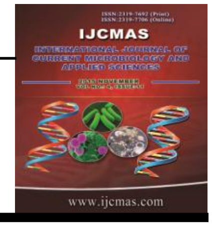

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Original Research Article

Molecular Insight of Halophilic Isolates for the Production of Potential Biomolecules under Stress Condition

A.B. Parameshwar, S.Y.Jayachandra, S.Anil, K. Mohan Reddy and M.B.Sulochana*

Department of Biotechnology, Gulbarga University, Kalaburagi-585106, India
**Corresponding author*

A B S T R A C T

Microorganism highly suited for the adaptation in the changing harsh inhabitable conditions. Extensive study provide insight into molecular moieties responsible for adaptation in such extreme situations. Halophilic environment known exert high osmotic pressure on the microorganisms, we have isolated the Halophilic bacteria from the western coastal region of India, A moderately halophilic, motile, Gram-negative, rod-shaped bacterium grown in halophic ATCC Media 213, microscopic and Biochemical and molecular study was done, optimal growth was noted that, the organism was able to grow at pH 6.0–11.0(optimum, pH 7.0–8.0), at 4–47^{o}C (optimum, 30–37^{o}C) and in the presence of 0.5–25% (w/v).NaCl (optimum, 1–3 %, w/v).Capable of producing the biocompatible molecule, Betaine, under NaCl stress conditions, betaine was extracted purified and characterized by UV-Visible Method, and FTIR.

Keywords

Halophiles, Betaines, Halomonas, FTIR and Compatible Solutes

Introduction

Harsh environmental conditions inhabitable to any living organisms, challenging for any organism to survive in extremely high or low environmental factors like pH, Salt, Heavy metal contaminated habitats, salinity pressure and temperature. The microorganisms adapted in such situations are categorically called extremophiles or poly extremophiles

Halophiles occurrence in high saline ecosystems such as sea water and saline soil, require Nacl for their growth, on the basis of their requirement for salt, halophiles are into three groups slight, Moderate and extreme halophiles (Kushner, 1998).

Extreme salinity exhibit more osmotic pressure on the microorganisms, Moreover, extremophiles in this group exhibit great potential for biotechnological exploitation: in fact, these bacteria have been used as a source of compatible solutes or hydrolytic enzymes (Mellado & Ventosa, 2003). Several studies have been conducted on the ecology, taxonomy, and phylogeny of halophiles' bacteria as well as their biotechnological applications (Lichfield and Gillevet, 2002).

Halomonadaceae interestingly achieve osmotic equilibrium in high saline situations by intracellular accumulation of compatible solutes, encounter the high osmotic imbalance and protect cell from damage (Ventosa *et.al.*,1998). There are range of compatible solutes that bacteria accumulates, like, glucosyl glycerol, quaternary amines and ectoine. Which are predominantly found in the *halomonadaceae* (Ventosa, *et.al.*, 1998).

The significant applications and biotechnological production of compatible solutes, like ectoine have been described in detail (Pastor *et al.*, 2010). The best-investigated compatible solute, ectoine, is biotechnologically produced by *Halomonas elongata* using a process named "bacterial milking", involving repetitive cycles of a fed-batch fermentation of *H. elongata* at 15% (w/v) NaCl to allow ectoine accumulation, followed by osmotic down shock at 3% (w/v) NaCl to release the osmolytes from the cells (Sauer and Galinski 1998). The physiology and genetics of ectoine biosynthesis in this bacterium have been studied in detail (Maskow and Babel 2001; Ono et al. 1999; Peters et al. 1990; Goller and Ofer 1998) as well as the glycine-betaine-synthesizing enzymes (Gadda and McAllister- Wilkins 2003). Also the compatible solute uptake system for ectoine accumulation (teaABC) from *H. elongata* has been characterized at the physiological and molecular level (Grammann *et al*. 2002; Tetsch and Kunte

Betaines, quaternary amine, are the compatible solutes occurring *halomonas sp.*, proved them as therapeutic important in the treatment and prophylaxis of adipose infiltration of the liver, which is the initial stage of cirrhosis (Detkova, E. N., *et al.*, 2007). Betaines decrease side effects of anti-inflammatory preparations. Their anticoagulant properties prevent thrombus formation and decrease the probability of heart attacks, infarctions and strokes (Messadek, J., *et al.*, 2005). They are useful in PCR amplification of GC-rich DNA templates to increase product yield and specificity. Betaine was shown to be a more effective cryoprotectant than serum albumin or trehalose/ dextran, particularly under conditions stimulating long-term storage (Cleland, D., e*t al.*, 2004).The present study describe the potential candidate for the production of betaines and purification and characterization from the *halomonas sp.*

