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ARTICLE

Purification and characterization of a 1-deoxy-D-xylulose 5-phosphate synthase from *Cymbopogon flexuosus*

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ABSTRACT Here we report purification and characterization of the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS) of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway from lemongrass (*Cymbopogon flexuosus*) leaves. The DXS catalyzes the condensation of pyruvate and glyceraldehyde 3-phosphate (G3P) to produce 1-deoxy-D-xylulose 5-phosphate (DOXP), which is the first and rate-limiting step of the MEP pathway. It is the main flux-controlling step and an attractive target to manipulate the formation of the MEP-derived products. The DXS was extracted from immature (15 days old) leaves of lemongrass cv. Suvarna and purified to homogenity using ion exchange DEAE column and gel filtration (Sephadex G-150) chromatography. The purified DXS was referred as *Cf*DXS. The *Cf*DXS had specific activity 8.56 U/mg. The K_m values for the two substrates, pyruvate and G3P were 4.4 and 8.8 µM, respectively and for the cofactor TPP 62 µM. The V_{max} of the *Cf*DXS was 20 µmol/min. The optimum pH and temperature of the *Cf*DXS were 7.5 and 40 °C, respectively. The *Cf*DXS activity enhanced significantly in the presence of Mg^{2+} (1 mM), whereas affected moderately by Mn^{2+} and Zn^{2+} (1 mM each). The enzyme was purified upto 11.64 fold with an yield of 32.34%. Its molecular weight was 130 kDa. The DXS was quite stable and retaining more than 80% of the initial activity upon storage at 4 °C in 100 mM Tris-HCl buffer (pH 8) for one month. **Acta Biol Szeged 61(2):149-156 (2017)**

KEY WORDS

2-C-methyl-D-erythriotol 4-phosphate
Cymbopogon flexuous
glyceraldehyde-3-phosphate
lemongrass
monoterpene
pyruvate

Introduction

Cymbopogon flexuosus (Nees ex Steud.) popularly known as East Indian lemongrass provides the lemon scented essential oil, which is referred as lemongrass oil (LO). Lemongrass oil is a complex mixture of several monoterpenes, of which citral, an acyclic monoterpene aldehyde is the dominating constituent (Ganjewala and Luthra 2010; Gupta and Ganjewala 2015a). The percentage of the citral in the LO range from 70-80% and it imparts unique lemon like aroma to the LO. Lemongrass oil owing to its lemon like aroma has wide applications in flavors, fragrances, perfumery, cosmetics, food and pharmaceuticals (Ganjewala and Gupta 2013). A number of reports have documented many useful bioactive properties from simple antimicrobial to anticancer and anti-HIV of the lemongrass oil and citral (Shah et al. 2011; Mirghani et al. 2012; Ganjewala et al. 2012; Ganjewala and Gupta 2013; Olorunnisola et al. 2014; Gupta and Ganjewala 2015a).

In lemongrass, citral is biosynthesized *via* recently elucidated 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Ganjewala et al. 2009; Gupta and Ganjewala 2015b). The first indication of the presence of the MEP pathway in lemongrass was came from earlier study conducted using mevinolin (a potent inhibitor of the hydroxymethyl-glutaryl-CoA reductase (HMGR) of the acetate-MVA pathway) (Ganjewala and Luthra 2007). At present, the MEP pathway has been elucidated from a number of plants, where it is utilized exclusively for the biosynthesis of monoterpenes (Eisenreich et al. 1998; Lichtenthaler 1999; Dudareva et al. 2005; Seemann et al. 2006; Ganjewala et al. 2009; Ganjewala and Luthra 2010). The MEP pathway was originally discovered from the *Escherichia coli* by Rohmer et al. (1993).

