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Isolation, purification and characterization of Cytosolic L-*myo*-Inositol-1-phosphate synthase from Marine macro Red Alga *Sebdenia flabellata* (J. Agardh) P.G. Parkinson

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Abstract

Inositols are a large class of poly-hydroxylated cycloalkanes, also called cyclitols. Out of nine structural isomers of inositol, *myo*-inositol is ubiquitously present in all organisms. It is a poly-hydroxy alcohol with six hydroxyl groups attached to each carbon atom of the hexose sugar which take part in metabolism namely signal transduction, cell membrane biogenesis, seed maturation, seed germination, growth, reproduction and as osmolytes to withstand abiotic stresses in plants. Marine water due to dissolved salts possesses low water potential which is a great challenge for normal plants for survival. Elevated level of *myo*-inositol has been reported from the plants living in highly saline environment like marine aquatic ecosystems. Rise in salinity units in the sea water of the littoral zones has shown considerable increase in the production of this *myo*-inositol. The principal enzymes that catalyze the biosynthesis of *myo*-inositol are two, L-*myo*-inositol-1-phosphate synthase (MIPS; EC 5.5.1.4) an isomerase and L-*myo*-inositol-1-phosphate phosphatase (MIPP; EC 3.1.3.25) which has been detected, purified and characterized from a number of biological systems ranging from microbial to higher plants and animals. The present work deals with the isolation of the first enzyme MIPS and its characterization from marine red macro alga *Sebdenia flabellata* (J. Agardh) P.G. Parkinson growing naturally in the elevated salinity levels at Arabian Sea coast of Okha, Gujarat, India. L-*myo*-inositol-1-phosphate synthase (MIPS) from *S. flabellata* was partially purified using cold centrifugation, ammonium sulphate salting out (0-25% and 25-75% saturation) followed by DEAE cellulose column chromatography and Sephadex G-200 gel filtration. 6.46 fold purification of MIPS was achieved which were pooled for

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characterization experiments. MIPS was active in a temperature range of 30°C to 40°C and unstable in high temperature or prolonged room temperature, active in the pH range of 6.5 to 7.5 and had a sole substrate D-Glucose-6-phosphate, V_{max} and K_m values for the enzyme were determined. NH_4^+, Mg^{2+}, Na^+, Ca^{2+} ions could promote and Pb^{2+}, Cd^{2+}, Hg^{2+} had inhibitory effect on the activity of the enzyme.

Key words : Cyclitols, *myo*-Inositol, compatible solute, MIPS, *Sebdenia flabellata.*

Living systems live under various environmental conditions. Adequate availability of nutrients and factors leads to successful growth, development and reproduction of all living systems. Episode of various abiotic stresses causes impairment in the metabolism and compromised growth and development. Salinity stress is a regular affair in coastal and on sea areas. Salinity stress has osmotic as well as cytotoxic effects and is a major abiotic stress to plant agriculture[37].

Plants upon episode of salinity stress produce osmolytes or compatible solute to lower the water potential of the cells and plant body as a whole so as to resist the unnecessary loss of fluids from the cells. And this process is called osmotic adjustment[35]. Cytosolic enzymes and several housekeeping proteins can be severely affected by the presence of undesirable ions in the environment. As a measure against such high salinity stress in plants[32] various polyhydroxy alcohols are produced in the cells like mannitol, pinitol, sorbitol, *myo*-inositol, 1D-1-O-Methyl- *myo*-inositol and other derivatives of *myo*-inositol. Methylated *myo*-inositol serves as osmoprotectant[8,18] during drought stress[26].

Among various structural isomers of inositols, the most widely found is the *myo*-form and important to all organisms. *myo*-Inositol ubiquitously occurs in all organisms play significant roles in signal transduction[34,36], seed maturation, seed germination, growth, reproduction and abiotic stress tolerance in the life cycle in plants ranging from the lower group of algal members to the most advanced angiosperms[7,14]. Phosphatidylinositol is an integral part of biological membranes often associated with the synthesis of phosphoinositol in higher plants[24].

Owing to its ubiquitous presence and crucial role served *myo*-Inositol has taken up a key position in both flowering and non-flowering plants[2]. It is an alcohol with six hydroxyl groups attached to each carbon atom of the hexose sugar which serve various metabolic works in plant system and present in all plant systems. In normal condition it takes part in the biogenesis of cell wall, polysaccharide biosynthesis[28], phospholipid synthesis [12,13,15,33], cell signalling through phosphoinositides[19] etc. As a response to abiotic stresses namely salinity stress[20,29] and drought stress[22], inositol and inositol derivatives[31] play important role

as compatible solutes towards minimising such abiotic stress by maintaining the ionic and osmotic homoeostasis in plant systems.

