retaining its rod shaped morphology (Fig. 7.3). Similar, observation was made by Takenaka, et al (2004) for *H. elongata* grown in media containing low (0.6M) and high (4.3M) NaCl.

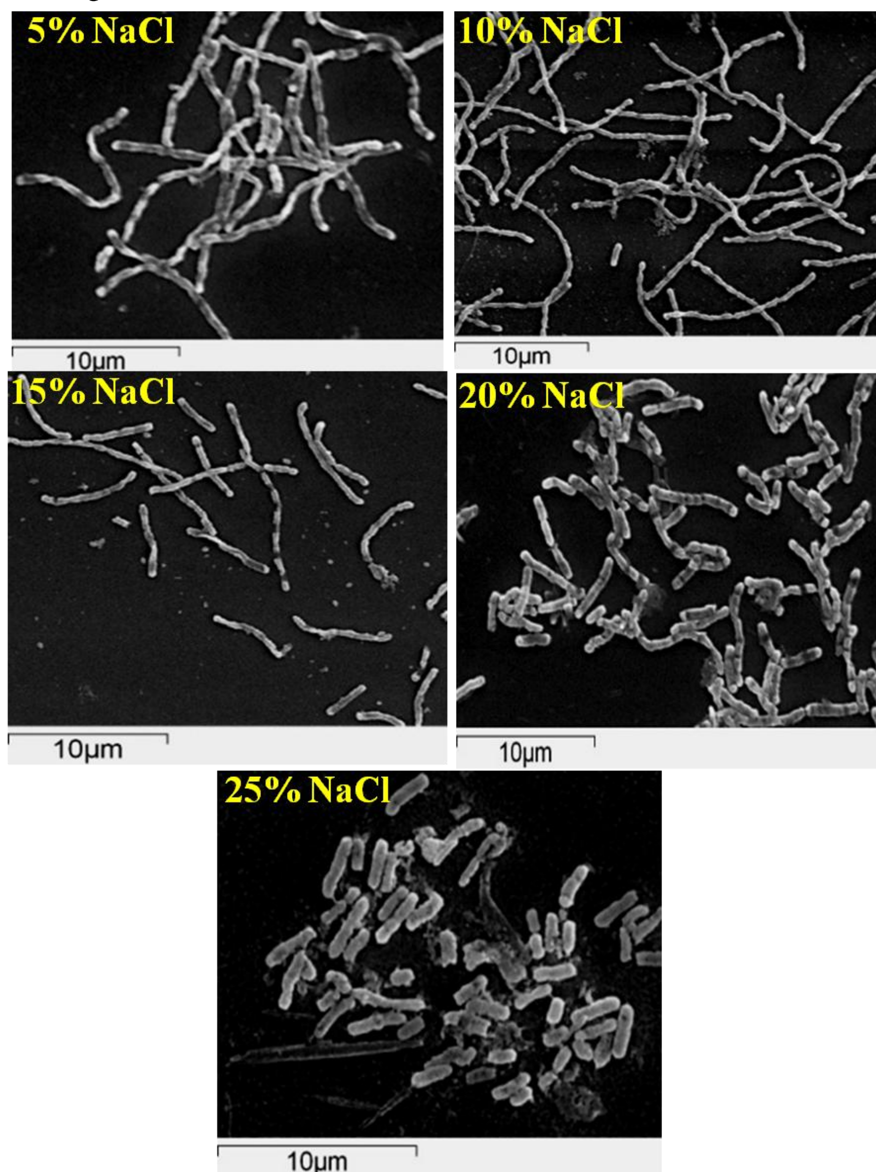

Fig. 7.3 Scanning electron micrographs depicting morphological changes of *H. elongata* strain M4 grown in E2 synthetic medium with varying NaCl concentrations.

7.3.3 HPLC analysis of the cell extracts

The HPLC profile of the total cells extracts of the *H. elongata* strain M4 in complex MHM and synthetic E2 media, respectively is represented in Fig. 7.4.In MHM, the cell extracts of *H. elongata* strain M4, grown in 5% and 20% NaCl, showed two sharp peaks at retention time of 2.9 and 3.6 min. Additionally, one broad peak was seen at retention time of 2.55 min. Though, the profiles of 5% and 20% were similar, the intensity of peaks varied ie. at 5% NaCl the first peak intensity is more compared to the second peak. However, at 20% NaCl the intensity of second peak is more as compared to the first peak. The two peaks represent two different osmolytes and this result clearly indicated that at different osmolarity / NaCl concentrations the type and amount of osmolytes accumulated / synthesized varied drastically. The profile obtained compared well with the retention time of standards, ie. retention time of first peak matched with ectoine (sigma) and second peak matched with betaine (sigma) (Fig. 7.4). In E2 medium, the cell extracts of isolate M4, grown in 5% and 20% NaCl, showed a single sharp peak at retention time of 3.3 and 2.5 min, respectively.

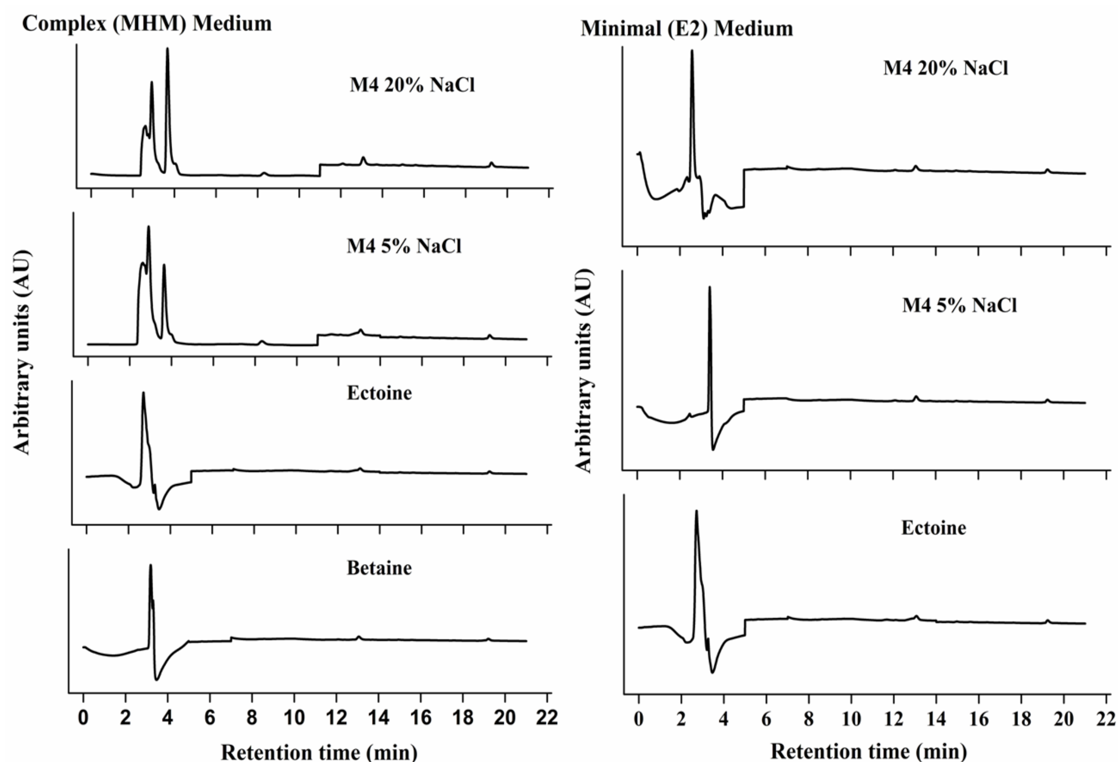

Fig.7.4 Comparison of HPLC profile of standards ectoine and betaine with the total cell extract of cells of *H. elongata* strain M4 grown in MHM and E2 media.

The HPLC profile of the FMOC derivatised total cells extracts of the halophilic archaea *Halogeometricum* strain TN9, *Halorubrum* strain BS17, and *Haloarcula* strain BS2, grown in NGSM is represented in Fig. 7.5. FMOC efficiently forms stable derivatives with primary / secondary amino acids and also with N-methyl amino acids at high temperature. It also helps in quantitative analysis of charged amino acids like glutamate. The profiles of cell extracts were compared with water and standard solutions of citrulline and alanine (Fig. 7.5). No additional peaks were corresponding to the standards were observed in the cell extracts, indicating that the halophilic archaea may not accumulate osmolytes when grown at high osmolarity (Kunte et al., 1993).

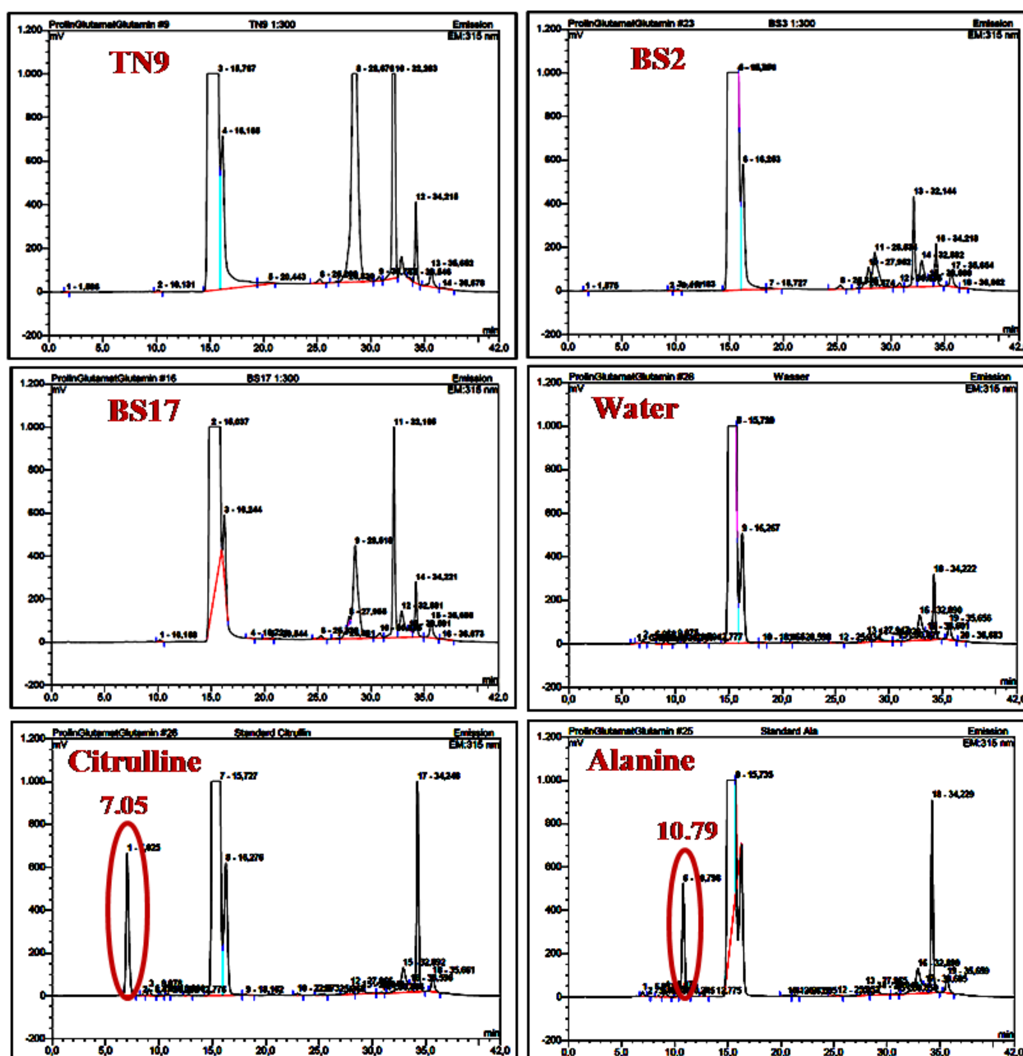

Fig.7.5 Comparison of HPLC profile of standard citrulline and alanine with the total cell extract obtained from cells of haloarchaeal isolates BBK2, BK20 and BS17 grown in NGSM.

