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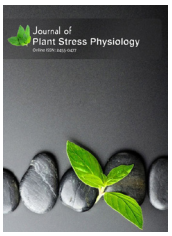

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Salinity endurance of marine macro Rhodophycean algae with special emphasis on *myo*-inositol biosynthesis: An enzymological analysis from *Halymenia venusta* Børgesen

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ABSTRACT

Altered salinity is one the most important perils encountered by marine plants inclusive of algae. Under hyper saline condition, plants accumulate several stress relieving osmolytes including *myo*-inositol, the most widespread cyclitol in plants. The present communication reports the occurrence of *myo*-inositol biosynthesis in six different Rhodophycean seaweeds growing under stressful intertidal habitats of the Okha coast (Gujarat, India), on the basis of a study conducted on two marker enzymes of *myo*-inositol biosysnthesis [L-*myo*-inositol-1-phosphate synthase (MIPS)and D/L-*myo*-inositol-1-phosphate phosphatase (MIPP)]. Both enzymes were partially purified from *Halymenia venusta* to about 27 fold (MIPS) and 39 fold (MIPP) over the homogenate following low-speed centrifugation, 30-75% ammonium sulphate fractionation, successive chromatography through DEAE-cellulose / CM-Cellulose, Sephadex G-200 and BioGel 0.5m / Ultrogel AcA 34 columns. The temperature and pH optima for MIPS and MIPP were similar and were recorded to be 35°C and 7.5 respectively. For MIPS, D-glucose-6-phosphate and NAD were the exclusive substrate and coenzyme respectively and D/L-I-1-P was the sole substrate for MIPP. The Km values for D-glucsoe-6-phosphate and β-NAD were recorded to be 3.599 mM and 0.2366 mM respectively, while the Km value for D-I-1-P was found to be 0.4070 mM. K^+ exhibited slight stimulatory effect while Li^+ was strongly inhibitory for both the enzymes. Ca^{2+} on the other hand exhibited a mild stimulatory and Cd^{2+} reduced activities of both MIPS and MIPP. MIPP was especially stimulated by Mg^{2+}. On the other hand Cu^{2+} and Hg^{2+} were strong inhibitors for both the enzymes. A steady and proportionate increase in the content of free *myo*-inositol was observed along with the elevated levels of recorded salinity.

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ABBREVIATIONS: CBB- Coomassie Brilliant Blue, G-6-P- D-Glucose-6-phosphate; D-I-1-P- D-*myo*-inositol-1-phosphate, I-1-P- *myo*-Inositol-1-phosphate; ME- 2-Mercaptoethanol; MIPP- D/L-*myo*-inositol-1-phosphate phosphatase, MIPS- L-*myo*-inositol-1-phosphate synthase; PSU- Practical Salinity Unit.

INTRODUCTION

Salinity is a major abiotic factor which is crucial to aquatic ecosystems and is responsible for the local and/or regional distribution of marine macrophytes. Unlike other abiotic factors viz, temperature, irradiance, nutrients and pH, salinity holds a special position and plays a key role in affecting not only the metabolism but also the growth and survival strategies of several marine macrophytes including seaweeds.

Seaweeds normally occupy rocky intertidal zones and are continuously subjected to desiccation, extreme temperatures and altered salinity. Eukaryotic marine algae are known to possess great plasticity and adaptability to most abiotic stresses including the stress imposed as a result of prolonged growth in hyper saline condition [1]. Hyper salinity affects aquatic macrophytes in several ways which includes osmotic stress, ion stress and ionic ratio imbalance [1]. Plants are known to accumulate several inositol derivatives in response to salt stress which act as osmolytes. These accumulated osmolytes include glycerol, betain, proline, trehalose, raffinose and *myo*-inositol [2]. *myo*- Inositol is the most widespread cyclitol in plants having a number of functions including amelioration of salinity stress [3]. It has been reported earlier that *myo* inositol along with its isomers and methylated derivatives are

interconnected with lenience to drought and/or salinity tolerance in plants [4]. Taking all these facts into consideration 'salinity-driven' enhanced synthesis of this cyclitol in plants has become an interesting and highly productive area of research in recent years even at the genetic level [5]. The biosynthesis of *myo* inositol is essentially reliant on two enzymes, out of which the first one is a rate limiting NAD^+-dependent oxidoreductase (L-*myo*-Inositol-1-phosphate synthase) and the second one is a Mg^{2+} dependent phosphatase (D/L- *myo*- Inositol-1-phosphate phosphatase).

L- *myo*-Inositol-1-phosphate synthase (D-glucose-6-phosphate-1L-*myo*-inositol-1-phosphate synthase; MIPS; EC: 5.5.1.4) is the prime enzyme that catalyzes the irreversible conversion of D-Glucose- 6-phosphate (G-6-P) to L-*myo*-inositol-1-phosphate (I-1-P) [6] which is subsequently dephosphorylated by inositol monophosphatase (MIPP; EC: 3.1.3.25) to produce free *myo*-inositol [7]. In plants two isoforms of MIPS have been reported till date [8] and a careful scrutiny of the available literature reveals that although studies on MIPS has been carried out from several prokaryotic as well as eukaryotic systems[8,9,10,11,12,13,14,15,16], investigations dealing with MIPP are less abundant and restricted. Regrettably, majority of the investigations involving these two marker enzymes have been carried out primarily in phanerogams [13]. Non vascular marine cryptogams, which occupy a vast biomass in any marine aquatic flora, have received very little attention in such studies.

Taking all these facts into consideration the present study was aimed towards understanding the relationship between inositol biosynthesis and salinity stress in marine macrophytes growing under hyper saline conditions.

MATERIALS AND METHODS

Plant Material

The experimental red algal samples, viz. *Coelarthrum muelleri*, *Gracilaria corticata*, *Grateloupia indica*, *Halymenia venusta*, *Kappaphycus alvarezii*, *Solieria robusta* were collected in winter (January 2019) season from the Arabian Sea coast of Okha, Gujarat (latitude 22°28′N and longitude 69°05′E ; Temperature range 21.5 to 30°C), India and [Table 1; Figure 1(a-f)]. The samples were brought to the laboratory in a portable ice-chamber and were kept frozen under -20°C until use. Specimens were identified using standard literature [17] and with direct comparison with herbarium specimens available in the Cryptogamic Section of 'Central National Herbarium (CAL)', Shibpur, Howrah (West Bengal, India). Voucher specimens were deposited in the Departmental herbarium of Barasat Govt. College.

Extraction of Enzymes comparing MIPS and MIPP activities in the Collected Sample

Sequestration of cytosolic MIPS and MIPP from experimental samples of was carried out by homogenization of tissues in two volumes of the extraction buffer [50 mM Tris-acetate, pH 7.5 containing 0.2 mM β-mercaptoethanol (ME)] followed by a centrifugation at 11,400 g for 30 min in a Remi C-24 BL cold centrifuge. Discarding the pellet the supernatant fraction was collected (Low-speed supernatant fraction). These preparations were dialyzed separately for 18 hours against 50 mM Tris-acetate, pH 7.5 containing 0.2 mM ME under 0 to 4°C. The dialyzed fractions were recovered from the corresponding dialysis bags and used as the enzyme source(s) for the preliminary screening experiments. The alga showing higher *myo*-inositol and cytosolic MIPS and MIPP activities were selected for further studies.

