International Journal of Biological Chemistry

ISSN 1819-155X

Academic Journals Inc.

www.academicjournals.com

International Journal of Biological Chemistry 9 (3): 133-141, 2015
ISSN 1819-155X / DOI: 10.3923/ijbc.2015.133.141

Medium Optimization, Purification, Characterization and Specificity Studies of Extracellular RNase from *Streptomyces* sp. (NCIM 2081)

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ABSTRACT

Streptomyces sp. (NCIM-2081) produced high levels of extracellular RNase (44.3 U mL^{-1}) activity when screened in MGYP medium. (Malt extract, Glucose, Yeast extract and Peptone) Medium optimization studies for RNase production showed that organic nitrogen source and initial pH had marked effect on enzyme activity. Maximum extracellular RNase activity (644.3 U mL^{-1}) was obtained in BG medium (Beef extract+Glucose) at 28°C and pH 6.0. RNase activity showed 14 fold increase (44.3-644.3 U mL^{-1}) and 7.4 fold increase in specific activity (295-2174 U mg^{-1}) after medium optimization studies. Purification of RNase was carried out using ion exchange chromatography and gel filtration using FPLC-Superose 12 column with 10%, recovery and 8 fold increase in specific activity. The M_r of the purified enzyme was 39.134 kDa. Optimum pH and temperature for RNA hydrolysis were 8.0 and 45°C. The RNase activity did not required metal ions for the optimum activity and was not affected by addition of EDTA. The enzyme was inhibited by divalent cations like Mn^{2+}, Zn^{2+} and Cu^{2+}. It was stable to high concentration of Urea but susceptible to low concentration of Sodium dodecyl sulphate and Guanidine-HCl. Km was 2.47 mg mL^{-1} and Vmax was 1.98×10^{6} μM min^{-1} mg^{-1}, respectively. The enzyme was non specific and cleaved RNA to 3'mononucleotides. The RNase was resistant to proteolytic enzymes like Papain, Trypsin and Pepsin but sensitive to low concentration of proteinase K.

Key words: Extracellular, medium optimization, production, purification, RNase

INTRODUCTION

The RNases are important analytical enzymes used in the determination of RNA structure. The RNases are also used for the removal of RNA in Single-cell protein preparations (Reddy and Shankar, 1993). RNase enzymes, which produced 5'GMP or 5'IMP as end products or having specificity are used in food industry (Joshi and Deshmukh, 2011). Plant viruses are responsible for numerous plant diseases. To control viral diseases biological treatments are superior to physicochemical methods (Zhou and Niu, 2009). Microbial RNases are considered alternatives to chemotherapeutic drugs because of its ability to inhibit animal tumors and viruses (Zhang *et al*., 2001).

Compared to fungal nucleases, few reports are available on Streptomyces nucleases and amongst them few reports are available on *Streptomyces* RNases compared to *Streptomyces* DNases. RNases reported during early 1960-80s showed specificity similar to RNase T1 and were

reported as guanylribonucleases (Joshi and Deshmukh, 2011). The DNase enzymes required metal ions for their activity so cannot be used in chelating buffers or in presence of metal chelators (Deshmukh and Shankar, 1999). The RNase enzymes exhibit sufficient activity in absence of metal ions. So *Streptomyces* cultures were screened to evaluate their RNase producing ability and *Streptomyces* sp. (NCIM 2081) showing high extracellular RNase activity was selected for purification, characterization and specificity studies.

MATERIALS AND METHODS

Materials: Acrylamide, Bis-acrylamide, RNA (Sisco Research Laboratories, Mumbai, India) Calf thymus DNA, Coomassie brilliant blue G-250, DEAE cellulose, CM cellulose, (Sigma Chemical Co St. Louis USA) bovine serum albumin, perchloric acid, D-glucose, methanol, acetic acid, potassium hydroxide and β-alanine (Qualigens Fine Chemicals Mumbai, India), SDS molecular weight markers (Fermentas Life Sciences, India), malt extract, yeast extract, beef extract, peptone, agar, and uranyl acetate, (Hi Media Laboratories Pvt Ltd Mumbai, India), All other chemicals used were of analytical grade.