7.3.4 Intracellular K^+ estimation

Halophilic archaea *Hfx. volcanii* strain BBK2, *Hcc. salifodinae* strain BK20, *Halorubrum* sp. strain BS17 accumulated 4.77, 8.14 and 10.12 of K^+/protein (μM/mg), respectively. *Hfx. volacnii* starin BBK2 was grown at 15% NaCl while *Hcc. salifodinae* strain BK20, *Halorubrum* sp. strain BS17 was grown at 20% NaCl. This could have influenced the difference in K^+ accumulation because of the fact that halophilic archaea maintains intracellular ion concentration with respect to the external ion concentration. This indicates that K^+ ion accumulation is the main osmoadaptation strategy of the halophilic archaeal isolates under investigation. *Hcc. hamelinensis s*till date is the only extremely halophilic archaeon which lack salt-in-cytoplasm(K^+ uptake) as osmoprotective strategy and instead accumulates compatible solutes like glycine betaine and trehalose (Goh et al., 2011). In this study no accumulation of compatible solutes was observed in *Hcc. salifodinae* strain BK20. *H. elongata* strain M4 at 17% salinity accumulated 2.1 K^+/protein (μM/mg). This clearly indicates that the intracellular K^+ ion accumulation has negligible role in osmoadaptation of *H. elongata*. The values obtained are listed in Table 7.3 and were correlated with study by Oren et al, (2002).

Table 7.3 K^+/protein ratio accumulated in halophiles.

Halophilic isolates	NaCl (%)	K^+/Protein (μM/mg)	Reference
Haloferax volcanii strain BBK2	15	4.77	Present study
Halanaerobium praevalens	13	6.3	Oren et al., 2002
Halomonas elongata strain M4	17	2.1	Present study
Halomonas elongata	10	1.1	Oren et al., 2002
Halococcus salifodinae strain BK20	20	8.14	Present study
Halorubrum sp. strain BS17	20	10.12	Present study
Salinibacter ruber M31	20	11.4	Oren et al., 2002
Salinibacter ruber pola-18	20	15.2	Oren et al., 2002
Haloarcula marismorti	20	13.2	Oren et al., 2002
Halobacterium salinarum	25	12	Oren et al., 2002

7.3.5 FT-IR analysis of cell exftracts

The infra red (IR) spectrum of the cell extract of *H. elongata* strain M4 grown in E2 synthetic medium and complex MHM is represented in Fig. 7.6. The spectrums of cell extracts obtained were comparable with that of the standard ectoine (Sigma). For all the spectrums, the hydrogen stretching region of N-H, C-H and N-H, respectively, around >2500cm^{-1} showed broad overlapping bands (Tanne et al., 2014). The fingerprint region (1500 to 500 cm^{-1}) of the FTIR spectra contains several peaks, based on which only tentative identification of the CS was possible. All the cell extract spectra showed prominent broad peaks between1639 to 1643 cm^{-1}, representing amide I group of C=O, C-N and N-H, due to stretching of amino acid (Parikh and Chorover, 2006). In addition to this, some prominent peaks around 1403 to 1407 cm^{-1} were also seen which represents COO^{-} group present in CS such as ectoine and / or hydroxyectoine. Minor peaks around 1129 cm^{-1} were observed, representing PO_2^-, the functional groups of cell envelope, which could have got co extracted along with CS (Dianawati, et al., 2012).

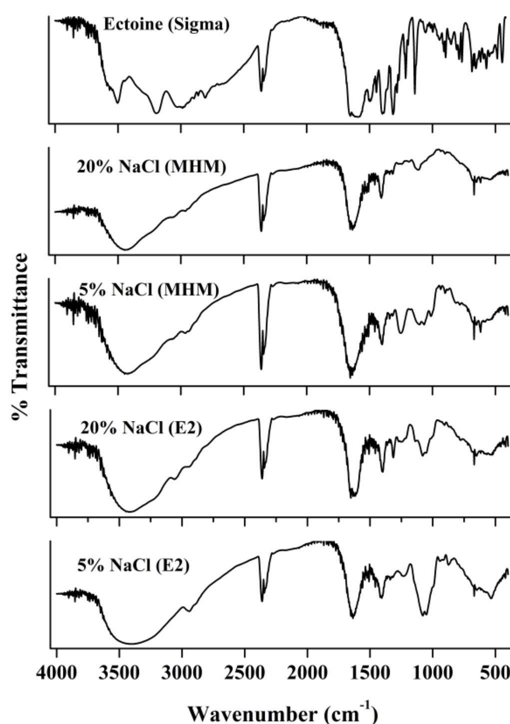

Fig.7.6 Comparison of FT-IR spectra of cell extracts of *H. elongata* strain M4 grow in MHM and E2 medium.

7.3.6. NMR analysis of the cell extracts

^{1}H NMR spectra of the cell extracts of *H. elongata* strain M4 grown in synthetic (E2) and complex (MHM) media are represented in Figs.7.7 and 7.8, respectively. In E2 medium, the isolate M4 grew lavishly in presence of 5% and 20% (w/v) NaCl. The ^{1}H NMR spectra of the cell extract, grown in 5% NaCl showed a single strong signal at around 1.96 ppm corresponding to ectoine. In addition to this, multiple distinct signals were observed between 3.0 - 4.0 ppm, corresponding to the disaccharide trehalose (3.4 – 3.8 ppm) and ectoine (3.37 – 3.16 ppm), respectively. Also, minute signals were seen at 1.94 and 2.31 ppm corresponding to γ-aminobutyrate (GABA). All this signals were well comparable to the osmolytes from halotolerant bacterium *Brevibacterium* sp. and extremely halophilic archaeon *Halococcus hamelinensis* (Nagata et al., 1996; Goh et al., 2011). The Compatible solutes accumulated by various strains of the genus *Halomonas* along a salinity gradient is represented in Table 7.4.

The ^{1}H NMR profile of the cell extract obtained from 5% and 20% NaCl were similar. However, the intensity of peaks corresponding to CS was lower at 20% as compared to 5% NaCl. Moreover, the peaks corresponding to GABA were missing at 20% NaCl. Therefore, from the above results, we can conclude that *H. elongata* strain M4, when grown in E2 medium at low osmolarity of 5% NaCl accumulated ectoine, trehalose and a synthetic amount of GABA. However, at high osmolarity (20%NaCl), GABA was not synthesized whereas ectoine and trehalose were synthesized at slightly lower levels.

The *H. elongata* strain M4 in MHM, grew efficiently at 5% and 20% NaCl. However, the cell extract at 5% accumulated lower CS in comparison with 20% NaCl. The ^{1}H NMR spectra of the cell extract, grown in 20% NaCl showed a strong signal at around 3.27 ppm and 2.24 ppm corresponding to glycine betaine (GB) and ectoine, respectively. In addition to this, signals at 3.9 ppm and 4.07 ppm also corresponded to GB and ectoine, respectively. Multiple distinct minor signals were observed between 2.05 - 2.15 ppm and 3.72 – 3.78 ppm, corresponding to glutamate. These signals were well comparable to the osmolytes reported by Nagata, et al., (1996). From the HPLC profile along with ^{1}H NMR data, one can clearly indicate that GB and ectoine are the major CS in *H. elongata* strain M4 when

grown in MHM which is in agreement with studies carried out by Guzmán et al., 2009 and Ono et al., 1999.

Table 7.4 Compatible solutes of genus *Halomonas*.

Culture	Growth medium	NaCl (%)	Compatible solute/s	Reference
Halomonas elongata strain M4	MHM	5 / 20	Glycine betaine, ectoine	Present study
	E2	5	Ectoine, trehalose, GABA	
		20	Ectoine, trehalose,	
Halomonas boliviensis LC1	HM medium	5	Ectoine, hydroxyectoine*	Guzman et al., 2009
Halomonas elongata DSM 142	Synthetic medium	15	Ectoine, glutamate ADABA, alanine, hydroxyectoine other amino acids	Sauer and Galinski, 1998
Halomonas elongata KS3	GMM	3-15	Ectoine, hydroxyectoine, ADABA, L-alanine	Ono et al., 1999
Halomonas elongata ATCC 33 1 73	Defined medium	8	Ectoine, glutamate	Wohlfarth et al., 1990

*synthesised at ≥10% NaCl; MHM: moderately halophilic medium; GABA : γ-amino butyric acid; ADABA: γ-*N*-acetyl-α,γ-diaminobutyric acid; GMM: Glucose mineral medium.

Ectoines are of prime importance because of their multitude functions as protein stabilizers in the presence of denaturants and at high temperatures (Lentzen and Schwarz, 2006). Hydroxyectoine, a derivative of ectoine is added in PCR reactions for maximizing the stability of DNA polymerases and for improving the quality of DNA microarrays (Schnoor et al., 2004). Ectoine prevents harmful effects of the ultraviolet radiation by inhibiting the singlet-oxygen mediated mechanism (Buenger and Driller, 2004).

Glycine betaine (N,N,N-trimethylglycine), was the first organic osmotic solute discovered in *Halorhodospira halochloris*an haloalkaliphilic anoxygenic phototroph (Galinskiand Truper 1982). Early studies by Imhoff and Rodriguez-Valera (1984) proved that certain halophilic microorganisms such as *Chromohalobacter marismortui*, *Salinivibrio costicola* and *Nesterenkonia halobia* produced glycine betaine (GB) as their sole osmotic solute to

combat the salinity stress. However, later it was proved that, in halophilic and halotolerant heterotrophic microorganisms, *de novo* biosynthesis of GB is rare as they lack the *bet* gene and instead have membrane transporters which help them assimilate GB from the surrounding. For example, *Vibrio cholera*, a halophilic facultative human pathogen, inhabiting marine environments is known to possess *OpuD* transporter involved in uptake of GB (Oren et al., 2013; Kapfhammer et al., 2005). Nevertheless, GB is produced by the most halophilic members of *Cyanobacteria* (Oren, 2012). Reports on synthesis of ectoine and hydroxyectoine with increase in salinity of 5-15% NaCl by the halophilic microorganisms, especially members of the genus *Halomonas* are known (Guzmán et al., 2009; Lentzen and Schwarz, 2006).

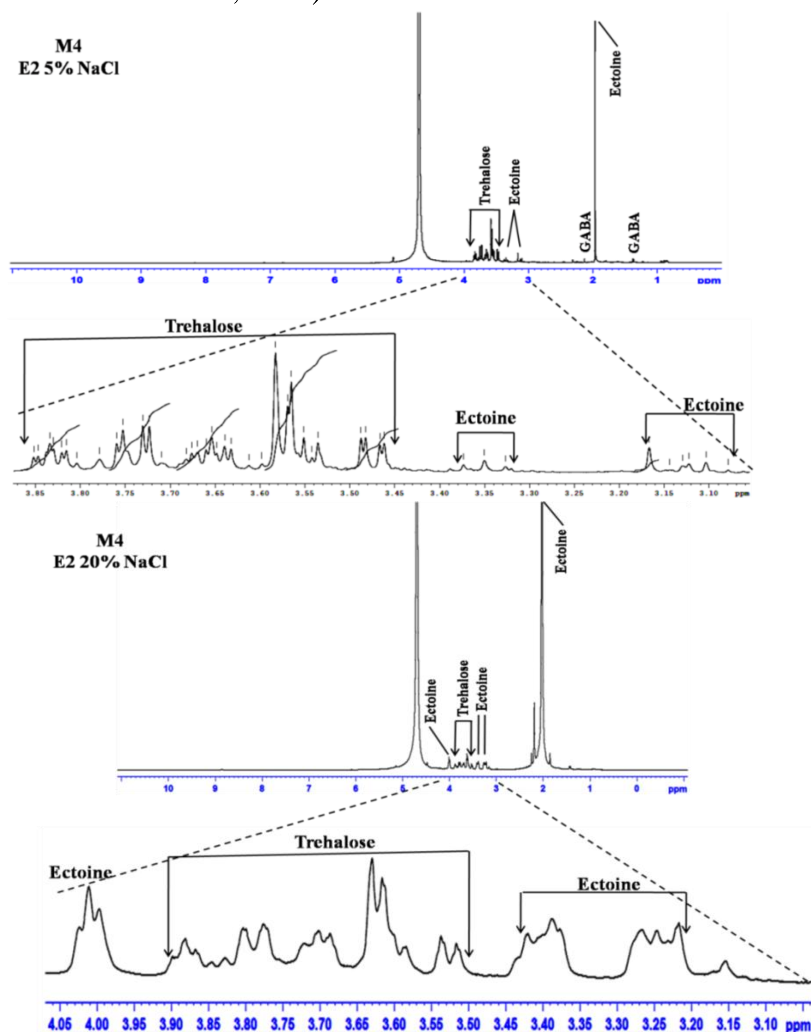

Fig.7.7 Comparison of ^{1}H NMR spectra of cell extract from *H. elongata* strain M4, grown in E2 medium supplemented with 5% and 20% NaCl.

