INTERNATIONAL JOURNAL OF INNOVATIVE RESEARCH & DEVELOPMENT

ISSN 2278 – 0211 (Online)

Extracellular RNase Production and Purification from *Streptomyces aureofaciens* (2614)

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Abstract:
Streptomyces aureofaciens (NCIM 2614) culture produced extracellular RNase (291U/ml) During screening among 9 cultures from NCIM division in MGYP medium.
Effect of media components on RNase production showed that organism could grow in simple PG (0.8% peptone and 1% glucose) medium to replace costly MGYP medium. Medium optimization studies resulted in 2.2-fold increase in RNase activity (291-669 U/ml) and 1.6-fold increase in specific activity (5836-9211). Production profile of Streptomyces aureofaciens (2614) in PG medium (pH 7.0) at 28^{o}C showed that organism produces maximum RNase (669U/ml) and DNase activity (153.3U/ml) in 72 hrs and thus can be classified as non-specific nuclease. The ratio of RNase to DNase is 4: 1 suggesting that organism prefers RNA to DNA as substrate. RNase activity could be detected in the absence of metal ions while DNase activity required metal ions.
During purification of RNase enzyme, heat treatment is not a suitable method as enzyme is not thermo stable. Ammonium sulphate precipitation and dialysis resulted in 89 % recovery with 1.13-fold purification. The enzyme eluted unbound on anion exchanger (DEAE sephadex) with 46.5 % recovery which was loaded on cation exchanger (CM cellulose) and eluted with buffer containing 0.2 M salt (11% recovery). The enzyme was further loaded on gel filtration column (FPLC Superose-12) with 11% recovery and 18.6-fold purification.

Keywords: *DNase, RNase, production, purification, specific activity.*

1. Introduction

All living systems contain nucleases, capable of interacting with nucleic acids and hydrolyzing the phosphodiester linkages. The enzymatic breakdown of nucleic acids was first observed in the early twentieth century, and the term 'nucleases' was coined for enzymes involved in this. The term nuclease was coined by Iwonhoff (1903). Kunitz (1950) classified nucleases into two groups, on the basis of sugar specificity into sugar specific and sugar nonspecific nucleases as DNases and RNases. Further Bernard (1969) and Laskowaski (1959, 1982) modified the classification.

Their classification classified the nucleases

1. On the basis of substrate hydrolyzed (DNA, RNA)
2. Type of nucleolytic attack (endonucleases, exonucleases)
3. Nature of bond hydrolyzed

The nature of hydrolytic products formed, mononucleotides or oligonucleotides terminating in 3' or 5' phosphate end Nucleases play an important role in replication, repair, recombination and restriction while extracellular enzymes have a role in nutrition. Rangarajan et al (2001). The genus Streptomyces, by their ability to degrade various biopolymers are known to produce a wide variety of extracellular hydrolases Czoch et al (1988).

However, very little information is available on the function of nucleases in the biology of these organisms. Hence, characterization of nucleases can be a preliminary step towards the genetic analysis of the function of these enzymes in this class of organisms. Nucleases are multifunctional enzymes and catalyze the hydrolysis of DNA and RNA.

The majority of these enzymes have been characterized with respect to their DNase activity; in comparison, little attention has been paid to the characterization of associated RNase activity. The present paper describes the production and purification of extracellular RNase from *streptomycesaureofaciens* (2614).

2. Materials and Methods

RNA, Casein and Acrylamide (Sisco Research Laboratories, Bombay, India), Malt extract, Yeast extract, Beef extract, Peptone, Soyabean meal and Bovine serum albumin (Hi media Laboratories Pvt Ltd, Bombay India), Peptone, (Sarabhai M. Chemicals Baroda, Pvt. Ltd India), Calf thymus DNA, Coomassie brilliant blue, G-250 and Sephadex G-80 (Sigma Chemicals Co. St. Louis, MO,USA), Tris (hydroxylmethyl methylamine), Peptone, Glucose, Perchloric acid, Silver nitrate, Sodium carbonate, Sodium Thiosulphate, Formaldehyde (Qualigens fine chemicals, Mumbai, India),Peptone and Uranyl acetate (LobaChemieIndoaustranal Co. Ltd Mumbai, India) Soluble starch (British Drug House) were used . All other chemicals used were of analytical grade.

Following cultures from National Collection of Industrial Microorganisms (NCIM) were used during screening.

i. *S. aureofaciens* (NCIM 2614)
ii. *S. atrofaciens* (NCIM – 2951)
iii. *S. albus* (NCIM – 2413)
iv. *S. aurofaciens* (NCIM – 2417)
v. *S. venezualae* (NCIM – 2215)
vi. *S. species* (CFTIR - 2081)
vii. *S.calestis* (NCIM-2870)
viii. *S. nevius* (NCIM-2502)
ix. *S. viridifaciens* (NCIM -2506)

Streptomyces cultures were routinely maintained on MGYP agar slant having composition-
Malt extract and Yeast extract - 0.3%, Glucose - 0.1%, Peptone- 0.5% and Agar- 2%.