Microorganisms: *Streptomyces aureofaciens* (2417), *Streptomyces caelestis* (2870), *Streptomyces* species (2081), *Streptomyces venezualae* (2215), *Streptomyces atrofaciens* (2951) and *Streptomyces albus* (2413) strains were obtained from NCIM (National Collection of Industrial Microorganisms) National Chemical Laboratory, Pune, India. These cultures were maintained on MGYP slants (Malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, glucose 1% and agar 2%).

Enzyme production: The inoculum was prepared by inoculating 10 mL of MGYP medium with a well sporulated piece of slant, followed by incubation at 28°C for 48 h. Fermentations were carried out in 250 mL Erlenmeyer flasks containing 50 mL MGYP medium and by transferring 10% inoculum followed by incubation at 28°C for 72 h with shaking (200 rpm). The extracellular broth collected by centrifugation (10000 rpm, 20 min) was used as source of enzyme. All experiments were performed in duplicate.

Methods

RNase activity: The RNase activity was carried out essentially according to Chacko *et al*. (1996). The total reaction mixture contains 0.75 mL containing 1.25 mg RNA in 200 mM Tris-HCl buffer pH 7.0 and appropriately diluted enzyme. The reaction was initiated by the addition of RNA followed by incubation at 37°C for 15 min. The reaction was arrested by the addition of 0.25 mL of Mac Fadyen's reagent (0.75% wt/vol uranyl acetate in 25% vol/vol perchloric acid) and the reaction was kept on ice for 5 min, the resulting precipitate was immediately removed by centrifugation (3000 rpm, 10 min), Subsequently 0.1 mL of the supernatant was diluted with 2.5 mL of distilled water. The amount of acid soluble ribonucleotides liberated was calculated by assuming a molar absorption coefficient of 10600 M^{-1} cm^{-1} for the ribonucleotides mixture (Curtis *et al*., 1966).

DNase activity: The DNase activity was estimated according to Apte *et al.* (1993). The standard reaction mixture of 1 mL contains 50 μg of native DNA in 30 mM tris-HCl buffer (pH 7.0) and appropriately diluted enzyme. The reaction was initiated by the addition of DNA followed by incubation at 37°C for 15 min the reaction was then terminated by the addition of 1 mL of 10%

chilled PCA and 1 mL of 0.2% BSA (Bovine Serum Albumin). The mixture kept in ice for 10 min and then centrifuge at 2500 rpm for 15 min. The acid soluble deoxyribonucleotides were measured at 260 nm. The amount of acid soluble deoxyribonucleotides liberated was calculated by assuming the molar absorption coefficient of 10000 M^{-1} cm^{-1} for deoxyribonucleotides mixture (Curtis *et al*., 1966).

One unit of RNase/DNase activity was defined as the amount of enzyme required to liberate 1 μM of acid soluble ribonucleotides or deoxyribonucleotides/min under the assay condition.

Determination of reducing sugar: Reducing sugar was determined by DNSA method of Miller (1959).

Protein determination: Protein concentration was estimated according to Sedmak and Grossberg (1977) using BSA as standard.

HPLC analysis of the reaction product

Action on RNA: The total reaction mixture of 1 mL containing 1.25 mg of RNA in 200 mM Tris-HCl buffer (pH 7.0) was incubated with 1 U of the purified RNase at 37°C. Subsequently, 1 U of enzyme was added twice after 6 h and incubated for 24 h. The reaction was terminated by the addition of 0.75 mL of ethanol and the mixture was left overnight at -20°C. The undigested RNA was removed by centrifugation (6000×g, 10 min). The supernatant was lyophilized, reconstituted in 100 μL water and subjected to HPLC.