M4 has an advantage over other strains of *H. elongata* as high levels of ectoine are produced at a relatively lower concentration of NaCl (5% (w/v) in synthetic media as compared to that used for *H. elongata* (10–15% w/v NaCl). *H. elongata* was also able to accumulate K^+ at higher osmolarity at 17% NaCl.

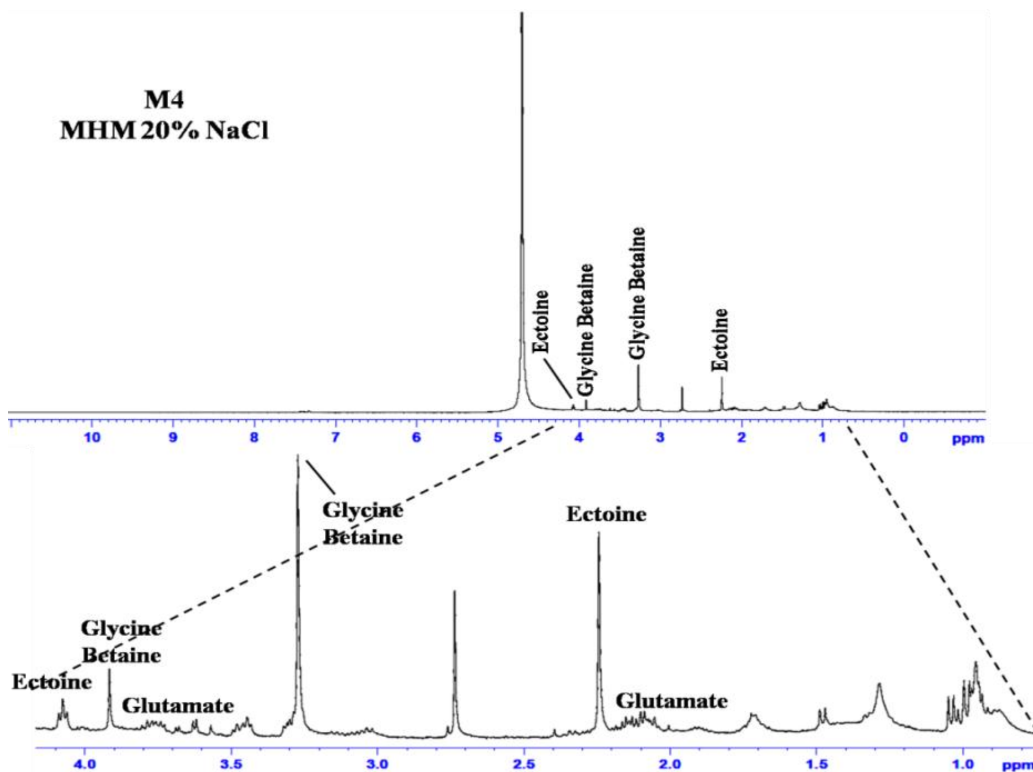

Fig.7.8 ^{1}H NMR spectra of cell extract from *H. elongata* strain M4, grown in MHM medium supplemented with 20% NaCl.

7.4 Conclusion

Osmoadaptation in halophilic microorganisms was studied by growing halophilic bacteria and archaea in E2 and NGSM/NH medium with varying NaCl concentrations. *Halomonas elongata* strain M4 showed the best resistance and growth in E2 medium over a range of NaCl concentrations, from 5% to 25% (w/v). The HPLC profile along with ^{1}H NMR spectra of the cell extracts concluded that, *H. elongata* strain M4 when grown in E2 medium at low osmolarity (5% NaCl) produces ectoine, trehalose and traces of γ-aminobutyrate (GABA). Whereas, at high osmolarity (20% NaCl), the osmolyte ectoine

and trehalose were accumulated in lower amount and GABA was not accumulated. In moderately halophilic medium (MHM), at high osmolarity (20% NaCl) the osmolyte glycine betaine (GB) was accumulated more than ectoine, whereas at low osmolarity (5% NaCl) the concentration of ectoine was higher than GB. No accumulation of osmolytes was seen in the total cell extracts of the haloarchaeal isolates *Haloferax* strain BBK2, *Halococcus* strain BK20 and *Halorubrum sp.* strain BS17. However an elevated intracellular K^+ concentration of 4.77, 8.14 and 10.12 of K^+/protein (µM/mg) was observed.

Summary of Results and Conclusion

Summary of Results and Conclusion

A quest for improved microbial metabolites coupled with enhanced industrial applications, halophiles have gained much interest in the past decade. Solar salterns are an excellent reservoir for studying halophilic prokaryotes and eukaryotes. In this study, we have successfully isolated halophilic bacteria and archaea producing biopolymers and compatible solutes. Water and sediment samples were collected from solar salterns located at Ribandar / Siridao, Goa and Marakkanam, Tamil Nadu. The obtained samples were spread plated on seven different media for the isolation of halophilic archaea and bacteria. The microorganisms were able to grow over a wide range of NaCl concentrations (0.5-30 %, w/v). A total of 31 moderately halophilic bacteria and 44 extremely halophilic archaea were obtained. Isolates were characterized morphologically by Gram staining and scanning electron microscopy (SEM) and showed varying cell morphologies such as rods in chains, cocci in groups, irregular cocci in groups, involuted oval discs, etc. The isolates were characterized by studying their response to antibiotics, metals, presence of specific polar lipids and carotenoid pigments. The halophilic archaea were susceptible to novobiocin and rifampicin and showed the presence of characteristic signature glycolipids and phospholipids such as sulfated diglycosyl diether (S-DGD), phosphatidylglycerol (PG), sulfated triglycosyl/tetraglycosyl diether (S-T/TeGD), phosphatidylglycerosulfate (PGS) and phosphatidylglycerophosphate (Me-PGP) when separated by thin layer chromatography (TLC). They harboured pink toorange to brick red pigment having characteristic absorption maxima at 496 nm and two shoulder peaks at 471 and 528 nm, characteristic of archaeal C-50 α-bacterioruberins and C-40 carotenoids. Most of the halophilic bacteriaand archaea were able to utilize carbohydrates such as D-glucose, sucrose, D-fructose, D-mannitol, D-maltose, glycerol and produced catalase and oxidase whereas some were able to hydrolyse substrates such as starch, skimmed milk, gelatin, cellulose and olive oil confirming their ability to produce extracellular enzymes such as amylase, protease, gelatinase, cellulase and lipase.

The halophilic bacterial isolates were screened for the production of polyhydroxyalkonates (PHAs) by Nile Blue / Nile Red staining and observing in UV light for fluorescence. Among the 31 moderately halophilic bacteria screened for PHA, three isolates H15, H16 and H26 showed the best PHA accumulation and were identified as *Bacillus megaterium* based on phenotypic and genotypic characterization.

H16 was able to accumulate PHA in the presence and absence of NaCl. In the absence of NaCl, PHA of 40.0% (w/w) of CDW was accumulated at 42 hrs of growth, whereas in presence of 5% w/v NaCl, the culture showed longer lag phase of up to 24 hrs and accumulated a maximum PHA of 39.0 % (w/w) of CDW at 54 h of growth. The FT-IR, XRD and ^{1}H NMR confirmed the polymer to be a homopolymer of 3-hydroxybutyrate (PHB). The TGA-DTA confirmed the polymer to be stable up to temperature of 160°C. This isolate can be exploited further for the production of PHA at an industrial scale due to its tolerance and growth in presence of up to 5% (w/v) of NaCl.

Among the 44 extremely halophilic archaea screened, TN9 and E3 isolates grew fast with the accumulation of PHA within 3–5 days, followed by isoates BBK2, TN4–7 and TN10 within 5–8 days. Based on phenotypic, chemotaxonomic and genotypic characterization, the organisms were grouped in five different genera namely *Halococcus*, *Haloferax*, *Haloarcula, Haloterrigena* and *Halogeometricum* of the family *Halobacteriaceae*. The isolates *Hgm. borinquense* strains TN9 and E3 showed maximal PHA accumulation of 14.0 and 73.5% (w/w) of CDW in NGSM supplemented with 0.2% nitrogen source and 2% carbon source. The crotonic acid assay, FT-IR, XRD, TGA-DTA and ^{1}H NMR analysis indicated the polymer produced by *Hgm. borinquense* strain TN9 to be a homopolymer of 3-hydroxybutyrate (PHB) while E3 accumulated a co-polymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)]. Industrial processes require organisms withstanding harsh physicochemical conditions like high salt concentration analogous to high ionic strength and low water activity (aw). *Hgm. borinquense* strain E3 could be engineered further for industrial production of PHA due to its robust growth at varying pH, temperature and NaCl concentration.

Optimization of culture conditions for *Hgm. borinquense* strain E3 showed that it could grow over a range of glucose concentrations, pH, temperature and salinity but the best cell mass to PHA yield was 5.8 ± 0.4 (g l^{-1}) and 4.0 ± 0.045 (g l^{-1}) respectively was obtained at 2.0% glucose, pH 7.0, 37°C and 20% NaCl. Chemical extraction using sodium hypochlorite (NaClO) with 4 % (w/v) chlorine could extract maximum PHA of 77.85 ± 0.3% (w/w) of CDW and soxhlet extraction using $CHCl_3$ gave PHA yield of 72.4 ± 3% (w/w) of CDW. The soxhlet extraction for polymer recovery is advantageous as upto 95% of $CHCl_3$ employed was recovered by rotary evaporator. ^{1}H NMR analysis of the [P(HB-*co*-HV)] revealed it to have 21.4% hydroxylvalerate (HV) units which on decolourisation with NaClO/acetone slightly reduced to 19.39 / 19.51% HV units.

Ability of extremely halophilic archaeon *Hgm. borinquense* strain E3 to use agro-industrial wastes for the production of PHA was investigated. *Hgm. borinquense* strain E3, was able to grow well on renewable agro-industrial wastes such as sugarcane bagasse (SCB), coconut oil cake (COC) and sago starch waste (SSW). SCB was selected for further studies as the culture showed best tolerance and grew well in NSM medium with 25% and 50% (v/v) SCB hydrolysate. The time-course of growth of *Hgm. borinquense* strain E3 in minimal medium with 25% and 50% (v/v) SCB hydrolysate showed maximum PHA of 50.4 ± 0.1% and 45.7 ± 0.19% (w/w) of CDW on the 7th day, respectively. The DSC thermogram showed the two melting endotherms in range of Tm_1 = 136.59 - 142.69 ^oC and Tm_2 = 149.4 - 155.27 ^oC. ^1H NMR analysis of the polymer obtained from SCB and SSW hydrolysates revealed it to be a co-polymer of [P(HB-*co*-HV)] comprising 13.29 and 19.65 % HV units. Various agro-industrial wastes that can be degraded by such halophilic microbes should be explored, for the production of biopolymers as this may help us in both, managing the agro-industrial wastes and cutting down the costs of commercial substrates. Since *Hgm. borinquense* strain E3 is an extremely halophilic archaeon requiring ≥ 25% NaCl for its growth, media sterilization costs can be cut down at industrial scale production.

Osmoadaptation in halophilic microorganisms was studied by growing halophilic bacteria and archaea in E2 and NGSM/NH media with varying NaCl concentrations. *Halomonas elongata* strain M4 showed the best resistance and growth in E2 medium over a range of NaCl concentrations, from 5% to 25% (w/v). The HPLC profile along with ^1H NMR spectra of the cell extracts concluded that *H. elongata* strain M4 when grown in E2 medium at low osmolarity (5% NaCl) produces ectoine, trehalose and traces of γ-aminobutyrate (GABA). Whereas, at high osmolarity (20% NaCl), the osmolyte ectoine and trehalose were accumulated in lower amount and GABA was not accumulated. In moderately halophilic medium (MHM), at high osmolarity (20% NaCl) the osmolyte glycine betaine (GB) was accumulated more than ectoine, whereas at low osmolarity (5% NaCl) the concentration of ectoine was higher than GB. No accumulation of osmolytes was seen in the total cell extracts of the haloarchaeal isolates *Haloferax* strain BBK2, *Halococcus* strain BK20 and *Halorubrum sp.* strain BS17. However an elevated intracellular K^+ concentration of 4.77, 8.14 and 10.12 of K^+/protein (µM/mg) was observed.