2.1. Enzyme Assays

2.1.1. Determination of RNase Activity

RNase activity was carried out essentially according to Tomoyeda et al (1969). The total reaction mixture of 0.75ml contained 1.25mg RNA in 200mM Tris-HCl buffer pH7.0 and appropriately diluted enzyme. The reaction was initiated by the addition of RNA followed by incubation at 37^{o}C for 15 min. The reaction was arrested by the addition of 0.25ml of Mac Fadyen's reagent (0.75%wt/vol Uranyl acetate in 25% vol/volperchloric acid) and the reaction mixture was kept on ice for 5min., the resulting precipitate was immediately removed by centrifugation (3000 rpm, 10min.). Subsequently 0.1ml of the supernatant was diluted with 2.5ml of distilled water and the acid soluble ribonucleotides were measured at 260 nm. The amount of acid soluble ribonucleotides liberated was calculated by assuming a molar absorption coefficient of 10600 Mol^{-1} cm^{-1} for the ribonucleotides mixture. One unit of RNase activity is defined as the amount of enzyme required to liberate 1µmol of acid soluble ribonucleotides/min under the assay condition.

2.1.2. Determination of DNase Activity

DNase activity was estimated according to Gite et al (1992). The standard reaction mixture of 1ml contained 50µg of native DNA in 30mM Tris-HCl buffer (pH 7.0) and appropriately dilutedenzyme. The reaction was initiated by the addition of DNA followed by incubation at 37^{o}C for 15min. The reaction was then terminated by the addition of 0.5ml of 10% chilled PCA and 0.5ml of 0.2% BSA (Bovine serum albumin). The reaction mixture is kept on ice for 10min and then centrifuge at 2500 rpm for 15min. The acid soluble deoxyribonucleotides were measured at 260nm. The amount of acid soluble deoxyribonucleotides liberated was calculated by assuming the molar absorption coefficient of 10000 Mol^{-1} cm^{-1} for deoxyribonucleotides mixture. One unit of DNase activity is defined as the amount of enzyme required to liberate 1µmol of acid soluble nucleotides / min. under the assay condition.

2.2. Protein Determination

Protein concentration was determined according to Bradford method (Sedmak J.J. and Grossberg S.E.1977) using BSA as standard.

2.3. Determination of Reducing Sugars

Reducing sugar was determined by the DNSA method of Miller (1959).

2.4. Microorganism and Growth

Screening was carried out by inoculating a piece of agar slant which contained well sporulated *Streptomyces* cultures in 50 ml MGYP medium in 250 ml Erlenmeyer flask followed by incubation at 28^{0}C for 72 h with shaking 200rpm .

2.5. Media Optimization

MGYP medium contained Malt extract and Glucose as carbon source and yeast extract and peptone as nitrogen source. During medium optimization studies one component from MGYP medium was deleted and finally PG medium was selected. In PG medium peptones of different companies were used. During different carbon and nitrogen source experiments, glucose and peptone of PG medium were replaced respectively. The extracellular broth collected by centrifugation (10000 rpm, 20min.) was used as source of enzyme. All experiments were performed in duplicate.

2.6. Purification of RNase

2.6.1. Heat Treatment

RNases are reported to be heat stable enzymes. When *Sterptomyceserythreus* (Tanaka 1966) RNase was heated at 80°C for 5 min, 100% activity was retained. So heat treatment was given at 50°C for 20 min to remove heat labile proteins.

2.6.2. Ammonium Sulphate Precipitation

Crude enzyme obtained after centrifugation was partially purified by ammonium sulphate precipitation method. Fractionation of protein was made by addition at 2-5^{o}C with stirring in addition of solid ammonium sulphate from the table given in the protocol of the protein purification by Deutscher (1988).

2.6.3. Ion Exchanger Chromatography

Ion exchange chromatography was used in next step of purification and enzyme was loaded on DEAE Sephadex (anion exchanger) column. The enzyme was eluted unbound and other impurities were removed from the enzyme since they bound to the column. The DEAE Sephadex unbound enzyme was then loaded on CM Cellulose (cation exchanger).

→ Salt Elutions

The protein bound to the matrix was eluted with of 10mM buffer for CM column containing 0.1 M to 0.5 M NaCl, collected in 10ml fractions. Active fractions having RNase activity were dialyzed and RNase activity and protein were determined again after dialysis. (A dialysis membrane such as cellophane (snake venom dialyzed bag) of 10 KD cut size is used for dialysis against 10mM Tris-HCl pH 7.0 at 2-4^{o}C)

2.6.4. Gel Filtration by FPLC (Fast Protein Liquid Chromatography)

The enzyme was then finally loaded on FPLC gel filtration column (Superose-12). The separation on FPLC is based on gel filtration chromatography. FPLC system (Biorad biologic Duo Flow), the matrix used was Superose 12 grade (composite of cross linked agarose) with the flow Rate of 0.17ml/min, fraction Volume collected was 1 ml for further studies.

