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Process Optimization for the Production and Purification of an Extracellular Ribonuclease from a Soil Bacterial Isolate RNS3 Using One-Factor-At-a-Time (OFAT) Approach

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Abstract: Ribonuclease (RNase; EC 3.1.2.6) hydrolyzes the phosphodiester linkage(s) of ribonucleic acid. Bacteria are the most preferred sources of RNase due to their broad biochemical diversity. The present study involved bacterium isolation, process optimization and purification of RNase from soil sample. Out of 14 bacterial isolates, one isolate (RNS3) was selected based on relatively higher extracellular RNase activity in the culture broth. The physicochemical parameters attempted to optimize the extracellular RNase production by the selected bacterial isolate RNS3 included incubation temperature, time of incubation, carbon & nitrogen sources, pH of the broth and salt (NaCl) concentration in the broth, and supplementation of glucose and peptone, respectively to the broth showed enhanced RNase production. Use of RNA as a substrate (10 µg/ml) to the reaction mixture and 45 minutes of incubation enhanced the RNase activity. A partially alkaline pH (8.5) was suitable for maximum enzyme production by the bacterial isolate RNS3. The extracellular RNase was purified by successive salting out with ammonium sulfate, dialysis and gel permeation on Sephadex G-50 column. The SDS/PAGE showed two protein bands having molecular weight of the ~28kDa and ~35kDa. It reflected the dimeric nature of enzyme having molecular weight of the ~65kDa.

Key words: Bacterial isolates RNS3; process optimization; extracellular RNase; SDS PAGE.

Introduction

Ribonucleases (RNases) are enzymes of ubiquitous nature that are involved in ribonucleic acid degradation in organisms. RNases participate in apoptosis and play significant roles in defense from viral infection. Such characteristics have made the RNases to be considered as important therapeutic enzymes for the treatment of cancers [1,7]. Besides, microbial RNases are used in pharmaceutical and food industries [24]. Interestingly, RNases purified from plants have been successfully tried as antiproliferative agents targeting human tumors grown in mice [20]. Onconase from the frog *Rana pipiens* has antitumor activity and clinical trials on patients with breast, kidney, lung and pancreatic cancers have been carried out [39]. Biotechnological advancement, in the area of gene therapy and DNA vaccines, increased the demand of production of RNases with high purity [30,36]. Applications of RNases are quite diverse and they have been found to play vital roles in the synthesis of some specific nucleotides [24], as well as undesirable RNA removal [35].

Yingying Wu found a novel RNase having antiproliferative activity from the mushroom [32]. Demir *et al.*, optimized the fermentative RNase production from *Streptomyces* sp. M49-1 and revealed its alkalotolerant and thermostable capability [5].

Ethical issue restricts the RNase production from the animal sources. Therefore, to produce a pharmaceutical grade plasmid DNA has a great

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challenge [36]. To date, more than 20 nucleases have been reported [15]. Bacterial RNases investigation remains limited [11,12,14,19,21,22,26,29,30,37].

Till date, few papers have reported optimization of production conditions for extracellular microbial RNases [3,8,33,37]. Optimized conditions enhance the production quantitatively as well as qualitatively. Effect of physicochemical factors on batch culture is an important tool to develop economically viable RNase production. The present manuscript describes the isolation of a potential RNAse producer from the soil, physicochemical parameters optimization, biochemical characterization of bacterial isolate and purification of single-stranded RNA-specific ribonuclease from bacterial isolate.

Materials and methods

Materials

Sodium dodecyl sulfate (SDS), Yeast ribonucleic acid (RNA), and Bovine serum protein were purchased from Sigma Chemical Co. (India). K_2HPO_4, KCl, $MgSO_4 7H_2O$, $FeSO_4 7H_2O$, $(NH_4)_2SO_4$ and Tris buffer are from HIMEDIA Laboratory Ltd., Mumbai, (India). LMW (Low molecular weight marker) were purchased from Life technologies (India). All of the other reagent and chemicals used were of analytical grade.

Methodology

Soil sample collection and screening for extracellular RNase producing bacterial isolates

The soil samples for isolation of extracellular RNase producing bacterial strains were collected from an open field from Himachal Pradesh University campus, Shimla, India. The pH and temperature of soil measured during collection of sample soil were found to be 8.0 and 25°C, respectively. The collected soil samples were processed on the same day.