Partial Purification MIPS and MIPP

Partial purification of cytosolic MIPS and MIPP from thalli of *Halymenia venusta* was carried out separately at 0 to 4°C. Buffer-washed *Halymenia venusta* thalli(40 g for each enzyme separately) was homogenized in 3 volumes of a buffer (50 mMTris-acetate, pH 7.5, containing 0.2 mM ME; hitherto termed as standard buffer). The homogenate was spun at 11,400 g for 30 min and the supernatant was collected (Low-speed supernatant). The low-speed supernatant was then fractionated with ammonium sulfate [$(NH_4)_2SO_4$] at the level of 30-75 % saturation. The consequential $(NH_4)_2SO_4$ pellet was dissolved in nominal volume of standard buffer and dialyzed for 18 hours against the same standard buffer. Chromatography of the dialyzed fractions of MIPS and MIPP were done individually expending an anion-exchange matrix DEAE-cellulose (for MIPS, obtained from GENEI, Bangalore, India) in a glass column (1.2 x12.0 cm) and cation-exchange matrix CM-cellulose (for MIPP, obtained from GENEI, Bangalore, India) in a glass column (1.2 x13.0 cm). The effluent was collected and the column was washed with one bed volume of the standard buffer. The elution of the adsorbed proteins was done by a linear gradient of 0 to 0.5 M KCl prepared in standard buffer. The MIPS active DEAE-cellulose

Table 1: Distribution of L-*myo*-inositol-1-phosphate synthase (MIPS) and D/L-*myo*-inositol-1-phosphate phosphatase (MIPP) in some members of marine Rhodophycean algae (whole plants)
[Specific activity defined as n mol I-1-P produced (mg protein)$^{-1}$ h^{-1} in a dialyzed 11.4 K supernatant for MIPS and n mole P_i hydrolyzed (mg protein)$^{-1}$ h^{-1} for MIPP; FW = Fresh Weight]

Samples	Family	Mean Salinity of water (PSU)	Free *myo*-inositol content (mg) g^{-1} FW	Total protein extracted [(mg) g^{-1} FW]	Specific activity of MIPS [n mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]	Specific activity of MIPP [n mol P_i hydrolyzed (mg protein)$^{-1}$ h^{-1}]
Halymenia Venusta	Halymeniaceae	37.68±0.48	3.316±0.318	6.159±0.406	355.73±15.18	167.42±19.27
Gracilaria corticata	Gracilariaceae	33.18±0.93	2.156±0.485	5.364±0.287	287.34±21.27	148.36±12.29
Solieria robusta	Solieriaceae	32.83±0.36	2.059±0.184	6.374±0.506	186.25±16.44	78.91±24.52
Grateloupia Indica	Halymeniaceae	30.38±1.97	1.872±0.234	4.643±0.604	108.63±17.16	64.21±17.60
Coelarthrum Muelleri	Rhodymeniaceae	34.87±2.16	2.343±0.521	3.826±0.409	278.88±7.92	153.46±24.84
Kappaphycus alvarezii	Solieriaceae	29.23±1.92	1.085±0.352	4.167±0.371	87.56±11.47	98.05±13.71

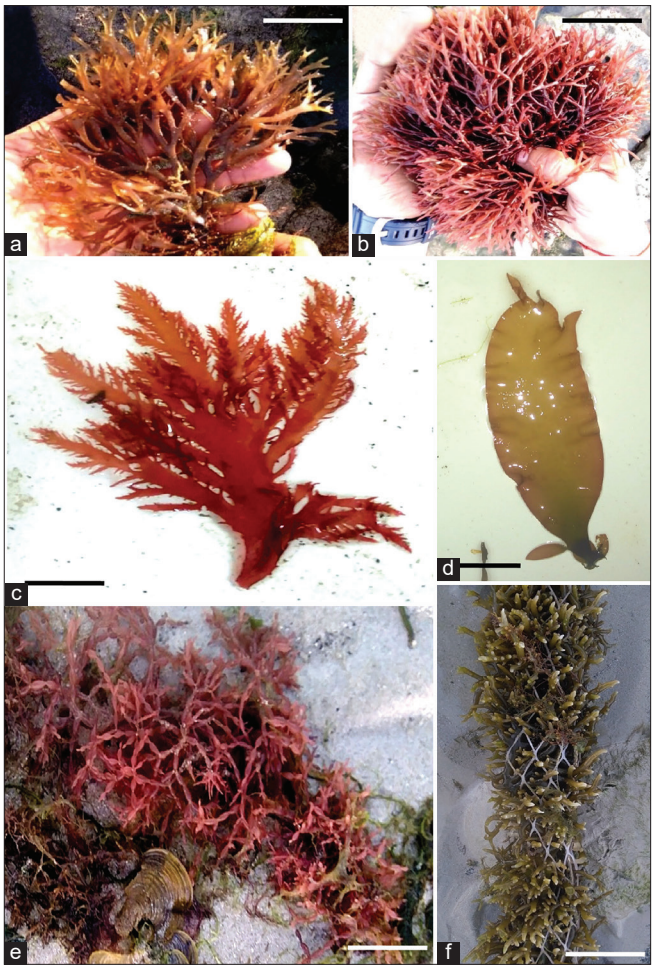

Figure 1: Exomorphology of the collected red algal samples [Scale bar: 5 cm]: 'a'–*Gracilaria corticata* J.Agardh; 'b'–*Solieria robusta* (Greville) Kylin; 'c'–*Halymenia venusta* Borgesen; 'd'–*Grateloupia indica* Borgesen; 'e'–*Coelarthrum muelleri* (Sonder) Børgesen; 'f'–*Kappaphycus alvarezii* (Doty) L.M.Liao

fractions and MIPP active CM-cellulose fractions were pooled together in separate experimental sets and subsequent gel filtration was carried out in subsequent separate experiments through Sephadex G-200 (obtained from Amersham Pharmacia Biotech, UK) columns (0.8 x 14.0 for MIPS/16.0 cm for MIPP). The respective MIPS and MIPP active Sephadex G-200 fractions were pooled separately and molecular sieve chromatography was performed through BioGel A0.5m (for MIPS, obtained from Bio-Rad, USA) column (0.8 x10.0 cm) and Ultrogel AcA 34 (for MIPP, Sigma, USA) column (0.8 x9.0 cm). The active MIPS BioGel A 0.5m fractions were pooled together as the MIPS concerned and used as the enzyme preparation for biochemical characterization of MIPS. The active MIPP Ultrogel AcA 34 fractions were pooled together as the MIPP preparation and used as the enzyme source for biochemical characterization of MIPP.

