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Purification and partial characterization of an extracellular alginate lyase from *Aspergillus oryzae* isolated from brown seaweed

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Abstract The extracellular enzyme alginate lyase produced from marine fungus *Aspergillus oryzae* isolated from brown alga *Dictyota dichotoma* was purified, partially characterized, and evaluated for its sodium alginate depolymerization abilities. The enzyme characterization studies have revealed that alginate lyase consisted of two polypeptides with about 45 and 50 kDa each on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and showed 140-fold higher activity than crude enzyme under optimized pH (6.5) and temperature (35°C) conditions. Zn^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+} and NaCl were found to enhance the enzyme activity while (Ca^{2+}, Cd^{2+}, Fe^{2+}, Hg^{2+}, Sr^{2+}, Ni^{2+}), glutathione, and metal chelators (ethylenediaminetetraacetic acid and ethylene glycol tetraacetic acid) suppressed the activity. Fourier transform infrared and thin-layer chromatography analysis of depolymerized sodium alginate indicated the enzyme specificity for cleaving at the β-1,4 glycosidic bond between polyM and polyG blocks of sodium alginate and therefore resulted in estimation of relatively higher polyM content than polyG. Comparison of chemical shifts in ^{13}C nuclear magnetic resonance spectra of both polyM and polyG from that of sodium alginate also showed further evidence for enzymatic depolymerization of sodium alginate.

Keywords Alginate lyase · *Aspergillus oryzae* · Fungus · PolyM · PolyG · Sodium alginate

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Introduction

Alginate occurs as a structural cell wall polysaccharide in a wide variety of brown seaweeds. It can also be obtained from bacteria such as *Azotobacter vinelandii* (Gorin and Spencer 1966) and *Pseudomonas aeruginosa* (Evans and Linker 1973) but with poor gelling characteristics. Alginates are linear unbranched polymers consisting of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) blocks, arranged as either homopolymeric (M–M or G–G blocks) or heteropolymeric (M–G and G–M blocks) random sequences (Gacesa 1992). Alginates are commercially important cell wall polysaccharides and widely used as stabilizers, viscosifiers, and gelling agents in diverse products such as food, beverages, and pharmaceuticals industries (Wong et al. 2000). The polyM has been well studied and reported as a potent inducer of cytokines under acute inflammatory responses (Jahr et al. 1997). In contrast, polyG inhibits the secretion of cytokines, resulting to the alleviation of the immunostimulation during tissue grafting and other autoimmune disorders (Otterlei et al. 1992). The alginate derivatives with sulfate groups have been reported to have high tumor inhibition activity against solid sarcoma 180 in vivo (Hu et al. 2004) in addition to tissue engineering applications (Kataoka et al. 2004). Further, the depolymerized products of alginate have also been stated to promote germination, growth, and development in crop plants (Cao et al. 2007).

Alginate lyases, characterized as either mannuronate (EC 4.2.2.3) or guluronate lyases (EC 4.2.2.11), catalyze the degradation of alginate. Alginates can be depolymerized into respective oligosaccharide fragments using either enzymatic lyases or acid hydrolysis. Alginate lyase uses a β-elimination in which a non-reducing unsaturated bond is produced during cleavage of the uronic acid, giving rise to

a strong absorption at 235 nm in the UV region (Song et al. 2003). There are 18 families of polysaccharide lyases (PL), including alginate lyases, reported (Maruta et al. 2008). The alginate lyases have been mainly included in the PL-5, PL-6, PL-7, PL-14, PL-15, PL-17 and PL-18 families. The alginate lyases belonging to the PL-5 and PL-7 families act on alginate polymers endolytically at specific sites to produce oligosaccharides (Yamasaki et al. 2005; Maruta et al. 2008). In contrast, acid hydrolysis results in random cleavage along the polysaccharide chain, producing polysaccharide fragments with unmodified hexuronic acid residues at both ends. Most recently, Chhatbar et al. (2009) reported a rapid microwave-assisted acid hydrolysis of alginate giving rise to corresponding oligosaccharide fragments as final products.