The first step of the MEP pathway involves the thiamine diphosphate (TPP) dependent condensation of the glyceraldehyde 3-phosphate (G3P) and pyruvate to produce 1-deoxy-D-xylulose 5-phosphate (DXP), which is catalyzed by the enzyme 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) (Fig. 1). This is also a regulatory or committed step of the MEP pathway, thus control and regulate the overall supply of the IPP derived products by the flux distribution between pyruvate and G3P (Farmer and Liao 2001; Lee et al. 2007). The DXS requires TPP as a cofactor and divalent metal ions Mg^{2+} or Mn^{2+} for the activity (Wang et al. 2014). The optimum pH and temperature of the DXS from *E. coli* has been reported

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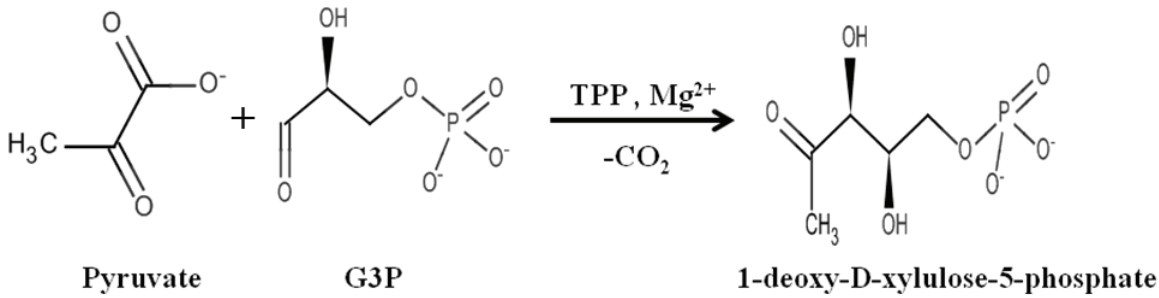

Figure 1. Reaction catalyzed by DXS.

to be 8.0 and 40 °C, respectively (Sprenger et al. 1997). The DXS has been cloned from several higher plants (Bouvier et al. 1998; Estevez et al. 2001) and bacteria *viz., Escherichia coli* (Sprenger et al. 1997) and *Agrobacterium tumefaciens* (Lee et al. 2007). Several studies have shown that the DXS significantly influences the rate of the biosynthesis of the IPP derived through the MEP pathway. The DXS could also be a promising target for the metabolic engineering in order to manipulate the monoterpene content and composition in plants. Currently, we have very limited knowledge of the enzymic regulation of MEP pathway in lemongrass, except our previous report highlighting the role of the DXR in the biosynthesis of the essential oil (Gupta and Ganjewala 2015c). In view of the important roles of the DXS, here we performed purification and characterization of this enzyme from lemongrass cv. Suvarna immature (15 days old) leaves to gain more deeper insight in to the regulation of the MEP pathway.

Materials and Methods

Chemicals

Glyceraldehyde 3-phosphate, sodium pyruvate, thiamine pyrophosphate (TPP), $MgCl_2$, $ZnCl_2$, Tris-base, EDTA-Na_2, 2-mercaptoethanol, ascorbic acid, sucrose, Sephadex G-150, DEAE cellulose, polyvinyl pyrophosphate (PVPP) were purchased from Sigma-Aldrich (Germany). Other chemicals and reagents used were of highest purity grade.

Plants

Cymbopogon flexuosus (Steud) Wats cv. Suvarna plants were grown in the Organic Farm House of the Amity University, Noida, Uttar Pradesh, India following the standard agronomic practices. Fully-grown lemongrass plants were harvested from 10 cm above the ground level. A fully-grown lemongrass plant has six enfolded leaves in the form a whorl. The leaves from inside to outside of the whorl are numbered from 1 to 6, which represent gradient increase in the leaf age. The innermost leaf represents the youngest leaf stage, while the outermost fully matured leaf stage. In the present study, second leaf (immature) was used for the extraction of the DXS enzyme.

Extraction of DXS

The DXS enzyme was extracted according to Wright and Phillips (2014). Leaf tissues (1 g) was homogenized in 50 mM Tris-HCl buffer (pH 8.0) consisting of 1 mM TPP, 50 mM sodium metabisulfite, 10 mM 2-mercaptoethanol, 10 mM ascorbic acid, 0.15 M sucrose, 1 mM EDTA, 10% glycerol and 1 mM NaF, polyvinyl pyrophosphate (PVPP) (50% of the tissue weight) was added prior to homogenization. The homogenate was filtered through four layers of muslin cloth and centrifuged at 13000 × g for 30 min at 4 ºC. The supernatant was collected in the screw capped graduated tubes and used as crude enzyme extract.