Materials and Methods

Bacterial Culture

The complex medium used for the isolation and maintenance of the bacterial strains contained 5g Tryptone, 10g Yeast extract, 3g Citrate sodium, 2g KCl and 20g MgSO4.7H2O dissolved in 1litre of distilled water and pH was adjusted to 7.5 and incubated at 37^{o}C for 48 hours.

Characterization of the Isolated Strains

To determine the colony morphology, bacterial cultures were grown on plates containing complex medium for 48 hours and plates were observed for pigmentation. A typical Gram's reaction was determined using 24 hours old culture.

PCR Amplification of 16S rRNA Gene Sequence

The 16S rRNA gene sequence of bacterial strain was amplified by PCR with the following forward and reverse primers: 5'-AGAGTTTGATCATGGCTCAG-3' (position 827) and 5'-CTACGGTTA CCTTGTTACGAC-3' (position 1492-1510) respectively. The PCR condition were as follow: 50 µl of reaction system, reaction cycles 35 cycles, 94°C pre-denaturation 5 min, 94^{o}C denaturation 1 min, 55°C annealing 30 sec, 72°C extension 1 min, 72°C final extension 8 min, 4^{o}C hold for 4 hrs. The PCR product was purified by DNA gel extraction kit and was sequenced in National Centre for Cell Science (NCCS), Pune.

Optimization of Growth Parameters

Effect of pH on Growth

Production medium was adjusted to different pH ranging from 5 to 13 using 0.1% Glucose with 10% NaCl (halo bacterium medium) was incorporated as a substrate after autoclaving.

Effect of Temperature on Growth

The optimum temperature for the growth and production ectoine by halophilic bacteria isolate was determined in production medium (halobacterium medium) by keeping it in temperature controlled shaker at 140 rpm range from 35^{o}C to 70^{o}C with increment of 5^{o}C.

Effect of NaCl on Growth

The effect of NaCl was studied on growth and ectoine production using production medium(halobacterium medium) containing different concentration of NaCl(0, 5,10,15,20 and 30% w/v) inoculated broth were kept under shaker at temperature 37$\pm$ 2^{o}C and pH 7.

Production of Betaine

Production of Betaine and Growth Conditions

Production of compatible solute by two-step fed-batch cultures *Halomonas sp.* was first grown in 100 mL of seed culture medium in a 250 ml flask at 37^{0}C and 200 rpm for 13 h. The culture broth was used to inoculate 500ml L of batch cultivation medium with 5%- 20% NaCl. After 18 h, cells were harvested from the culture medium by centrifugation at 6000 RPM for 10 min at 4^{o}C and used to inoculate a batch. The concentration of NaCl in the feed solution was modified accordingly. Temperature during the cultivations was maintained at 37°C while pH was kept constant at 7 using HCl and NaOH.

Purification of Betaine

The samples (200 mg) were mixed at 4 °C with 5 mL of distilled water (method 1), 5 mL of a mixture of methanol/ chloroform/water 12:5:3 v/v (method 2), or 5 mL of a mixture of ethanol/water90:10 v/v (method 3). Extracts were dried under reduced pressure at 35 °C. Purified sample was detected by UV scan absorbance at 230-231nm indicate the presence of betaine and further characterization by FTIR

Characterization of Betaine by FTIR Analysis

The FTIR analysis of betaine analyte was performed on the instrument from the 400 to 4000 cm^{-1}, KBR was mixed with the sample and KBR-Sample Pellet was prepared for the analysis.

Results and Discussion

Cell colonies are cream coloured, convex and mucoid. Gram negative bacilli, rod shape, non-spore forming and motile microorganism. Halophilic bacteria capable of growing at NaCl concentration range from 3–25% (w/v), with optimum growth occurring at about 10 % and grows within a temperature range of up to 45^{0}C at pH values of between 5 and 10, the optimum values being pH 7–8.