Two specific and committed enzymes namely L-*myo*-Inositol-1-phosphate synthase (MIPS) and D/L *myo*-Inositol phosphate phosphatase (MIPP) by successive actions Chen and Eisenberg[11], lead to the synthesis of *myo*-inositol in cytoplasm as well as in chloroplasts. Cytosol and chloroplasts both serve as locales of MIPS and MIPP in marine macro green alga *Enteromorpha intestinalis*[1,5]. Enhanced levels of chloroplastidial MIPS activity owing to salinity stress were reported earlier from marine green alga *Ulva lactuca*[6].

Indian Western Coastal belt of Okha, (Fig. 1A) Gujarat is home to more than 80 types of marine red algal members of which most number are macrophytes[25]. Salinity in this region is higher around 33.18 to 34.46 PSU than Bay of Bengal due to high temperature, less precipitation by tropical cyclones, and draining by a vast river system. This hypersalinity condition may result in the elevated biogenesis of *myo*-inositol from which methyl inositol, D-ononitol due to stress condition[30].

Free *myo*-inositol is synthesized in cell upon successive reactions from phosphorylated hexose sugar D-Glucose-6-phosphate (G-6-P) in an irreversible reaction catalyzed to L-*myo*-inositol-1-phosphate (MIP), an isomer of G-6-P by the action of an isomerase, MIPS which requires NAD for its catalysis. Thereafter, phosphate group of MIP is hydrolysed to *myo*-inositol by the action of the Mg^{2+} dependent enzyme D/L *myo*-inositol phosphate phosphatase (MIPP)[27].

Study on the cytosolic MIPS were chosen from *Sebdenia flabellata* (J. Agardh) P.G. Parkinson as still there is a major dearth of information on the availability and report of this enzyme from red algal members except from *Halymenia venusta*[29]. And therefore partial purification of the enzyme was done in laboratory by cold low speed centrifugation, ammonium sulfate salting out, column chromatography using DEAE-cellulose and ultimately gel filtration by Sephadex-G-200 gel. Pool of the partially purified enzyme was used for the characterization of the enzyme.

1 *Plant material :*

The experimental red algal sample alga *Sebdenia flabellata* (Fig. 1B) was collected by specimen collector Aarif Ibrahimbhai Qureshi, Specimen Collector and staff of Fisheries Research Station, Okha, Gujarat. Immediately after collection the specimen were kept in plastic zipper pouch and ice boxes. The specimen was sent by Okha – Shalimar Express in cold condition. From Shalimar, Howrah, West Bengal the specimen were transported to the Plant Physiology and Plant Biochemistry Laboratory, Post Graduate Department of Botany, Barasat Government College. All the specimens were kept in -21°C freezer for research purpose. All the red algal species were identified by the staff of Fisheries Research Station, Okha, Gujarat as well as specialist in Phycology at Central National Herbarium (CAL), Howrah. Samples are kept in the P.G. Department of Botany, Barasat Government College for future reference.

