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Research Article

INFLUENCE OF VARIOUS ENVIRONMENTAL STRESSES IN THE GROWTH AND ACCUMULATION OF COMPATIBLE SOLUTES IN MARINE HALOPHILE

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ABSTRACT
Compatible solutes are osmoregulating organic compounds accumulate to high levels by halotolerant and halophilic organisms. A study was conducted to evaluate the intracellular accumulation of compatible solutes e.g. Proline and trehalose from the marine moderately haloalkaliphilic *Pseudomonas stutzeri*. In response to the environmental stress, maximum proline accumulation was reported nearly 8.18µM/mg of cell mass at the 4% salt (NaCl) concentration, 14.5µM/mg at the 8.5 pH and 8.73 µM/mg at the 50 ºC temperature. When growth media supplemented with 1µg/mL concentration of α-ketoglutaric acid, proline was reported to amass 66.07µM/mg of cell mass due to the utilization of α-ketoglutaric acid as a precursor. Extraction and detection of proline were done by TLC followed by the identification using HPLC. Similarly, trehalose accumulation was also evaluated in the presence of various stresses. Maximum trehalose accumulation was reported nearly 2.67µg/mg of cell mass at 4% salt (NaCl) concentration and 3.46µg/mg at the 8.5 pH. The temperature was drastically affected the accumulation of trehalose. Maximum 6.75µg/mg was estimated at 50ºC temperature. Acquisition of tolerance to salinity is due to the increased in proline concentration, whereas thermostability was due to the high accumulation of trehalose. The study will be helpful for the extraction of compatible solute from the waste biomass at the industrial scale to isolate the byproducts and thereby makes the bioprocess more viable.

Keywords: *Environmental Stresses, Compatible Solutes, Halophile*

INTRODUCTION
Halophilic and halotolerant microorganisms have evolved two basic mechanisms of osmoadaplation for the survival in harsh condition. First is by accumulating the K^+ ions and second is by synthesis of compatible-solutes, the latter representing a very flexible mode of adaptation making use of distinct stabilizing properties of compatible solutes (Galinski and Truper, 1994). Compatible solutes are an organic osmolytes that can be amassed by the bacterial cell in exceedingly high concentrations without disturbing vital cellular functions and the correct folding of cellular proteins. They are also referred as a kosmotropic organic solute or an osmoprotectant due to its vital role as an osmosensing and osmoregulatory system of the bacterial cells (Wood *et al.*, 2001; Kempf and Bremer, 1998). These low-molecular-weight organic solutes accumulate to high levels in halotolerant and halophilic organisms to survive in the saline environments (Martins *et al.*, 1996). To flourish in saline and hypersaline environments, various halophiles and haloalkaliphiles indicated the accumulation of the stress reducing compatible solutes as an adaptive strategy. Compatible solutes can make important contributions to the restoration of the turgor under conditions of low water activity by counteracting the efflux of water from the cell. They are also responsible for the protein stabilization and support the correct folding of polypeptides under *in vitro* and *in vivo* conditions. The alternative roles which compatible solutes may also play as intracellular reserves of carbon, energy and nitrogen, and released compatible solutes to the environment may influence global climate, due to the production of the trace gases, methane and dimethylsulfide (Welsh, 2000). Various types of such solutes have been reported from bacteria, including

proline, trehalose, glycine betaine, ectione, glutamate, glutamine, D-mannitol, carnitine etc. (Ameur *et al.*, 2011).

Proline is zwitterionic osmolyte accumulated by the halophiles in response to salinity. The biosynthesis and regulation of proline in cells have been studied in several different bacteria and archaea. Proline is commonly reported under the salinity in bacterial species, including *Streptomycetes griseus and S. californicus* (Killham and Firestone, 1984), *Palaeococcus ferrophilus* (Neves *et al.*, 2005), *Halobacillus halophilus* (Saum and Muller, 2008). Marine bacterial species specifically *pseudomonas* species are well reported in the proline production. Accumulation of proline is depending on the types and variety of stresses. Temperature, pH and salinity are the common environmental stresses occur in the habitats. Proline is accumulated in halophilic bacteria to the response of increasing salinity and even dominates over glutamine and glutamate as the major compatible solute at higher salinity due to the common α-ketoglutaric acid synthesis pathway.