The strain was identified on the basis of morphological, Biochemical and molecular characters of strain was, the biochemical tests of isolated strain is as shown in the Table1 and Pic 1. All these observations were compared with Bergey's Manual of Bacteriology, 2004 edition and Volume II and was reported earlier in our laboratory, and this bacteria strain belongs to this genus *Halomonas.* The organism was identified as *Halomonas sp.*SAV2 by 16S rRNA sequencing, the Genbank Accession. no JQ906722and accumulate solute called betaine. Optimization of pH, temperature and salt concentration for production of betaine from *Halomonas sp* SAV2 has been carried out.

Identification of Strain for the Production of Osmolytes

Reports on isolation of novel isolates from alkaline habitats were few with respect to the organic osmolytes. Today, many of this alkalophilic isolates are of considerably industrial importance, partially for use in pharmaceutical and cosmetic industries and also in anti- aging molecule, anti-stress molecule. In our study, we have selected one strain showing most promising for the production betainewith optimum activities at pH 8.0 and temperature of 45^0 C and upto 25% salt concentration which is desirable for industrial application. Further, this strain was characterized based upon the phylogenetic analysis, morphology, biochemical and 16S rRNA.

The halophilic microorganism's adaptation to changing extracellular osmolarity. Osmolytes are accumulated as a consequence of increased extracellular salinity and their intracellular concentration is reduced. The concentration of osmolytes in the cell can range from millimolars to 1–2 M in response to the extracellular osmolarity. This means that some osmolytes are tolerated by the macromolecular machinery of the cell over a wide concentration range, hence, these compounds were also termed compatible solutes (Brown, 1976).

The biosynthesis and release of osmolytes is tightly regulated. In general, the microorganism can accumulate osmolytes either by de novo synthesis or by uptake from the environment. Moderately halophilic, heterotrophic bacteria growing in high salt concentrations can accumulate betaine even in molar concentrations (Imhoff and Rodriguez-Valera, 1984).Most often the compatible solute accumulation has been observed in halophilic bacteria, gradient concentration of salt in the growing media impede the growth of bacteria, thus, they accumulate betaine substantial concentration gradients in osmoregulating bacterial cells.

However,The betaine formation from simple carbons sources widely observed in phototrophic bacteria and halophilic phototrophic anaerobes (Severin *et al.*, 1992) and for halophilic cyanobacteria (Mohammed *et al.*, 1983; Mackay *et al.*, 1984; Reed *et al.*, 1984; Gabbay-Azaria *et al.*, 1988). *Halomonas sp.* SUBG004, isolated from little ran of Kutch which provides insights for salt stress adaptation through betaine synthesis (Jigna H. Patel, 2015).

***Halomonas Sp.* Characteristics**

Grams Test:	Negative
Shape	Rod
Endospore	Positive
Motility	Positive
Oxidasae	Positive
Catalase	positive
VP	Positive

Physicochemical Parameters

Temperature

$7^{O}C$	+Ve
$40^{O}C$	+Ve
$50^{o}C$	+Ve

Growth in Medium Ph

5	+Ve
6	+Ve
7	+Ve
8	+Ve
9	+Ve
10	+Ve
11	+Ve

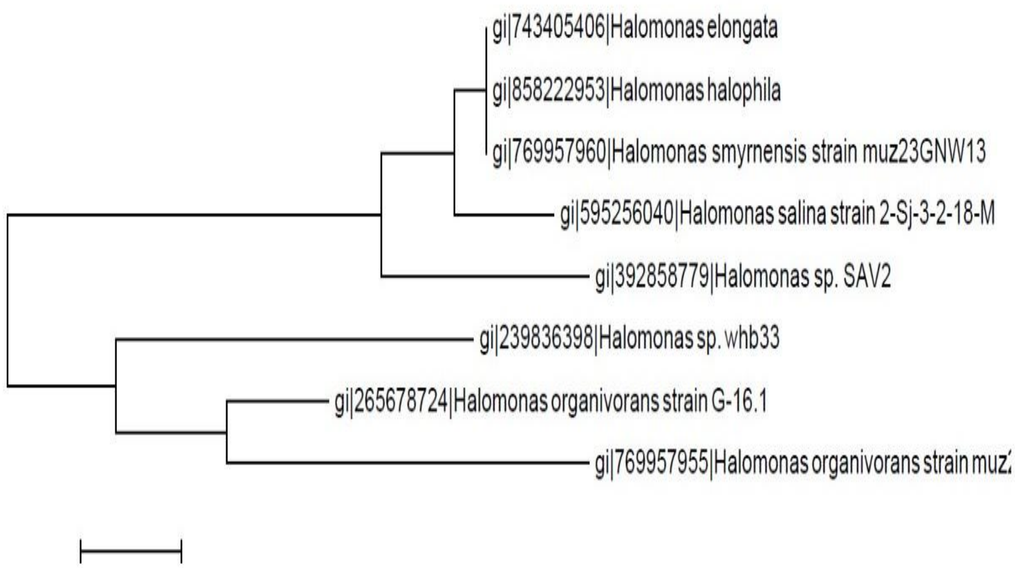
Growth in the Presence of NaCl (w/v)