Assay of L-*myo*-inositol-1-phosphate Synthase

Assay of L-*myo*-inositol-1-phosphate synthase was performed by the method of Barnett et al. [18]. In a total volume of 0.5 ml, the incubation mixture comprised of 500 mM Tris-acetate (pH 7.5), 140 mM NH_4Cl, 8 mM NAD, 50 mM ME, 50 mM G-6-P and an appropriate protein aliquot (30-100 µg). The reaction was initiated by incorporation of substrate (G-6-P) promptly after the addition of the enzyme. Duplicate tubes were run along with a blank (minus enzyme) and also a zero minute control in which 200 µl of 20 % (w/v) chilled trichloroacetic acid (TCA) was loaded in the incubation mixture prior to addition of the enzyme. The enzymatic incubation was allowed for 60 min at 37^{0}C in a thermostatic digital incubator. After 60 min the reaction was stopped by the addition of 200 µl of chilled 20 % (w/v) TCA. Two such sets (set A – periodate and set B – non-periodate) were run concurrently each having one blank, one zero minute control and two experimental tubes. The quantity of the enzymatic product was estimated by periodate oxidation followed by the estimation of inorganic phosphates.

On completion of the enzyme incubation, this was treated with 0.7 ml of 200 mM sodium metaperiodate ($NaIO_4$) and further incubated for 60 min at 37 ^{0}C. Then, 1.4 ml of 1(M) Na_2SO_3 (freshly prepared) was added in case of set–A to destroy excess of $NaIO_4$. In set-B, distilled water was added instead of $NaIO_4$ and Na_2SO_3 to maintain the volume with that of set-A.

Inorganic phosphate was hydrolysed from *myo*-inositol-1-phosphate during oxidation which could be subtracted considering the blank or zero minute control from the experimental value in set-A. Hydrolysis of phosphate from G-6-P by contaminating phosphatase (if any) was measured by subtracting the value of the blank or zero minute control from the experimental value in set-B. Product dependent cleavage of inorganic phosphate was estimated by subtracting the corrected value of set-B from that of set-A. Inorganic phosphate was estimated by the method of Chen et al. [19].

Assay of *Myo*-inositol-1-phosphate Phosphatase

The *myo*-inositol-1-phosphatase activity was assayed by the method of Eisenberg Jr. [7]. In a total volume of 1.0 ml, the incubation mixture contained 500 µl of 0.67 M Tris-HCl (pH 7.4) containing 50 mM $MgCl_2$, 400 µl of 154 mM KCl, 50 µl of 100 mM D-*myo*-inositol-1-phosphate and an appropriate protein aliquot (50 µl / 50 -200 µg). The MIPP reaction was initiated by adding of substrate immediately after the enzyme. The complete assay mixture was maintained along with an appropriate blank (sans enzyme) and a zero-minute control in which 250 µl of 20% (w/v) chilled TCA was added prior to the addition of the enzyme. The enzymatic incubation was carried out for 60 min at 37^{0}C. After 60 min of incubation, the reaction was terminated by 250 µl of 20 % (w/v) chilled TCA. Two such sets (set A- experimental and set B- control), where *myo*-inositol- 1-phosphate was replaced by an identical volume and concentration of D-fructose-6-phosphate were carried out simultaneously each having one blank, one zero-minute control and one with complete assay mixture. Inorganic phosphate was estimated by the method of Chen et al. [19]. As 1 mol of *myo*-inositol-1-phosphate contains 1 mol of inorganic phosphate, the total mole number of inorganic phosphate released was equal to the total mole number of *myo*-inositol-1-phosphate hydrolyzed.

The specific activity was defined either as n mol *myo*-inositol-1-phosphate released / hydrolyzed $(mg)^{-1}$ protein h^{-1}.

Determination of Protein

Protein was determined according to the method of Bradford (1976) with BSA as a standard [20].

Determination of Molecular Weights of MIPS and MIPP

Approximate molecular weight of the native MIPS and MIPP obtained from the respective samples was determined by gel-filtration through Sephadex G-200. The Sephadex G-200 was suspended in 50 mM Tris- acetate (pH 7.5) and packed in a column of suitable size and calibrated with 1 ml each of marker proteins e.g., catalase (221.6 kDa); bovine serum albumin dimer (133.352 kDa); phosphorylase-b (97.4 kDa); ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.3 kDa). The void volume was determined with blue dextran 2000 (1 mg per ml). All standards were loaded in the column separately and fractions (0.75 ml) were collected at a flow rate of 0.75 ml per 6 min. Each individual protein peak was located by spectrophotometric scanning at 280 nm in a Systronics UV-Visible spectrophotometer. A standard curve was prepared by plotting relative elution volume of proteins against their respective log molecular weights.

Gel Electrophoresis and Slice Assay

Polyacrylamide gel electrophoresis of MIPS and MIPP under non-dissociating condition followed by gel-slice assay was performed using 1.5 mm thick gel slab following the method of Bollag et al. [21].

For the MIPS/MIPP assay from the gel, replicate gels were run. One of the gels was stained with CBB (R-250) in order to visualize the protein bands and the other was sliced to 5 mm fragments. The enzyme from each of the slice was then extracted with 250 µl of 50 mM Tris-acetate buffer (pH 7.5) for 30 min at 0 ^{0}C to 4 ^{0}C.

Isolation and Quantification of Free *Myo*-inositol

Free *myo*-inositol was isolated by the method of Charalampous and Chen [22] and free *myo*-inositol was estimated spectrophotometrically according to the method of Gaitonde and Griffiths [23].

Stability of Enzymes under Laboratory Condition

Both MIPS (obtained from 11, 400 g supernatant fraction and BioGel 0.5m fraction) and MIPP (obtained from 11, 400 g supernatant fraction and Ultrogel AcA 34 fraction) preparations were separately stored in properly labelled Eppendorf tubes (12 x 4). Each tube contained 0.3 ml and was stored at -20°C until further use. Assay of both enzymes were performed from 0 day to day 60 day (maintaining an interval of 5 days) taking protein from each individual vial.

Determination of Salinity

Sea water of different locales (algal sample collection spots) were collected and stored in properly labelled sterile vials and brought to the laboratory under chilled condition. The practical salinity unit (PSU) values were measured in the laboratory using a Systronics Compact Water Analyzer (Model no: 371). This equipment used the measurements of electrical conductivity (EC) and temperature to provide a measurement of salinity of water, i.e., Practical Salinity Units (PSU). All measurements were recorded in triplicate.

Determination of Km and Vmax Values

The effect of substrate concentration and kinetic analyses for partially purified MIPS were carried out using D-glucose-6-phosphate (substrate) concentrations in the range of 0.0 to 10.0 mM at an interval of 1.0 mM. All data regarding specific activity of MIPS corresponding to the respective substrate concentration were analyzed by means of non linear regression kinetics using Prism 5 (Grapg pad) software package. Similar experiments for MIPP were also carried out using D-*myo*-inositol-1-phosphate (substrate) concentrations in the range of 0.0 to 1.0 mM at an interval of 0.1 mM. The data regarding specific activities of MIPP corresponding with respective substrate concentrations were also analyzed using Prism 5. The effect of coenzyme concentration (NAD) and kinetic analysis for MIPS was carried out using NAD in the range of 0.0 to 1.0 mM (maintaining an interval of 0.1 mM). Enzyme incubation and assay was carried out following methods described earlier. Specific activities of MIPS corresponding with the respective coenzyme concentrations were also analyzed using Prism 5 (Grapg pad). All results were obtained directly following software analyses.

Determination of pH Reliance

MIPS and MIPP incubation were carried out separately with Tris-acetate (for MIPS) and Tris-HCl (for MIPP) buffer having pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 keeping other assay components unchanged in all experimental sets.