Apart from amino acids as osmolyte, various sugars have also been reported to accumulate in the various extremophiles including trehalose, sucrose and mannitol. Trehalose is withstood with alkali and acidity as well as it is thermostable sugar. Trehalose accumulation has been reported in different species bacteria, including *Rhodothermus marinus* and *Rhodothermus obamensis* (Silva *et al.*, 1999) *Rhodobacter sphaeroides* (Tsuzuki *et al.*, 2011), and *Mycoleptodiscus terrestris* (Dunlap *et al.*, 2010). Accumulation of the trehalose has been shown to be associated with increased thermotolerance of bacterial, yeast, fungal and slime mould and their spores. In baker's yeast, *S. cerevisiae*, mild heat shock induces the rapid biosynthesis of trehalose to high cytoplasmic concentrations and the increased thermotolerance of the cells to subsequent heat treatments is directly related to their trehalose content (Hottiger *et al.*, 1987). The greater efficacy of trehalose compared to other compatible solutes *in vivo* may be due to its interaction with membrane lipids, which are a critical target for heat treatments. The present study was conducted to identify and purify proline and trehalose osmoprotectants from marine isolated haloalkaliphilic *Pseudomonas stutzeri*. The effects of environmental stresses including salt, pH and temperature were also evaluated to know the accumulation status.

MATERIALS AND METHODS

Sampling and Isolation of Bacteria

Soil sample was collected from the coastal region of Dandi, Gujarat state, India (N: 21°19'60˙ and E: 72°37'60˙). The sample was collected during day time at afternoon from the 10cm depth and it was brought to the laboratory in sterile polyethylene bags on the same day. 1gm of soil sample was inoculated in previously prepared 250ml of gelatin casein (GC) liquid media containing (g/L) 30g gelatin powder, 10g casein enzymatic hydrolysate, 13g dehydrated power of nutrient broth, 40g NaCl and pH 9.0 was adjusted with sterile 20% w/v Na_2CO_3 and incubated on shaker at 150rpm for 24hrs at 37°C temperature followed by the isolation of morphologically distinguished colonies on the same solid medium by streaking the 24hrs old broth. Dominating colonies of isolate DD8 were selected and further streaked on gelatin casein agar plate for the pure culture.

Morphological and Biochemical Heterogeneity of Isolate

Isolate DD8 was grown on GC agar plates to study their basic colonial and morphological characteristics. Gram's reaction and colony characteristics were examined by standard method. Log phase cells were used for the cell morphology analysis using scanning electron microscopy (SEM), Log phase indicating cells were washed with sterile normal saline and mounting on copper stubs followed by dehydration using the dryer and examined under SEM at 2700X. The sugar utilization pattern was determined using carbohydrate utilization kit (HiMedia Laboratories Pvt. Limited, India),

Identification of Bacteria

DD8 isolate was identified by the molecular method using sequencing of amplified of 16s rRNA gene by the polymerase chain reaction (PCR) (Eppendorf thermocycler).Universal primer of 16s rRNA gene was used to amplify the 1180bp fragment followed by the sequencing using ABI 3110 sequencer. A 10pmol of forward primer RINF 5`GCTCAGATTGAACGCTGGCG3` and 10pmol of reverse primer U2R

5`ACATTTCACAACACGA-GCTTG3` were used in the 25 µL PCR reaction. The reaction was performed using 33 cycles. Cycle steps include initial denaturation at 94 °C for 5min. followed by 33 cycles of denaturation at 94 °C for 45sec., annealing at 72 °C for 45sec. and synthesis at 72°C for 45sec. Amplified products were subjected to the sequencing and similarity search using BLASTn to identify up to the species level.

Mass Multiplication and Estimation of Intracellular Accumulated Proline

Isolate DD8 was grown in a 250ml flask of liquid media containing (g/L) 2ml glycerol, 5g glucose, 5g sucrose, 3.1g K_2HPO_4, 1g $(NH_4)_2SO_4$, 2g KCl, 0.01g $MgCl_2$, 40 g NaCl and pH 9.0. It is incubated at 37°C temperatures for 96hrs, followed by the extraction and estimation of proline. Extraction was carried out by harvesting the bacterial cells at 8000rpm for 15min. and 0.25mg cell pellet was collected and treated with 1mL 80% ethanol after that it was incubated at 100°C temperature in waterbath for 20 min. followed by centrifugation at 7000 rpm for 10 min. Proline was extracted in supernatant fraction.