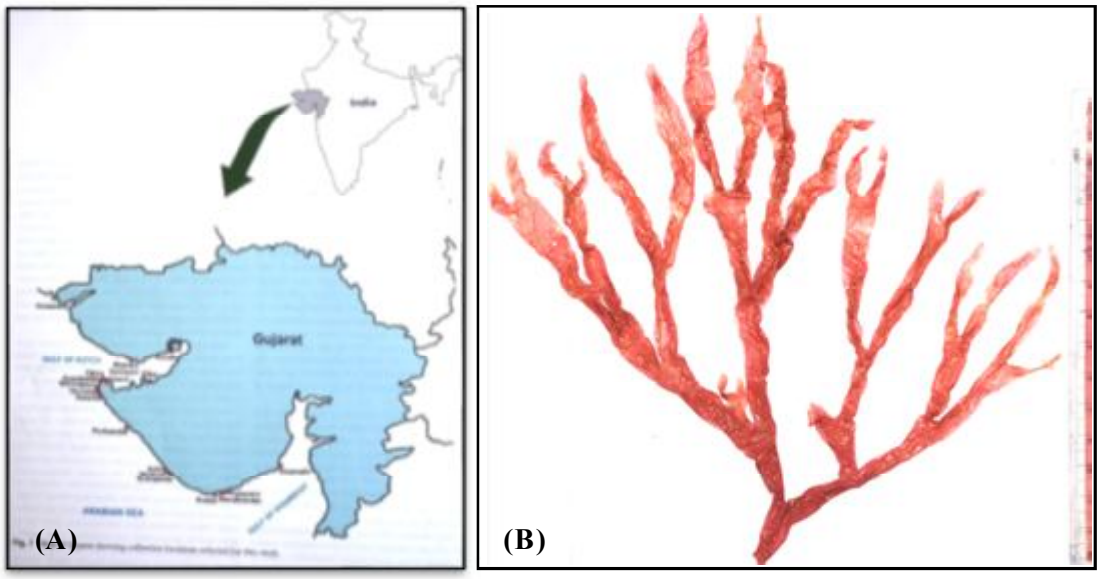


Figure 1 (A) Location of Okha in coastal Gujarat, India. (B) Exomorphology of the collected algal specimen *Sebdenia flabellata* (J. Agardh) P.G. Parkinson, with vibrant red colour and distinct and regular dichotomous branching.

Systematic position of the specimen Sebdenia flabellata (J. Agardh) P.G. Parkinson *:*

Phylum: Rhodophyta
Class: Florideophyceae
Order: Sebdeniales
Family: Sebdeniaceae
Genus: *Sebdenia*
Species: *Sebdenia flabellata*
Classification and nomenclature were as per https://www.algaebase.org/search/species

2. *Extraction of myo-Inositol phosphate synthase (MIPS) :*

Extraction was done by tissue maceration under cold condition using 0.5mM Tris-acetate extraction buffer (pH 7.5) containing 0.2 mM β-mercaptoethanol (β-ME) followed by cold centrifugation at 10,000 rpm for 30 minutes in REMI C-24 cold centrifuge. Pellet was discarded, ammonium sulphate salting out in two phases (0-25% and 25% to 75% saturation) were performed with the supernatant followed by dialysis of the resultant pellet at 0-4°C for 48 hours using extraction buffer for preliminary experiments.

3. *Partial purification of MIPS :*

Partial purification of cytosolic MIPS enzyme from the thalli of *S. flabellata* was done separately at 0-4°C. Around 40 g of plant body was taken homogenized separately in 50mM Tris-acetate buffer (TAB), pH 7.5 with 0.2 mM β-ME. The homogenate was spun at 10,000 rpm for 30 min and low speed supernatant was collected and fractionated with ammonium sulphate $[(NH_4)_2SO_4]$ salting

out in two phases 0-25% and 25-75% saturation. The resultant ammonium sulphate pellet was dissolved in small volume of extraction buffer followed by dialysis at 0-4°C for 48 hours. Chromatography of the dialyzed fractions of MIPS was done with the help of anion exchange matrix of DEAE-cellulose in a glass column (1.2X12.0 cm). The effluents were collected and the column was washed with one bed volume of extraction buffer. Elution of the adsorbed protein was done by linear gradient of 0 to 0.5M KCl prepared in extraction buffer. In both the cases fractions were pooled together separately with which gel filtration using Sephadex G-200 was performed. The respective MIPS active Sephadex G-200 fractions were pooled separately and characterization experiments were performed with the pooled enzyme (Table-1).

4. *Assay of L-myo-inositol-1-phosphate synthase (MIPS) enzyme :*

The *myo*-inositol synthase activity was assayed by the procedure of Barnett *et al.*[3] with slight modifications as per Adhikari *et al.*[1].

In a total volume of 0.5 mL, the incubation mixture contained 500 mM Tris-acetate (pH 7.5), 140 mM NH_4Cl, 8 mM NAD, 50 mM β-ME, 50 mM G-6-P and an appropriate protein aliquot (100-200 µg). The reaction was started by addition of substrate immediately after the addition of the enzyme with proper mixing. Duplicate tubes were run along with an appropriate blank (without enzyme) and a zero minute control in which 200 µL of 20 % chilled TCA was added prior to the addition of the enzyme. The enzymatic incubation was carried out for 60 min at 37°C. After 60 min the reaction was terminated by the addition of 200 µL of chilled TCA (20 %). Two such sets (set I – periodate and set II – non-periodate) were run simultaneously each having one blank, one zero minute control and two experimental tubes.

After completion of the enzyme incubation, the resultant supernatant was subjected to a treatment with 0.7 mL of 200 mM sodium metaperiodate ($NaIO_4$) and incubated for 60 min at 37 °C. Then, 1.4 mL of 1M Sodium sulphite (Na_2SO_3), prepared immediately before use, was added in case of set–I to destroy excess of $NaIO_4$. In set II, water was added instead of $NaIO_4$ and Na_2SO_3 to maintain the volume equal with the set I.