2.6.5. Molecular Weight Determination by SDS- PAGE

SDS–PAGE was carried out at pH 8.3, according to Laemmli (1970). After electrophoresis, the bands were visualized by silver staining according to Blum et al. (1987)

3. Results and Discussion

3.1. Media Optimization:

3.1.1. Screening of Streptomyces cultures

Streptomyces cultures were grown in 50 ml MGYP medium in 250 ml Erlenmeyer flask followed by incubation at 28^{0}C for 72 h at pH 7 with shaking (200rpm)

Culture	RNase (U/ml)	Protein (mg/ml)	RNase Specific activity (U/mg)	DNase (U/ml)	DNase Specific activity (U/mg)	Final pH
***S. aureofaciens* (NCIM – 2614)**	**558.0**	**0.21**	**2657.1**	**26.2**	**124.7**	**6.4**
S. atrofaciens (NCIM – 2951)	36.9	0.30	123.0	119.0	395	7.8
S. albus (NCIM – 2413)	14.2	0.07	202.8	3.73	51.8	6.9
S. aurofaciens (NCIM – 2417)	7.5	0.02	375	1.9	96.5	7
S. venezualae (NCIM – 2215)	270	0.08	3375	56.8	710	5.4
S. species (CFTIR - 2081)	257	0.06	4283.3	50	833	6.9
S.calestis (NCIM-2870)	4.2	0.02	210.0	1.6	80.0	6.3
S. nevius (NCIM-2502)	1.6	0.2	8	5.4	27	8
S. viridifaciens (NCIM -2506)	1.7	0.79	2.2	1.7	2.2	7.7

Table 1: Screening of Streptomyces cultures

When different Streptomyces cultures from NCIM division were screened for RNase activity, *Streptomyces aureofaciens* (NCIM 2614) showed maximum RNase activity (558U/ml) so selected for further studies.

3.2. Effect of Media Components on RNase Production

Streptomyces cultures were grown in 50 ml medium as shown in table in 250 ml Erlenmeyer flask followed by incubation at 28^0C for 72 h at pH 7 with shaking (200rpm)

Media components	RNase (U/ml)	Protein (mg/ml)	RNase Specific activity (U/mg)	DNase (U/ml)	DNase Specific activity (U/mg)	Final pH
MGYP	669.8	0.11	6089.1	106.0	963.6	6.5
MYP	38.5	0.09	427.8	56	583.3	8.5
YPG	628.9	0.092	6835.9	34.33	373.2	6.3
YG	66.6	0.06	912.3	12	200	6.4
PG (0.5%)	97.9	0.05	1958	32.3	687.2	6.7
PG (0.8%)	**669.8**	**0.072**	**8930.6**	**65.3**	**906.9**	**6.5**

Table 2: Effect of different media components on RNase production

MGYP medium contained Malt extract and Glucose as carbon source. Omission of glucose in the medium (MYP) resulted in very low activity (38.5U/ml), suggesting that glucose is essential for RNase production. However, when malt extract was omitted from the medium (YPG) higher RNase activity (628.9U/ml) was detected, suggesting that malt extract is not essential for RNase production. Therefore, malt extract was removed in further experiments. This will be helpful in reducing the cost of the medium. MGYP medium contains yeast extract and peptone as nitrogen source. In PG medium where yeast extract and malt extract were removed from the medium, highest RNase activity (669.8 U/ml) and highest specific activity (8930.6 U/mg) is detected. But very poor RNase activity (66.6U/ml) is detected in YG medium in which peptone and malt extract is removed.

3.3. Effect of Different Peptones on RNase Production

Streptomyces cultures were grown in 50 ml PG medium in 250 ml Erlenmeyer flask followed by incubation at 28^0C for 72 h at pH 7 with shaking (200rpm)

Peptone	RNase (U/ml)	Protein (mg/ml)	RNase Specific activity (U/mg)	DNase (U/ml)	DNase Specific activity (U/mg)	Final pH
Hi media	182.3	0.062	2941	40.3	650.5	7.3
Sarabhai	**421.4**	**0.080**	**5267.5**	**76.3**	**953.7**	**7.1**
Qualigens	685.5	0.150	4570	31.8	244.6	7.1
Loba	150.3	0.060	2505	13.7	228.3	7.0
Bengal Immunity	8.2	0.180	45.5	2.9	15.7	6.9
S.D. Fine	254.7	0.130	1959.2	14.6	110.6	7.5

Table 3: Effect of different peptones on RNase Production

4. Results and Discussion

Apte et al (1993), while studying the influence of various organic nitrogen sources on extracellular DNase production by *R. stolonifer*, noted that among the various peptones used, only one, i.e. the one obtained from Sarabhai M. Chemicals, supported growth and enzyme production. Effect of different peptones like Hi media, Qualigens, Loba, S.D Fine, Bengal Immunity and Sarabhai chemicals was studied. In case of *Streptomyces aureofaciens*. Although peptone of Qualigenes showed highest RNase activity (685.5U/ml), specific activity was low (4570). Sarabhai peptone gave highest specific activity (5267.5U/mg). So this peptone was used in PG medium for further studies.