Estimation of proline was carried out by Carillo *et al.,* (2008) method. 1mL of 1% (w/v) ninhydrin prepared in the 1:1 mixture of 60% acetic acid:20% ethanol was added in 0.5mL of ethanolic extract of proline and shake vigorously. The mixture was then incubated 95°C temperature in the water bath for 20 min followed by the centrifugation at 10000rpm for 1min and supernatant was collected to measure the OD at 520nm. Concentration of proline was calculated by plotting the standard curve of proline with aliquots ranging from 10mM to 100mM.

Purification of Proline

Purification of extracted proline was carried out by thin layer chromatography (TLC). Silica Gel plate (Merck Limited, India) was activated at 100°C for 5 min and cool down at room temperature. 20µl alcoholic extracted proline sample along with 20µl standard proline samples were applied to the charged TLC plate and allowed for separation in a solvent system containing the mixture of 3:1:1 ratio of n-butanol: acetic acid: sterile distilled water. The detection of amino acid separation on TLC plate was observed by spraying of 0.1% ninhydrine solution. Spots were identified by comparing with a standard and calculating the *Rf* values. Observed spots on the TLC were extracted and dissolve in methanol for the HPLC analysis. Dissolved sample was injected almost 50µL in the column C18 at 30ºC temperature. Elution was performed by creating the elution gradient with 50 to 90% Elute-A containing H_2O: 0.1% TFA (Trifluroacetic acid) to the 10 to 50% Eluent-B containing CH_3CN: 0.1% TFA with a flow rate 1mL/min. Detection was done by the UV detector at 215nm and the final result was obtained graphically.

Effect of Environmental Stresses and Precursor to Proline Biosynthesis

Effects of NaCl concentration ranging from 0% to 10% (w/v) was studied by supplementing the respective concentration of salt in the growth media. Effect of pH was studied with adjusting the pH of the growth medium with sterile Na_2CO_3 ranging from 7.5 to 10.0 Effects of temperature was examined by incubating the culture flask at different temperature including 30, 37, 40, 45 and 50°C. α-ketoglutaric acids act as a precursor for the biosynthesis of proline. An effect of precursor was studied by supplementing in growth media with different concentration ranging from 0.0 to 4.0µg/mL. Effects on each parameter were studied over 96 hour's incubation and proline was estimated as described in an earlier section.

Mass Multiplication and Estimation of Intracellular Accumulated Trehalose

Media for mass multiplication and estimation of intracellular trehalose and proline was similar, as describe in an earlier section. Extraction of trehalose was performed by the method described by Managbanag and Torzilli, (2002). After 96 hrs cells were harvested by centrifugation at 8000 rpm for 15min. and 0.25mg cell pellet was collected and treated with chilled and sterile double distilled water, followed by incubation in water bath at 95°C temperature for 20 min. Then supernatant was collected by centrifugation at 7000 rpm for 10 min. and used for estimation of trehalose.

Estimation of trehalose carried out by taking 100µL aqueous extracted supernatant dried at 100°C. The residues were dissolved in 0.3mL 1N H_2SO_4 and heated at 100°C in boiling water bath for 10 min. and cool it at room temperature, followed by the addition of 0.2mL 6N NaOH. Again treated at 100°C in boiling water bath for 10min and allowed to cool down it at room temperature. Then 3mL of Anthrone

reagent (prepared by dissolving 0.025g anthrone powder into 50mL of concentration H_2SO_4) was added and kept it into boiling water bath for 10 min. Measurement of trehalose was carried out at 620 nm (Jin *et al.*, 2011) and quantification was done using standard curve of trehalose.

Purification of Trehalose

Purification of aqueous extracted trehalose was carried out on activated TLC plate. 20µl of trehalose sample and 20µl standard trehalose samples containing 1µg/µl concentration were applied to the charged TLC plate and allowed for the separation in defined solvent system prepared by mixing 30:50:8:4 ml of chloroform: methanol: acetic acid: sterile distilled water (Nobre *et al.*, 2008). TLC plate was developed by spraying of 0.5% aqueous $KMnO_4$ solution (Managbanag and Torzilli, 2002). Developed spots were identified by calculating the *Rf* values of standard spot of trehalose followed by extraction of observed spots to dissolve into sterile deionized water for the HPLC analysis. A 50µL dissolved sample was injected into column C18 at 30 ºC temperature. Elution was performed by creating the elution gradient with 60 to 98% Elute-containing deionized water in the 2 to 40% Eluent-B containing 0.1% TFA with a flow rate 1mL/min. Detection was done by the UV detector at 215nm and the final result was obtained graphically.