Inorganic phosphate was liberated from *myo*-inositol-1-phosphate during oxidation. Cleavage of G-6-P also took place but it was thought to be extremely low (2 m mol of P_i mol^{-1} of G-6-P) which could be subtracted considering the blank or zero minute control from the experimental value in set I. 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 II. Product specific cleavage of inorganic phosphate was estimated by subtracting the corrected value of set II from that of set I.

Inorganic phosphate was estimated by the method of Chen *et al.*,[10] with slight modifications. P_i reagent (2.8 mL) was added to the reaction mixture (total volume now became 5.6 mL) and incubated at 37°C for 60 min. The P_i reagent was prepared immediately

before use at 10°C by adding H_2SO_4 (6N), ascorbic acid (10 % w/v), chilled ammonium molybdate (2.5 % w/v) and H_2O (1:1:1:2) in same order. After incubation, the blue colour developed was measured at 820 nm in a Systronics-106 spectrophotometer. The inorganic phosphate released was estimated with the help of a standard curve prepared from different known quantities (0-20 µg) of phosphorous (using K_2HPO_4). As 1 mole of *myo*-inositol-1-phosphate contains 1 mole of inorganic phosphate, the total mole number of inorganic phosphate released was equal to the total mole number of myo-inositol-1-phosphate produced.

5. *Estimation of Protein :*

Estimation of protein was done using protocol of Bradford[9] with BSA as standard.

6. *Thermal sensitivity of MIPS :*

The thermal sensitivity conferring the MIPS reaction was investigated at a temperature range of 0 – 60°C with intervals of 10°C in the laboratory.

7. *Determination of K_m and V_{max} 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 7 (Graphpad) software package.

8. *Enzyme action under variable pH buffers:*

MIPS incubation were carried out separately with Tris-acetate 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.

9. *Effect of different metal ions :*

MIPS incubation were carried out in presence of different metal ions to observe the promoting or inhibitory effects keeping other assay components unchanged in all experimental sets.

10. *Recognition involving enzyme MIPS in marine-alga Sebdenia flabellata from sub-littoral zone of* Okha, Gujarat *:*

It has been revealed that enzymatic biosynthesis of *myo*-inositol through the MIPS enzyme has been documented among different members of genera starting from unicellular microorganisms to higher plants and animals; there is a dearth of information regarding one of the prime or marker inositol synthesizing enzyme (MIPS) in marine red algae for which a member of red algae was chosen for the work.

11. *Purification of MIPS from the isolated from thallus of Sebdenia flabellata :*

The enzyme purified was extracted from *S. flabellata* thalli by the procedure described in section 3 stating purification procedure. This is clear as per Table-1 enzyme taken for the study was purified to about 6.46 fold at Sephadex G 200 level. This was based on the specific activity of the enzyme done

Table-1 Partial purification of L-myo-inositol-1-phosphate synthase from isolated of *Sebdenia flabellata*

Fraction	Total volume (mL)	Protein concen-tration (mg/ mL)	Total protein (mg)	Specific activity [n mole MIP produced $(mg)^{-1}$ $proteinh^{-1}$]	Total activity [n mole MIP produced h^{-1}]	Reco-very (%)	Fold purifi-cation
Homogenate-fraction	21.00	1.03	21.63	331.65	7173.58	100.00	1.00
11,400 x g supernatan-tfraction	18.50	0.99	18.31	359.23	6577.50	91.69	1.08
0-75% $(NH_4)_2$ SO_4 fraction	29.00	0.55	15.95	402.11	6413.65	89.40	1.21
DEAE cellulose fraction	27.0	0.38	10.26	522.34	5359.20	74.70	1.57
Sephadex G-200 fraction	12.00	0.22	2.64	2145.22	5663.38	78.94	6.46

through assay at each step of purification. The recovery of the enzyme based on total activity was about 78.94 %.

12. *Enzymatic characterization of MIPS from S. flabellata :*

Requirements for MIPS activity :

Various requirements of the enzyme MIPS extracted from *Sebdenia flabellata* are given as underneath in Table -2 in which 50mM Tris-Acetate Buffer (TAB), 140 mM Ammonium chloride, 0.8mM NAD, β-mercaptoethanol, and G-6-P and required amount of protein from the pool called the full set of reaction mixture. When all full set of chemicals were added to the reaction it recorded the maximum activity. In absence of the specific substrate of the enzyme no activity was exhibited by the enzyme whereas absence of TAB was not present in the reaction mixture about 7.57% reduction in the activity was noticed, in absence of the substrate no activity of the enzyme was noticed. Without the presence of the specific coenzyme NAD 67.26% reduction the activity of the enzyme was noticed this confirms the necessity of the coenzyme for the enzyme taken for the study without which a fall in the enzyme activity was observed. In absence of ammonium chloride about 21.59 % reduction in the enzymatic activity was noticed. Taking out of 2-ME (β-mercaptoethanol) led to just 5.35% reduction in the activity of the enzyme.