Protein estimation

Protein content of the crude enzyme extract was estimated by Bradford method (Bradford 1976).

DXS assay

A coupled spectrophotometric assay was used for rapid screening of the DXS activity. The DXS activity was determined using a reaction mixture (3 ml) containing 50 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM TPP, 0.1 M NADPH, 0.1 M sodium pyruvate, 0.05 M DL-glyceraldehyde 3-phosphate (G3P), 1 mM NaF and 0.2 mg/ml DXR at pH 8.0. The reaction was initiated by adding the enzyme extract to the assay mixture.

In this assay, DXP generate by DXS is further converted to MEP in a NADPH-dependent reaction by an excess of the DXR. The change in absorption at 340 nm due to conversion

of NADPH in to NADP was monitored. One unit of the DXS activity (U) was defined as the amount of the enzyme required to produce 1 µM of DXP second^{-1} under the conditions described above (Altincicek et al. 2000). Enzyme activities were expressed as nkatal/ml. The molar extinction coefficient value for NADPH 6220 $M^{-1}cm^{-1}$ at 340 nm was used for the calculation. A control with the boiled enzyme was also run simultaneously.

Purification of DXS enzyme

The DXS was purified in three steps. All purification procedures were carried out at 4 ºC. In the first step, the crude enzyme extract (25 ml) prepared from immature leaves was fractionated by ammonium sulfate ($(NH4)_2SO_4$) precipitation as 0-40 and 40-80% saturation. Proteins precipitating at all the $(NH4)_2SO_4$ saturation steps were collected by centrifugation at 13000 x g for 15 min, suspended in extraction buffer (50mM Tris-HCl buffer, pH 8.0) and proceeded to the dialysis.

The desalting was done using dialysis bags (Himedia; AV width-42.44 nm, AV diameter-25.4 mm, capacity-5.07 ml/cm) as per molecular weight cut off size of the membrane (25 kDa) against three changes of the buffer, which was used for extraction of the DXS. The dialyzed sample was applied to the pre-equilibrated (Tris-HCl 10 mM, pH 8.0) DEAE cellulose matrix packed in a column (Borosil, 200 x 10 mm column). The fractions (2 ml each) were collected by washing the matrix with Tris-HCl buffer (10 mM, pH 8.0).

Thereafter, the column was eluted by salt step gradient method, using increasing concentration of NaCl (50 mM, 100 mM, 200 mM, 300 mM, 400 mM in 10 mM Tris-HCl buffer, pH 8.0). Total 120 fractions (2 ml each) were collected. Peaks were obtained during the elution process, and the fractions corresponding to the peaks were pooled and specific activity was determined. Pools with maximum values were further dialyzed and processed to the gel filtration chromatographic separation using the Sephadex-G150 packed in a column (Borosil, 200 x 10 mm column). The column was washed with equilibration buffer (0.01 M Tris-HCl buffer pH 8.0). The pool obtained from the previous chromatographic procedure was loaded on to the pre-swelled and pre-equilibrated Sephadex G-150 matrix and eluted by using Tris-HCl 0.01 M, pH 8.0) 36 fractions (2 ml each) were collected and assayed for the DXS activity.

Determination of molecular mass

Appropriate molecular mass of the DXS was determined by the SDS-PAGE technique according to Laemmli (1971) using 10% polyacrylamide gel of 1 mm thickness. The purified protein was loaded into the wells in the gel along with a standard protein molecular weight marker (Merck Millipore, Germany). Separation was carried out in electrophoresis device (Double-sided Vertical Gel Electrophoresis System, Genetix-SCZ) at current of 15 mA for approximately 4 h. Protein bands were visualized by staining with Coomassie Brilliant Blue G-250 according to a previously published report. The molecular mass of the protein bands was determined by the comparison with the standard molecular marker set.