This study, for the first time explored the ability of extremely halophilic archaeon *Halogeometricum borinquense* to accumulate a homopolymer of PHB and copolymer of P[HB-*co*-HV] using glucose and starch as commercial substrates. The study also explored the production of biopolymers by *Hgm. borinquense* strain E3 by utilising cheap agro-industrial wastes.

Future Prospects

Future Prospects

(i) PHAs are an alternative to conventional plastics produced from renewable sources. However, the high cost of production and other downstream processing hampers its use in commercial applications. We have reported for the first time a copolymer produced by *Halogeometricum*. This polymer can be further studied for various medical applications like tissue engineering and as scaffolds in organ culture.

(ii) Since India's economy is dominated by agriculture and agro based industries, large amounts of agro industrial wastes are being generated. In this study the ability of extremely halophilic archaea to use the agro-industrial wastes for the production of biopolymers. Various agro-industrial wastes that can be degraded by such halophilic microbes should be explored, for the production of biopolymers as this may help us in both, managing the agro-industrial wastes and cutting down the costs of commercial substrates.

(iii) Molecular basis of the PHA production should be studied in depth to explore the possibility of engineering their metabolic pathways. This can result in enhanced and high quality polymer. Elucidating the metabolic basis of PHA production by *Halogeometricum* may serve as a model organism and allow us to understand the PHA production pathways in other halophilic archaea.

(iv) Osmolytes from halophilic microorganisms could be further explored for their potential biotechnological applications.

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Appendix

Appendix I : Media and Stains

1) NGSM Media (NaCl Glucose Synthetic Medium)

Ingredients	**Grams/litre**
$NaCl$	200.0
$MgCl_2.6H_2O$	13.0
KCl	4.0
$CaCl_2.2H_2O$	1.0
$NaHCO_3$	0.2
NH_4Cl	2.0
$FeCl_3.6H_2O$	0.005
KH_2PO_4	0.5
Glucose	2.0

pH 7.0 (Adjust using 1M KOH)

2) E2 mineral medium (Lageveen *et al.,* 1988)

Ingredients	**Grams/litre**
Basal medium	
Microcosmic salt ($NaNHPO_4$)	3.5
Dipotassium hydrogen phosphate	7.5
Potassium dihydrogen phosphate	3.7
$MgSO_4.7H_20$ (100 mM)	10 ml
MT stock	1.0 ml
Yeast extract	0.004
Glucose	20
Distilled water	989 ml
pH	7.2

MT stock

$FeSO_4.7H_20$	2.78 g
$MnC1_2.4H_20$	1.98 g
$CoSO_4.7H_20$	2.81 g
$CaCl_2.2H_20$	1.47 g
$CuC1_2. 2H_20$	0.17 g
$ZnSO_4.7H_20$	0.29 g
Distilled water	1.0 L

The basal medium, glucose, magnesium sulfate solution, and MT stock were sterilized separately and added to medium prior to use.

3) NH Media (Norberg and Hofstein)

Ingredients	Grams/litre
NaCl	200.0
$MgCl_2.6H_2O$	10.0
KCl	5.0
Yeast Extract	1.0
Glucose	2.0

pH 7.0 (Adjust using 1M KOH)

4) NA Medium (Nutrient Agar)

Ingredients	Grams/litre
Paptic Digest of Animal Tissue	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Agar	18.0

pH 7.2 (Adjust using 1M NaOH)

5) TSB Medium (Tryptone Soya Broth)

Ingredients	Grams/litre
Pancreatic Digest of Casein	17.0
Papaic Digest of Soyabean Meal	3.0
Sodium Chloride	5.0
Dipotassium Hydrogen Phosphate	2.5
Dextrose	2.5
Agar	18.0

pH 7.2 (Adjust using 1M NaOH)

6) Simmons citrate agar

Ingredients	Grams/litre
Tri sodium citrate	1.0
Magnesium sulphate	1.2
Diammonium hydrogen phosphate	0.5
Potassium chloride	1.0
Trace elements	40.0 ml
Agar agar	15.0
Distilled water	920.0 ml
Phenol Red (0.04%)	20.0 ml
PH	6.8

Staining solutions

1) Gram staining reagent's

a) Crystal violet stain (Primary stain)

Crystal violet	1.0 g
Ethanol	10.0 ml
Distilled water	90.0 ml

b) Gram's Iodine (Fixative)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

Grind the Iodine granules to fine powder using motor and pestle, add distilled water and shake the solution thoroughly so as to dissolve the ingredients.

c) 70% ethanol (Decolourizer)

Ethanol	70.0 ml
Distilled water	30.0 ml

d) Safranine (Counterstain)

Safranine	0.5 g
Distilled water	100 ml

2) Endospore Staining (Schaeffer and Fulton's method)

a) Malachite Green stain

Malachite Green stain	5.0 g
Distilled water	100 ml

Malachite Green stain was dissolved in distilled water, the solution was kept for 30 minutes at 37°C, and filtered through filter paper.

b) Safranine Stain

Safranine	0.5 g
Distilled water	100 ml

Safranine stain was dissolved in distilled water and the solution was filtered through filter paper.

3) PHA staining (Ostle and Holt, 1982)

Nile Blue A	1.0 g
Distilled water	100 ml

The Nile Blue A solution was mixed by thoroughly shaking and mildly heated (45-55°C) to fully dissolve the stain. The solution was filtered before use.

4) Sudan black B Staining (Norris, and Swain, 1971)

a) Sudan black B stain

Sudan black B stain	0.3 g
Ethanol	70 ml
Distilled water	30 ml

The stain was completely dissolved in 70 % ethanol by thoroughly shaking anf filtered before use.

b) Safranine Stain

Safranine	9.5 g
Distilled water	100 ml

Safranine stain was dissolved in distilled water and the solution was filtered through filter paper.

5) Catalase test reagent

Hydrogen peroxide (30%)	10.0 ml
Distilled water	90.0 ml

6) Oxidase test reagent

N,N, N',N'- Tetramethyl-p-phenylene diamine dihydrochloride	1.0 g
Distilled water	100.0 ml

The reagent is light sensitive, so it was freshly prepared in a covered container just before use.

7) Kovac's reagent (Indole test)

KOH	40.0 g
Creatine	0.3 g
Distilled water	100.0 ml

8) Omeara's reagent (Voges-Proskauer test)

Iso-amyl alcohol	150.0 ml
p-dimethyl-l-aminobenzaldehyde	10.0 g
HCl (concentrated)	50.0 ml

9) Methyl Red test reagent

Methyl Red	0.1 g
Ethyl alcohol (95%)	300.0 ml
Distilled water	200.0 ml

10) Griss-Illosway's reagent for nitrate reductase activity

a) Reagent A

Sulphanillic acid	0.5 g
Acetic acid (5N)	100 ml

b) Reagent B

α-napthylamine	0.8 g
Acetic acid (5N)	100 ml

Appendix II : Reagents for Genomic DNA Extraction and PCR Amplification

1) SET (Sucrose EDTA Tris) buffer

Sodium chloride	75mM
EDTA	25 mM
Tris	20 mM
pH 7.5	

2) SDS solution

SDS	10 gm
Distilled water	100 ml

3) TE Buffer

Tris (pH 7.3)	10 mM
Sodium-EDTA	1.0 mM

4) Phenol-chloroform-isoamyl alcohol (PC1)

Phenol (pH 8.0)	25.0 ml
Chloroform	24.0 ml
Isoamyl alcohol	1.0 ml

5) Chloroform-isoamyl alcohol (C1)

Chloroform	24.0 ml
Isoamyl alcohol	1.0 ml

Appendix III : Estimation Methods

1] PHA estimation (Law and Slepecky, 1961)

Reagents:

Stock solution: 1 mg ml^{-1} PHB (Sigma-Aldrich) in concentrated sulphuric acid

Diluent: Concentrated sulphuric acid

Procedure:

Dilution of the PHB were made in µg range from a working stock of 100 µg ml^{-1} of PHB

↓

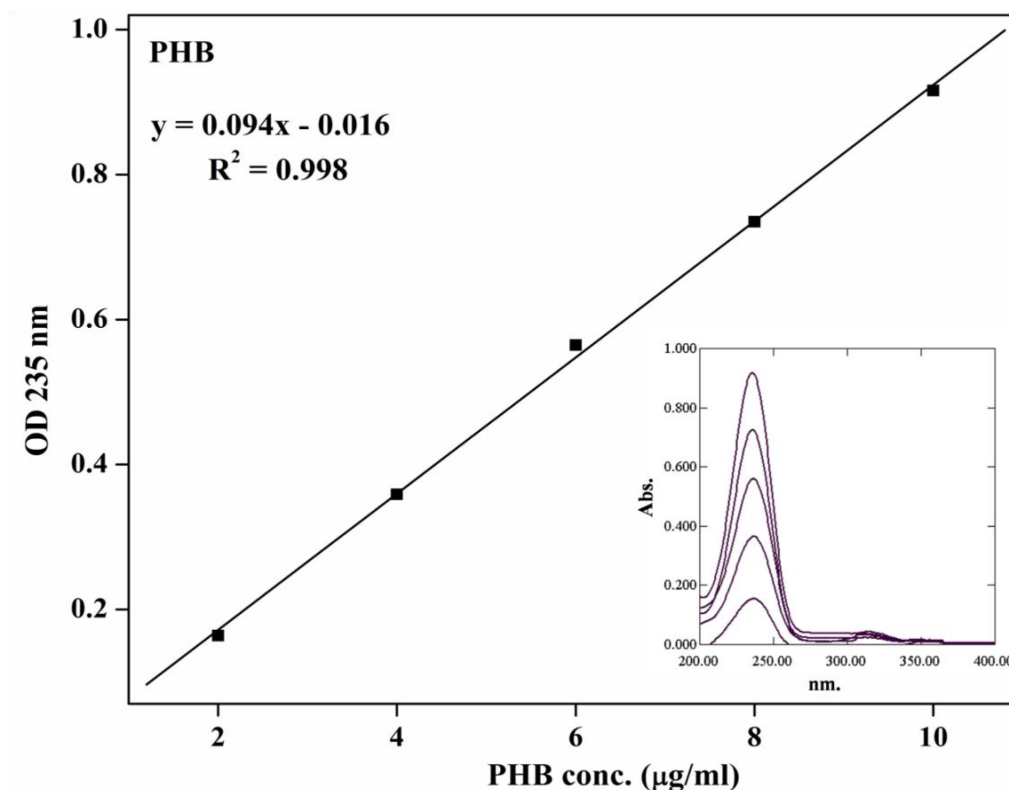
Keep in boiling H_2O bath for 10 min

↓

Solution was cooled and mixed vigorously

↓

Record absorbance at 235 nm



Standard curve for PHB and the inset representing the spectrophotometric scans of crotonic acid derived from PHB

2] <u>Total carbohydrates estimation</u> (Dubios *et al.,* 1956)

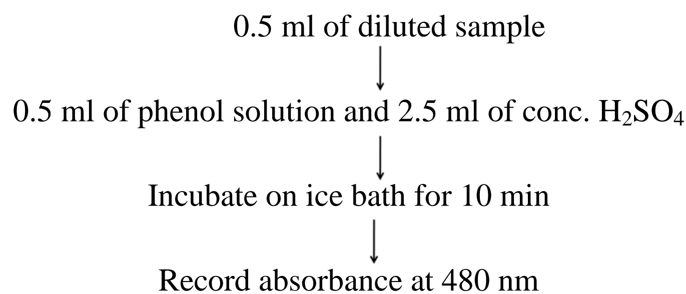
Reagents:

Phenol solution: 5% (w/v) phenol solution was prepared by dissolving 5 g of phenol in 100 ml distilled water.