The enzymatic lyases of alginates have continued to be of interest to researchers as they provide defined oligosaccharide products that can be exploited for applications in various industrial, agricultural, and medical fields (Hu et al. 2005). Alginate lyases that have been reported earlier were mostly from crustacean (Shun et al. 1984), bacterial (Cao et al. 2007), viral (Davidson et al. 1977), and terrestrial sources. However, there are also quite a few from marine fungi (Wainwright and Sherbrock-Cox 1981; Schaumann and Weide 1990; Sarrocco et al. 2004; Orgaz et al. 2006).

The present study reports production of an extracellular alginate lyase that specifically cleaves at the β-1,4 glycosidic linkage between polyM–G blocks of sodium alginate producing homopolymeric blocks of polyM and polyG from the marine fungus *Aspergillus oryzae* isolated from the brown alga *Dictyota dichotoma* that specifically cleaves at the β-1,4 glycosidic linkage between polyM–G blocks of sodium alginate producing homopolymeric blocks of polyM and polyG. The enzyme was further purified and evaluated for its sodium alginate depolymerization abilities. The enzymatic depolymerized products were estimated and characterized by using different analytical tools such as thin-layer chromatography (TLC), Fourier transform infrared (FT-IR), and ^{13}C nuclear magnetic resonance (NMR) and compared with the findings of acid hydrolysis.

Materials and methods

The fungus-infected brown alga *Dictyota dichotoma* was collected from Kalubhar island (22° 29′ N and 69° 37′ E), Gulf of Kutch, Gujarat (India), and brought to the laboratory under cool conditions. The infected alga was left in moistened condition in the culture lab at 25±1°C in Petri plate for a week to allow adequate microbial growth. Fungi were isolated from degrading algal parts by inoculating its mycelium on solid-state media of potato dextrose agar (PDA) plates under aseptic conditions on bio-clean bench (MCV-13BSF, Sanyo, Japan). The plates were incubated at room temperature (35±1°C) for 4 days; pure cultures of each fungal strain were prepared by repeated streaking. The pure fungal strains thus obtained were screened for alginate lyase activity by subculturing on basal salt medium (pH 6.5) containing 3% NaCl, 0.2% KCl, 0.01% $MgSO_4$, 0.01% yeast extract, and 0.05% peptone on (*w*/*v*) base in distilled water solidified with 2% (*w*/*v*) sodium alginate as substrate for enzyme production. Subsequently, the plates were incubated at 35±1°C for 3 days and then flooded with 5% $CaCl_2$ solution. The zone of clearance indicated alginate lyase activity while non-degraded sodium alginate is precipitated.

The optimum temperature for the growth of the isolated fungus was determined by culturing their conidia (200 conidia 100 μL^{-1}; counted by hemacytometer) at different temperatures (4° to 40±1°C) in 20 mL PDA medium plate for 3 days. Later, the growth pattern/curve was plotted based on turbidity measurements (405 nm) of 100 mL PD broth inoculated with 100 μL conidial suspension having 200 conidia (Dabur et al. 2007) for 4 days. During growth studies, the flasks were incubated at optimum temperature on orbital shaker at 150 rpm.

Isolation and purification of extracellular alginate lyase

Fungal conidial suspension (200 conidia 100 μL^{-1}) was inoculated in 100 mL of basal salt medium and incubated at 35°C on an orbital shaker for 4 days as above. The medium was centrifuged at 12,000 rpm for 30 min at 4±1°C. The enzyme was concentrated from the cell-free broth by salting out with ammonium sulfate (80% saturation). The protein precipitated was re-suspended in 25 mL of 0.2 M phosphate–citric acid buffer (pH 6.5) and dialyzed against the same buffer. The dialyzed sample was applied to DEAE cellulose column (1.6×20 cm, Sigma) equilibrated with 400 mL of 0.2 M phosphate–citric acid buffer at pH 6.5. The elution was performed with a linear gradient of 0.0 to 1 M NaCl in phosphate–citric acid buffer (pH 6.5) with a flow rate of 0.5 mL min^{-1} at 4±1°C. Fractions which exhibited alginate lyase activities were pooled and concentrated. The concentrate was further fractionated using a Sephadex G-50 column (Sigma-Aldrich, Germany), equilibrated as mentioned earlier. Elution was performed by 0.2 M phosphate–citric acid buffer (pH 6.5), at a flow rate of 0.5 mL min^{-1}. Fractions possessing highest specific activity were pooled and used for enzyme characterization. Protein concentration was determined by folin phenol method (Lowry et al. 1951).