Table-2 Requirements for L-myo-Inositol-1-phosphate synthase activity from *S. flabellata*

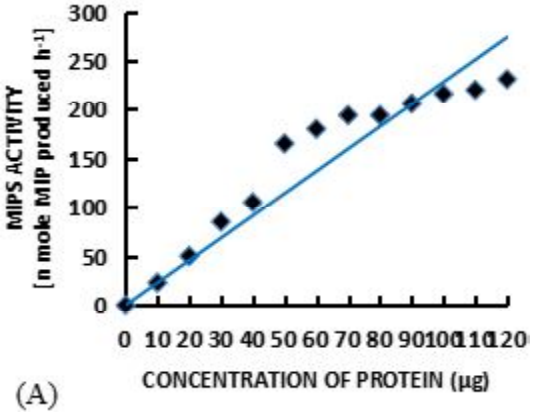
Assay condition	Specific activity [n mole MIP produced $(mg)^{-1}$ protein h^{-1}]	Percent activity
Complete set	2045.12	100.00
Minus substrate (G-6-P)	0.00	0.00
Minus buffer	1890.37	92.43
Minus coenzyme (NAD)	690.22	33.74
Minus NH_4Cl	1603.61	78.41
Minus 2-mercap-toethanol	1935.77	94.65
Heat killed enzyme	0.00	0.00

13. *Progress of reaction with respect to protein concentration :*

Using increasing concentrations of the enzyme protein ranging from 0-120 µg as given the figure 2(A) purified from *S. flabellata*. The assay of the enzyme was done by progressively varying concentrations of the enzyme. It was observed that activity of the enzyme increased linearly with the increase in the enzyme concentration keeping substrate concentration constant.

14. *Progress of the MIPS reaction with time:*

With the enzyme pool extracted from *S. flabellata* assay was carried out taking different incubation time starting from 0min, to 90 minute with an interval of 15 min. Through this characterization experiment it was found that an incubation time of 60 minute to 75 minute was optimum for the enzyme to show maximum activity as shown in the Fig. 2(B). In case of 0 min the reaction mixture was added with 20% TCA to stop the reaction. By adding 20% TCA after 15 min the need for actual incubation time was ascertained. It was also found that after 60 minute of incubation time very low increase in the catalytic activity of the enzyme as a result at plateau like curve was obtained.



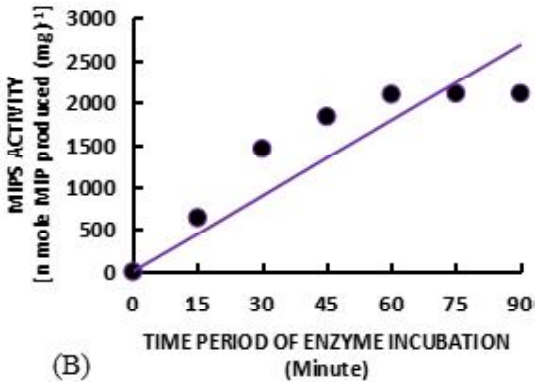


Figure 2(A) Effect of protein concentrations on *S. flabellata* on MIPS activity, (B) Time course of *S. flabellata* MIPS activity

15. *Thermal sensitivity of the enzyme :*

Temperature is a very significant criterion for enzymatic action. To study the effect of temperature the enzyme thus extracted or partially purified to Sephadex G-200 level was assayed taking all reaction mixture required together for one hour standard incubation. Separate reaction mixtures were kept in different temperature to study the optimum temperature for the enzyme to perform starting from 0 °C to 50 °C in an interval of 10 °C. The results of this characterization experiment is shown in Figure 3. It was found that the optimum temperature range in which the enzymatic activity is from 30 °C to 40 °C, beyond which there was a decrease in the specific activity of the enzyme. Though, the enzyme was active in the temperature range from 20 °C to 40 °C.

16. *Specificity for substrate :*

MIPS extracted from *S. flabellata* was assayed against substrates in addition to its specific substrate D-Glucose-6-Phosphate as depicted in the Table-4 given underneath with similar concentration to ascertain whether other similar compound can act as its substrate or not. It was found that the enzyme has no activity with other substrates. This experiment confirms the fact that the enzyme has D-G-6-P as its sole and specific substrate.