Effect of Environmental Stresses to Trehalose Biosynthesis

Effects of NaCl concentration ranging from 0% to 10%, w/v was studied by supplementing the respective concentration of salt in the growth media. Effect of pH was evaluated by adjusting the pH of the growth medium with sterile Na_2CO_3 ranging from 7.5 to 10.0. Temperature influence was examined by incubating the culture flask of the isolate at different temperature including 30, 37, 40, 45 and 50°C. Effects on each parameter were studied after 96 hours' incubation.

RESULTS AND DISCUSSION

Results

Sampling and Isolation of Bacteria

Sample site was found to saturate with the salt and the presence of white salt crystal on the surface of the moist soil surface indicated the salinity rich soil. The soil samples were found alkaline, with pH 8.5 to 9.0 and temperatures during sampling was recorded 30 °C. DD8 isolate was isolated on GC media supplemented with 4% NaCl, pH 9.

Morphological and Biochemical Heterogeneity of Isolate

The isolate was Gram negative, short rod, producing opaque, smooth and flat colony on GCA plate (Figure 1). Based on the SEM analysis, isolate was found bacilli rod shape with 1.7 to 1.8 µm long and 0.4 to 0.5µm wide (Figure 2). This isolate could grow well at 4% salt concentration and pH 9.0 However, it can tolerate up 6% salt and 10.0 pH. The isolate was able to use 14 different sugars and hydrolyzed the esculin and citrate due to its broad catabolic efficiency (Table 1).

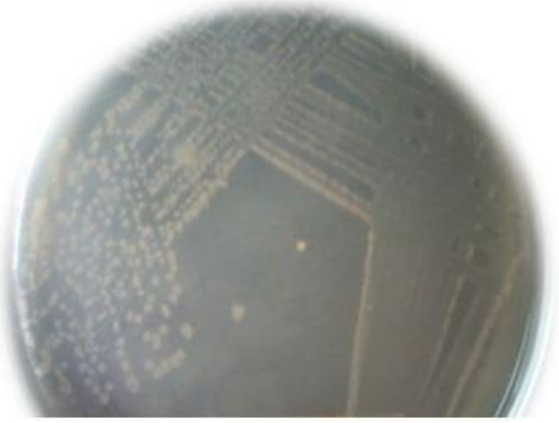

Figure 1: Isolated DD8 strain on GCA plate

Table 1: Sugar utilization pattern of isolate using Carbohydrate kit

No	List of Carbohydrates	Result	Observation
1	Lactose	-	
2	Xylose	-	
3	Maltose	+	
4	Fructose	+	
5	Dextrose	+	
6	Galactose	-	
7	Raffinose	-	
8	Trehalose	+	
9	Melibiose	-	
10	Sucrose	+	
11	L-arabinose	+	
12	Mannose	+	
13	Inuline	+	
14	Sodium gluconate	-	
15	Glycerol	+	
16	Salicin	+	
17	Dulcitol	-	
18	Inositol	+	
19	Sorbitol	+	
20	Mannitol	+	
21	Adonitol	-	
22	Arabitol	-	
23	Erythritol	-	
24	α-methyl-D glucoside	-	
25	Rhamnose	-	
26	Cellobiose	+	
27	Melezitose	-	
28	α-methyl-D mannoside	-	
29	Xylitol	-	
30	ONPG	-	
31	Esculin hydrolysis	+	
32	D arabinose	-	
33	Citrate utilization	+	
34	Malonate utilization	-	
35	Sorbose	-	

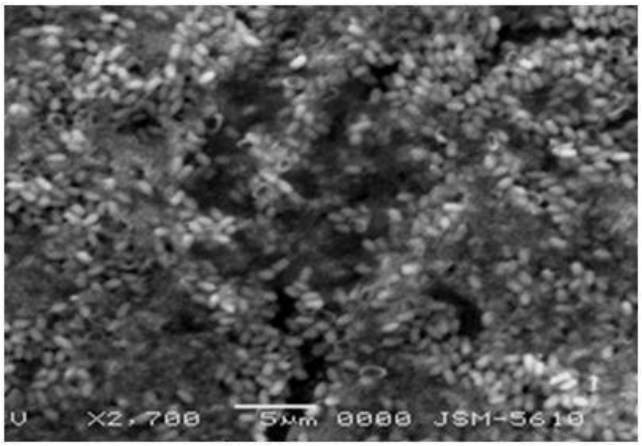

Figure 2: Cells morphology of log phase cells using scanning electron microscopy at 2700X

Identification of Bacteria

Isolate was identified by 16s rRNA gene sequence analysis using BLASTn. This strain found 99% similar to *Pseudomonas stutzeri*. The results of morphological and biochemical characterization was also supporting the molecular identification.