4.1. Effect of initial temperature on RNase production

Streptomyces cultures were grown in 50 ml PG medium in 250 ml Erlenmeyer flask followed by incubation at 28^0C – 50^0C for 72 h at pH 7 with shaking (200rpm)

Initial temperature	RNase (U/ml)	Protein (mg/ml)	RNase Specific activity (U/mg)	DNase (U/ml)	DNase Specific activity (U/mg)	Final pH
28^0C	**291.8**	**0.05**	**5836.0**	**63.0**	**126.0**	**6.7**
37 ^{0}C	209.4	0.08	2617.5	18.0	226.0	7.1
42 ^{0}C	30.8	0.05	616.0	13.8	276.0	7.1
50 ^{0}C	18.8	0.07	268.6	5.6	80	6.0

Table 4: Effect of initial temperature on RNase production

When initial temperature of PG medium was adjusted from 28-50^0C, maximum RNase was obtained at 28^0C(291.8U/ml) and specific activity (5836.0 U/mg). As the fermentation temperature increased RNase as well as specific activity decreased progressively.

4.2. Effect of pH on RNase production

Streptomyces cultures were grown in 50 ml PG medium in 250 ml Erlenmeyer flask followed by incubation at 28^0C for 72 h at pH 5 to 9 with shaking (200rpm).

Initial pH	RNase (U/ml)	Protein (mg/ml)	RNase Specific activity (U/mg)	DNase (U/ml)	DNase Specific activity (U/mg)	Final pH
5	327.0	0.08	4087.0	8.8	110.0	6.9
6	389.9	0.09	4332.2	22.0	244.4	6.5
7	**525.5**	**0.094**	**5590.0**	**20.6**	**219.1**	**6.4**
8	503.1	0.10	5031.0	11.6	116.0	4.9
9	437.1	0.09	4855.0	8.9	50.0	6.0

Table 5: Effect of initial pH on RNase production

When initial pH of PG medium was adjusted from 5-9, maximum RNase was obtained at pH-7 (525.5 U/ml) and specific activity (5590.0 U/mg).

4.3. Effect of different nitrogen sources on RNase production

Streptomyces cultures were grown in 50 ml PG medium in 250 ml Erlenmeyer flask followed by incubation at 28^0C for 72 h at pH 7 with shaking (200rpm)

Nitrogen sources	RNase (U/ml)	Protein (mg/ml)	RNase Specific Activity (U/mg)	DNase (U/ml)	DNase Specific activity (U/mg)	Final pH
PG	**669.8**	**0.092**	**7275.0**	**65.3**	**709.8**	**6.5**
BG	352.2	0.076	4634.2	4.3	56.6	6.3
Casein	29.9	0.04	747.5	1.0	25.0	4.4
Soybean	399.0	0.074	5392.1	22.8	308.1	7.0
NH_4Cl	12.9	0.039	330.8	1.3	33.3	5.0
NH_4NO_3	14.4	0.014	1028.6	2.6	185.7	4.6
YPG	638.3	0.103	6197.1	59.7	579.6	6.3
Glucose	28.3	0.023	1230.4	1.9	82.6	5.0

Table 6: Effect of different nitrogen sources on RNase production

Horitsu et al (1974) reported that inorganic nitrogen sources like NH_4Cl and NH_4NO_3 are more suitable than organic nitrogen sources for RNase production in *Aspergillus niger*. Peptone was used as a nitrogen source in case of *S. cinereofuscu*s (Verzilov 1972) and *S. aureofaciensB*96 (Zuzana 2005) and beef extract for *S. thermonitrificance* (Deshmukh S. 2007). In *S. erythreus* RNase production soyabean meal, corn-steep liquor and amino acids were used along with yeast extract (Tanaka 1966). In the present study effect of different organic nitrogen sources and inorganic nitrogen sources was seen on RNase production. Organic nitrogen sources are better than inorganic nitrogen sources for RNase production. Among the different organic nitrogen sources tested peptone (0.8%) showed maximum RNase activity (669.8U/ml) and maximum specific activity (7275.0U/mg). Thus PG (pH 7.0) medium in which Sarabhai peptone (0.8%)was used as nitrogen source and glucose as carbon source is selected for further studies.

4.4. Effect of different carbon sources on RNase production

Streptomyces cultures were grown in 50 ml PG medium in 250 ml Erlenmeyer flask followed by incubation at 28^0C for 72 h at pH 7 with shaking (200rpm)

Carbon sources	RNase (U/ml)	Protein (mg/ml)	RNase Specific activity (U/mg)	DNase (U/ml)	DNase Specific activity (U/mg)	Final pH
0.5% glucose	380.5	0.088	4323.9	26.4	300.0	7.6
1% glucose	**654.0**	**0.071**	**9211.0**	**10.7**	**150.7**	**6.6**
2% glucose	293.6	0.119	2467.2	12.6	105.9	5.8
Peptone (- G)	20.3	0.06	338.3	5.06	84.3	8.4
Soluble starch	213.8	0.032	6681.3	5.7	178.1	7.8

Table 7: Effect of different carbon sources on RNase production

When different carbon sources like glucose and soluble starch were used, it was noted that glucose was better than starch for enzyme production. Effect of different % of glucose like 0.5% glucose, 1% glucose, 2% glucose were used 1% glucose gave highest activity (654.0 U/ml) and specific activity (9211.0 U/mg).