Effect of Monovalent and Divalent Cations

The different monovalent and divalent cations was incubated separately at 37°C (concentration of 5 mM each)with chloride anions of the respective metals in addition to all assay components in individual experimental set for both the enzymes separately keeping a control set also where no metal ion was added.

Statistical Data Analysis

All experiments were repeated thrice and quantitative data were expressed as means ± SE of three replicates. As far as the evaluation of the kinetic parameters of MIPS and MIPP are concerned, the Michaelis–Menten constant (Km) was analysed. In order to establish relation between the substrate (D-G-6-P / D-MIP for MIPS and MIPP respectively), coenzyme (NAD for

MIPS) and the rate of the enzyme catalysed reaction, all the parameters were calculated by nonlinear regression method using the software Prism 5 (GraphPad).

RESULTS

Marine Rhodophycean macro algal samples were collected from different spots (mid and low intertidal pools) of the Okha coast (within a radius of 10 Km) in the month of January, 2019 when the seaweed growth is at its peak and the salinity levels are also higher. Specimens collected from locations of highest recorded salinity were chosen for experiments. Preliminary experiments were conducted to screen the selected specimens for their inositol synthesizing potential and the two marker enzymes were assayed following the methods described earlier. The results depicted in Table 1 show that both the enzymes of *myo*-inisitol biosynthesis were functional with variable specific activities in all representative species tested. The results also elucidate the fact that maximum activities of MIPS and MIPP were recorded in *Halymenia venusta* and such elevated activity also corresponded with the higher free *myo*-inositol content (Table 1). It is noteworthy to mention here that during collection, all specimens were housed in isolated intertidal pools and/or puddles under varying salinity regimes (PSU value ranged from 29.23 to 37.68) of the near shore environment.

Starting from the crude homogenate both MIPS and MIPP were gradually purified using low speed centrifugation, ammonium sulphate fractionation (30-75%) and chromatography on DEAE-cellulose / CM cellulose, Sephadex G-200 and BioGel 0.5m / UltrogelAcA 34 (Table 2A and 2B). Comparative elution profiles are depicted in Figure 2 (a-f). The overall purification of cytosolic MIPS and MIPP was around 27- and 39-folds respectively over homogenate with about 40% and 35% yields (Table: 2A and 2B). In case of this red alga, D-glucose-6-phosphate served as the principal substrate for MIPS. However, a very faint activity was recorded when D-glucose-6-phosphate was replaced by D-glactose-6-phosphate (Table 3). In contrast, D-*myo*-inositol-1-phosphate served as the exclusive substrate for MIPP (Table 3). Other hexose phosphates (viz, D-glucose- 6- phosphate, D-fructose-6- phospahte, etc.) under identical concentrations were completely ineffective as substrates (Table 3). It is noteworthy to mention here that although partially purified cytosolic MIPS from *H. venusta* exhibited coenzyme specificity primarily on NAD, a basal level of activity was observed in presence of few other coenzymes tested (viz, FAD, NADP and

Table 2A: Partial purification of MIPS from *Halymenia venusta* (40 g plant material) Results are mean±SE (in the parenthesis) of three replicates

Fraction	Total protein (mg)	Specific activity [n mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]	Total activity [n mol I-1-P produced h^{-1}]	Recovery (%)	Fold purification
Homogenatefraction	246.00 (16.47)	338.43 (18.25)	83253.78 (125.41)	100.00 (22.19)	1.00 (0.08)
11,400 x g supernatant fraction	160.095 (6.45)	386.11 (14.36)	61814.28 (85.32)	74.24 (15.36)	1.14 (0.23)
30-75 % $(NH_4)_2SO_4$fraction	100.162 (17.63)	553.52 (21.44)	55441.67 (53.73)	66.59 (8.42)	1.63 (0.71)
DEAE-cellulosefraction	31.320 (13.11)	1606.75 (20.31)	50323.41 (132.49)	60.44 (5.64)	4.74 (1.08)
Sephadex G-200 fraction	14.530 (3.24)	2941.17 (30.78)	42735.20 (312.64)	51.33 (3.12)	8.69 (2.34)
Bio Gel A 0.5mfraction	3.634 (1.052)	9205.68 (436.10)	33453.44 (1217.25)	40.18 (1.86)	27.20 (4.16)

Table 2B: Partial purification of MIPP from *Halymenia venusta* (40 g plant material) Results are mean±SE (in the parenthesis) of three replicates

Fraction	Total protein (mg)	Specific activity [n mol$P_i$hydrolyzed (mg protein)$^{-1}$ h^{-1}]	Total activity [n mol$P_i$hydrolyzed h^{-1}]	Recovery (%)	Fold purification
Homogenate fraction	236.67 (20.16)	154.32 (18.26)	36522.29 (65.47)	100.00 (14.38)	1.00 (0.06)
11,400 x g supernatant fraction	148.64 (11.51)	186.74 (24.94)	27757.03 (46.82)	76.00 (10.41)	1.21 (0.17)
30-75% $(NH_4)_2SO_4$fraction	92.43 (23.27)	267.41 (34.36)	24716.70 (140.58)	67.67 (7.68)	1.73 (0.33)
CM-cellulose fraction	36.85 (8.55)	611.75 (56.64)	22542.98 (161.87)	61.72 (3.88)	3.96 (0.84)
Sephadex G-200	10.36 (4.06)	1338.07 (21.90)	13862.40 (237.12)	37.95 (3.04)	8.67 (1.52)
UltrogelAcA 34 fraction	2.14 (0.42)	6122.81 (58.24)	13102.81 (316.40)	35.87 (1.77)	39.67 (3.84)

Table 3: Specificity of substrates (MIPS & MIPP) and coenzyme (MIPS) [Results are mean ± SE of three replicates]

Substrate	Coenzyme	MIPS specific activity	MIPP specific activity
D-Glucose-6-phosphate	-	9134.46±512.74	0.00
D-Galactose-6-phosphate	-	365.48±58.67	0.00
D-Fructose-6-phosphate	-	0.00	0.00
D-Mannose-6-phosphate	-	0.00	0.00
D-Ribose-5-phosphate	-	0.00	0.00
D-*myo*-inositol-1-phosphate	-	-	5837.26±412.83
-	NAD	8968.61±621.45	-
-	NADP	326.41±38.56	-
-	FAD	412.23±18.90	-
-	FADP	310.16±37.16	-