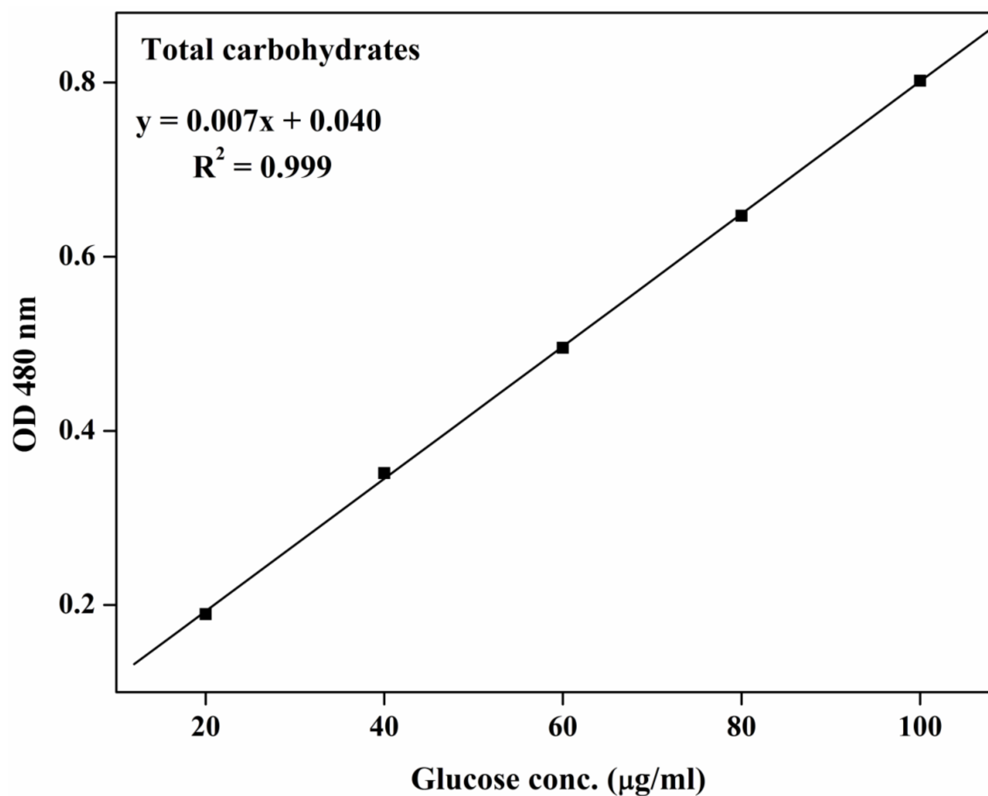
Stock solution: 1 mg ml^{-1} glucose in sterile distilled water

Diluent: Distilled water

Procedure:



The standard curve for total carbohydrate was made using glucose



Standard curve for total carbohydrates

3] <u>Reducing sugar estimation</u> (Miller, 1959)

Reagents:

Dinitro salicylic acid solution (DNSA)

4% of NaOH solution was made by dissolving 4 g of NaOH in 100 ml distilled water. To this 0.4 g of phenol and 0.4 g of DNSA was added with constant stirring until the ingredients are completely dissolved. Just before use, add 0.05 g of sodium sulfite and mix thoroughly.

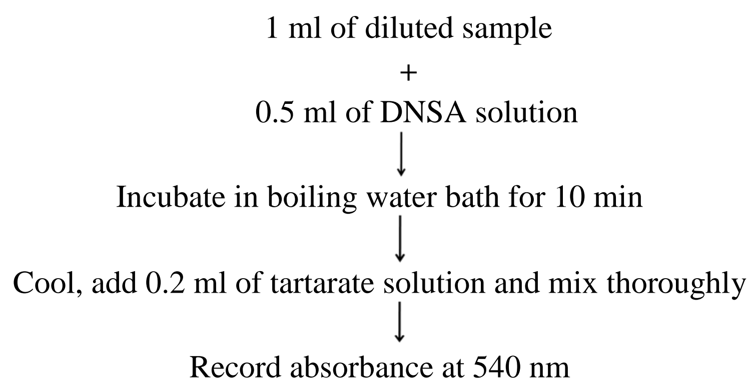
Tartarate solution

33.0 g of potassium sodium tartarate was dissolved in 100 ml of distilled water.

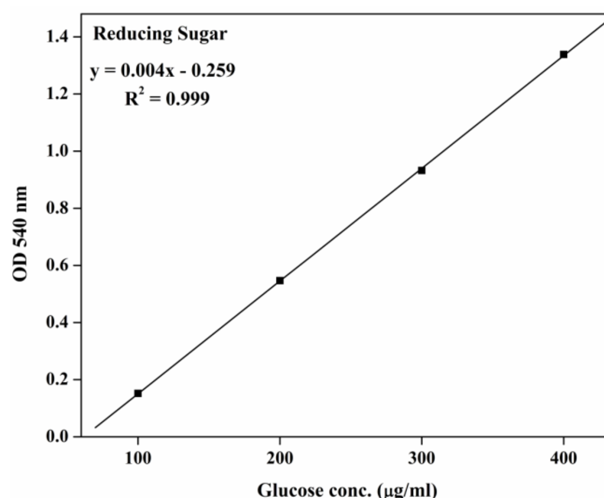
Stock solution: 1 mg ml^{-1} glucose in sterile distilled water

Diluent: Distilled water

Procedure:



The standard curve for reducing sugars was made using glucose



Standard curve for reducing sugars

Appendix IV : List of Publications

(1) **Bhakti B. Salgaonkar** and Judith M. Bragança (2015) **Consumption of sugarcane bagasse as substrate by extremely halophilic archaeon strain E3 for biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate).** (Communicated to *Journal of Applied Microbiology*).

(2) **Bhakti B. Salgaonkar** and Judith M. Bragança (2015) **Biosynthesis of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Halogeometricum borinquense* strain E3.** *International Journal of Biological Macromolecule* (doi 10.1016/j.ijbiomac.2015.04.016).

(3) **Bhakti B. Salgaonkar,** Deepthi Das and Judith M. Bragança (2015) **Resistance of extremely halophilic archaea to zinc and zinc oxide nanoparticles.** *Applied Nanosciences* (doi: 10.1007/s13204-015-0424-8).

(4) **Bhakti B. Salgaonkar,** Kabilan Mani and Judith M. Bragança (2013) **Accumulation of polyhydroxyalkanoates by halophilic archaea isolated from traditional solar salterns of India.** *Extremophiles* 17: 787-795. (doi: 10.1007/s00792-013-0561-5).

(5) **Bhakti B. Salgaonkar,** Kabilan Mani and Judith M. Bragança (2013) **Characterization of polyhydroxyalkanotes accumulated by a moderately Halophilic Salt pan isolate *Bacillus megaterium* strain H16.** *Journal of Applied Microbiology* 114:1347-1356. (doi:10.1111/jam.12135).

(6) **Bhakti B. Salgaonkar,** Kabilan Mani, Anjana Nair, Sowmya Gangadharan and Judith M. Bragança (2012) **Interspecific interactions among members of family *Halobacteriaceae* from natural solar salterns.** *Probiotics and Antimicrobial Proteins*. 4(2):98-107. (doi: 10.1007/s12602-012-9097-8).

(7) **Bhakti B. Salgaonkar,** Kabilan Mani and Judith M. Bragança (2011) **Sensitivity of Haloarchaea to eubacterial pigments produced by *Pseudomonas aeruginosa* SB1**. *World Journal Microbiology Biotechnology*. 27:799–804. (doi: 10.1007/s11274-010-0519-z).

List of other Publications

(1) Kabilan Mani, Sivaraman Chandrasekaran, **Bhakti B. Salgaonkar,** Srikanth Mutnuri, Judith M. Bragança (2014) **Comparison of bacterial diversity from solar salterns and a simulated laboratory study.** *Annals of Microbiology* (doi: 10.1007/s13213-014-0944-6).

(2) Deepthi Das, **Bhakti B. Salgaonkar,** Kabilan Mani and Judith M. Bragança (2014) **Cadmium resistance in extremely halophilic archaeon *Haloferax* strain BBK2.** *Chemosphere* 112: 385–392. (doi: 10.1016/j.chemosphere.2014.04.058).

(3) Vidhya Prabhudessai, **Bhakti B. Salgaonkar,** Judith M. Bragança and Srikanth Mutnuri (2014) Pretreatment of Cottage Cheese to Enhance Biogas Production. *BioMed Research International* Article ID 374562, 6 pages. (doi: 10.1155/2014/374562).

(4) Kabilan Mani, **Bhakti B. Salgaonkar,** Deepthi Das and Judith M. Bragança (2012) **Community solar salt production in Goa, India.** *BMC Aquatic Biosystems*. 8:30. (doi:10.1186/2046-9063-8-30).

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International Journal of Biological Macromolecules xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Halogeometricum borinquense* strain E3

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ARTICLE INFO

Article history:
Received 8 January 2015
Received in revised form 27 March 2015
Accepted 9 April 2015
Available online xxx

Keywords:
Halophile
Archaea
Halogeometricum borinquense strain E3
Polyhydroxyalkanoates
poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

ABSTRACT

Polyhydroxyalkanoates (PHA's) can be a key solution for pollution problems caused by plastics derived from petrochemical sources. Extremely halophilic archaeon *Halogeometricum borinquense* strain E3 showed maximum PHA accumulation of 73.51% ±1.7 of cell dry weight (CDW) with 2% glucose. The crotonic acid assay, XRD, FT-IR and ^{1}H NMR analysis revealed that the polymer was a co-polymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)] comprising of 21.47% HV units. This is the first report on P(HB-*co*-HV) production by an extremely halophilic archaeon *Hgm. borinquense* strain E3.

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UNCORRECTED PROOF

1. Introduction

Q3 High usage of conventional plastics viz. polyethylene, polypropylene and poly(vinyl alcohol), derived from non-renewable petro-chemical resources have various environmental problems [1]. The need of sustainable plastics, obtained from renewable resources like starch, protein and cellulose is the need of the hour [2]. Plastics made from these materials are either used directly or in combination with conventional plastics, still resulting in pollution because of their partial degradation. Therefore, there is a continuous search for biodegradable plastics, which can efficiently replace conventional plastics. Presently, polyhydroxyalkanoates (PHAs) are the only 100% biodegradable microbial plastics [3], accumulated intracellularly by numerous microorganisms [3]. In spite of its huge potential, PHAs are more expensive than synthetic plastics due to the high production cost [4].

PHAs are accumulated in response to stress by many genera of bacteria such as the Gram-positive genera *Bacillus*, *Streptomyces*, etc. and Gram negative genera *Cupriavidus*, *Halomonas*, *Pseudomonas*, etc. and a few members of archaea viz. halophilic archaea [5]. Gram-negative bacteria have limitations due to the co-extraction of lipopolysaccharide (LPS) endotoxin along with the PHA polymer. Such a polymer is highly unsuitable for biomedical applications as LPS can elicit strong inflammatory responses in individuals. In spite of the existing methods on the removal of LPS, the treatment process itself changes the polymer properties resulting in an overall increase in the production cost. Lack of LPS in Gram-positive bacteria gives them an advantage over their Gram-negative counterparts however the relative production of PHAs is lesser [6]. These issues make haloarchaea interesting candidates to look for biodegradable polymers.

Archaea are a huge untapped resource of potential industrially important metabolic products with interesting environmental applications. Among the members of archaea, halophilic archaea have gained much attention due to easier handling and culturing techniques. Halophilic archaea are commonly inhabit hypersaline environments like salt lakes and solar salterns. Employing haloarchaea for PHA production have added advantages, as they are grown in hyperosmotic environments, intracellular accumulated PHA can be easily harvested from the cells by suspending them into low osmalarity solutions including water [7]. Use of haloarchaeal strains requiring 20–25% (w/v) salt for its growth can cut down media sterilization costs as well [8,9].

As of November 2014, the family *Halobacteriaceae* is reported to have 49 genera and 182 species [10–12], among which only few genera are reported to accumulate PHA. The haloarchaeon, *Haloferax mediterranei* is the most widely studied representative of

Abbreviations: Hfx., *Haloferax*; Har., *Haloarcula*; Hgm., *Halogeometricum*; PHA, polyhydroxyalkanoates; PHB, polyhydroxybutyrate; NaClO, sodium hypochlorite; CDW, cell dry weight; P(HB-*co*-HV), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate); HB, hydroxybutyrate; HV, hydroxyvalerate.