4.5. Production profile of Streptomyces aureofaciens (NCIM 2614) in PG medium

Streptomyces cultures were grown in 50 ml PG medium in 250 ml Erlenmeyer flask followed by incubation at 28^{0}C for 24 to 96 h at pH 7 with shaking (200rpm)

EXTRACELLULAR:							
Time	**RNase**	**Protein**	**Ranse Specific activity**	**DNase with Mn2+**	**DNase specific activity**	**Sugar utilized**	**Ratio**
(Hrs)	**(U/ml)**	**(mg/ml)**	**(U/mg)**	**(U/ml)**	**(U/mg)**	**(%)**	**RNase / DNase**
24	98.7	0.082	1203	96.3	1174.4	48	1.1:1
48	437.1	0.113	3868.1	**260**	**2300.9**	73	1.7:1
72	**654**	**0.07**	**9211.3**	153.3	2190	80.3	4.3:1
96	371	0.143	2594.4	189.3	1323.8	**84**	2:1
INTRACELLULAR:							
24	42.7	0.413	103.3	2.5	6.1	-	17:1
48	51.3	0.155	330.6	6.4	41.3	-	8:1
72	**62.9**	**0.151**	**416.5**	**9.2**	**60.3**	-	6.8:1
96	53.8	0.278	193.4	8.2	29.5	-	6.5:1

Table 8: Production profile

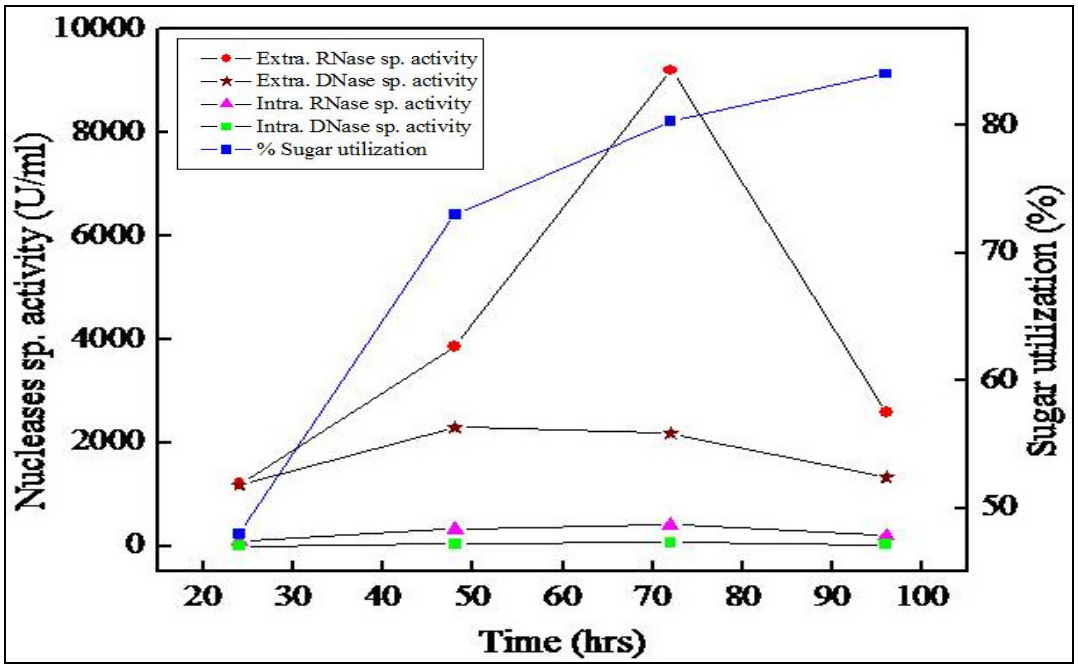

Figure 1: Production profile

The time course of cultivation of *Streptomyces aureofaciens* (NCIM 2614) in PG (0.8%) medium is shown in Figure-1. Extracellular RNase activity (654.0U/ml), and specific activity (9211.3U/ml) was maximum in 72 hr. Maximum sugar (84%) was utilized in 96h and 80% sugar was utilized in 72hr. Extracellular DNase activity (260U/ml) and DNase specific activity (2300.9) was also detected maximum in 48 hr suggesting that *Streptomyces aureofaciens* is a non-specific nuclease. Like extracellular RNase, *Streptomyces aureofaciens* produces maximum intracellular RNase (62.9U/ml) and specific activity (416.5U/mg) in 72h. Intracellular DNase activity (9.2U/ml) is and specific activity (60.3U/mg) was maximum in 72h.