Effects of substrate concentration

The optimum concentration of substrates (G3P and sodium pyruvate) required for the maximum activity of the DXS was determined in terms of V_{max} and K_m. The rate (V_0) of the DXS catalyzed reaction was measured using different concentrations of G3P (10-50 µM), sodium pyruvate (20-100 µM) and cofactor TPP (200 µM-1 M). The V_{max} and K_m values were determined from the double reciprocal (Lineweaver-Burk) plot.

Effects of temperature and pH

Temperature and pH optima were determined by performing the enzymatic reaction at different temperature 0-100°C and pH 6.0-9.0.

Effect of metal ions

Effect of the various metal ions such as $MgCl_2$, $CoCl_2$, $MnCl_2$, $CuCl_2$, $CaCl_2$ and $ZnCl_2$ on activity of the DXS enzyme was evaluated. The enzymatic reaction was performed in the presence of 1 mM concentration of each metal ion. Effective metal ion, $MgCl_2$ as a cofactor was checked for optimum DXS enzyme activity.

Results

Purification of the DXS

The DXS was purified in three steps. Results of the purification procedure are summarized in Table 1. In the first step, the crude enzyme extract was purified by ammonium sulfate fractionation and dialysis. Results revealed that approximately 60% of the DXS activity was precipitated between 0-40% $(NH_4)_2SO_4$ saturation with specific activity recorded 2.44 U/mg protein^{-1}. The purification fold was increased three times compared to the crude enzyme extract. Dialysis of the 0-40% fraction against Tris-HCl buffer (50 mM, pH 8.0) resulted in a significant increase in the specific activity of the DXS from 2.44-3.37 U/mg protein^{-1} with a yield of ~ 58%. The dialyzed fraction was subjected to the ion exchange chromatography using DEAE column and eluted with increasing concentration

Table 1. Purification of DXS from *C. flexuosus* cv. Suvarna leaves.

Step	Total protein (mg)	Total activity (U/min)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Crude enzyme extract	120.0	87.60	0.73	100	1.0
Ammonium sulfate 0-40%	71.07	173.41	2.44	59.23	3.31
Ammonium sulfate 40-80%	42.82	76.65	1.79	35.69	2.29
Dialysis of 0-40% fraction	69.94	235.70	3.37	58.29	4.59
DEAE-cellulose (pool 5)	55.66	291.66	5.24	46.39	7.14
DEAE-cellulose (pool 5) dialyzed	54.97	328.17	5.97	45.81	8.13
Sephadex G-150 (pool 3)	38.80	332.13	8.56	32.34	11.64

of NaCl. The elution profile (Fig. 2A, B) depicts prominent peaks of the DXS protein and DXS activity with increasing concentrations of the NaCl.

It showed that the pool number 5 comprising of fractions 67-72 (100 mM NaCl-200 mM NaCl) (Fig. 2B) had most of the DXS activity. By the end of ion exchange chromatography, specific activity of the DXS significantly increased from 3.37 to 5.97 U/mg protein^{-1} with 7 times increase in the purification fold.

Finally, concentrated sample was dissolved in 10 mM Tris-HCl buffer for further purification by gel filtration chromatography using the Sephadex G-150 column (100 x 10 mm). Elution profiles (Fig. 3A, B) indicated the highest concentration of the DXS protein and its activity in the pool 3 comprising of fractions number 21-26. At the end, specific activity of the DXS increased markedly from 5.97 to 8.56 U/mg protein^{-1} with almost 12% rise in the purification fold. Homogenity of the DXS containing fraction was evaluated by SDS-PAGE. A single band corresponding to the 130 kDa was detected in the gel electrophoretogram, which was identified as the DXS and named as *Cf*DXS (Fig. 4).