Mass Multiplication and Estimation of Intracellular Accumulated Proline

The enough cell mass of isolate was achieved using cultivation in triple sugars containing medium. Estimation of proline was performed in triplicate and cumulative data were calculated to lessen the errors.

Purification of Proline

Separation of the extracting proline sample from the cell mass was observed on TLC plate, which is compared with the standard proline sample (Figure 3). The R_f value of standard proline was 2.05 and similarly extracted proline samples was separately run by HPLC and R_f value was found 2.07. Both R_f were observed similar, indicating the presence of proline in the sample (Figure 4).

Figure 3: Separation of proline with standard sample. (a) Yellow spot of the standard proline sample and (b) indicated the yellow diffused spot of extracting proline from cell mass

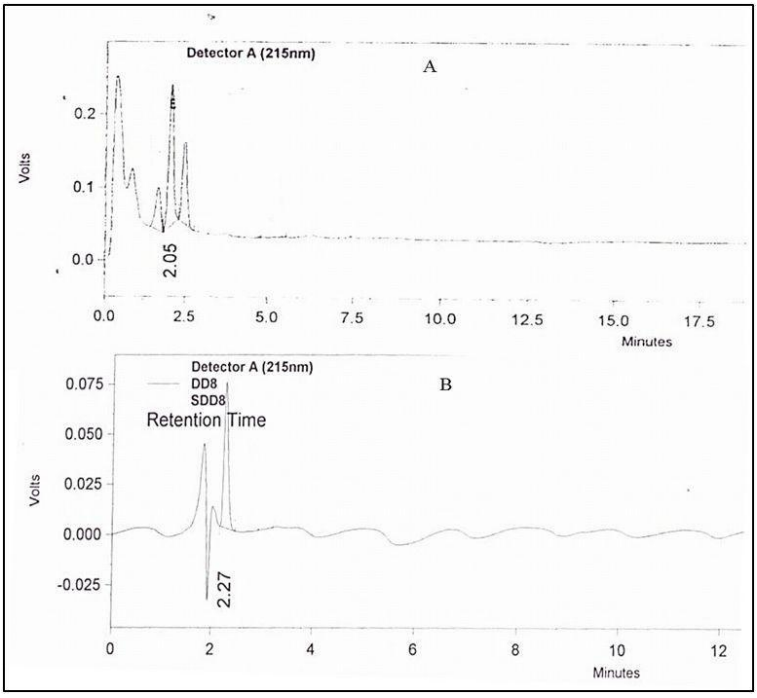

Figure 4: Chromatogram of proline generated by HPLC. (a) Standard proline sample and (b) extracted proline sample

Effect of Environmental Stresses and Precursor to Proline Biosynthesis

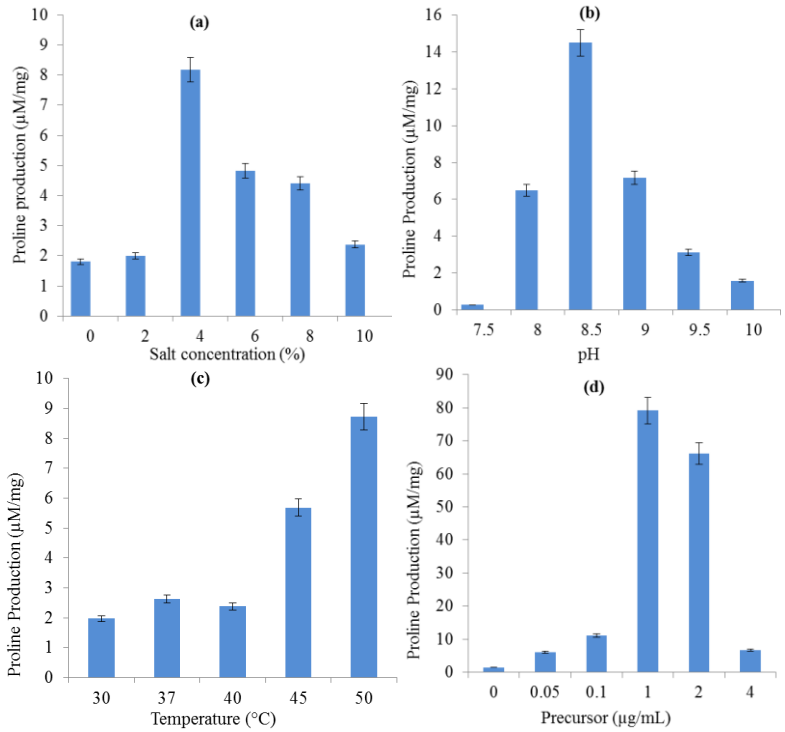

Figure 5: Effect of various stresses in proline synthesis (a) Salt (NaCl) concentrations (b) pH (c) Temperature (d) Precursor (α-ketoglutaric acid) concentrations