4.6. Purification of RNase

4.6.1. Heat Treatment: This Was the First Step of Purification

Crude RNase at 37^0 C (U/ml)	**Heat treatment at 50^{0}C RNase (U/ml)**
433.9	157.2 (74 %loss)

Table 9

RNases are reported to be heat stable enzymes. When *Sterptomyceserythreus* (Tanaka 1966) RNase was heated at 80°C for 5 min, 100% activity was retained. So heat treatment was given at 50°C for 20 min to remove heat labile proteins. However, at this temperature RNase activity decreased by 74% suggesting that enzyme is not stable at high temperature and heat treatment is not a suitable method for purification of the enzyme.

4.6.2. Ammonium Sulphate Precipitation

Enzyme	Total RNase (U)	Total Protein (mg)	Specific activity (U/mg)	Recovery%	Fold Purification
Crude	55340	9.8	5646.9	100	1
90% Ammonium sulphate precipitation and dialysis	49428	7.7	6419.2	89	1.13

Table 10

Since heat treatment did not prove to be a suitable method for purification of enzyme, ammonium sulphate precipitation was used as the first step of purification. After dialysis the enzyme showed 89% recovery with 1.13-fold purification. Thus, ammonium sulphate precipitation and dialysis was the first step of purification.

4.6.3. Ion Exchanger Chromatography

In the next step of purification, ion exchange chromatography was used and the enzyme obtained in the first step of purification was loaded on ion exchanger column.

→ DEAEcellulose: Diethyl Amino Ethyl (DEAE)-cellulose which is known as Anion exchanger was used as second step for enzyme purification.

Enzyme	Total RNase (U)	Total Protein (mg)	Specific activity (U/mg)	Recovery%	Fold Purification
Crude	56407	15.9	3527.0	100	1
90% Ammonium sulphate precipitation and dialysis	40080	7.9	5099.2	71.1	1.4
DEAE unbound	19468	2.4	8111.6	34.5	2.2
DEAE bound	20612	5.5	3775	36.5	1.1
0.2M salt elution and dialysis	3044	2.54	1198.4	5.4	0.33
1M salt elution and dialysis	248	1.82	136.3	0.43	0.04

Table 11

Ammonium sulphate precipitated and dialyzed enzyme having 40080 units and 7.9 mg protein was loaded on DEAE cellulose column equilibrated with 10mM Tris-HClbuffer (pH 7.0). DEAE bound enzyme (20612 units) was eluted with the same buffer containing 0.2M and 1M salt but eluted enzyme after dialysis showed less specific activity (1198.4 and 136.6) and recovery (5.4 and 0.43%) than loaded enzyme i.e. ammonium sulphate precipitated and dialysed. DEAE unbound enzyme showed more specific activity (8111.6) with 2.2-fold purification.

DEAE Sephadex

As DEAE loaded enzyme showed less recovery, the enzyme was bound to DEAE Sephadex matrix instead of DEAE.

Enzyme	Total RNase (U)	Total Protein (mg)	Specific activity (U/mg)	Recovery%	Fold Purification
Crude	55340.0	14.2	3897.2	100	1
90% Ammonium sulphate precipitation and dialysis	49428.0	7.7	6419.2	89	1.6
DEAE Sephadex unbound	25714.3	3.9	6593.4	46.5	1.7

Table 12

Like DEAE the enzyme is eluted unbound and specific activity (6593.4) has increased (1.7 fold) In case of DEAE Sephadex unbound enzyme more recovery (46.5%) was obtained, therefore DEAE Sephadex matrix is preferred over DEAE Cellulose.

CM Cellulose: -The DEAE Sephadex unbound and lyophilized enzyme was then loaded on Carboxy Methyl (CM) Cellulose which is known as cation exchanger. The CM bound enzyme was eluted with the same buffer containing 0.2M and 1M salt and used as third step of enzyme purification.

Enzyme	Total RNase (U)	Total Protein (mg)	Specific activity (U/mg)	Recovery%	Fold Purification
Crude	78131.0	23.44	3333.4	100	1
90% Ammonium sulphate precipitation and dialysis	57972.0	14.2	4076.8	74	1.2
DEAE Sephadexlyophilised	29915.0	5.3	5622.6	38	1.7
CM 0.2M and dialysed	9054.0	1.1	8230.9	11	2.4

Table 13

DEAE Sephadex lyophilized enzyme having 29915.0 units and 5.3mg protein was loaded on CM cellulose column pre-equilibrated with 10mM Acetate buffer (pH 5.0). The bound enzyme was eluted with buffer containing 0.2M NaCl, out of 29915.0 units loaded only 9054.0 units were recovered on elution and dialysis with 1.1 mg protein. Recovery at this stage was 11 % and 2.4-fold purification was obtained.

4.6.4. Gel Filtration By FPLC

CM 0.2M lyophilized enzyme was further loaded on gel filtration column of FPLC (Superose12). Column was equilibrated with 10 mMTris- HCl buffer (pH -7). Single peak is obtained at 20/21 fractions which showed recovery (10.9 %) with 18.6-fold purification.