Characterization of DXS, substrate concentration

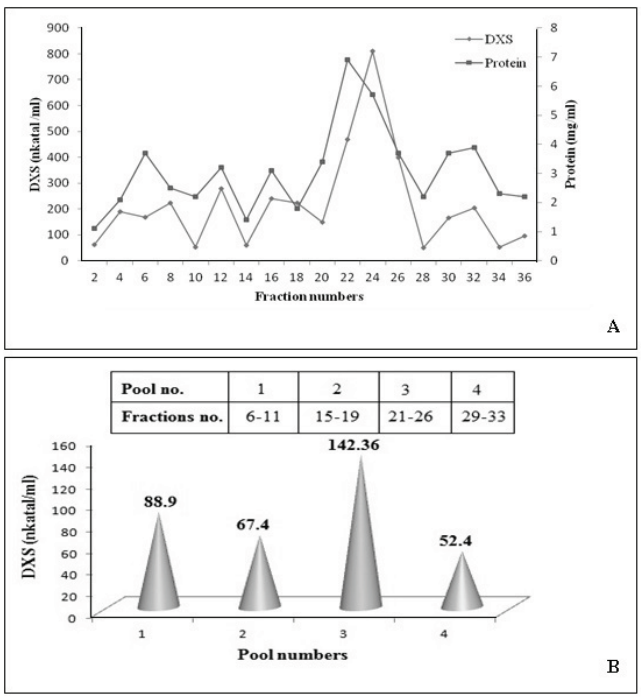

Figure 2. (A) DEAE-cellulose anion-exchange chromatogram (200×10 mm) showing elution profile of the dialyzed ammonium sulfate fraction (0-40%) from *C. flexuosus*; eluted by step ascending gradient method, using NaCl (50-400 mM) in Tris-HCl buffer (10 mM, pH 8.0); **(B)** DXS activities in ion exchange chromatography purified fractions.

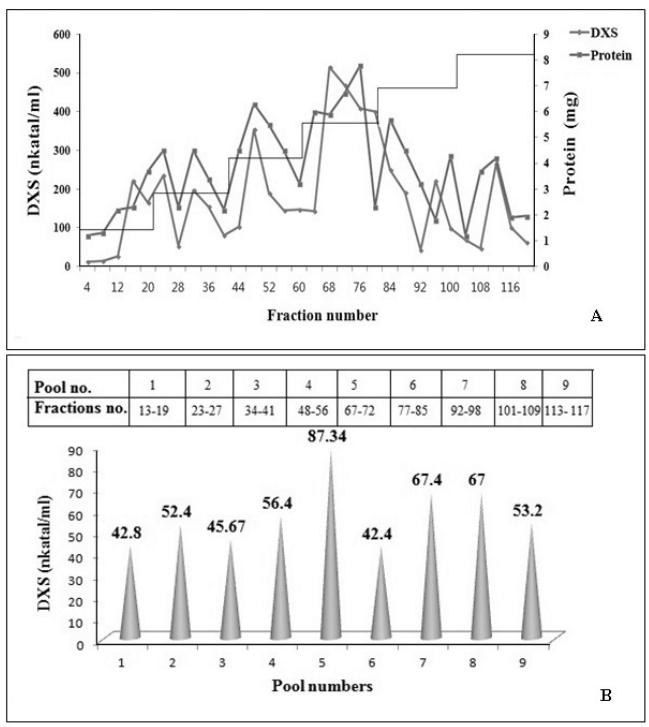

Figure 3. (A) Elution profile of the DXS enzyme on Sephadex G-150 (200 ×10 mm) by gel filtration chromatography. Column was eluted by using Tris-HCl 10 mM, pH 8.0. **(B)** DXS activities in gel chromatography fractions.