Separation of the reaction products: High performance liquid chromatography (Waters model fitted with 515 HPLC pump) was carried out on a symmetry C18 column (250×4.6 mm, 5 μm, Waters, USA). The mobile phase comprising a linear gradient of acetonitrile in 100 mM triethylammonium acetate (pH6.2), 0-2.5% v/v for 20 min and 2.5 and 50% v/v for 10 min followed by 50-100% v/v for 5 min was used at 25±1°C at a flow rate of 0.8 mL min^{-1}. Ten micro Liter standard or 40 μL sample solution was injected onto the column and the nucleotides were detected at 254 nm, using Waters 2487 Dual Absorbance Detector. The nucleotides were eluted in the order of 3'CMP, 3'UMP, 3'GMP and 3'AMP with retention time of approximately 16.34, 21.2, 26.76 and 33.8 min, respectively (data not shown).

RESULTS AND DISCUSSION

All the values in the tables are mean of three sets

Screening: All the cultures were grown in MGYP* medium. As shown in Table 1, *Streptomyces* sp. (NCIM-2081) showing highest extracellular RNase activity was selected for further studies.

Table 1: Screening of *Streptomyces* cultures for extracellular RNase production

Culture No.	RNase (U mL^{-1})	Protein m (g mL^{-1})	Specific activity (U mL^{-1})
Streptomyces sp. (NCIM-2081)	44.3	0.15	295.3
Streptomyces atrofaciens (NCIM 2951)	3.9	0.22	17.7
Streptomyces albus (NCIM 2413)	4.4	0.13	33.8
Streptomyces calestis (NCIM 2870)	2.3	0.09	25.5
Streptomyces aurofaciens (NCIM-2417)	1.8	0.43	4.2

*Composition of MGYP medium was malt extract (0.3%), yeast extract (0.3%), peptone (0.5%) and glucose (1%)

Effect of media components on RNase production: During medium optimization studies, MGYP medium was modified by eliminating one component at a time and effect on RNase activity was checked. Table 2 showed that elimination of glucose (MYP medium) showed marked decrease in RNase activity from 44.3-19.6 U mL^{-1} suggesting that glucose was essential in production medium. Elimination of malt extract (YPG medium) showed increase in RNase activity (90 U mL^{-1}) but specific activity was marginally increased (366.7 U mg^{-1}). In YG medium where peptone was eliminated from YPG medium, lowest RNase activity (5.7 U mL^{-1}) was detected. In PG medium where yeast extract and malt extract were eliminated from MGYP medium highest RNase activity (195 U mL^{-1}) and specific activity (1406 U mg^{-1}) was detected. The PG medium was thus selected for further studies.

Effect of initial pH on RNase production: Initial pH of PG medium was adjusted from 5-9. Highest RNase activity and specific activity was detected at pH 6.0, so PG medium (pH 6.0) was used in further studies. This was different than *Actinomyces levoris* 2789 (*Streptomyces levoris*) RNase which was produced in alkaline media (Table 3) (Bezborodov *et al*., 1967b).

Effect of nitrogen sources on RNase production: The MGYP medium without nitrogen source (MG) showed 7.5 U mL^{-1} of RNase activity suggesting that nitrogen source was essential for RNase production. In *S levoris* DNase production inorganic nitrogen sources like NH_4Cl and NH_4NO_3 were reported to be most suitable nitrogen sources (Bezborodov *et al*., 1967a) but in the present studies, organic nitrogen sources were better for RNase production (55-539 U mL^{-1}) than inorganic nitrogen sources (7.7-10.5 U mL^{-1}). Among the different organic nitrogen sources tested (Table 4) beef

Table 2: Effect of media components on RNase production

Media components	Rnase (U mL^{-1})	Protein (g mL^{-1})	Specific activity (U mL^{-1})
MGYP*	44.3	0.15	295.3
MYP[a]	19.6	0.06	326.6
YPG[b]	90.5	0.25	366.7
YG[c]	5.7	0.08	73.1
PG[d]	195.0	0.05	1406.0