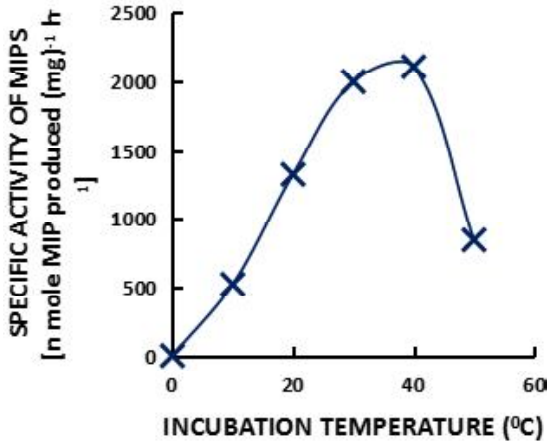


Figure 3. Effect of incubation temperature on *Sebdenia flabellata* MIPS activity

Table-3. Substrate specificity of L-myo-Inositol-1-phosphate synthase from *S. flabellata*

Substrate	Concentration (mM)	Specific activity [n mole MIP produced $(mg)^{-1}$ protein h^{-1}]	Percent activity
D-Glucose-6-P	5	1985.34	100.00
D-Fructose-6-P	5	0.00	0.00
D-Galactose-6-P	5	0.00	0.00
D-Mannose-6-P	5	0.00	0.00

17. *Effect of substrate (G-6-P) concentration on MIPS activity and determination of K_m and V_{max} values :*

Effect of substrate concentration :

Activity of the Sephadex G 200 purified enzyme from *S. flabellata* was assayed at varying and progressive increase of its specific substrate concentration (ranging 0mM to 10mM G-6-P) and it was observed that the enzymatic activity increased sharply with the increase in the substrate concentration which was followed by very feeble change in the activity up to 10mM concentration in the X axis. The Km value for the substrate of MIPS was 1.078 mM as given in the following Fig. 4.

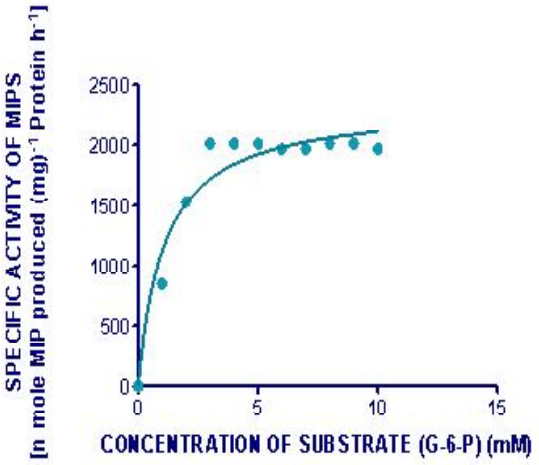


Figure 4. Effect of substrate concentrations and determination of K_m value for G-6-P of *S. flabellata* MIPS by Non-linear Regression Kinetics (Graph Pad Prism 7 software)

Table-4. Analysis of Kinetic data by GraphPad Prism 7 Software

Michaelis-Menten	Best fit values
Vmax	2338
Km	1.078
Standard error Vmax	133.6
Standard error Km	0.3049
Vmax range	2036 to 2640 nmole MIP produced per mg protein per hr
Km range	0.3881 to 1.767 mM G-6-P
Degrees of freedom	9
Number of points analyzed	11

18. *Specificity for the Coenzyme :*

MIPS enzyme from most of the sources catalyze in presence of a specific coenzyme nicotinamide adenine dinucleotide (NAD). In absence of this coenzyme in the reaction substantial decrease in the enzymatic activity was noticed in the section "Requirements for MIPS Activity" Table 3.2 in which only 33.74% activity of the enzyme was observed when NAD was absent in the reaction mixture. In addition to NAD, NADP was used as the coenzyme and about 27.59% of the enzymatic activity was observed in the latter case. This confirms the fact that the enzyme from *S. flabellata* works best in presence of NAD as its coenzyme and NADP though could replace NAD but not to a satisfactory level. Two parallel experiments were carried out one of which contained NAD as coenzyme and another had NADP as the coenzyme in the reaction mixture and in NAD set optimum specific activity was observed unlike NADP (Table-5).