Enzyme	Total RNase (U)	Total Protein (mg)	Specific activity (U/mg)	Recovery%	Fold Purification
Crude	189878	53.6	3450.5	100	1
90% Ammonium sulphate precipitation and dialysis	147480.0	31.7	4652.4	77	1.3
DEAE Sephadexlyophilised	64071.0	11.4	5620.3	33.7	1.6
CM 0.2M and dialysed	25430.0	1.6	15993.7	13.4	4.6
FPLC column (Superose-12)	20776.0	0.32	64925.0	10.9	18.6

Table 14

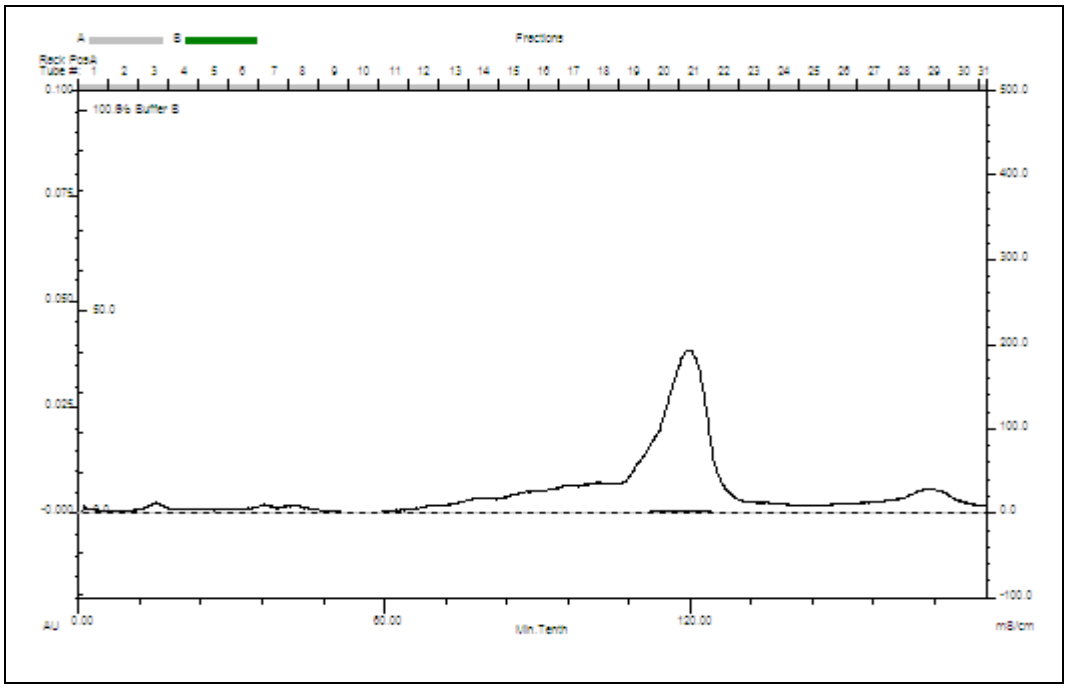

Figure 2: Gel filtration by FPLC

4.7. Molecular Weight Determination by SDS- PAGE

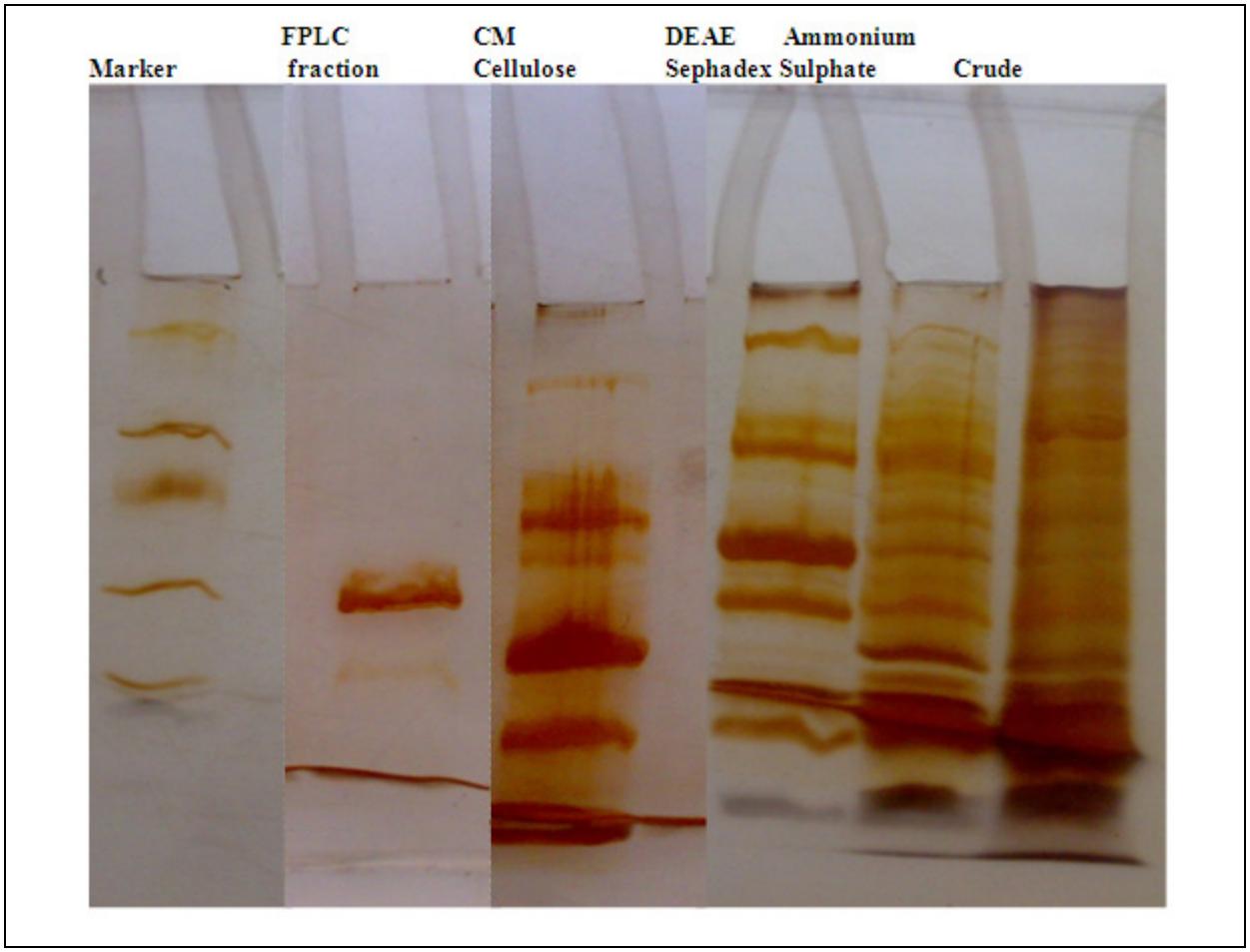

Figure 3

4.8. Molecular Weight Markers

Markers	Molecular weight
Myosin	200000
Beta galactosidase	116250
Phosphorylase B	97400
Serum albumin	66200
Ovalbumin	45000
Carbonic anhydrase	31000
Trypsin inhibitor	21500
Lysozyme	14400
Aprotinine	6500

Table 15

5. Conclusion

Among the nine *Streptomyces* cultures from NCIM (National Centre for Industrial Micro-organisms, National Chemical Laboratory, Pune) screened for RNase production in MGYP (Malt extract, Glucose, Yeast extract, Peptone) medium *Streptomyces aureofaciens* (NCIM – 2614) culture showed 558 units of extracellular RNase and was selected for further optimization studies. Among the different media tested for maximum RNase activity, *Streptomycesaureofaciens* (NCIM – 2614) showed highest RNase production in MGYP medium. Hence, this medium was further selected for optimization studies. In optimization studies effect of different media components on RNase production was studied which resulted in modification of MGYP medium to PG (Peptone, Glucose) medium which is simple and cost effective with 1.2-fold increase in RNase activity (669U/ml- 558U/ml) and 3.4-fold increase in specific activity(8930U/mg-2657U/mg). Effect of different nitrogen sources on RNase production was studied and peptone was found to be the most suitable organic nitrogen source with maximum RNase activity (669.8U/ml) and maximum specific activity (7275.0U/mg).