*Composition of MGYP: Medium was malt extract 0.3%, yeast extract 0.3%, peptone 0.5% and glucose 1% YPG: Medium contained yeast extract 0.3%, peptone 0.5% and glucose 1%,MYP: Medium contained malt extract 0.3%, yeast extract 0.3% and peptone 0.5%, YG: Medium contained yeast extract 0.3% and glucose 1% and PG: Medium contained peptone 0.5% and glucose 1%

Table 3: Effect of initial pH on RNase production

pH	RNase (U mL^{-1})	Protein (mg mL^{-1})	Specific activity (U mL^{-1})
5	341.1	0.13	2583.0
6	506.3	0.09	5819.5
7	396.2	0.10	3962.0
8	316.0	0.13	2358.0
9	5.09	0.05	103.9

Table 4: Effect of organic and inorganic nitrogen sources on RNase production

Medium	RNase (U mL^{-1})	Protein (mg mL^{-1})	Specific activity (U mg^{-1})
MG*	7.5	0.03	250.0
Organic nitrogen sources:			
YPG	114	0.17	670.5
YG	55	0.13	423.0
PG	100	0.11	909.0
BG[a]	539	0.30	1796.7
CG[b]	10.3	0.06	171.6
SG[c]	36.4	0.12	303.3
Inorganic nitrogen sources:			
(NH_4Cl)G	7.7	0.03	256.6
(NH_4NO_3)G	10.5	0.03	350.0

MG: Medium was (malt extract 0.3% and glucose 1%), BG: Medium was (beef extract 0.8% and glucose 1%), CG: Medium was (casein 0.8% and glucose 1%), SG: Medium was (soyabean meal 0.8% and glucose 1%). YPG: Medium contained yeast extract 0.3%, Peptone 0.5% and glucose 1%

extract was best compared to peptone as maximum RNase activity (539 U mL^{-1}) and specific activity was obtained (1796.7). In *Streptomyces erythreus* RNase production (Tanaka, 1966), soyabean meal, corn steep liquor, yeast extract and casamino acids were used as nitrogen source. Compared to this, only one nitrogen source (beef extract) was used in the present medium, which will reduce the production cost. In the present studies, soyabean meal showed less RNase compared to beef extract. In *S. levoris* RNase production amino acids like phenylalanine, arginine and lysine were used as nitrogen source (Bezborodov *et al*., 1967b) compared to beef extract in *Streptomyces* sp. RNase. Thus MGYP medium used initially for screening was finally modified to BG medium (beef extract+glucose) for production profile experiment.

Effect of carbon source, temperature and metal ions did not showed any increase in RNase activity.

Production profile of RNase from *Streptomyces* sp. (NCIM 2081): Production profile of *Streptomyces* sp. in BG medium showed that extracellular RNase activity (644.3 U mL^{-1}) and protein (0.3 mg mL^{-1}) were maximum in 72 h at pH 6.0 and 28°C Sugar utilization was 97% in 72 h. Extracellular DNase activity was also detected (28.5 U mL^{-1}) suggesting that microorganism produces non specific nuclease (Table 5). The RNase activity do not require metal ions but DNase activity required Mn^{2+} metal ion for the activity. The ratio of RNase/DNase activity in 72 h was 22.6:1 suggesting that organism prefers RNA as substrate compared to DNA (Fig. 1).

Table 5: Production profile of *Streptomyces* sp. (NCIM2081) RNase

Time (h)	RNase (U mL^{-1})	Protein (mg mL^{-1})	RNase specific activity (U mg^{-1})	Sugar (%) utilization	DNase+Mn^{2+}(U mL^{-1})	RNase/DNase ratio
24	60.0	0.19	315.7	45.0	69.7	0.86
48	189.6	0.27	702.2	74.0	58.9	3.22
72	644.3	0.30	2147.6	97.0	28.5	22.61
96	467.0	0.26	1796.1	98.4	32.8	14.24