The isolate was found moderately haloalkaliphilic. Maximum proline was accumulated nearly 8.18 µM/mg of cell mass at the 4% salt and decreased with increased in salt concentration (Figure 5a). The effect of pH was very effective for accumulation of high concentrations of pH. A 14.5µM proline/mg of cell mass was reported at 8.5 pH, above and below 8.5 pH the concentration was decreased drastically (Figure 5b).

Cells were accumulated the 8.73µM proline/mg of cells at the 50ºC temperature, which was three times more than at the 40ºC temperature (Figure 5c). Proline is synthesized by α-ketoglutaric acid pathway via glutamate formation. When growth media supplemented with 1µg/mL and 2µg/mL concentration of α-ketoglutaric acid, the 79.16 and 66.07µM/mg proline production was reported respectively (Figure 5d).

Mass Multiplication and Estimation of Intracellular Accumulated Trehalose

Isolated was found haloalkliphic and maximum growth was achieved by cultivation in medium supplemented with 4% NaCl and pH 9.0. Estimation of trehalose was performed in triplicate and cumulative data were calculated to lessen the errors.

Purification of Trehalose

Separation of cell extracted trehalose observed as a colored spot on TLC plate, which is compared with the standard trehalose sample (Figure 6). Aqueous solution of 0.1 mM standard trehalose was injected into the C18 column. The R_f value of standard proline was 2.98 and similarly extracted trehalose samples was separately run by HPLC and R_f value was found 2.98 confirm the presence of the trehalose in the sample (Figure 7).

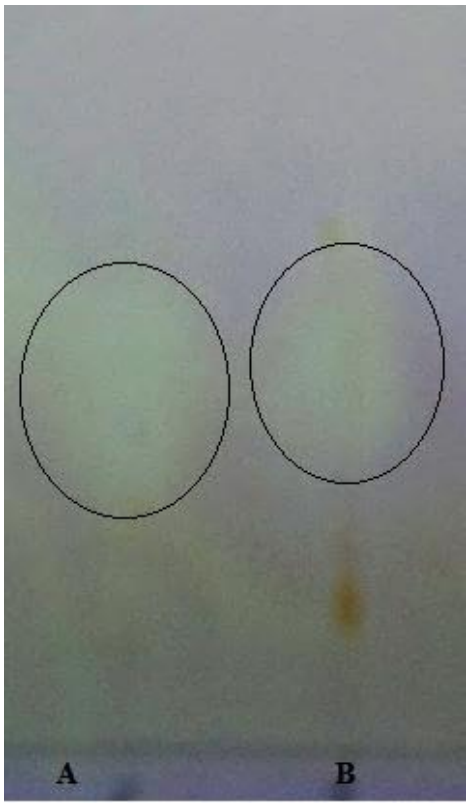

Figure 6: Separations of trehalose with standard sample. (a) A colored diffused spot of the standard trehalose sample and (b) indicated the similar spot of extracted trehalose from cell mass