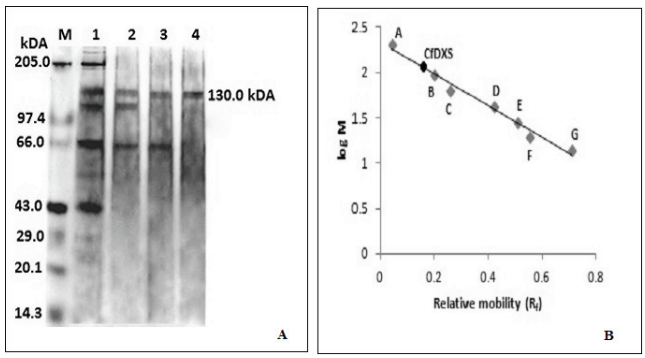

Figure 4. SDS-PAGE analysis of the *Cf*DXS enzyme **(A)** Lane M, protein marker with the indicated molecular masses; lane 1, crude enzyme extract; lane 2, ammonium sulfate precipitated sample; lane 3, DEAE-Cellulose; lane 4, Sephadex G-150 purified *Cf*DXS. **(B)** Calibration curve for the determination of the *Cf*DXS molecular weight by SDS-PAGE (10%). Marker proteins used for calibration: A : Myosin, rabbit muscle (205.0 KDa); B: phosphorylase b (97.4 KDa); C: bovine serum albumin (66.0 KDa); D: ovalbumin (4E: carbonic anhydrase (29.0 KDa); F: soyabean trypsin inhibitor (20.1 KDa); F: lysozyme (14.3 KDa); *Cf*DXS (130.0 KDa).

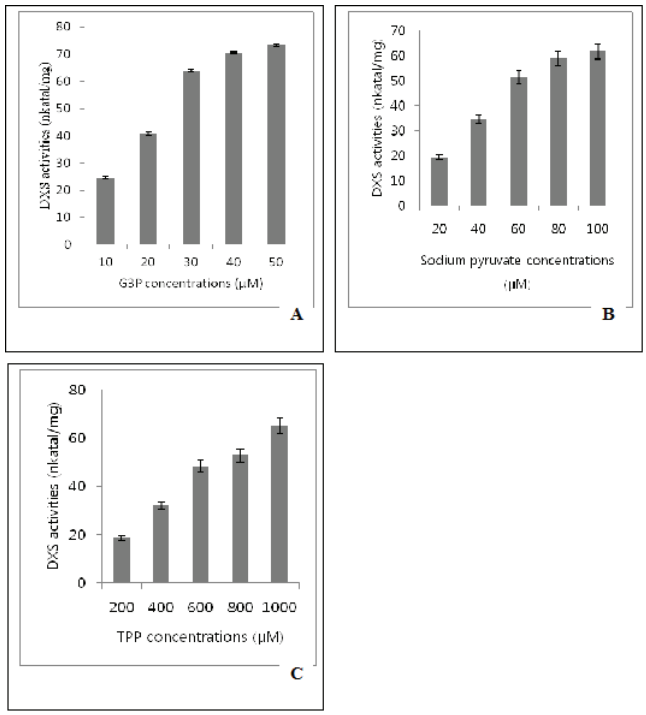

Figure 5. Effects of substrates (A) G3P (B) sodium pyruvate and (C) cofactor TPP on *Cf*DXS activity.

The optimum concentration of substrates G3P and sodium pyruvate measured were 50 and 100 µM, respectively, while that of cofactor TPP 1 mM (Fig. 5). Concentrations > 50 µM (for G3P) and 100 µM (for sodium pyruvate) were also tested, however, it did not result in any increment in the DXS activities (data not shown). After the optimum substrate concentration point, no increment in the enzyme activity was seen, which suggested the highest activity of any enzyme is observed only at the optimum substrate concentration. However, other factors like pH, temperature and cofactors also influence activity of any enzyme.

Temperature and pH

The pH and temperature optima determined for the *Cf*DXS were 8.0 and 40 °C, respectively (Fig. 6). The pH stability of the *Cf*DXS was found to be 6.0 to 9.0 in 50 mM Tris-HCl buffer, whereas the temperature stability 20 to 60 °C. The *Cf*DXS was quite stable (up to one month) when kept in 50 mM Tris-HCl buffer (pH 8.0) at 4 °C in the refrigerator retaining about 70% of the activity.