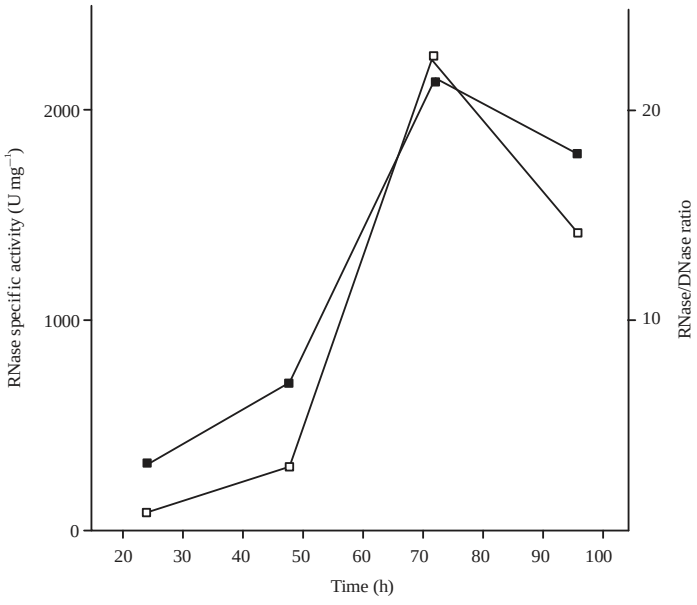

Fig. 1: Production profile of *Streptomyces* sp. RNase (a) RNase specific activity, (b) RNase/DNase ratio

Purification: Unless otherwise mentioned all operations were carried out at 4°C. The enzyme was purified by using ion exchange chromatography on DEAE cellulose where the enzyme was eluted unbound with 87% recovery and 1.1 fold purification. This step was important in removing coloured impurities. The enzyme bound to CM cellulose and eluted with 0.5 M salt. The enzyme after dialysis showed 16.6% recovery and 3 fold purification. The enzyme was further loaded on FPLC gel filtration column (superpose 12) after lyophilization. The final recovery was 10% with 8 fold purification (Table 6). The FPLC loaded enzyme showed 2 peaks (Fig. 2) suggesting that two RNase were produced by the organism but recovery of second RNase was very low. Only the properties of peak 1 were reported in the Table 7. Figure 3 showed homogeneity of RNase peak 1 and SDS molecular weight compared with standard markers.

Properties

Optimum temperature, pH and stability: Optimum temperature was 45°C and enzyme was stable for 20 min. Optimum pH was 8.0 and enzyme was stable from pH 6-9 for 20 min.

Table 6: Purification of *Streptomyces* sp. RNase

Enzyme	Total RNase (U mL^{-1})	Total protien (mg mL^{-1})	Specific activity (U mg^{-1})	Recovery (%)	Fold purification
Crude	24624.0	16.18	1521.8	100.0	1.0
DEAE unbound	21525.7	12.84	1676.4	87.0	1.1
CM (0.5 M NaCl) elution and dialysis	4083.0	0.87	4691.1	16.6	3.08
Superpose-12	2462.0	0.2	12310 .0	10.0	8.09