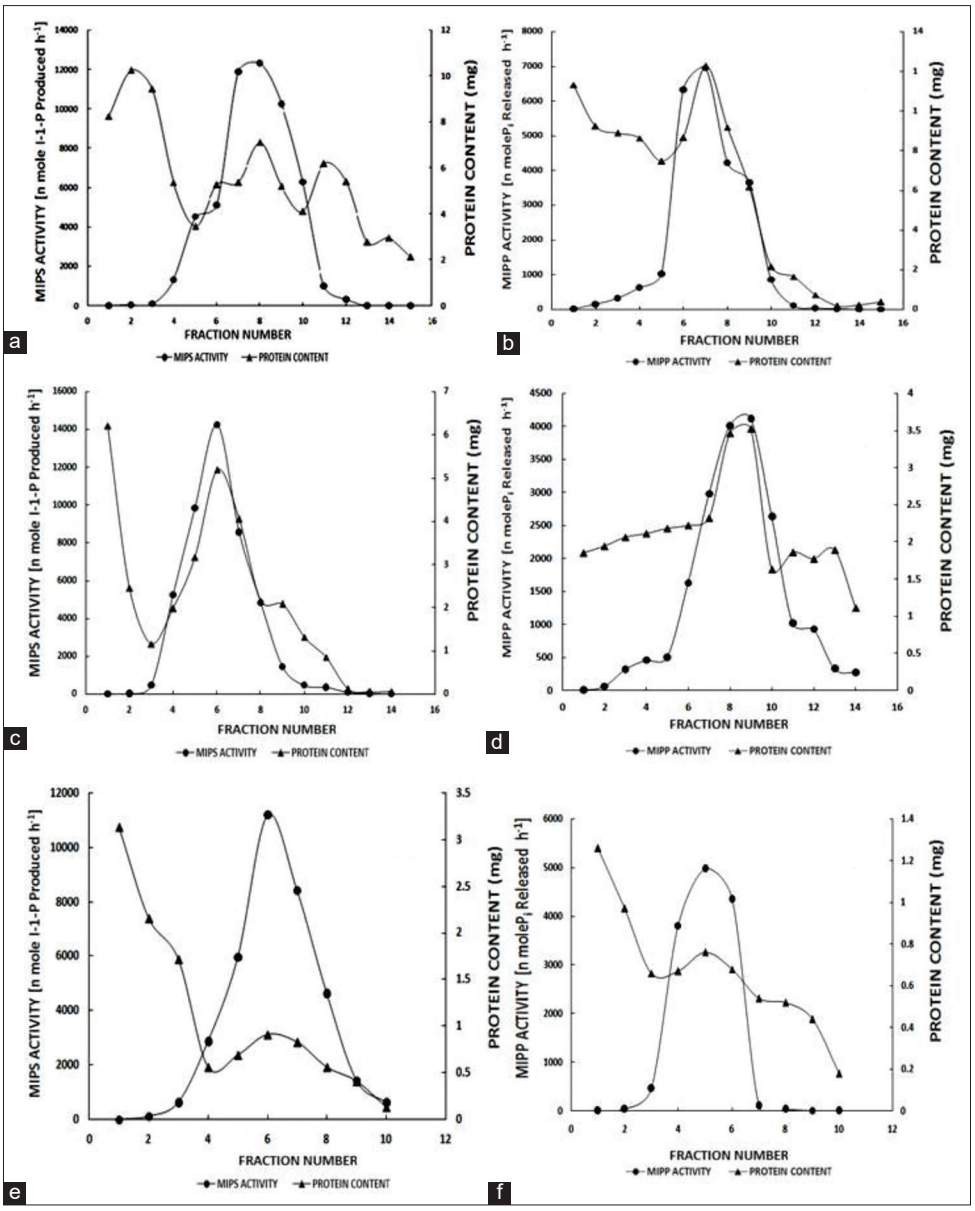

Figure 2: Typical elution profiles of *Halymenia venusta* cytosolic MIPS, MIPP and proteins chromatographed through different columns - 'a' - DEAE cellulose; 'b' - Sephadex G-200; 'c'-Biogel A-0.5m; ''d'-CM Cellulose; 'e'-Sephadex G-200; 'f'-Ultrogel AcA 34

FADP). This is indicative of the possible presence of a 'bound NAD' in this system. However, such 'built-in' NAD was in no way associated with thermo-tolerance, since no significant result could be obtained on removal of this endogenous NAD by means of charcoal treatment as conducted previously in animal systems [11].

The apparent molecular weights of cytosolic MIPS and MIPP as determined by gel filtration on a Sephadex G-200 column were 198 kDa and 193 kDa respectively. Native PAGE of partially purified cytosolic MIPS from *H. venusta* exhibited only one high intensity band along with few insignificant bands. By means of gel slice assay the major band was found to coincide with the enzymatic activity (Figure 3a). The PAGE profile of MIPP on the other hand exhibited two major high intensity bands along with two low intensity insignificant bands. Slice assay was effectively used to determine the actual band of interest (Figure 3b).

The stability of algal MIPS and MIPP varied at different stages of purification. The 11, 400 g supernatant retained activity for about 10-15 days in case of MIPS and 50-60 days for MIPP when stored at -20^{0}C. However, the BioGel 0.5m or Ultrogel AcA 34 fractions of MIPS and MIPP retained their activity up to 20 days and 60 days respectively when stored at identical temperature. Repeated freezing and thawing resulted in considerable loss of activity particularly in case of MIPS but not in MIPP.

When assay was carried out using increasing concentration of MIPS protein (0-90 μg) and MIPP protein (0-70 μg) it was revealed that the activity of both the enzymes increased linearly with respect to protein concentration of about 75 and 80μg respectively