Enzyme assay

Enzyme activity was determined in 1 mL assay mixture containing 0.2 M phosphate–citric acid buffer (pH 6.5), 0.15 mL sodium alginate (1% *w*/*v*), and 50 μL enzyme solution. The assay mixture was incubated at 35°C for 30 min, and the absorbance was recorded at 235 nm. One unit of lyase activity was defined as the amount of enzyme that increased the absorbance at 235 nm by 0.01 units after 1 min.

Determination of optimum pH, temperature and stability

The optimum pH for alginate lyase activity was determined in the pH ranges of 2.0–10.5, using the 0.2 M phosphate–citric acid buffer for pH 2.0–8.5 and 0.2 M glycine–NaOH buffer for pH 8.0–10.5. The optimum temperature for alginate lyase activity was determined by varying the incubation temperatures from 4°C to 50°C. The thermal stability of enzyme was studied at optimum pH by incubating the enzyme at different temperature ranges for 1 h. The residual activity was determined by adding the substrate and carrying out the enzyme assay under optimum reaction conditions. Activity obtained for the enzyme stored at 4°C was considered as 100%.

Determination of enzyme kinetic and effects of different substrates

PolyM and polyG were prepared according to Haug et al. (1966, 1967) and subsequently used for estimating the lyase capacity of the alginate lyase. The kinetic properties of the enzyme were determined using different substrates such as sodium alginate, polyM, and polyG at different concentrations ranging from 0 to 40 mg mL^{-1} using Lineweaver–Burk double reciprocal plot. The apparent K_m and V_{max} values were calculated using curve-fitting software (FindGraph 2.18, UNIPHIZ Lab, Russia).

Effects of metal ions and chelators on the enzyme activity

The effect of additives including metal ions (Ca^{2+}, Cd^{2+}, Zn^{2+}, Hg^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+} and Sr^{2+}), reducing agent glutathione and chelators (EDTA and EGTA) on enzyme activity was tested at a concentration of 2 mM each and NaCl at different concentrations (2, 20, 100 and 150 mM). The enzyme activity was measured by adding these metal ions and chelator together with the substrate, and then enzyme assay was carried out under the optimum conditions. An enzyme assay reaction without any additive was used as control and activity obtained for this was considered as 100%.

Electrophoresis

The purity of the alginate lyase was analyzed by 10% native and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). The proteins were developed by silver staining.

Acid hydrolysis of sodium alginate

For acid hydrolysis, sodium alginate (10 mg) from Sigma was dissolved in 50 mL of 1 M oxalic acid and heated at 80°C for 4 h. Subsequently, the polyM and polyG blocks were isolated using pH gradient method (Sakugawa et al. 2004). To check the stability of sodium alginate against the heating process, a control experiment was performed under the same conditions except using acid reagent.

Viscometric assay and TLC

Viscometric study was carried out for 16 days at a regular interval of every 2 days for both acid and enzymatic treatments using a Brookfield DV-II+ Pro viscometer with spindle number SC-18 at 100 rpm. The degraded products after acid and enzymatic treatments were analyzed by TLC. Five-microliter sample of both was used for spotting onto silica plates (silica gel-60, Whatman) and separated simultaneously by ascending chromatography using the solvent system 1-butanol/acetic acid/water (3:2:2, *v*/*v*/*v*). Plates were sprayed with 10% sulfuric acid in ethanol and heated at 110±1°C for 45 min (Warren 1960).

FT-IR and NMR spectroscopy

A pellet of 0.75 mg of degraded sodium alginate (acid- and enzyme-hydrolyzed) was prepared with KBr (200 mg) followed by pressing the mixture into a 16-mm-diameter mold. FT-IR spectrum was recorded on Perkin Elmer (Spectrum GX, USA) with a resolution of 4 cm^{-1} in the 4,000–400-cm^{-1} region. Approximate contents of polyM and polyG were determined by spectroscopy (Haug and Larsen 1962; Ji et al. 1981).