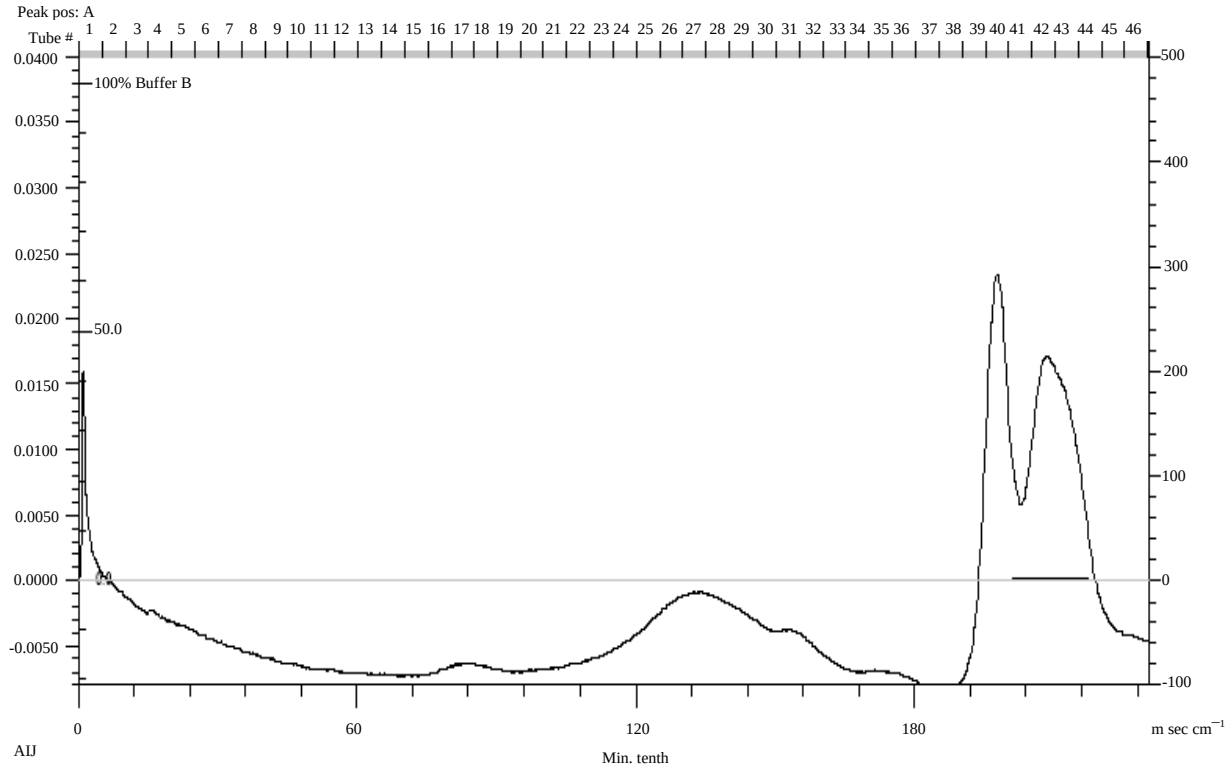

Fig. 2: FPLC profile of *Streptomyces* sp. RNase on superpose 12 column

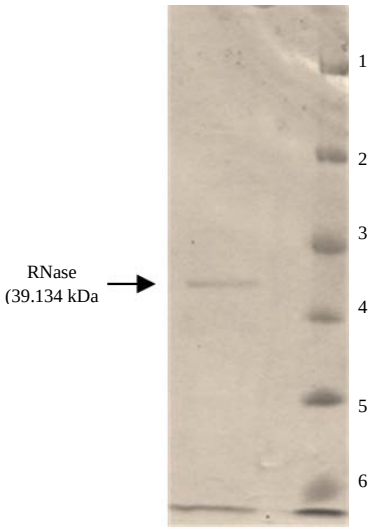

Fig. 3: SDS-PAGE: Lane 1: Purified RNase, Lane 2: molecular weight markers: (1) Beta galactosidase (116 kDa), (2) Bovine serum albumin (66.2 kDa), (3) Ovalbumin (45 kDa), (4) Lactate dehydrogenase (35 kDa), (5) Rease Bsp98I (25 kDa), (6) Beta-lactoglobulin (18 kDa)

Table 7: Properties of the purified *Streptomyces* sp. RNase

Properties	RNase -1
Optimum pH	8.0
Optimum temperature	45°C
Effect of metal ions	No increase
Effect of NaCl concentration (50 and 100 mM)	100%
SDS-Mol. Wt	39 kDa
Km and vmax	2.47 mg mL^{-1},1.98×10^{6}
Effect of protein denaturants (SDS) 0.05%	Total loss
Guanidine hydrochloride (200 mM)	51%
Urea (2 M)	Increase

Effect of metal ions: RNase activity do not required metal ions for the activity. Zn^{2+}, Cu^{2+} and Mn^{2+} ions were inhibitory for RNase activity but DNase activity of the same enzyme required Mn^{2+} ions for the activity. RNase activity was not inhibited by addition of EDTA.