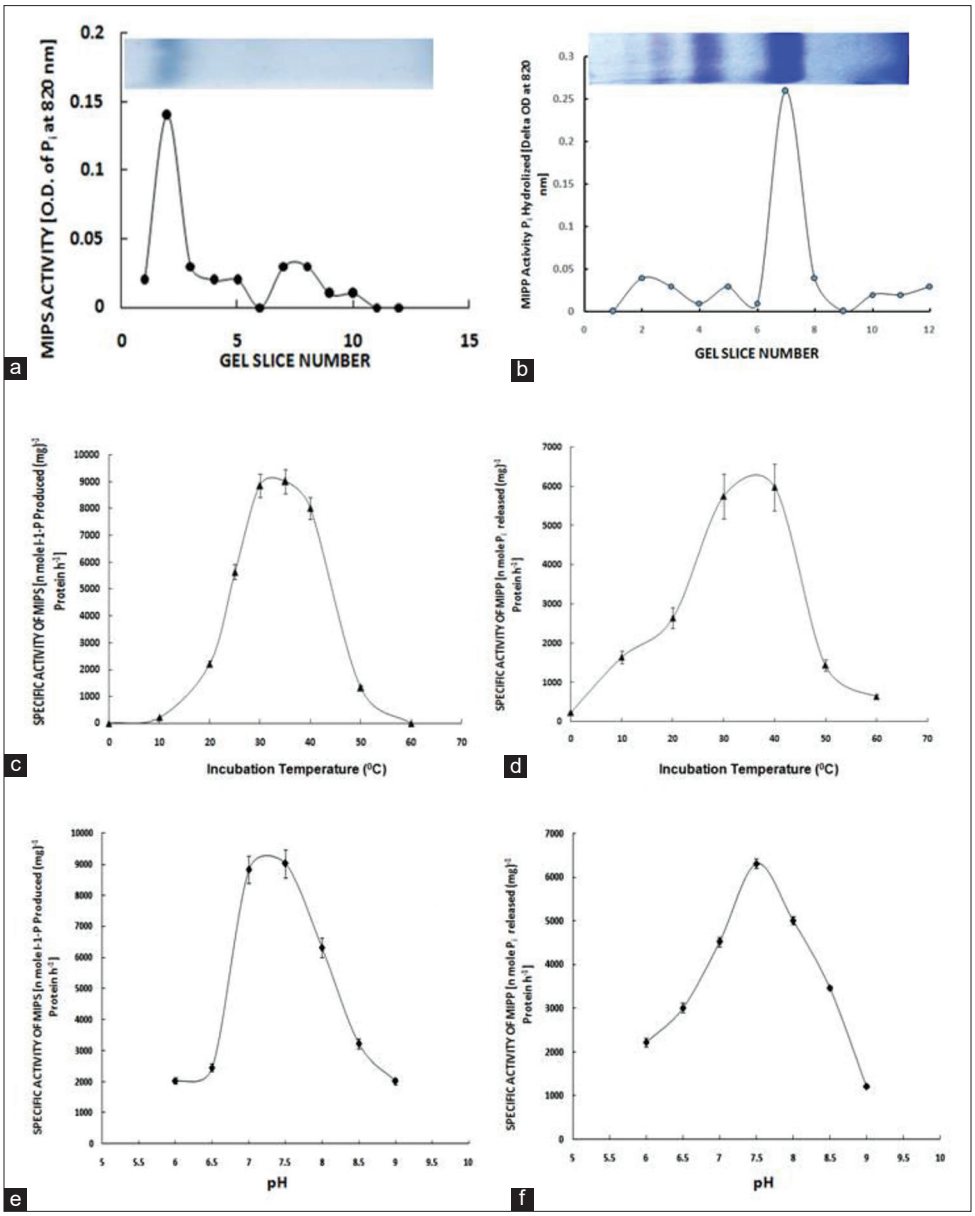

Figure 3: 'a'-Native PAGE profile showing the *myo*-inositol-1-phosphate synthase (MIPS) activity of the corresponding band; 'b'-Native PAGE profile showing the *myo*-inositol-1-phosphate phosphatase (MIPP) activity of the corresponding band; 'c'-Effect of incubation temperature on *Halymenia venusta* MIPS; 'd'-Effect of incubation temperature on *Halymenia venusta* MIPP; 'e'-pH dependence of *Halymenia venusta* MIPS; 'f'-pH dependence of *Halymenia venusta* MIPP

(Figure 3). Under standard assay conditions it was also observed that the MIPS and MIPP reactions proceeded linearly with time up to a maximum of 75 min in case of MIPS and 90 min in case of the MIPP (Figure 3). When thermal sensitivity experiments conferring to the cytosolic MIPS and MIPP was conducted at a temperature range of 0-60°C (with intervals of 5°C) it was revealed that both MIPS as well as MIPP were notably active between the temperature range of 20-40°C. The temperature maxima for MIPS were found to be 35-40°C, while that of the MIPP was 35°C. Minimum activity for both MIPS and MIPP was recorded at 10°C and 50°C respectively (Figure 3c,d).When 50 mMTris-acetate buffer was used in the pH range of 6.0-9.0, the algal MIPS exhibited optimum activity at pH 7.5, while optimum activity of MIPP was detected in between pH 7.0 – 7.5 (Figure 3e,f).

In case of MIPS, among the monovalent cations tested, NH_4^+ was highly stimulatory, K^+ exhibited an insignificant stimulatory role and in contrast, Li^+ was strongly inhibitory to this enzyme. Using similar concentrations of divalent cations it was observed that Mg^{2+} played an ephemeral stimulatory role and Ca^{2+} exhibited inhibitory effect (Figure 4a).

Among the monovalent cations tested, in case of MIPP, K^+ played a fleeting stimulatory role; Li^+ on the other hand was strongly inhibitory. Using identical concentrations of divalent cations it was observed that Mg^{2+} played an appreciable stimulatory role while Ca^{2+} exhibited moderate inhibitory effect. (Figure 4b).

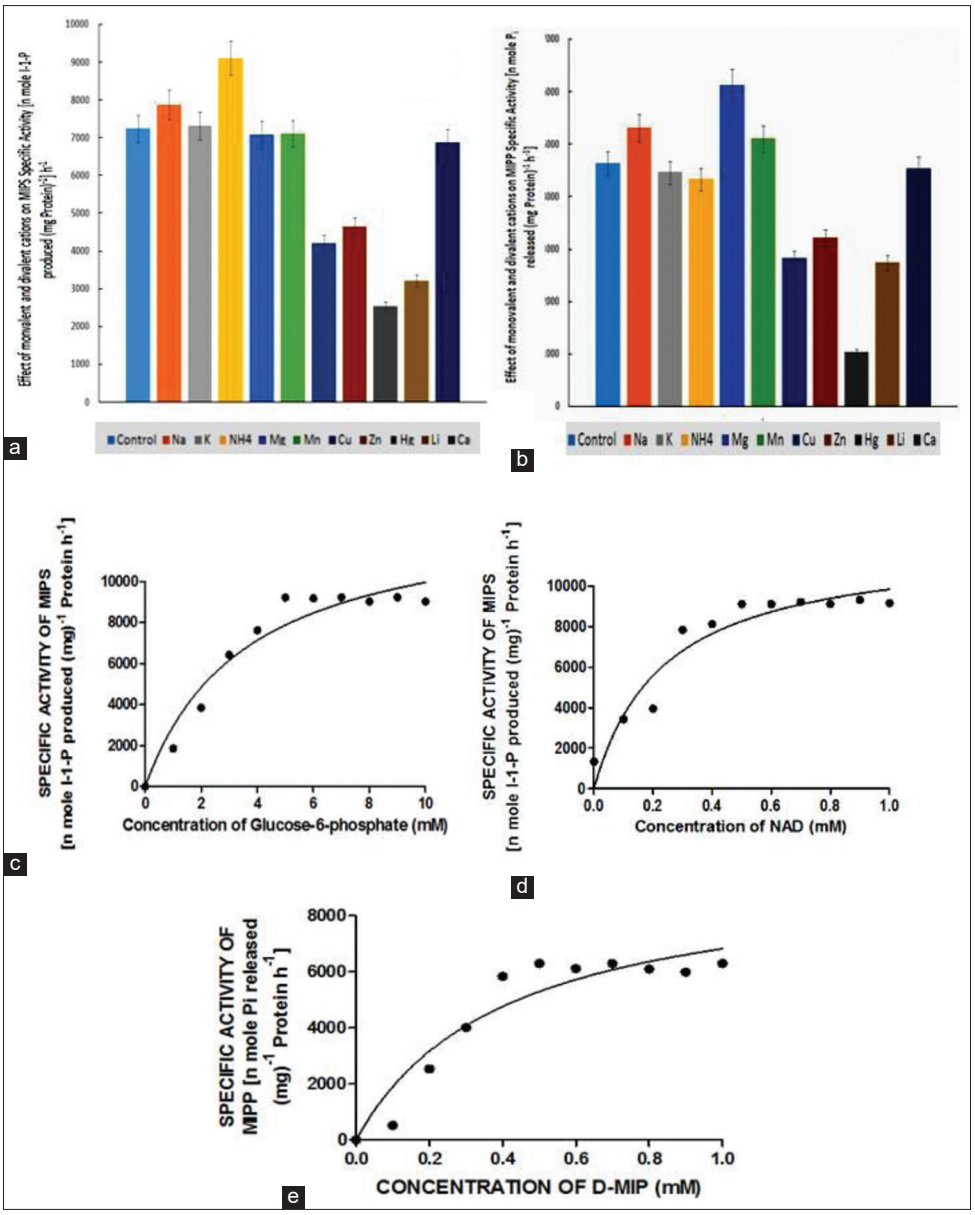

Figure 4: Kinetic parameters and effect of different metal ion concentrations on partially purified cytosolic MIPS and MIPP from *Halymenia venusta* **-** 'a'-Effect of monovalent and divalent cations on L-*myo*-inositol-1-phosphate synthase expressed as n mol-1-1-P produced per mg protein per h; 'b' - Effect of monovalent and divalent cations on MIPP activity as n mol MIP hydrolysed per mg protein per h; 'c'-Determination of the Km value for glucose-6-phosphate (G-6-P) of MIPS by non-linear regression kinetics, activity is expressed as n mol MIP produced per mg protein per h; 'd'- Determination of the Km value for the coenzyme NAD of cytosolic MIPS by non- linear regression kinetics, activity is expressed as n mol MIP produced per mg protein per h; 'd'-Determination of Km value for D-MIP of *H. venusta* MIPP by Nonlinear Regression Kinetics