Noise-decoupled ^{13}C-NMR spectra were recorded on a Bruker Advance-II 500 (Ultra Shield) spectrometer, Switzerland, at 500 MHz. Sodium alginate and enzymatic degradation products (polyG and polyM) were dissolved in the *d*-NaOH (50 mg mL^{-1}), and spectra were recorded at 25°C with 5,000–5,200 accumulations, pulse duration of 5.9 μs, acquisition time of 1.2 s, and relaxation delay of 6 μs using DMSO as internal standard (approximately δ 39.7).

Phylogenetic analysis

The identification of fungal strain was carried out by sequencing of nuclear encoded ribosomal internal transcribed spacer (ITS) region. DNA extraction using the existing CTAB protocol (Doyle and Doyle 1987) and polymerase chain reaction (PCR) amplification for ITS region was carried out with the primer pair IF (TCC GTA GGT GAA CCT GCG G) and IR (TCC TCC GCT TAT TGA TAT GC). PCR amplification was performed with the following cycling program: denaturation for 6 min at 94°C, 30 amplification cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, with a 10-min extended elongation step. The PCR product was purified with QIAquick PCR purification kit (Qiagen, Germany) as per manufacturer's instructions. Forward and reverse DNA sequencing reaction of PCR amplification was carried out using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Partial consensus sequence of ITS region was generated after alignment of forward and reverse sequence data using Aligner software. The consensus sequence was then subjected to BLAST for homology search in the National Center for Biotechnology Information (NCBI) GenBank database. Phylogenetic tree was inferred by Molecular Evolutionary Genetics Analysis (MEGA4) software. The sequence obtained for the fungal strain was submitted to NCBI GenBank.

Results

Identification and characterization of alginate-degrading fungus

Among the two isolated fungi, only one showed alginate lyase activity with clear zone around the fungal colony. The homology search of the partial consensus sequence of 623 bp of ITS region revealed the strain as *A. oryzae* (accession number GU385811) (Fig. 1). The phylogenetic tree was constructed using the NJ method (Saitou and Nei 1987) conducted in MEGA4 (Tamura et al. 2007). The bootstrap consensus tree was inferred from 500 replicates (Felsenstein 1985). There were a total of 599 positions in the final dataset. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates were collapsed. The evolutionary distances were computed using the Kimura two-parameter method (Kimura 1980) and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

The fungus growth curve analysis showed a long lag phase of about 20 h followed by log phase of 28 h. The log phase was typically characterized with maximum conidia germination and mycelial growth. After 48 h, fungus attained its stationary phase. The optimal temperature for fungal growth was found to be 35±1°C.

Purification of alginate lyase

The culture fluid for crude enzyme preparation was collected before the onset of the stationary growth phase of *A. oryzae*. The two-step purification of crude enzyme increased the specific activity as much as 140 times higher with a yield of 21.11% (Table 1).

The characterization of alginate lyase from the 10% SDS-PAGE revealed that it was comprised of two polypeptides with molecular weight of about 45 and 50 kDa each (Fig. 2a). The 10% native PAGE analysis clearly indicated the purity of enzyme with a single band (Fig. 2b).

Effects of pH and temperature on enzyme activity and stability

The enzyme activity (67.24 U mg^{-1} protein) was optimum at 35±1°C and decreased by almost 20% at both 25°C and 40°C while its activity was completely lost at 50°C (Fig. 3a). The optimum pH was 6.5 for the enzyme activity which (Fig. 3b). Moreover, the relative enzyme activity was observed to increase by 20% after preincubation of 30 min at 35±1°C compared to the activity that was obtained using the enzyme stored at 4°C. The enzyme kinetic study depicted a hyperbolic curve with different concentrations of the sodium alginate. The alginate lyase was found capable of degrading only sodium alginate and was inefficient in the degradation of polyM and polyG (Fig. 3c). The K_m and V_{max} values of alginate lyases evaluated from double reciprocal curve of Lineweaver–Burk plot, were 21.52 mg mL^{-1} and 222.68 U mg^{-1} proteins, respectively.

Effects of metal ions and chelator on the enzyme activity

The alginate lyase activity was found to increase by supplementing metal ions of Zn^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, and Mg^{2+} to the enzyme assay medium. In contrast, Ca^{2+}, Sr^{2+}, Ni^{2+}, Fe^{2+}, Cd^{2+}, EGTA, and EDTA decreased the activity, while Hg^{2+} exerted a significant inhibitory effect on the enzyme activity. The reducing agent glutathione decreased activity by 77%. The addition of 2 mM or higher concentration of NaCl (20, 100 and 150 mM) to bioassay medium (pH 6.5) also increased the alginate lyase activity (Table 2).