Effect of metal ions

Effects of metal ions such as Co^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Ca^{2+} and Zn^{2+} were evaluated on the *Cf*DXS activity. The results revealed that the *Cf*DXS activity enhanced significantly (~15 fold) in the presence of 1 mM Mg^{2+} (Fig. 7). Two metal ions namely, Zn^{2+} and Mn^{2+} moderately increase the *Cf*DXS

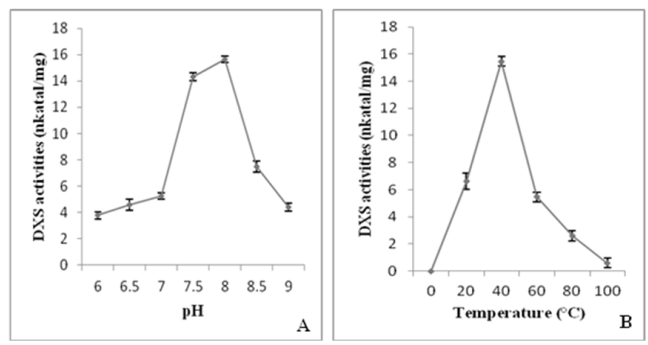

Figure 6. Effects of (A) pH and (B) temperature on *Cf*DXS activity.

activity, while the remaining metal ions Ca^{2+}, Co^{2+} Cu^{2+} and Fe^{2+} did not show any effect on the *Cf*DXS activity. The different concentrations (0.1 to 1 mM) of Mg^{2+} were used. The most effective concentration was found to be 1 mM.

The K_m for substrate pyruvate and G3P determined were 4.4 and 8.8 µM, respectively. The K_m for the cofactor TPP was measured 62 µM. The V_{max} of the *Cf*DXS measured were 20, 17 and 200 $\mu M^{-1} min^{-1}$, respectively of the pyruvate, G3P, pyruvate and TPP.

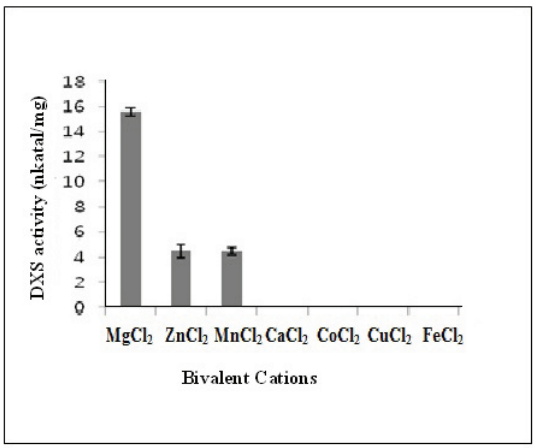

Figure 7. Effects of various metal ions (1mM) on *Cf*DXS activity.

Discussion

Very recently, the MEP pathway has been elucidated from the lemongrass (Gupta and Ganjewala 2015), which is utilized for the biosynthesis of the citral, a major monoterpene constituent of the lemongrass oil. The first step, which is also a regulatory step of the MEP pathway is catalyzed by the DXS enzyme, which is believed to control and regulate the over all rate of the biosynthesis of the citral. In view of the regulatory roles of the DXS in the MEP pathway, here we carried out its purification and characterization from the lemongrass cv. Suvarna immature leaves. Earlier, we carried out purification and characterization of the DXR enzyme, which catalyzes the second regulatory step of the MEP pathway (Gupta and Ganjewala 2015c).

The purification and characterization of both these regulatory enzymes have provided deeper knowledge of their kinetic parameters and catalytic principles, which has been very useful for understanding their roles in control and regulation of the substrates/precursor supply *via* the MEP pathway in lemongrass and other plants. Here, we used the similar three step procedures for purification of the *Cf*DXS, which was used earlier for the C*f*DXR with minor modifications. Due to the rate limiting nature of the DXS, we extracted it from the second leaf of a fully-grown tiller of lemongrass. The second leaf represents rapidly growing stage of the leaf development and biogenetically most active in the synthesis and accumulation of the essential oil. Hence, a very high CfDXS activity was expected in the second leaf.

Despite, the DXS being a key enzyme of the MEP pathway is present at very low concentration in cellular systems, therefore highly sensitive methods are required to detect the DXP production. The quantification of the DXP in living cells is performed by using recombinant technique (Lois et al. 1998), spectrofluorometer (Querol et al. 2001) and spectrophotometric assay (Altincicek et al. 2001). The yield of purified *Cf*DXS was 32.34% with specific activity of 8.56 U/mg protein^{-1}. At the end of the purification procedure, purification fold was increased 12 times compared to crude enzyme extract.