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http://dx.doi.org/10.1016/j.ijbiomac.2015.04.016
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Appl Nanosci
DOI 10.1007/s13204-015-0424-8

ORIGINAL ARTICLE

Resistance of extremely halophilic archaea to zinc and zinc oxide nanoparticles

Bhakti B. Salgaonkar · Deepthi Das · Judith Maria Bragança

Received: 7 January 2015 / Accepted: 23 February 2015
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Abstract Industrialization as well as other anthropogenic activities have resulted in addition of high loads of metal and/or metal nanoparticles to the environment. In this study, the effect of one of the widely used heavy metal, zinc (Zn) and zinc oxide nanoparticles (ZnO NPs) on extremely halophilic archaea was evaluated. One representative member from four genera namely *Halococcus, Haloferax, Halorubrum* and *Haloarcula* of the family *Halobacteriaceae* was taken as the model organism. All the haloarchaeal genera investigated were resistant to both $ZnCl_2$ and ZnO NPs at varying concentrations. *Halococcus* strain BK6 and *Haloferax* strain BBK2 showed the highest resistance in complex/minimal medium of up to 2.0/1.0 mM $ZnCl_2$ and 2.0/1.0–0.5 mM ZnO NP. Accumulation of $ZnCl_2$/ZnO NPs was seen as *Haloferax* strain BBK2 (287.2/549.6 mg g^{-1}) > *Halococcus* strain BK6 (165.9/388.5 mg g^{-1}) > *Haloarcula* strain BS2 (93.2/28.5 mg g^{-1}) > *Halorubrum* strain BS17 (29.9/16.2 mg g^{-1}). Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM–EDX) analysis revealed that bulk $ZnCl_2$ was sorbed at a higher concentration (21.77 %) on the cell surface of *Haloferax* strain BBK2 as compared to the ZnO NPs (14.89 %).

Keywords Halophilic · Archaea · *Halococcus* strain BK6 · *Haloferax* strain BBK2 · *Halorubrum* strain BS17 · *Haloarcula* strain BS2 · $ZnCl_2$ · ZnO nanoparticles · Metal tolerance · Growth kinetics

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Published online: 10 March 2015

Introduction

Rapid industrialization and other anthropogenic activities have resulted in drastic environmental pollution. Natural processes like surface runoffs, weathering and/or erosion and anthropogenic activities like mining, industrial effluents, agricultural runoffs and sewage have led to the accumulation of toxic metals and their derivatives like nanoparticles in the environment (Paula et al. 2013; Zhao et al. 2012). Zinc and zinc oxide nanoparticles (ZnO NPs) are of high concern because of their increasing demand in electroplating, galvanization, cosmetics, sunscreens, paints, food industry, anticancer drugs, antimicrobials, ceramics, and semiconductors (Monteiro et al. 2011; Li et al. 2011).

ZnO NPs have been extensively studied for their antifungal (*Candida albicans*), antibacterial (*Escherichia coli*) and antiviral (bacteriophages MS2) effect (Lipovsky et al. 2011; You et al. 2011). There are numerous reports on resistance and/or tolerance of metals by bacterial strains either individually or in consortium (non halophilic) (Gadd 2009). Various mechanisms of Zn resistance such as physical bioadsorption/sorption, ion exchange, bioprecipitation and intracellular accumulation in microorganisms such as bacteria (Gram-positive and Gram-negative), cyanobacteria (*Microcystis aeruginosa*) archaea (*Halobacterium saccharovorum*) and eukarya (diatoms) have been revealed (Gadd 2009; Green-Ruiz et al. 2008; Zeng and Wang 2009; Guine et al. 2006; Mangold et al. 2013; Williams et al. 2013; Gelabert et al. 2006).

Li et al. (2011) studied the antibacterial activity of ZnO NPs on bacterial cells and found that Gram-negative bacteria (*Pseudomonas putida* and *E. coli*) are more resistant than Gram-positive bacteria (*Bacillus subtilis*). On the other hand, Sinha et al. (2011) investigated the toxic effect of silver and zinc oxide nanoparticle on mesophilic and

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Extremophiles
DOI 10.1007/s00792-013-0561-5

ORIGINAL PAPER

Accumulation of polyhydroxyalkanoates by halophilic archaea isolated from traditional solar salterns of India

Bhakti B. Salgaonkar · Kabilan Mani · Judith Maria Bragança

Received: 25 March 2013 / Accepted: 18 June 2013
© Springer Japan 2013

Abstract Extremely halophilic archaeal isolates obtained from brine and sediment samples of solar salterns of Goa and Tamil Nadu, India were screened for accumulation of polyhydroxyalkanoates (PHA). Seven polymer accumulating haloarchaeal strains (TN4, TN5, TN6, TN7, TN9, TN10 and BBK2) were selected based on their growth and intensity of fluorescence when grown on 20 % NaCl synthetic medium supplemented with 2 % glucose and incorporated with Nile red dye. The polymer was quantified by conversion of PHA to crotonic acid which gave a characteristic absorption maxima at 235 nm. On the basis of phenotypic and genotypic characterization the cultures TN4, TN5, TN6, TN7, TN10 and BBK2 were grouped under genus *Haloferax* whereas isolate TN9 was grouped under the genus *Halogeometricum*. Growth kinetics and polymer accumulation studies revealed that the culture *Halogeometricum borinquense* strain TN9 accumulates PHA maximally at the mid-log phase, i.e. 5th day of growth (approx. 14 wt% PHA of CDW). Analysis of the polymer by IR, ^{1}H NMR and ^{13}C NMR confirmed it to be a homopolymer of 3-hydroxybutyrate.

Keywords Solar salterns · Haloarchaea · Polyhydroxyalkanoates (PHA) · 16S rRNA · *Halogeometricum borinquense* strain TN9 · Polymer characterization

Communicated by F. Robb

Electronic supplementary material The online version of this article (doi:10.1007/s00792-013-0561-5) contains supplementary material, which is available to authorized users.

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Abbreviations

PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
NTYE	NaCl tryptone yeast extract
NT	NaCl tri-sodium citrate
NSM	NaCl synthetic media
DMSO	Dimethylsulfoxide
PI	Propidium iodide
w/v	Weight by volume
EPS	Exopolysaccharide
CDW	Cell dry weight
rpm	Revolutions per minute
IR	Infra red
NMR	Nuclear magnetic resonance
TGA	Thermo gravimetric analysis
DTA	Differential thermal analysis

Introduction

Microorganisms cope with stress by various responses like accumulation of osmolytes, volutin granules, spore formation, etc. (Roberts 2005; Seufferheld et al. 2011; Osman et al. 2008). Accumulation of polyhyroxyalkanotes (PHA) is also a stress response employed by many bacteria and few members of halophilic archaea. The nutrient limiting stress conditions will usually be an excess carbon substrate while deficient in other elements like nitrogen and phosphorus. PHAs are potential renewable and biodegradable polyesters synthesized by numerous bacteria as an intracellular carbon and energy storage granules (Rehm 2007; Tian et al. 2009). Interest in production of PHAs arose due to its unique characteristics of being biodegradable and

Published online: 26 July 2013

Journal of
Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Characterization of polyhydroxyalkanoates accumulated by a moderately halophilic salt pan isolate *Bacillus megaterium* strain H16

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Keywords
Bacillus megaterium strain H16, halophiles, polyhydroxyalkanoates, polymer characterization, salterns.

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2013/1611: received 10 September 2012, revised 21 December 2012 and accepted 2 January 2013

doi:10.1111/jam.12135

Abstract

Aim: Characterization of polyhydroxyalkanoates (PHA) accumulated by halophilic bacteria isolated from solar salterns.
Methods and Results: Twenty-six halophilic isolates were obtained from solar salterns of Goa, India. They were screened for accumulation of PHA by Sudan black B, Nile blue A and Nile red stains. Strains H15, H16 and H26 were selected based on their intensity of Nile blue A/Nile red fluorescence. On the basis of phenotypic and genotypic characterization, the three isolates were identified as *Bacillus megaterium*. Growth kinetics and polymer accumulating capacity of strain H16 were studied in E2 mineral media with 2% glucose with/without NaCl. In the absence of NaCl, strain H16 accumulated PHA to 40·0% (w/w) of cell dry weight (CDW) at 42 h of growth, whereas in presence of 5% w/v NaCl, the culture showed longer lag phase of up to 24 h and accumulated a maximum PHA of 39% (w/w) CDW at 54 h of growth. The infrared spectra of both the polymers exhibited peaks at 1733·9 cm^{-1} characteristic of C=O. Scans of ^{1}H nuclear magnetic resonance (NMR) showed a doublet at 2·5 ppm corresponding to methylene group (-CH_2), the signal at 5·3 ppm corresponded to methine group (-CH-), and another signal at 1·3 ppm corresponded to the methyl group (-CH_3). Scans of ^{13}C NMR showed prominent peaks at 20, 40, 67–68 and 170 ppm, indicating the polymer to be homopolymer of 3-hydroxybutyrates. The polymer is stable up to a temperature of 160°C.
Conclusion: Three moderately halophilic isolates (strain H15, H16 and H26) capable of accumulating PHA were isolated from solar salterns of Ribandar Goa, India, and identified as *B. megaterium* based on phenotypic and genotypic characterization. Strain H16 accumulated polyhydroxybutyrate in the presence and absence of NaCl up to 40% of its CDW.
Significance and Impact of the Study: This strain would be better suited for production of PHA at industrial level due to its tolerance to high concentration of NaCl.

Introduction

Polyhydroxyalkanoates (PHAs) are a family of polyhydroxyesters synthesized by numerous microorganisms from various carbon sources as intracellular carbon and energy storage compounds to overcome stress under nutrient-limiting conditions (Steinbuchel and Schlegel 1991; Tian *et al.* 2009). Research on PHA is gaining momentum due to its varied applications. It is an eco-friendly green material, completely biodegradable and biocompatible thermopolyester with material properties similar to plastics, which are obtained from nonrenewable petrochemical sources. PHAs from diverse sources with various chemical structures find attractive applications in medical

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Probiotics & Antimicro. Prot.
DOI 10.1007/s12602-012-9097-8

Interspecific Interactions Among Members of Family *Halobacteriaceae* from Natural Solar Salterns

Bhakti B. Salgaonkar · Kabilan Mani · Anjana Nair · Sowmya Gangadharan · Judith M. Braganca

© Springer Science + Business Media, LLC 2012

Abstract Solar salterns are crystallizer ponds with highly diverse extremophilic microbial flora living individually or in consortium. Twenty-four culturable haloarchaeal isolates were obtained from solar salterns of Goa, which were grouped under *Halococcus*, *Haloferax*, *Haloarcula* and *Halorubrum*. Cell-free supernatants of different isolates were checked against each other by pour plate technique combined with agar well diffusion method. This resulted in a zone of growth inhibition or stimulation around wells, indicating that some isolates had antagonistic and/or a beneficial effect on the other genera. Thus, members of family *Halobacteriaceae* were found to secrete extracellular metabolites, which can act as growth enhancers or repressors.

Keywords *Halobacteriaceae* · Cell-free supernatant · Halocin · Growth factors · Inhibition · Exhibition · Antagonistic · Beneficial

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Abbreviations

CFS	Cell-free supernatant
Har	*Haloarcula*
Hfx	*Haloferax*
Hcc	*Halococcus*
Hrr	*Halorubrum*
AWDM	Agar well diffusion method
BLAST	Basic Local Alignment Search Tool
PAGE	Polyacrylamide gel electrophoresis

Introduction

A solar saltern is a man-made ecosystem providing a salinity gradient for the production of solar salt. Based on microbial diversity, flow of nutrients and other environmental conditions, salterns can be described as a semi-closed chemostat [12]. Salinity in the saltern varies from 0.59 M NaCl to saturation (5.9 M NaCl). Thus, microbes inhabiting these salt pans are divided into extreme halophiles (3.0–5.0 M NaCl), borderline extreme halophiles (2.0–3.0 M NaCl), moderate halophiles (0.5–2.0 M NaCl) and halotolerant organisms (0.2 M NaCl) [19].

In a natural ecosystem, no organism exists in absolute isolation, and therefore, all organisms need to respond to each other through biological interactions (i.e. mutualism, commensalism, amensalism or parasitism). This phenomenon of biological interaction is very important for the survival of organisms as well as functioning of any ecosystem as a whole. The organisms in solar salterns too may thrive symbiotically. For instance, available organic nitrogen is converted to ammonia by microbes, which in turn is utilized by *Dunaliella* species, which again supports the growth of brine shrimps (*Artemia* species). Further, the

Published online: 29 March 2012

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World J Microbiol Biotechnol (2011) 27:799–804
DOI 10.1007/s11274-010-0519-z

ORIGINAL PAPER

Sensitivity of Haloarchaea to eubacterial pigments produced by *Pseudomonas aeruginosa* SB1

B. B. Salgaonkar · M. Kabilan · J. M. Braganca

Received: 3 February 2010 / Accepted: 26 July 2010 / Published online: 1 August 2010
© Springer Science+Business Media B.V. 2010

Abstract *Pseudomonas aeruginosa* SB1 was isolated from conventional well water. It produced an extracellular fluorescent bright green pigment which diffused into the growth medium. The purified pigment showed absorption maxima of 690, 370, 312 nm and was identified as pyocyanine, a known inhibitor of eubacterial and eukaryotic growth. The effect of this pigment when tested on orange-red pigmented halophilic archaeal isolates showed remarkable inhibitory activity and resulted in total growth inhibition at 4.67 mg/ml pyocyanine in 24 h. The present work is the first report on eubacterial *Pseudomonas aeruginosa* SB1 showing anti-Haloarchaeal activity.