The effect of substrate concentration and kinetic analyses of both MIPS and MIPP were carried out using D-G-6-P and D-MIP in the range of 0.0-10.0 mM and 0.0-1.0 mM respectively. The reaction rate was noted to increase with respect to D-G-6-P and D-MIP concentration of 1.0 to 4.0 and 0.1 to 0.2 mM correspondingly. The Km value for G-6-P was 3.599 mM (Figure 4c,d) and NAD was 0.2366 mM (Figure 4d) for MIPS. The Km value for D-I-1-P of MIPP was 0.4070 mM as determined by nonlinear regression kinetics (Figure 4e).

DISCUSSION

The coast of Okha situated in the 'Gulf of Kutch' is one of the most important places of interest for natural algal growth in India. This area is characterized by the presence of flat rocky intertidal belt, clear waters, several tide pools, puddles and crevices which offer an ideal habitat for various Phaeophytes and Rhodophytes. Taking into account their diversity, antiquity, presence of variable morphology, growth forms, capacity of sustaining hyper saline environments, great abundance in the

coast of Okha and also the paucity of information regarding their inositol synthesizing potential, members of Rhodophyceae were selected for the present investigation.

Table 1 portrays substantial activity of MIPS, MIPP along with the proportionate increase in the content of free *myo*-inositol in all the experimental algal genera. Scrutinizing the results depicted in Table 1, it appears that both the enzymes are functionally active in all the samples with variable titre. In this context, it has been observed that highest activity of both MIPS and MIPP was detected in *H. venusta* on the other hand the commercial red seaweed *Kappaphycus alvarezii* exhibited lowest activity of MIPS. Other experimental samples also exhibited comparatively lesser activity for both the enzymes (Table 1). Variation in the titre of MIPS and MIPP in the experimental samples may be due to genotypic and physiological differences and other accompanying factors like local salinity fluctuations in the tidal pools which are either partially or entirely isolated from the adjacent sea. It has been reported earlier that open oceans are characterized by a remarkable constancy in their composition [24]. In contrast, the inshore and near shore marine environments are subjected to influence of tides, freshwater inputs, evaporation and various anthropogenic contaminants [24,25,26].These results in considerable variability in salinity level on daily and seasonal basis. The results obtained in the present communication thus confirm the fact that fluctuating salinity is a local parameter which strongly influences the distribution, diversity and physiological performance of seaweeds, at least in case of red sea weeds. Work done by previous workers indicate that *myo*-inositol and other related cyclitols undergo isomerisation and methylation to produce O-methyl inositols, which play vital roles in salinity related responses and are known to accumulate upon salt stress in several halotolerant plant species including algae [4, 13, 26, 27, 28, 29]. The results obtained in the present investigation clearly elucidate the fact that *Halymenia venusta* along with other Rhodophyceae members also support the salt-induced enhancement of MIPS and MIPP as reported earlier in higher plants by Ray Choudhury et al. [30]. However, it cannot be stated with certainty whether *myo*-inositol is the only cyclic polyol which is involved in stress protection, the possible involvement of other cyclic polyols (D-ononitol, D- pinitol etc.) along with *myo*-inositol cannot be ruled out.

Since the cytosolic enzyme MIPS exhibited a basal activity in absence of NAD^+(Table 3) as an assay component, it can be presumed that this partially purified MIPS from *Halymenia venusta* did possess a 'built in' NAD^+ associated with thermal stability in its molecular architecture, like the MIPS obtained from mammalian adult brain [11] and from *Euglena gracilis* [31]. During the course of the present investigation we attempted to detect its actual role in the investigated sample following the method of activated charcoal treatment and non-treatment experiments, as reported earlier [11]. Incidentally, even after several repetitive trials, concurrence with the previous results could not be obtained. Hence, the rationale of the presence of such 'built-in' NAD^+ in *Halymenia venusta* is still obscure and its real metabolic significance is yet to be unravelled.

The MIPS and MIPP exhibited temperature optima of 35°C, which is in complete congruence with majority of the published reports in various plant and animal systems [13]. The enzymes exhibited maximum activity within a pH range of 7.0 to 7.5 with pH optima of 7.5. This is also in complete agreement with previous reports [7, 29, 30, 31].

In case of cytosolic MIPS obtained from *Halymenia venusta*, the K_m value for G-6-P was recorded to be 3.599 mM. This is in slight disagreement with previous reports obtained in lower eukaryotes and in mammalian systems. In case of rat testes enzyme, the K_m was found to be 3.89 mM [9], while in case of *Euglena gracilis* [31] and yeast [32], the recorded K_m values were much lower (2.1 mM and 1.18 mM respectively) in comparison to the value obtained in the present study. With respect to the concentration of the coenzyme (NAD) of MIPS, the K_m value for NAD^+ was found to be 0.2366 mM, which is nearly identical in comparison to the values recorded for the same enzyme from other sources [13]. The K_m value for MIP was found to be 0.4070 mM which appeared much higher than vascular cryptogamic MIPPs viz., 0.068 mM (recorded in *Lycopodium clavatum*) and 0.076 mM (recorded in *Selaginella monospora*) [33].

Among the monovalent cations tested K^+ had trivial stimulatory effect, Na^+ played an impartial role and Li^+ was sturdily inhibitory to these enzymes. It has been mentioned earlier that both MIPS and MIPP have two isoforms located in cytosol and chloroplasts in various higher plant systems [8,30]. However, during the course of the present investigation the study of chloroplastidial MIPS and MIPP was not attempted.

The tropical Indian waters harbour a diversity of habitats which provide excellent support for a plethora of seaweeds in different proportions and densities, many of which are effectively utilized as important phycocolloid sources; few are consumed as food while the rest are insufficiently known. Members of Rhodophyceae are considered as unique and valuable from economic as well as from eco-physiological stand points. Although various aspects on red seaweed research have been carried out for decades, enzymological investigations in red algae have been a highly neglected area of research. The present study thus not only proves the existence of inositol biosynthesis in marine Rhodophyceae members for the first time but also establishes a connection between enhanced sugar–alcohol synthesis and salinity stress. However, it must be taken into account that our study has been conducted primarily with the cytolosic part of the red algal cells as the enzyme source. The possible contribution of chloroplasts in this context have to be essentially unveiled in future for understanding the correlation between variable ecological rhymes and stress related cyclitol synthesis in red algal group. The existence of diverse pigment system, complex carbohydrate components like agar and carrageenan, demand an extensive study of carbohydrate metabolism in this group using radio-labelled precursors. A molecular approach can also possibly resolve the unsolved questions if suitable DNA probes or sequence information is available.