Viscometric assay, FT-IR, NMR spectroscopy and TLC

The sodium alginate degraded with both acid hydrolysis and enzymatic lyase was separately investigated for the intrinsic viscosity (Fig. 3d). The acid-hydrolyzed products

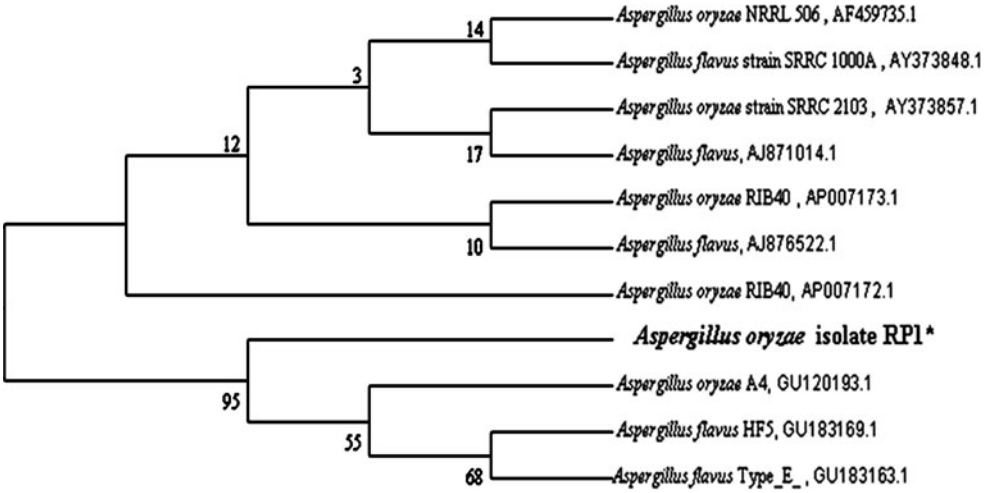

Fig. 1 Phylogenetic tree based on neighbor-joining analysis of ITS sequence (* present study)

showed nearly constant viscosity (3.8 cP) during the study period of 16 days. On the other hand, enzymatic depolymerization of sodium alginate showed a rapid decline in viscosity (5.9 to 2.2 cP) during the initial 8 d and decreased further to 1.8 cP at the end of the experiment (16 days).

The FT-IR spectrum of the sodium alginate showed characteristic peaks at 893 and 821 cm^{-1} for polyG and at 944 cm^{-1} for polyM. However, the FT-IR spectrum of lysed products had peaks at 880, 803 and 931 cm^{-1} corresponding to polyM and peaks at 1,728 and 1,748 cm^{-1} corresponding to polyG, indicating the depolymerization of sodium alginate. The enzymatically depolymerized sodium alginate revealed that it had a twofold increase in the yield of polyM blocks and a 0.64 fold decrease in the yield of polyG compared to that of acid hydrolysis as quantified from pH-dependent precipitation. This, in turn, increased the M/G ratio by 1.6-fold in enzymatic lysed reaction compared to acid hydrolysis (Table 3).

Further, the TLC R_f value of 0.28 that was observed for enzymatic lysed products coincided with the R_f value of polyM blocks obtained after acid hydrolysis of sodium alginate (Fig. 4). However, no spots were found for sodium alginate and polyG (data not shown). Chemical shifts for the ^{13}C NMR spectra obtained for the sodium alginate (*d*-NaOH) were δ=100.69 (C-1 of MM), 71.46 (C-2 of MM), 70.18 (C-3 of MM), 76.69 (C-4 of MM), 72.49 (C-5 of MM), 176.01 (C-6 of MM), 100.36 (C-1 of GG), 80.81 (C-4 of GG), 72.31 (C-5 of GG), and 176.38 (C-6 of GG), and enzyme-depolymerized polyG (δ=101.11, 99.62, 66.61, 70.27, 71.43, 76.75, 75.62, 67.0, 86.10, 175.12) and polyM (δ=99.77, 99.68, 69.79, 66.96, 71.43, 70.25, 79.09, 76.83, 75.56, 175.24) were found to be comparable with the report of Chhatbar et al. (2009).