Keywords *Pseudomonas aeruginosa* SB1 · Fluorescent cell free supernatant · Haloarchaea · Eubacteria

Introduction

Pigment production constituting of phenazine compounds is an important characteristic of the genus *Pseudomonas* (Turner and Messenger 1986). The pigment serves two major functions such as siderophore and/or secondary metabolite with antibiotic-like activity (Baron and Rowe 1981, Davison 1986; Defago and Hass 1990). The phenazine compounds produced by *Pseudomonas* species as a blue pigment known as pyocyanine are biologically active metabolites that function in microbial competitiveness (Mazzola et al. 1992) and are known to posses a broad spectrum of antibiotic activity against Gram-positive and Gram-negative bacteria, fungi and mamalian tissue/cells (Gobbetti et al. 1997; Saha et al. 2008; Laursen and Nielsen 2004; Mavrodi et al. 2006; Dwivedi and Johri 2003), probably by generation of reactive oxygen species (Hassan and Fridovich 1980). It strongly inhibits the growth of various green microalgae and cyanobacteria (Dakhama et al. 1989, 1992). It also shows inhibitory effects on human T and B lymphocytes and in vitro lymphocyte proliferation (Ulmer et al. 1990; Sorensen et al. 1983).

Haloarchaea are salt-loving archaea that thrive in NaCl-saturated environments such as the Great Salt Lake, Dead Sea, brines, marine and rock salt, natural and artificial salterns (Javor 1989) and are members of the family *Halobacteriaceae* (Grant and Larsen 1989). They grow optimally in the presence of 2.5–5.2 M NaCl (Kushner and Kamekura 1988) and are known to produce carotenoid pigments which protects the cells from harmful effects of u.v. light (Shahmohammadi et al. 1998) and give them peculiar red–orange coloration. The present work focuses on the anti-Haloarchaeal activity of our *Pseudomonas aeruginosa* isolate SB1.

Materials and methods

Micro-organisms and culture media

A wellwater sample from an active conventional well of Calangute Goa-India, was aseptically plated on nutrient agar plates (NA) (pH 7.0). The plates were incubated at room temperature for 24 h. A colony showing intense green pigmentation was picked up and re streaked on NA plates for purification. The culture was thereafter maintained on nutrient agar slopes at room temperature (28°C) and/or 4°C and labeled as *Pseudomonas aeruginosa* SB1.

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Chemosphere 112 (2014) 385–392

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Cadmium resistance in extremely halophilic archaeon *Haloferax* strain BBK2

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HIGHLIGHTS

- *Haloferax* strain BBK2 grew in media supplemented with up to 4 mM Cadmium.
- *Haloferax* grew at a pH range of 6–10 and salinity range of 10–25% NaCl in 1 mM Cd.
- The cells showed a maximal accumulation of 15.19% in presence of 1 mM Cd.

ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form 26 March 2014
Accepted 22 April 2014

Handling Editor: A. Gies

Keywords:
Growth
Salinity
Haloarchaea
Metal tolerance
Resistance

ABSTRACT

Halophilic archaea are prevalent in highly saline habitats. *Haloferax* strain BBK2 is an orange pigmented, exopolysaccharide (EPS) producing extremely halophilic archaeon, isolated from solar salterns of Ribandar, Goa, India. It grew in varying pH (5–10) and NaCl concentration (10–30%). The isolate grew well in complex (NTYE) and minimal media (NGSM) in presence of heavy metal cadmium (Cd) up to 4.0 mM (805.28 mg L^{-1}) concentration. The optimum growth in the presence and absence of Cd was seen at a pH range of 7–9 and salinity of 15–25%. The growth kinetics of the isolate in NTYE showed a specific growth rate (μmax) of 0.352 with generation time of 1.968 days. In presence of 1 mM Cd, the μmax was 0.325 day^{-1} and generation time was 2.132 days. In NGSM, the μmax decreased from 0.517 day^{-1} (in control) to 0.265 day^{-1} in 1 mM Cd while, the doubling time increased from 1.34 days in control to 2.615 days in presence of 1 mM Cd. SDS PAGE of the whole cell protein extracts showed overexpressed proteins of 74.14 and 40 kDa. The scanning electron microscopy, energy dispersive X-ray spectroscopy (SEM–EDX) analysis of the intact cells and cells disrupted by dialysis revealed that Cd was bound onto the cells, which was further confirmed by AAS, FTIR and XRD analysis.

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1. Introduction

Metals are ubiquitous in the environment due to natural processes like weathering and/or erosion. However, most of the toxic metals are released into the water bodies as a result of anthropogenic activities such as sewage and industrial disposals, agricultural waste and mining. There have been world-wide reports on metal (Cd, Cr, Cu, Mn, Pb, Zn, Ni and Fe) contamination of soil (sediments) and water bodies like rivers, lakes, estuaries, etc. due to industrial processes (Jain et al., 2007; Amin et al., 2009; Kaushik et al., 2009; Rath et al., 2009; Uluturhan, 2010).

Cadmium is released in the environment as a byproduct of the zinc and lead mining industries. It is also used as pigment in paints, in electroplating and galvanizing, in batteries, etc. It is a non-essential element, highly toxic, highly water soluble heavy metal, which can accumulate in living tissues and hence can easily enter the food chain resulting in bioaccumulation (Gonzalez et al., 2006; Garcia et al., 2008; Khan et al., 2008; Yang et al., 2009). Because of its toxic, persistent and xenobiotic nature it is a potential threat to both environment and human health (Raju et al., 2011). Although most of the microorganisms are susceptible to Cd, a number of bacteria, fungi and mosses are capable of tolerating Cd (Green-Ruiz et al., 2008; Sari et al., 2008; Xiao et al., 2010; Saluja et al., 2011; Rehman and Anjum, 2011; Zhou et al., 2013).

Halophilic (salt loving) archaea belong to the family *Halobacteriaceae* and are the predominant microorganisms of the extreme hypersaline environments like the Great Salt Lake, Dead Sea, crystallizer ponds of solar salterns, etc. These halophilic organisms

Abbreviations: EPS, Extracellular polysaccharide; SEM, scanning electron microscopy; EDX, energy dispersive X-ray spectroscopy; XRD, X ray diffraction; NTYE, NaCl Tryptone Yeast Extract; NGSM, NaCl Glucose Synthetic Medium.

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http://dx.doi.org/10.1016/j.chemosphere.2014.04.058
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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 374562, 6 pages
http://dx.doi.org/10.1155/2014/374562

Research Article

Pretreatment of Cottage Cheese to Enhance Biogas Production

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Received 15 February 2014; Accepted 19 May 2014; Published 3 June 2014

Academic Editor: José Luis Campos

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This study evaluated the possibility of pretreating selected solid fraction of an anaerobic digester treating food waste to lower the hydraulic retention time and increase the methane production. The study investigated the effect of different pretreatments (thermal, chemical, thermochemical and enzymatic) for enhanced methane production from cottage cheese. The most effective pretreatments were thermal and enzymatic. Highest solubilisation of COD was observed in thermal pretreatment, followed by thermochemical. In single enzyme systems, lipase at low concentration gave significantly higher methane yield than for the experiments without enzyme additions. The highest lipase dosages decreased methane yield from cottage cheese. However, in case of protease enzyme an increase in concentration of the enzyme showed higher methane yield. In the case of mixed enzyme systems, pretreatment at 1 : 2 ratio of lipase : protease showed higher methane production in comparison with 1 : 1 and 2 : 1 ratios. Methane production potentials for different pretreatments were as follows: thermal 357 mL/g VS, chemical 293 mL/g VS, and thermochemical 441 mL/g VS. The average methane yield from single enzyme systems was 335 mL/g VS for lipase and 328 mL/g VS for protease. Methane potentials for mixed enzyme ratios were 330, 360, and 339 mL/g VS for 1 : 1, 1 : 2, and 2 : 1 lipase : protease, respectively.

1. Introduction

Food waste is the single largest component of waste stream by weight. About 135.5 million tons per year of municipal solid waste is generated in India and food waste alone constitutes about 30–40% [1]. Anaerobic digestion is a proven technology that offers significant environmental benefits and has been considered as one of the most viable options for managing solid organic waste [2]. Anaerobic digestion is a process, where complex particulate organic material is broken down into simpler soluble compounds which are taken up by microbial cells and ultimately converted into methane and carbon dioxide.

Food waste is characterized by its high organic content, most of it being composed of easily biodegradable compounds carbohydrates, proteins, and, in some cases, small amount of lipids. The anaerobic biodegradability of organic matter depends on its composition and the amount of methane produced depends on the biochemical nature of the waste [3]. For instance, carbohydrates, proteins, and fats show different methane production rates [4]. Food waste contains variable type and amount of organic matter whose behaviour in digester depends on the biodegradation of organic pools characterized by different methane production rates. Although food waste has been regarded as readily biodegradable because of its high volatile fraction (90% of total solids), its hydrolysis reaction is still a rate limiting step [5]. Enhancement of the hydrolytic reaction during anaerobic digestion could shorten the hydraulic retention time and thus improve the economics of the process. During recent years, various studies have been conducted on pretreatment of food waste, such as mechanical and sonication [6], thermal [7, 8], acid [9], alkaline [10], and enzymatic [5, 11, 12].

We had set up a horizontal plug flow type of anaerobic digester handling one ton of food waste/day and generating 60 m^3 of biogas/day. The food waste for this plant is from the institute's cafeteria catering to 2500 students. The commonly seen undigested solid fractions in the outlet of the digesters are cottage cheese, whole potatoes, and whole eggs. Studies have shown that digestate can still contain a high biogas potential, mainly as a consequence of residual and undigested volatile solids [13]. Digested solid fraction, with its biogas

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Ann Microbiol
DOI 10.1007/s13213-014-0944-6

ORIGINAL ARTICLE

Comparison of bacterial diversity from solar salterns and a simulated laboratory study

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Received: 8 January 2014 / Accepted: 14 July 2014
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Abstract Bacterial diversity in solar salterns and a simulated solar saltern under laboratory conditions was studied. The two systems were compared at the pre-salt harvesting phase and salt harvesting phase using Denaturing Gradient Gel Electrophoresis (DGGE). Bacterial composition was dominated by Gammaproteobacteria and more specifically with members of *Alteromonas*, *Vibrio*, *Pseudomonas*, *Tolumonas*, *Marinobacter*, *Pseudoalteromonas* and novel uncultured bacteria. The Shannon–Weaver index (*H*), Simpson diversity index (*D*) and Equitability index (*E*) values showed that salterns can support a wide range of microbes during the pre-salt harvesting phase (3–4 % salinity) when compared to the salt harvesting phase (21–29 % salinity). Range-weighted richness (*Rr*) values indicated that solar salterns are habitable only by a limited group of microbes as they have medium richness, indicated by physico-chemical characteristics. Principal Coordinate Analysis (PCO) of the simulated study showed a variation in diversity at a large scale with increase in salinity. Solar salterns as well as the simulated tank showed more diversity during the pre-salt harvesting phase.

Keywords Solar salterns · Halophiles · Bacterial diversity · DGGE · 16S rRNA

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Introduction

Hypersaline environments like salt lakes and solar salterns are extreme ecosystems in which the change in salinity is the dictating factor which determines microbial diversity at any given point of time (Rodríguez-Valera et al. 1985). Prokaryotic diversity in any ecosystem is an important factor to be considered because of its role in nutrient turnover, element recycling and as a potential hub for recovery of microorganisms for industrially important metabolic products (Garcia-Martinez et al. 1999; Lorenz and Eck 2005). Solar salterns serve as a good model for studying the changes in biodiversity over salinity, thereby providing us with information on extremes of life. Since diversity studies on hypersaline environments are gaining momentum in the past decade, the chances of obtaining novel isolates are relatively high when compared with any other ecosystem. Novelty of isolates is not limited to bacteria and archaea but includes eukaryotes like fungi, protists and algae (Casamayor et al. 2013).