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REFERENCES

1. Krist GO. Salinity tolerance of eukaryotic marine algae. Annu. Rev. Plant Physiol. Plant Mol. Biol. 1989; 40: 21-53.
2. Taji T, Takahashi S, Shinozaki K. Inositols and their metabolites in abiotic and biotic stress responses. Biology of Inositols and Phosphoinositides. Springer, Boston, MA; 2006. 239-264.
3. Bohnert HJ, Nelson DE, Jensen RG. Adaptations to environmental stresses. Plant Cell. 1995; 7(7): 1099–1111.
4. Sengupta S, Patra B, Ray S, Majumder AL. Inositol methyl tranferase from a halophytic wild rice, *Porteresia coarctata* Roxb. (Tateoka): regulation of pinitol synthesis under abiotic stress. Plant, Cell & Environment. 2008; 31(10): 1442-1459.
5. Joshi R, Ramanarao MV, Baisakh N. *Arabidopsis* plants constitutively overexpressing a *myo*-inositol 1-phosphate synthase gene (SaINO1) from the halophyte smooth cord grass exhibits enhanced level of tolerance to salt stress. Plant Physiol. Biochem. 2013; 65: 61-66.
6. Wells WW, Eisenberg Jr. F. Cyclitols and Phosphoinositides Academic Press, NY; 1978.
7. Eisenberg F. Jr. D-*myo*-inositol 1-phosphate as product of cyclization of glucose 6-phosphate and substrate for a specific phosphatase in rat testis. J. Biol. Chem. 1967; 242(7): 1375-1382.
8. Adhikari J, Majumder AL, Bhaduri TJ, Das Gupta S, Majumder A L. Chloroplast as a locale of L-*myo*-inositol-1-phosphate synthase. Plant. Physiol. 1987; 85(3): 611-614.
9. Maeda T, Eisenberg F. Purification, structure, and catalytic properties of L-*myo*-inositol-1-phosphate synthase from rat testis. J. Biol. Chem. 1980; 255(18): 8458-8464.
10. Loewus FA, Loewus MW. *Myo*-inositol: its biosynthesis and metabolism. Annual Review of Plant Physiology. 1983; 34(1): 137-161.
11. Adhikari J, Majumder A L. Differences in thermal stability of the foetal and adult brain *myo*-inositol-1-phosphate synthase: Probable involvement of NAD. FEBS lett. 1983; 163(1): 46-49.
12. Gumber SC, Loewus MW, Loewus F A. Further Studies on *myo*-Inositol-1-phosphatase from the Pollen of *Lilium longiflorum* Thunb. Plant Physiology. 1984; 76: 40–44.
13. Majumder AL, Biswas BB. Biology of inositols and phosphoinositides (Vol. 39). Springer Science & Business Media; 2006.
14. Lohia A, Hait NC, Majumder AL. L-*myo*-Inositol 1-phosphate synthase from *Entamoeba histolytica*. Mol. Biochem. Parasitol. 1999 ;98(1): 67-79.
15. Chen L, Zhou C, Yang H, Roberts MF. Inositol- 1-phosphate synthase from *Archaeoglobus fulgidus* is a class II aldolase. Biochemistry. 2000; 39: 12415–12423.
16. Patra B. Dastidar KG, Maitra S, Bhattacharyya J, Majumder AL. Functional identification of sll1383 from *Synechocystis sp* PCC 6803 as L-*myo*-inositol 1-phosphate phosphatase (EC 3.1. 3.25): molecular cloning, expression and characterization. Planta. 2007; 225(6):1547-1558.
17. Jha B. Reddy CRK, Thakur MC, Rao MU. Seaweeds of India: the diversity and distribution of seaweeds of Gujarat coast (Vol. 3). Springer Science & Business Media; 2009.
18. Barnett JEG, Brice RE, Corina DL. A colorimetric determination of inositol monophosphates as an assay for D-Glucose-6-phosphate-1L-myo-InositoL-1- phosphate cyclase. Biochem. J. 1970; 119: 183-186.
19. Chen RS, Toribara TY, Warner H. Micro determination of phosphorous. Annals of Biochemistry. 1956; 28: 1756–1758.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. – Annals of Biochemistry. 1976; 72: 248–254.
21. Bollag DM, Rozycki MD, Edelstein SJ. Protein Methods. 2nd edn. Wiley-Liss. New York; 1996.
22. Charalampous F, Chen IW. Inositol-1-phosphate synthase and Inositol 1-phosphatase from yeast. Meth. Enzymol. 1966; 9: 698-704.
23. Gaitonde MK., Griffiths M. A spectrophotometric method for the determination of micro quantities of free Inositol in biological material. Anal. Biochem. 1966; 15: 532-535. 24.
24. Sverdrup HU, Johnson MW, Fleming RH. The Oceans: Their physics, chemistry, and general biology (Vol. 7). New York: Prentice-Hall; 1942.
25. Wright J, Colling A. Open University Course Team. Seawater: Its Composition, Properties and Behaviour; 1995.
26. Elliott M, Whitfield AK. Challenging paradigms in estuarine ecology and management. Estuarine, Coastal and Shelf Science. 2011; 94(4): 306-314.
27. Wolanski E, Elliott M. Estuarine ecohydrology: an introduction. Elsevier; 2015.
28. Vernon DM, Bohnert HJ. Increased expression of a *myo*-inositol methyl transferase in *Mesembryanthemum crystallinum* is part of a stress response distinct from Crassulacean acid metabolism induction. Plant Physiology. 1992; 99(4): 1695-1698.
29. Ishitani M, Majumder AL, Bornhouser A, Michalowski C B, Jensen RG, Bohnert H J. Coordinate transcriptional induction of *myo*-inositol metabolism during environmental stress. The Plant Journal. 1996; 9(4): 537-548.
30. Raychaudhuri A, Hait NC, Das Gupta S, Bhaduri TJ, Deb R, Majumder AL. L-*myo*-Inositol 1-phosphate synthase from plant sources (characteristics of the chloroplastic and cytosolic enzymes). Plant Physiol. 1997; 115(2): 727-736.
31. Dasgupta S, Adhikari J, Majumder AL. *Myo*-inositoL-1-phosphate synthase from lower plant groups: purification and properties of the enzyme from *Euglena gracilis*. Physiol. Plant. 1984; 61: 412-416.
32. Donahue TF, Henry SA. *myo*-Inositol-1-phosphate synthase. Characteristics of the enzyme and identification of its structural gene in yeast. J. Biol. Chem.1981a; 256: 7077–7085.
33. Basak A, Jha TB, Adhikari J. Biosynthesis of *myo*-inositol in lycopods: characteristics of the pteridophytic L-myo-inositol-1-phosphate synthase and L-myo-inositol-1-phosphate phosphatase from the strobili of *Lycopodium clavatum* and *Selaginella monospora*. Acta Physiol. Plant. 2012; 34(4): 1579-1582.