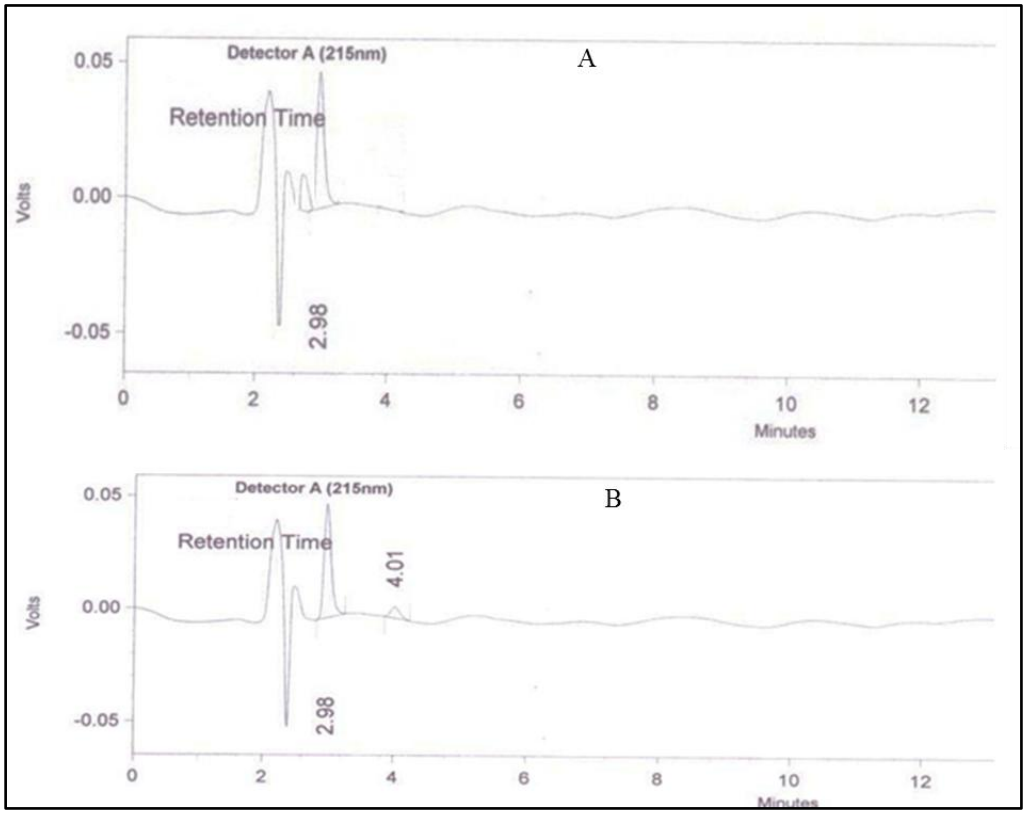

Figure 7: Chromatogram of trehalose generated by HPLC. (a) Standard trehalose sample and (b) extracted trehalose sample

Effect of Environmental Stresses to Trehalose Biosynthesis

Growth of isolate at 4% salt concentration was indicated the maximum trehalose accumulation which was reported nearly 2.67µg/mg of cell mass, whereas, at the lesser than or greater than 4% salt concentration, trehalose was very negligible (Figure 8a). Isolate was found to accumulate 3.46µg/mg trehalose at the 8.5 pH, while less accumulation was reported at other tested pH values. (Figure 8b) Temperature was drastically affected the accumulation of trehalose. Maximum 6.75µg/mg trehalose was testified at 50 ºC temperature followed by 6.62 µg/mg at 45 ºC, 4.47 µg/mg at 40 ºC, 1.13µg/mg at 37 ºC and 0.96 µg/mg at 30 ºC (Figure 8c).

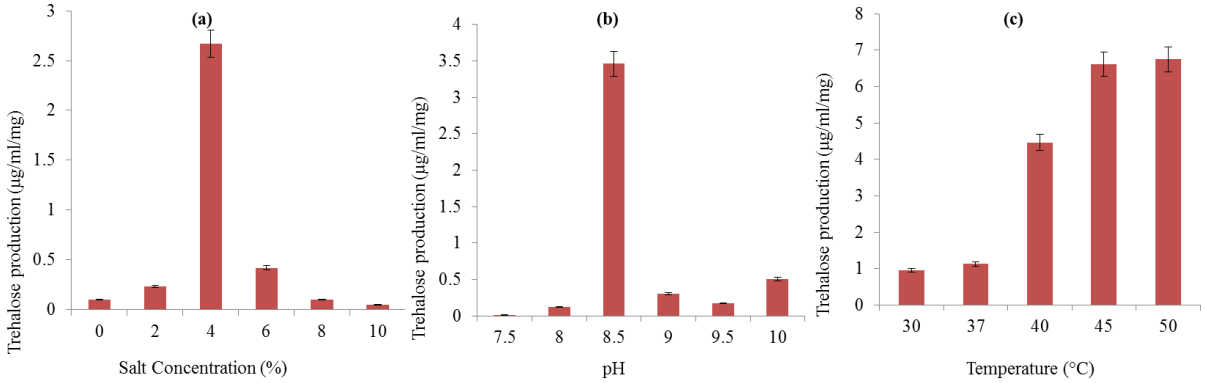

Figure 8: Effect of various stresses in trehalose synthesis (a) Salt (NaCl) concentrations (b) pH (c) Temperature