Effect of salt concentration: Addition of NaCl up to 100 mM concentration showed no effect on RNase activity but further concentration showed decrease in RNase activity.

Stability to protein denaturants: Effect of protein denaturants like SDS showed complete loss at very low concentration (0.05%), while Guanidine hydrochloride at 200 mM concentration showed 51% RNase activity. Urea at 1 and 2 M conc. showed increase in activity and up to 4 M concentration 85% activity remained.

Properties of peak 1 like optimum temperature, effect of metal ions, effect of salt concentration, effect of denaturants and mol. wt were similar to *Streptomyces thermonitrificans* α-RNase (Deshmukh, 2007) stability was less. Optimum pH was different but comparable to Streptomyces DNases (Joshi and Deshmukh, 2011).

Molecular weight, Km and Vmax determination: The Mol. wt by SDS gel electrophoresis was 39.134 kDa, which was comparable to *Streptomyces glaucescens* (Aparicio *et al*., 1992) and

S. thermonitrificans nuclease (Deshmukh and Shankar, 2004). This was different than most of the reported RNase, which belong to T1 family and showed mol. wt <15 kDa. Km and Vmax values were 2.47mg mL^{-1} and 1.98×106 μmol min^{-1} mg^{-1}.

Effect of proteolytic enzymes: RNase enzymes are used as antiviral agents. So resistance to proteolytic enzymes would be an added advantage. *Streptomyces* sp. RNase activity increased when treated upto 200 mM pepsin, trypsin and papain. Compared to *Bacillus cereus* RNase, where pepsin inhibited 85% activity, our enzyme was better and showed increase in RNase activity. However like *Bacillus cereus* RNase *Streptomyces* sp. RNase was sensitive to proteinase K treatment (Zhou and Niu, 2009).

Specificity: RNase reported during 1960-80's gave preference to specificity studies and reported to be guanylribonucleases (Yoneda, 1964a). The HPLC analysis of hydrolytic products after exhaustive digestion (24 h) of RNA with *Streptomyces* sp. RNase showed that 3' mononucleotides were liberated with the order 3'UMP>3'GMP>5'UMP>3'AMP. The amount of 3'CMP produced was less (data not shown) indicating its resistance to hydrolysis. Like *Streptomyces aureus* RNase (Yoneda, 1964b) 18, which produced 5' nucleotides our enzyme also showed 5'UMPas one of the end product along with 3' nucleotides. *S. thermonitrificans* α RNase (Deshmukh, 2007) showed preference for adenylic acid linkages from initial stages of hydrolysis and *Streptomyces aureofaciens* RNase (Bacova *et al*., 1971) was guanine specific. HPLC analysis showed *Streptomyces* sp. The RNase enzyme was different than all above mentioned enzymes and non specific. Expecting specificity in enzyme, we had carried out purification and characterization studies of *Streptomyces* sp. The RNase (NCIM 2081) but results predict that structural and cytotoxicity studies will be more interesting in future.

CONCLUSION

Due to high extracellular RNase activity during screening, *Streptomyces* sp. (NCIM 2081) culture was selected. During medium optimization studies, initial pH (6.0) and organic nitrogen source (beef extract) showed marked effect on RNase production. Maximum RNase activity (644.3 U mL^{-1}) was obtained in the BG medium, pH 6.0 at 28°C. Purified RNase showed same properties and non specific behavior like reported Streptomyces nucleases. The Km values showed high affinity for RNA substrate. The end product of hydrolysis was 3' nucleotides. Increase in RNase activity when treated with Urea (2 M) would be helpful during structural studies. Increase in RNase activity when treated with proteolytic enzymes like pepsin, papain and trypsin would be an interesting point during antiviral studies.

ACKNOWLEDGMENT

We thank Mr. Vaibhav Mhaindarkar for his help during manuscript preparation.

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