2%	+Ve
5%	+Ve
7%	+Ve
10%	+Ve
15%	+Ve
20%	+Ve
25%	+Ve

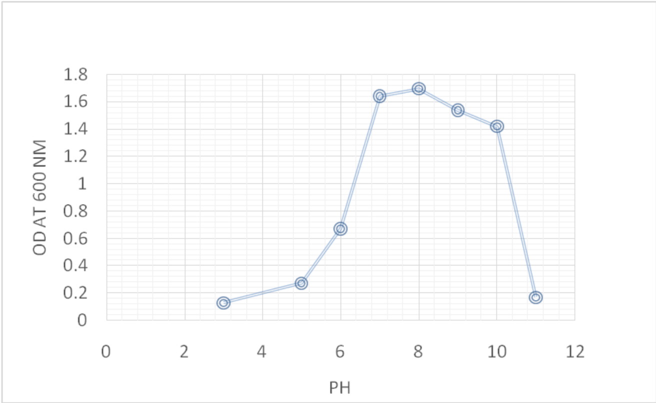
Acid Produced from

D-glucose	+Ve
L-arabinose	+Ve
D-xylose	+Ve
D-mannitol	+Ve
Utilization of citrate	+Ve
Indol reaction	+Ve
starch	+Ve
casein	+Ve

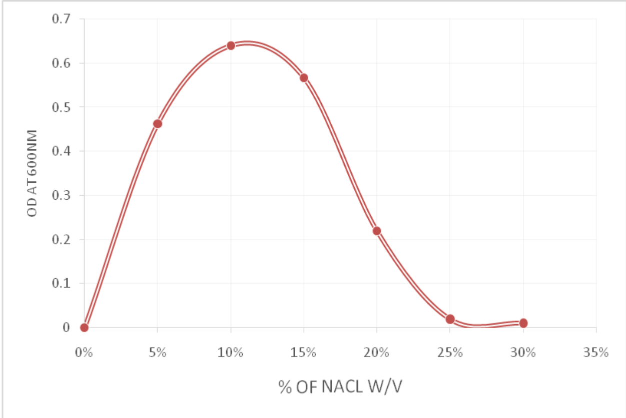
Picture 1 Phylogenetic Tree Contracted using MGA4 Software by Neighbour Joining Method



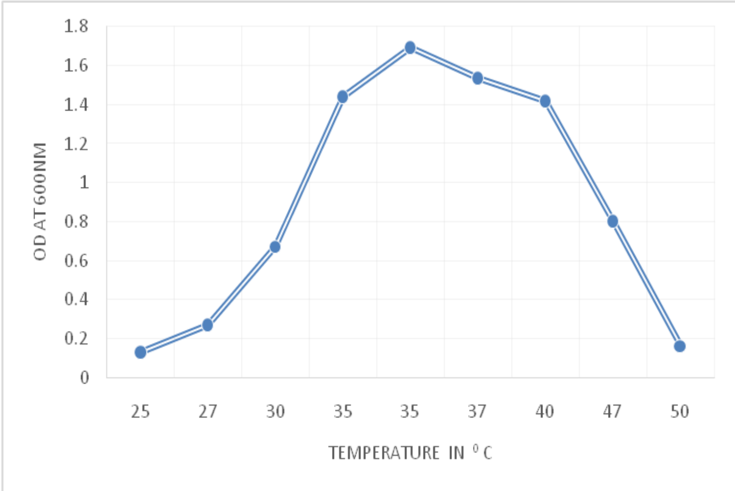
Graph.1 Effect of pH on Growth of the *Halomonas* sp