Table-5. Replacement of NAD by NADP as the coenzyme on MIPS activity

Coenzyme	Concentration (mM)	Specific activity [n mole MIP produced $(mg)^{-1}$ protein h^{-1}]	Percent activity
NAD	0.8	2134.25	100.00
NADP	0.8	589.04	27.59

19. *Effect of Coenzyme (NAD) concentration on MIPS activity and determination of K_m and V_{max} values :*

The activity of Sephadex G-200 purified *S. flabellata* L-myo-inositol-1-phosphate synthase was found to increase with respect to the concentration of NAD up to 0.4 mM when tried between concentration ranges of 0.1 to 1.0 mM coenzyme. Thereafter, the enzymatic activity remained unchanged up to 1.0 mM (Figure 5). The results were calculated using Non-linear Regression Kinetics (GraphPad Prism 7 software). The K_m value for NAD of MIPS was 0.09226 mM (Figure 5 and Table-5).

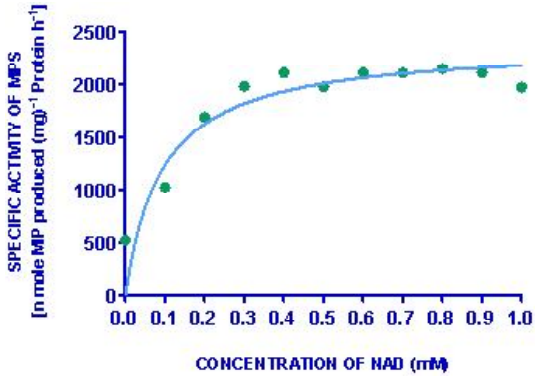


Figure 5. Effect of coenzyme concentrations and determination of K_m value for NAD of *S. flabellata* MIPS by Non-linear Regression Kinetics (GraphPad Prism 7 software)

Table-6. Analysis of enzyme kinetics data by GraphPad Prism 7 Software

Michaelis-Menten	
Best-fit values	
Vmax	2383
Km	0.09226
Std. Error	
Vmax	170.4
Km	0.03580
95% Confidence Intervals	
Vmax	1997 to 2768
Km	0.01128 to
	0.1732
Goodness of Fit	
Degrees of Freedom	9
R square	0.8490
Absolute Sum of Squares	430215
Sy.x	218.6
Constraints	
Km	Km > 0.0
Number of points	
Analyzed	11

20. *pH Sensitivity :*

To study the effect of variable pH on the enzymatic activity assay of the MIPS enzyme was performed using different pH of 100mM Tris-Acetate buffers ranging from 5.0 to 8.5. It was observed that the enzyme taken from the Sephadex G-200 pool of *S. flabellata*

exhibited considerably high activity at a pH range of 6.5 to 7.5, beyond which the enzymatic activity declined. This confirm the fact that the enzyme from *S. flabellata* bear optimum pH range for catalysis at 7.0 to 7.5 range as depicted in the Fig. 6 (A) underneath.

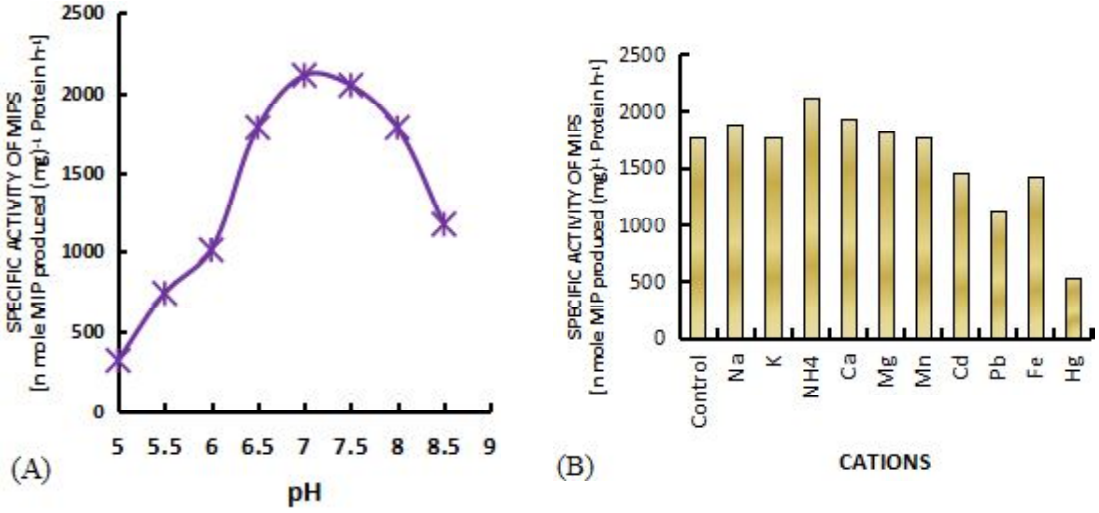


Figure 6 (A) pH dependence of *Sebdenia flabellata* MIPS activity. (B) Activity of the pooled enzyme in presence of various metal ions