Discussion

The filamentous fungus *A. oryzae* has been increasingly recognized as a commercially important organism and extensively used in the industrial production of sake, miso and shoyu (Machida 2002). The US Food and Drug Administration and WHO have accorded the status of 'generally regarded as safe' to *A. oryzae* due to its continued use in food production (Machida 2002). *A. oryzae* has also been reported to produce a wide variety of industrially important enzymes like α-amylase and keratinase (Farag and Hassan 2004).

The present study reports the production of an extracellular alginate lyase from fungus *A. oryzae* for the first time. The enzyme in the present study is unique by having its specific cleavage site at the β-1,4 glycosidic bond between polyM and polyG blocks of sodium alginate. Consequently, the enzymatically depolymerized sodium alginate resulted in higher polyM/polyG ratio as compared to acid hydrolysis. Traditionally, bacteria are considered to be a major commercial source of alginate lyase. The earlier studies have been largely involved in screening of various fungal species for alginolytic activity from terrestrial source (Sarrocco et al. 2004). Among the *Aspergillus* species from terrestrial

Table 1 Summary of alginate lyase purification

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Crude	1,800	860	0.48	100	1
$(NH_4)_2SO_4$	140	613	4.38	71	9.33
DEAE cellulose	14	363.2	25.94	42	54.04
Sephadex G - 50	2.7	181.57	67.24	21.11	140.08

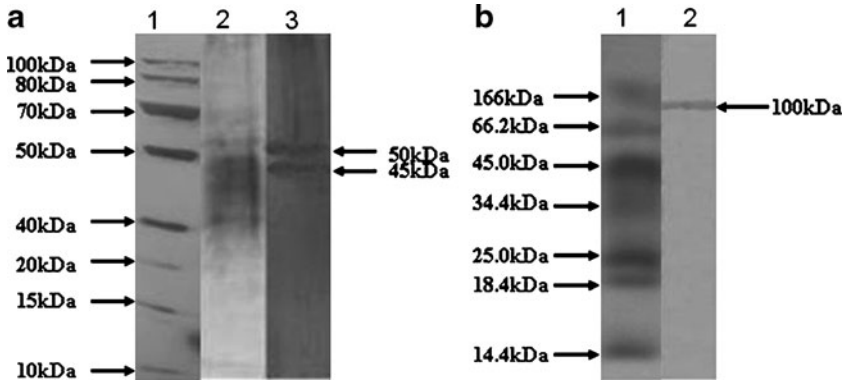

Fig. 2 Electrophoretic profile of alginate lyase, **a** 10% SDS-PAGE: (*1*) molecular weight standards, (*2*) crude supernatant alginate lyase, (*3*) purified lyase from molecular exclusion chromatography and gel filtration; **b** 10% native PAGE: (*1*) molecular weight standards, (*2*) purified lyase from molecular exclusion chromatography and gel filtration. The *arrow* indicates the position of purified lyase band

sources, *A. flavipes*, *A. fumigates*, and *A. ustus* and the ascomycete (*Corollospora intermedia*) are known as a sources of alginate lyase. A few species from deuteromycetes (*Asteromyces cruciatus*, *Dendryphiella arenaria* and *D. salina*) from marine habitats have also been reported to have alginolytic activity (Schaumann and Weide 1990). All the lyases that have been investigated for alginate depolymerization to date belong to the class of polyM, polyG or oligoalginate lyase. The alginate lyases from animals such as the abalone *Haliotis* (Suzuki et al. 2006) and *Pseudomonas syringae* Pv. *syringae* (Preston et al. 2000) belong to polyM lyase while those from bacteria such as *Klebsiella aerogenes* (Lange et al. 1989) and *Streptomyces* sp. A5 (Cao et al. 2007) are polyG lyase. The lyase from the bacterial strain *Sphingomonas* sp. A1 (Yamasaki et al. 2005) is regarded as an oligoalginate lyase. The alginate lyases that have been studied earlier from marine fungi were mainly of polysaccharide lyases specifically cleaved at either polyM or polyG blocks.