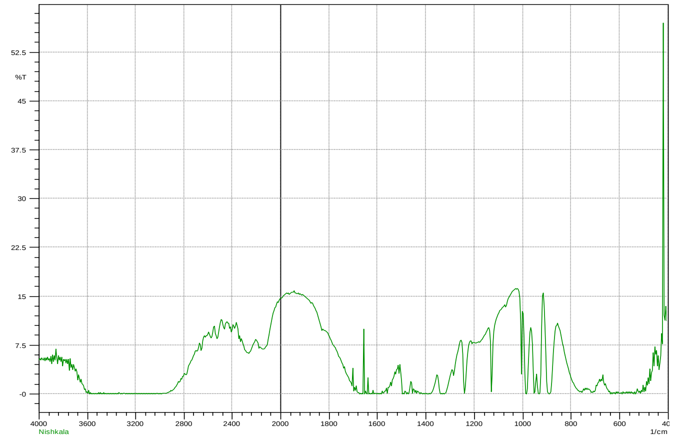
Graph.2 Effect of NaCl (W/V) on Growth of the *Halomonas* sp



Graph.3 Effect of Temperature on Growth of the *Halomonas* sp



Graph.4 FTIR Analysis of the Purified Betaine



In present study the betaine accumulation was observed to accumulate the higher concentration was detected further the purified betaine was characterized by FTIR analysis, on comparison with the NIST data for FTIR, results are similar, thus betaine was confirmed a compatible solute accumulated in the strain *Halomonas. Sp.*SAV2. strain able to tolerate maximum 25 % W/V NaCl concentration extracellular.
Cyanobacteria use to accumulate betaine under high salt concentration, halophilic cyanobacterium *Synchocystis* DUN52 produces 3.0 M betaine when the bacterium is cultivated at 200 g sea salt · l-1 (Mohammed *et al.*, 1983). Whereas the halophilic strains synthesize the sugars and glycosyl glycerol under salt stress conditions (Mackay *et al.*, 1984). Eubacteria halophilic *methanogenic archaebacteria* (*Methanohalophilus*-strains) also synthesize betaine as their osmolyte (Lai *et al.*, 1991).

Hyper saline conditions of the growth medium, influences the accumulation extremely high concentration of Betaine in their cytoplasm, archaebacterium *Methanohalophilus* Z7302reported to accumulate 4.1 M betaine at 4.4 M NaCl (Lai and Gunsalus, 1992). Sulphur reducing extremely halophilic bacteria *Ectothiorhodospira halochloris* synthesizes 2.5 mol betaine at 240 g NaCl (Galinski and Herzog, 1990). The pathways of *de novo* synthesis of betaine in bacteria have not been fully characterized. The studies based on 13C-NMR are consistent with betaine being synthesized *via* the glycine methylation pathway in the methanogenic archaebacterium *Methanohalophilus portucalensis* FDF1 (Roberts *et al.*, 1992). Enzyme activities of the threefold methylation of glycine have been found in the cell extract prepared from this strain (Lai *et al.*, 1999). Complex media components, such as yeast extract and peptone, contain considerable amounts of betaine (1 - 3% of the dry weight) that can be taken up by heterotrophic bacteria (Galinski and Trüper, 1994). In fact, betaine synthesis from simple carbon sources has proven to be quite rare among heterotrophic bacteria (Galinski and Trüper, 1994). some of these newly described organisms *Salinibacter ruber* shows that may necessitate us to revise our understanding on how the different physiological and phylogenetic groups of microorganisms have solved the problem, how to cope with hyper saline concentrations in their environment (Anton 2002). Hyper saline environments are extremely diverse, and so are the microorganisms that inhabit them. It is well possible that the upper salinity at which different metabolic types of prokaryotes are found in nature depends to a large extent on the balance between the amount of energy that is available to the cells and the cost of production of organic solutes needed to provide osmotic balance (Oren 1999)

These findings suggest that this *Halomonas sp*. has evolved a sophisticated mechanism to regulate the accumulation of compatible solutes to survive and grow in habitats with extremely gradient saline concentrations. The halophilic microbial world is tremendously diverse, and novel types of halophiles are being discovered at ahigh pace.

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