21. *Activity of the enzyme in presence of different metal and other ions :*

In the last characterization experiment for extracted MIPS from the thallus of *S. flabellata* the specific activity of the enzyme was determined in presence of different metal chelators name Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cd^{2+}, Pb^{2+}, iron and mercury ions. Taking different salts of these mono-valent and divalent ions in place of ammonium chloride (an essential chemical in the reaction mixture) assay was done. It was observed as depicted in the Fig. 6 (B) higher activity in presence of ammonium ions, presence of Na^+, K^+, NH^{4+}, Ca^{2+}, Mg^2 did not inhibit the enzymatic reactions whereas presence Cadmium, lead, mercury caused decrease or slump in the enzymatic activity. This experiment gives an idea regarding enzyme inhibition of MIPS.

Direction of *myo*-inositol biosynthesis in the salinity dominating aquatic flora of at the coast of Okha, Gujarat could be established in *Sebdenia flabellata*. This was done through the study of the principal enzyme associated with the biosynthesis of *myo*-inositol viz. MIPS. Starting from the collection of the red algal specimen to the penultimate step of characterization involved a chain of works. The thalli were homogenized in the cold with 100% recovery and 1 fold purification Table-1 which was followed by low speed centrifugation in the cold at 10,000 rpm and in this phase 91.69% recovery was attained. Unwanted protein were salted out during ammonium sulphate cut at 0-25% and 25% to 75% and an increase in the fold purification was observed which was

about 1.21 fold. In the last step of gel filtration using Sephadex G 200 about 6.46 fold purification was obtained.

As stated in earlier sections of characterization experiments, optimum temperature for the activity of the enzyme was around 40°C. It was observed that MIPS is not at all a thermo-stable enzyme and slightest increase in temperature of prolonged keeping in the room temperature could cause loss in the viability of the enzyme. In connection of the optimum incubation period for the functioning of the enzyme it was 60 to 75 min as stated earlier. NAD being the sole and indispensable coenzyme required for the catalysis of the enzyme taken for the study.

The enzyme activity increased linearly with respect to protein concentration to about 100 µg and with respect to time up to 60 minutes. Temperature optimum was found at 40°C. This enzyme exclusively exploited D-glucose-6-phosphate as its substrate. The apparent K_m for D-glucose-6-phosphate of *Sebdenia flabellata* L-myo-inositol-1-phosphate synthase was determined to be approximately 1.078 mM as determined by means of non-linear regression kinetics (GraphPad Prism 7 software). Using Non-linear Regression Kinetics (GraphPad Prism 7 software), the K_m value for NAD of MIPS was 0.09226 mM. The enzyme was functional in a constricted pH range of 6.5 to 7.5 giving maxima at pH 7. 0. Among the monovalent cations tested NH_4^+ and Na^+ had stimulatory role. K^+ was ineffective. Using the analogous concentrations of divalent cations, it was revealed that Mg^{2+} exhibited slight stimulatory function. Mn^{2+} and Ca^{2+} are also minor stimulators to this enzyme. Cd^{2+} moderately inhibited; Fe^{2+} and Pb^{2+} and Hg^{2+} evidently inhibited the enzyme activity. This data can definitely throw some light on the activity and control of the enzyme like role of lithium ion on *myo*-inositol phosphate phosphatase (MIPP)[21].

Purification procedure whether enzyme or protein requires constant and uninterrupted regime of laboratory works. Starting from collection of the specimen from a high salinity niche region of marine ecosystem of a distant area followed by different phases of purification and characterization, chain of events took place. Appreciable magnitude of specific activity of the enzyme MIPS taken for the study was observed. Considerable amount of specific activity in each step of purification throw light on the biosynthesis of *myo*-inositol in this genus under osmotically stressed environment. Study of biosynthesis of *myo*-inositol in marine red macro algae was not adequately reported earlier as a result *S. flabellata* was chosen, high specific activity of MIPS in the collected specimen may be attributed to the hyper-saline environment. Comparison with the fresh water green macro algae could offer a clearer notion on the synthesis of *myo*-inositol as a compatible solute or as a regular metabolite. Marine red macro-algal population can serve as a natural source of *myo*-inositol and its various chemical derivatives. Also they can throw light on the novel genes, expression or over expression of these genes during salinity induced heightened biogenesis of *myo*-inositol in cytosol. These results can help meet various challenges like drought, salinity stress, drought induced heat stress in crop plants which has become a

regular affair owing to climate change, change in average temperature, altered rainfall, drought and desertification.

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