The *Cf*DXS was characterized by studying different parameters *viz.,* effect of substrate concentrations, pH, temperature and metal ions on the *Cf*DXS activity. These studies have revealed that the *Cf*DXS is similar to other DXSs reported from plants, *Mentha piperita* (Bouvier et al. 1998; Lange et al. 1998), *Arabidopsis thaliana* (Estévez et al. 2001; Flores-Perez 2008), *Zea mays* (Cordoba et al. 2011), *Oryza sativa* (Kim et al. 2005) and bacteria like *Escherichia coli* (Sprenger et al. 1997) and *Agrobacterium tumefaciens* (Lee et al. 2007). Like other DXSs, the *Cf*DXS is also a TPP-dependent enzyme with high catalytic efficiency (Lee et al. 2007). Studies of kinetic parameters of the *Cf*DXS showed relatedness with a class of TPP-dependent enzymes and displayed the properties of pyruvate decarboxylases and transketolases (Hahn et al. 2001). Reaction catalyzed by the DXS proceeds in two steps. In the first step, TPP-dependent decarboxylation of pyruvate occurs, which is analogous to decarboxylation catalyzed by the pyruvate decarboxylase. In the second step, enzyme-bound thiamine-stabilized acetyl anion is then transferred to the aldehyde moiety, which is analogous to trans-ketolase reaction (Hahn et al. 2001).

The present study revealed that the *Cf*DXS was dependent on Mg^{2+} ions for its activity. The Zn^{2+} and Mn^{2+} ions too moderately affected the *Cf*DXS activity, but Ca^{2+}, Cu^{2+}, and Fe^{2+} ions showed no effect on the *Cf*DXS activity. The order (descending) of the effectiveness of metal ions was $Mg^{2+} > Zn^{2+} > Mn^{2+}$. These characters of the *Cf*DXS have been consistent with several previously reported DXSs from *A. tumefaciens* (Lee et al. 2007), *R. capsulatus* (Hahn et al. 2001), *M. tuberculosis* (Bailey et al. 2002) and *A. thaliana* (Flores-Perez et al. 2008), which required Mg^{2+} as a cofactor for the activity. It is reported that the Mg^{2+} helps the DXS to maintain its activity in native form, and its removal does lead to the permanent loss of the DXS activity (Bailey et al. 2002). The TPP helps in the decarboxylation of pyruvate (Hahn et al. 2001). Here, the optimum concentration (1 mM) of the TPP determined matched with the earlier published reports (Hahn et al. 2001; Lee et al. 2007; Wright and Phillips 2014). The pH optima for *Cf*DXS was 8.0 and small deviation in it caused significant decline (60 to 80%) in the *Cf*DXS. These results are in complete agreement with several previous studies, which reported the similar pH optima 7.5-8.0 for the DXS (Sprenger et al. 1997; Bouvier et al. 1998; Hahn et al. 2001; Lee et al. 2007; Wang et al. 2014; Wright and Phillips 2014).

The optimum temperature for the *Cf*DXS was 37-40 ºC. The K_m for G3P (20 µM) and sodium pyruvate (4.4 µM) were found to be the same as reported previously (Lee et al.

2007; Wang et al. 2014). Relative molecular mass *Mr* of the DXS from plants such as tomato (Paetzold et al. 2010) and bacteria *viz., Haemophilus influenza* (Matsue et al. 2010), *Streptomyces* sp. strain CL190 reported was ranged from 65-70 kDa. The *Mr* of the C*f*DXS as determined by SDS-PAGE was 130 suggesting that it is most likely a dimeric enzyme. Similar dimeric DXSs with *Mr* 130 kDa have been reported previously from *Haemophilus influenzae* (Matsue et al. 1997), *Plasmodium vivax* (Handa et al. 2013) and *Streptomyces* sp. strain CL190 (Kuzuyama et al. 2000). To the best of our knowledge, this is for the first time the DXS enzyme has been purified and characterized from a member of the *Cymbopogon* genus.

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