Discussion

Pseudomonas stutzeri is a ubiquitous bacterium with a high degree of physiological and genetic adaptability. It is present in a large number of different natural environments ranging from garden soil to pollutant soil and from sea sediment to aquifer. Like other *Pseudomonas* species, *P. stutzeri* is involved in environmentally important metabolic activities. Some of its major tasks are metal cycling and degradation of biogenic and xenobiotic compounds and metabolite production. Marine isolates required the salt concentration and alkaline environment was remarkable properties to sustain (Lalucat *et al.*, 2006). The species has a wide range of growth temperatures. Most strains grow at 40°C to 41°C, some at 43°C. The optimum temperature for growth is approximately 35°C. (Palleroni *et al.,* 1970), and it is concur with present investigation. The strains are nutritionally versatile, using some carbon compounds seldom utilized by other *Pseudomonas*. Nutritional studies of more than 150 carbon substrates used by *P. stutzeri* strains have been carried out by Stanier *et al.,* (1966). So, the versatility of *P. stutzeri's* metabolism allows it to grow in a variety of conditions, including marine habitat by accumulation of osmolytes.

Halophilic bacteria equilibrate their osmotic potential via the accumulation of inorganic ions and have consequently adapted their cellular environment to tolerate high internal concentrations of inorganic ions, e.g. Proline, trehalose, betain etc. (Eisenberg and Wachtel, 1987; Galinski and Truper, 1994; Ventosa *et al.*, 1998). Isolate is indigenous to saline soils of marine habitat; tolerance to high salinity is associated with intracellular accumulation of free amino acids and under extreme salt stress, with selective internal concentration of proline. *Pseudomonas stutzeri* is accumulated the intracellular proline by the synthesis of α-ketoglutaric acid to glutamate followed by glutamate to proline pathway. Proline is the main organic osmotic solute in certain Gram-positive species e.g. *Salinicoccus roseus* and *Salinicoccus hispanicus* (Galinski and Trüper, 1994; Severin *et al.*, 1992). Intracellular proline production in isolate was comparable in stress condition to the available reports of *Streptomyces griseus* and *Streptomyces californicus,* (Killham and Firestone, 1984). Over production of proline is due to the activation and mutation in ProB gene encoded for γ-glutamyl kinase, which is the first enzyme of the proline synthesis pathway (Sleator *et al.,* 2001). The pH is the key stress in the accumulation of intracellular proline by isolate. Alkaline pH specifically 8.5 causes the maximum accumulation. Salt effect in proline accumulation was similar to the earlier investigation (Qurashi and Sabri, 2013) and temperature affect marginally in accumulation of proline. Precursor effect was found positive suggest the synthesis is carried out by α-ketoglutaric acid pathway by isolate.

Trehalose has been proposed to play an important role in the ability of yeast to withstand environmental stresses (Managbanag and Torzilli, 2002). Trehalose produced as an osmoprotectant is commonly used as protein stabilizers (Azizi *et al.,* 2011). Trehalose thought to be synthesized either UDP-glucose pathway or by maltooligosaccharide pathways (Teramoto *et al.,* 2008). The isolate was found to ferment the maltose and trehalose sugars suggest the positive inputs in the synthesis of trehalose. Accumulation of trehalose can protect proteins and cell membranes from inactivation or denaturation caused by a variety of stress conditions, including desiccation, oxidation, heat, cold, and dehydration (Elbein *et al.,* 2003). However, heat or high temperature causes the high accumulation of trehalose in yeast (Attfield, 1987). Similarly, in isolate, the greater trehalose accumulation at 50°C than at alkaline pH and high salt concentration was reported. The reason is the high cellular concentration of trehalose contribute to the acquisition of stress tolerance (Torzilli, 1997) and important in the cellular induction of thermotolerance (Singer and Lindquist, 1998).

Conclusion

Marine bacteria are subjected to periodic wetting and drying by salt water and variation of pH and temperature leads to amassing the intracellular stress tolerant proteins and compatible solutes. Isolated moderately haloalkiphilic *Pseudomonas stutzeri* strain was a vital source of proline and trehalose. Alkaline pH increased the accumulation of more proline, while high temperature was reported to cause the high accumulation of trehalose and thereby contribute to the acquirement of stress tolerance of bacterium against stresses. Biomass of *Pseudomonas stutzeri* are the byproduct at industrial application can be potential sources of proline and trehalose. However the economic extraction and viable

purification processes need to be optimized before applied in large scale. Positives impact of proline precursor to induce the intracellular proline concentration and confirmation of proline by HPLC will help for the development of prototypes for large scale exploration of isolate.

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