Comparison of chemical shift in the ^{13}C NMR spectra of both polyM and polyG with that of sodium alginate shows evidence for depolymerization of the substrate. The ^{13}C NMR spectra and FT-IR findings of enzymatically depolymerized sodium alginate support the fact that the alginate lyase obtained in the present study has got specificity for cleaving the polymer at the β-1,4 glycosidic link (Chhatbar et al. 2009).

The higher specificity of the enzyme towards the polyM–G link is also been further supported by the viscometric analysis. The viscometric results showed a decline in viscosity of sodium alginate with time at 35± 1°C. The decrease in the viscosity (5.9 to 1.8 cP) continued until 16 days and thus suggests that the alginate lyase has depolymerized the substrate more efficiently than acid

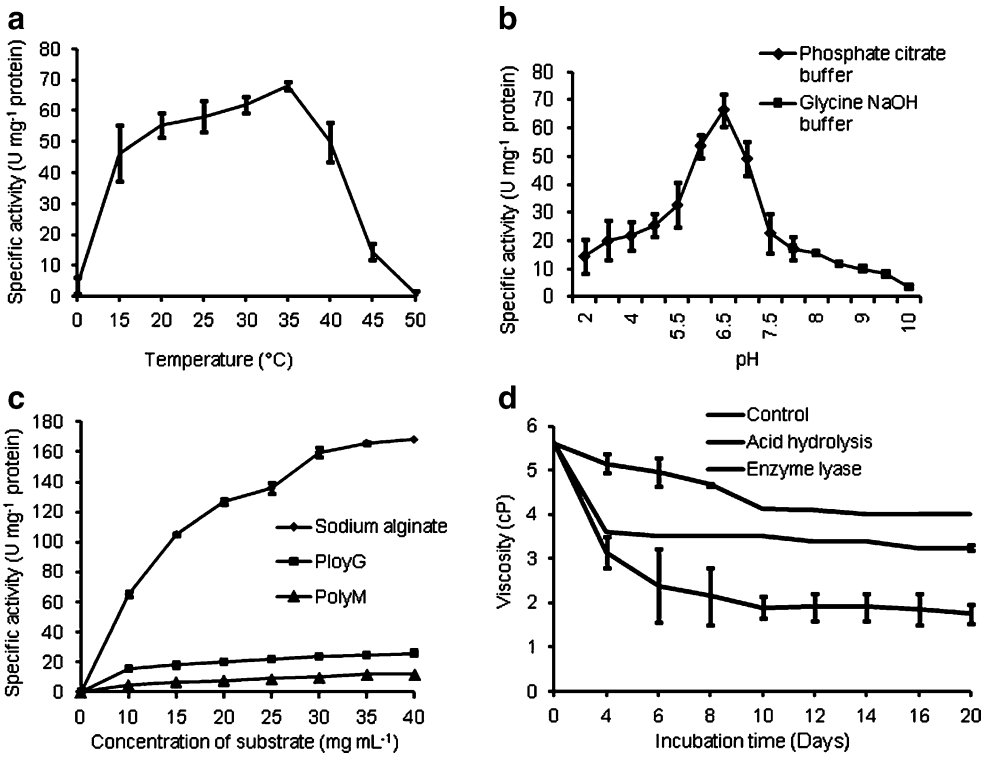

Fig. 3 Optimization of different parameters for obtaining maximum activity of alginate lyase, **a** temperature, **b** pH, **c** different concentrations of substrate, and **d** viscosity profile versus incubation time for the sodium alginate solution degraded by both alginate lyase and acid hydrolysis

Table 2 The effect of metal ion on the activity of alginate lyase

Additive	Concentration (mM)	% Relative activity
Control		100±1
NaCl	2	110±1
NaCl	20	137±1
NaCl	100	193±1
NaCl	150	196±1
$MnCl_2$	2	103±1
$ZnCl_2$	2	105±1
$HgCl_2$	2	10±1
$FeCl_2$	2	30±1
$CdCl_2$	2	25±1
$CaCl_2$	2	95±1
$MgCl_2$	2	101±1
$NiCl_2$	2	65±1
$CuSO_4$	2	104±1
$CoCl_2$	2	105±1
$SrCl_2$	2	70±1
EGTA	2	23±1
EDTA	2	15±1
Glutathione	2	23±1

±1 indicates the deviation of value from mean (OD) of the three replicates

hydrolysis (Fig. 3d). Similar findings have also been reported by Schaumann and Weide (1990) for enzymatically depolymerized sodium alginate. TLC further confirmed the enzymatic cleavage of polymer at the β-1,4 glycosidic link. The R_f values of both acid-hydrolyzed and enzymatically degraded polyM were similar, whereas polyG could not move on the TLC plate which may be possibly due to the long chain of guluronic acid that resulted in the increase of molecular weight.