Temperature of 28^0C and pH 7 was suitable for enzyme production. Effect of different carbon sources on RNase production was also studied and 1% glucose gave highest activity (654.0 U/ml) and specific activity (9211.0 U/mg) was found to be the most suitable carbon source.

Hence this medium with 0.8% peptone and 1% glucose was selected for RNase production.

The production profile for RNase activity was studied from 24 to 96 hours using PG medium and maximum extracellular and intracellular RNase activity and specific activity was detected at 72 hours. Extracellular RNase activity (654.0U/ml), and specific activity (9211.3U/ml) was maximum in 72 hr. Maximum sugar (84%) was utilized in 96h and 80% sugar was utilized in 72hr. Extracellular DNase activity (260U/ml) and DNase specific activity (2300.9) was also detected maximum in 48 hr in presence of metal ions suggesting that *Streptomyces aureofaciens* is a non-specific nuclease.

Purification of the enzyme showed that heat treatment is not a suitable method for purification since it resulted in loss of RNase activity. Ammonium sulphate precipitation was the first step of purification. Ion exchange chromatography was used in next step of purification and enzyme was loaded on DEAE Sephadex (anion exchanger) column. The enzyme was eluted unbound and other impurities were removed from the enzyme since they bound to the column. The DEAE Sephadex unbound enzyme was then loaded on CM Cellulose (cation exchanger). The enzyme was then finally loaded on FPLC gel filtration column (Superose-12) and the final recovery was 10.9%. The specific activity of the enzyme increased 18.6 fold at this step of purification.

6. Acknowledgement

The authors thank the Department of Biochemical Sciences, National chemical laboratory Pune, India, for making this research possible.

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