Many reports have focussed on the occurrence of halophilic archaea in hypersaline regions (Munson et al. 1997; Ochsenreiter et al. 2002; Ahmad et al. 2008; Braganca and Furtado 2009; Oh et al. 2010; Zafrilla et al. 2010). However, information on what happens to the bacterial community in increasing salinity gradients from 2 to 30 % are limited (Henriques et al. 2006; Demergasso et al. 2008; Deshmukh et al. 2011; Boujelben et al. 2012). It is generally accepted that diversity decreases with increase in salinity, as the case with any extreme ecosystem. Previous studies employing genetic fingerprinting techniques like clone library construction, DGGE, T-RFLP and RISA analysis has shown the general trend of decrease in diversity with increase in salinity. Furthermore, these studies have shown that a diverse group of bacteria dominates salterns in low (4 %), moderate (15 %) and high (32 %) salinity ponds (Casamayor et al. 2002). But trends in archaea are different with few detected in low salinity

Published online: 02 August 2014

Mani et al. Aquatic Biosystems 2012, 8:15
http://www.aquaticbiosystems.org/content/8/1/15

AQUATIC BIOSYSTEMS

SHORT REPORT Open Access

Culturable halophilic archaea at the initial and crystallization stages of salt production in a natural solar saltern of Goa, India

Kabilan Mani, Bhakti B Salgaonkar and Judith M Braganca*

Abstract

Background: Goa is a coastal state in India and salt making is being practiced for many years. This investigation aimed in determining the culturable haloarchaeal diversity during two different phases of salt production in a natural solar saltern of Ribandar, Goa. Water and sediment samples were collected from the saltern during pre-salt harvesting phase and salt harvesting phase. Salinity and pH of the sampling site was determined. Isolates were obtained by plating of the samples on complex and synthetic haloarchaeal media. Morphology of the isolates was determined using Gram staining and electron microscopy. Response of cells to distilled water was studied spectrophotometrically at 600nm. Molecular identification of the isolates was performed by sequencing the 16S rRNA.

Results: Salinity of salt pans varied from 3-4% (non-salt production phase) to 30% (salt production phase) and pH varied from 7.0-8.0. Seven haloarchaeal strains were isolated from water and sediment samples during non-salt production phase and seventeen haloarchaeal strains were isolated during the salt production phase. All the strains stained uniformly Gram negative. The orange-red acetone extract of the pigments showed similar spectrophotometric profile with absorption maxima at 393, 474, 501 and 535 nm. All isolates obtained from the salt dilute phase were grouped within the genus *Halococcus*. This was validated using both total lipid profiling and 16S rRNA data sequencing. The isolates obtained from pre-salt harvesting phase were resistant to lysis. 16S rRNA data showed that organisms belonging to *Halorubrum*, *Haloarcula*, *Haloferax* and *Halococcus* genera were obtained during the salt concentrated phase. The isolates obtained from salt harvesting phase showed varied lysis on suspension in distilled water and /or 3.5% NaCl.

Conclusion: Salterns in Goa are transiently operated during post monsoon season from January to May. During the pre-salt harvesting phase, all the isolates obtained belonged to *Halococcus sp*. During the salt harvesting phase, isolates belonging to *Halorubrum*, *Haloarcula*, *Haloferax* and *Halococcus* genera were obtained. This study clearly indicates that *Halococcus sp.* dominates during the low salinity conditions.

Keywords: Archaea, Haloarchaea, Hypersaline, Solar saltern

Findings

Marine solar salterns are thalassohaline hypersaline environments located in tropical and subtropical areas worldwide, consisting of shallow ponds for the production of common salt from seawater during summer. The method of making salt through natural evaporation dates back to pre-historic times. This traditional approach of salt production involves construction of series of rectangular ponds, each connected to the other through a common opening [1-4].

Goa (15°34′60N, 74°0′0E) is a coastal state in India and salt making is being practiced for many years. Saltpans are found in Pernem, Bardez, Tiswadi and Salcete talukas of Goa. Saltpans are inundated by sea water from estuaries during high tides. Sea water is retained in every pond for certain time to facilitate evaporation. As concentration of NaCl gradually increases, first component to precipitate is

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Mani et al. Aquatic Biosystems 2012, 8:30
http://www.aquaticbiosystems.org/content/8/1/30

AQUATIC BIOSYSTEMS

REVIEW Open Access

Community solar salt production in Goa, India

Kabilan Mani, Bhakti B Salgaonkar, Deepthi Das and Judith M Bragança*

Abstract

Traditional salt farming in Goa, India has been practised for the past 1,500 years by a few communities. Goa's riverine estuaries, easy access to sea water and favourable climatic conditions makes salt production attractive during summer. Salt produced through this natural evaporation process also played an important role in the economy of Goa even during the Portuguese rule as salt was the chief export commodity. In the past there were 36 villages involved in salt production, which is now reduced to 9. Low income, lack of skilled labour, competition from industrially produced salt, losses incurred on the yearly damage of embankments are the major reasons responsible for the reduction in the number of salt pans.

Salt pans (*Mithagar or Mithache agor*) form a part of the reclaimed waterlogged *khazan* lands, which are also utilised for aquaculture, pisciculture and agriculture. Salt pans in Goa experience three phases namely, the ceased phase during monsoon period of June to October, preparatory phase from December to January, and salt harvesting phase, from February to June. After the monsoons, the salt pans are prepared manually for salt production. During high tide, an influx of sea water occurs, which enters the reservoir pans through sluice gates. The sea water after 1–2 days on attaining a salinity of approximately 5ºBé, is released into the evaporator pans and kept till it attains a salinity of 23 - 25ºBé. The brine is then released to crystallizer pans, where the salt crystallises out 25 - 27ºBé and is then harvested.

Salt pans form a unique ecosystem where succession of different organisms with varying environmental conditions occurs. Organisms ranging from bacteria, archaea to fungi, algae, etc., are known to colonise salt pans and may influence the quality of salt produced.

The aim of this review is to describe salt farming in Goa's history, importance of salt production as a community activity, traditional method of salt production and the biota associated with salt pans.

Keywords: Salt pan, Goa, Estuary, Community, *Khazan*, Tidal influx, India, Salt production

Background

Goa, together with Daman and Diu, was a province under the Portuguese rule from 1510 and referred to as *Estado da India*. Goa was annexed by India on 19th December 1961 and liberated from the Portuguese rule [1]. Solar salt production in Goa had been an important activity through its history.

Goa experiences a tropical monsoon climate with hot summers followed by long monsoons from June to October. Goa has 9 rivers, most of them forming estuaries, the major being river Mandovi and Zuari. These rivers experience high tidal influx during summers and therefore the salinity varies during monsoon (2–3ºBé) and non-monsoon times (4–5ºBé) [2]. Various factors such as favourable climatic conditions and easy accessibility to sea water have aided salt production through natural evaporation in Goa.

Today, solar salt production has become a declining industry due to low income generated, competition from industrially produced iodized salt, yearly damage and repairs of the embankments and pollution. Currently there are 9 villages producing salt each having a few operational salt pans.

Historical background

Solar salt production in Goa, described as a traditional village industry, has been practiced for the past 1,500 years by various communities [3,4]. Since most of the rivers form estuaries and experience tidal influxes, salt production was started mainly in the coastal villages. Salt served as an important trade commodity too, playing an important role in the economy of Goa. The salt

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Appendix V : List of Conferences

(1) Bhakti B. Salgaonkar and Judith M. Bragança (2014) ***Bacillus megaterium* strain H16 Producing Polyhydroxybutyrate. (Poster Presented)** at *Indo – UK International Workshop on Advanced Materials and Their Applications in Nanotechnology (AMAN 2014)*, 17th - 19th May 2014, BITS Pilani K K Birla Goa Campus, India.

(2) Deepthi Das, **Bhakti B. Salgaonkar**, Kabilan Mani and **Judith M. Bragança** (2013) **Resistance of Extremely Halophilic Archaea to Various Heavy Metals.** (Poster Presentation) HALOPHILES 2013 Conference, 23rd – 27th June 2013, University of Connecticut, Storrs, USA.

(3) Bhakti B. Salgaonkar, Kabilan Mani and Judith M. Bragança (2012) **Accumulation of Polyhydroxybutyrate by Halophiles from Solar Salterns of India.** (Poster Presented) at *9th International Congress on Extremophiles 2012*, 10th-13thSeptember 2012, Sevilla, Spain).

(4) Kabilan Mani, **Bhakti B. Salgaonkar** and **Judith M. Bragança** (2011) **Molecular Microbial ecology of solar salterns of Goa and the isolation of culturable Haloarchaea.** (Oral presentation at National symposium on "Microbial Diversity and its Application in Health, Agriculture and Industry", March 4th – 5th 2011) organised by Indian Council for Agricultural and Scientific Research (ICAR) CSIR research complex Goa sponsored by Dept. of Science and Technology (DST), Govt. of India.

(5) Bhakti B. Salgaonkar, Kabilan Mani and **Judith M. Bragança** (2010) **Biodiversity of Haloarchaea in Hypersaline Environments of Saltpans of Goa–India**. (Paper presented at the 9th International Conference of Halophilic Microorganisms HALOPHILES 2010), 29th June - 3rd July 2010, Beijing China.

(6) Bhakti B. Salgaonkar and Judith M. Bragança (2009) **Characterisation of Extracellular Pigment produced by *Pseudomonas* species**. (Poster Presented at AMI, 16th-19th December 2009, National Chemical Laboratory NCL- Pune).

Appendix VI : Brief Biography of the Candidate

Personal Details

Name : Ms. Salgaonkar Bhakti Balkrishna

Education : M.Sc. (Microbiology), Goa University (2009)

: B.Sc. (Microbiology), St. Xavier's College, Goa University (2007)

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Work experience

1) Worked as a Research Fellow on a **University Grants Commission (UGC)** sponsored Major Research Project (MRP) entitled "Studies of Haloarchaea Producing Polyhydroxyalkanoates" Reference no. 34-500/2008(SR) from 2nd September 2009 to 31st March 2012.

2) **Council of Scientific and Industrial Research,** Senior Research Fellowship (CSIR-SRF), 09/919(0016)/2012-EMR-I for the period of three years from 1st April 2012 to 31st March 2015.

Achievements

- **Department of Biotechnology (DBT)** Conference, Travel, Exhibition and Popular Lectures (CTEP), DBT/CTEP/02/201200602, Government of India awarded travel grant for attending 9th International Congress on Extremophiles from 10th-13th September 2012, Seville Spain.

No. of publications : 7 (as first author) and 5 (as co-author)

No. of conferences / workshops attended : 6

Appendix VII : Brief Biography of the Supervisor

Prof. Judith M. Bragança completed her Ph.D. from Goa University in 2003 as a CSIR Fellow. Her Doctoral work elucidated the biodiversity of halophilic archaea and explored their mechanisms of resistance to arsenic and cadmium. Dr. Bragança joined the Department of Biological Sciences, BITS Pilani, K K Birla Goa Campus as a faculty in 2004 when BITS Pilani established its third campus in Goa. Since then she is involved in teaching a number of courses at the First Degree and Higher Degree Level. She has also been a Principal Investigator for in house as well as externally funded research projects. Prof. Bragança has over 20 publications in reputed peer reviewed journals and a number of conference proceedings to her credit.

Her research interests include Biodiversity of Halophiles, Biotechnological applications of halophilic archaea and bacteria and Metal tolerance in microorganisms. Under the BITS Pilani – University Immersion Scheme she visited Goethe-University Frankfurt, Germany during June – July 2013, and worked with Prof. Dr. Volker Muller, Dept. of Molecular Microbiology and Bioenergetics, Biozentrum. Prof. Bragança is a member of American Society of Microbiology (ASM), International Society for Salt Lake Research (ISSLR) and Association of Microbiologists of India (AMI). She has been actively involved as a reviewer of international journals from Elsevier, FEMS, Springer, etc. Presently she has three registered Ph.D. students under her tutelage, besides numerous thesis, dissertation and project students working with her.

Besides teaching, Prof. Bragança has been associated with BITS Pilani administration assuming multifarious responsibilities, mainly as Faculty-In-charge, Publications and Media Relations, Convener-Departmental Research Committee, besides being a member of various committees such as the Standing Committee for Students Discipline, Senate, Cross-campus Departmental Committee for Academics, Academic Counseling Board etc.