Furthermore, the degradation of sodium alginate with alginate lyase resulted in actual estimation of polyM and polyG contents compared with that of acid hydrolysis, allowing a realistic estimation of copolymers present in the sodium alginate (Table 3). Therefore, the increased M/G ratio in the resultant depolymerized sodium alginate

Table 3 Comparison between enzymatic lyase and acid hydrolysis of sodium alginate (10 mg)

Enzymatic degradation			Acid hydrolysis		
PMA%	PGA%	M/G ratio	PMA%	PGA%	M/G ratio
54	46	1.17	30	70	0.43

Percentage of PGA and PMA was calculated by UV-Vis spectroscopy as described by Haug and Larsen (1962) and Ji et al. (1981)

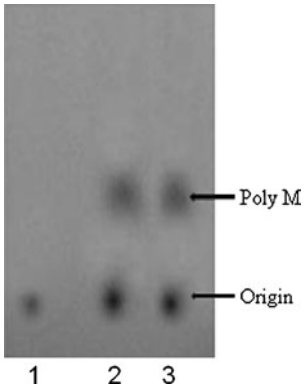

Fig. 4 TLC of (*1*) sodium alginate, (*2*) polyM of enzymatic degradation, and (*3*) acid hydrolysis

compared with acid hydrolysis could be due to relative abundance of polyM blocks over polyG blocks while the same was different with acid hydrolysis due to random cleavage of the sodium alginate.

Most of the alginate lyases that have been studied earlier consisted of a single subunit with molecular weight ranging from 25 to 100 kDa (Wong et al. 2000). The alginate lyase characterized in this study had two polypeptides with molecular weight of about 45 and 50 kDa each on 10% SDS-PAGE. There is only one report (Kaneko et al. 1990) describing an alginate lyase from two soil bacteria having two polypeptide chains, each having 35, 20, 50, and 38 kDa protein, respectively. However, there is no report of alginate lyase with two polypeptide chains from any fungal source. Thus, the alginate lyase isolated from *A. oryzae* in the present study is new and unique. The optimum temperature for maximum activity of the bacterial alginate lyases of *Streptomyces* sp. A5 (Cao et al. 2007) and *Sphingomonas* sp. A1 (Yamasaki et al. 2005) is 37°C which is quite close to the 35°C optimum in the present study. Preincubation of alginate lyase at 35°C increased the activity, possibly due to the expansion of the enzyme active site. Alginate lyases from different origins have a maximum functionality at pH near neutral with pH optima of 7.0–8.0 with a few exceptions (Gacesa 1992). The optimum pH for alginate lyase studied in this study is in accordance with earlier reports showing maximum activity at pH 6.5. The maximum activity of the alginate lyase under optimized conditions in the present study makes it distinct from other alginate lyases known from other sources. Further, the alginate lyases reported so far require low concentrations of divalent metal ions for the maximum activity. Similarly the enzyme activity of the enzyme reported here also enhanced by addition of certain divalent metal ions (Zn^{2+}, Mn^{2+}, Cu^{2+} Mg^{2+} and Co^{2+}) while glutathione, EDTA, EGTA, Ca^{2+}, Hg^{2+} and Cd^{2+} ions were found to suppress enzymatic activity. Addition of NaCl (up to 150 mM, pH 6.5) also increased the alginate lyase activity. The increased activity in the presence of divalent metal cations could be due to cross-bridging the anionic carboxyl group of sodium alginate and the nucleophilic

amino acid side chain in the active site of the enzyme (Gacesa 1992).

The alginate lyase with two polypeptide subunits (45 and 50 kDa each) and high thermal stability is new and unique from a fungal source. The polyM and polyG blocks as obtained due to enzyme lyase from sodium alginate could be of potential use in the biomedical industry.

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