Screening of the bacterial isolates for RNase activity

A total of 14 bacterial isolates coded as RNS1 to RNS14 were isolated from soil samples. Bacterial isolates were screened on the basis of hydrolysis zone on the Nutrient Agar NA Petri plates containing 0.1 % (w/v) RNA [13]. The width of this clear zone was related to the amount of the exocellular enzyme produced. Efficient bacterial isolate(s) which showed large visual zones of hydrolysis were selected. The bacterial isolate(s) were cultured in modified culture medium described by Gundampati *et al*., [10] at 25°C in basal salt medium having Yeast extract 5.0 (g/l); Potassium chloride 0.6 (g/l); Magnesium sulphate heptahydrate 2.0 (g/l); Dipotassium hydrogen phosphate 0.1 (g/l); Ferrous sulphate heptahydrate 0.2 (g/l); Ammonium sulphate 2.0 (g/l) maintaining the pH 8.0 that could permit isolate growth. Briefly after 24 h of growth at 25°C, the cells were retained by centrifugation at 12,000 g for 10 min. The physicochemical tests were performed in order to characterize the RNase producing bacterial isolate RNS3. These morphological and chemical tests included Endospore, Gram staining, growth, margin, shape and color.

RNase activity assay

The activity of extracellular RNase was analysed by measuring the A_{260} as reported earlier [16]. The enzyme activity describe as One Unit (U) of ribonuclease activity was defined as an amount of enzyme capable of giving an increase of 1.0 A_{260} under the standard assay conditions. The 24 h culture broth was centrifuged at 12,000 g for 10 min at 4°C. The extracellular and intracellular RNase activity were assayed using supernatant and pellet, respectively.

Growth and RNase activity profile of isolate RNS3

The seed culture (24 h old) at 10 % (v/v) concentration was inoculated in the production broth (50 ml final volume taken in 250 ml Erlenmeyer flask). This flask was incubated at 25°C in an incubator shaker (160 rpm). At 8 h intervals, 4 ml of inoculated broth was aseptically sampled up to 56 h post inoculation. A_{660} value of each of the sample was recorded to determine the growth of the bacterial strain. Growth and activity profile was plotted against time.

Production conditions optimization for RNase

Different carbon sources (Lactose, Starch, Sucrose, Glucose, Maltose, Galactose and Fructose) were tested for RNase production by bacterial isolate RNS3. Production broth was cultivated in

250 ml Erlenmeyer flasks with 50 ml medium (initial pH 8.0) at 25°C and 150 rpm for 24 h. To evaluate the effect of different concentration of glucose as carbon source for RNase production by bacterial isolate RNS3, the culture broths (50 ml) were supplemented with concentration between 0.2 and 2.2 % w/v of glucose and inoculated with 10 % (v/v) of seed culture followed by incubation for 24 h at 25°C having pH 8.0 under shaking (160 rpm). Selected carbon source and its concentration were used for optimizing nitrogen source. Ammonium sulphate, sodium nitrate, casein, peptone, tryptone, urea, ammonium chloride and ammonium nitrate (1.0 %; w/v) were analyzed for there effect on maximum RNase production by bacterial isolate RNS3. For selection of optimum NaCl concentration for production of RNase, the concentartion of NaCl was varied from 0.5 to 4.5 mM by keeping the remaining parameters same. The optimum pH for enzyme production was selected by varying the pH of the production broth from pH 6 to 10 whereas other parameters were unaltered.

To determine the optimum cultivation temperature for the production of RNase, the temperatures were varied from 25 to 55°C while keeping the remaining parameters same.

Effect of incubation time and substrate concentration on RNase activity

The effect of pH of the reaction system and the incubation time on RNase assay were studied to find out optimal values of pH and temperature. The incubation time was ranging from 15 to 60 min at 15 min interval while the concentration of substrate (yeast extract) was varied from 2 to 18 µg/ml in the reaction mixture.

Purification by gel permeation chromatography on Sephadex G-50 column

RNase was partially purified by ammonium salt precipitation and column chromatography. The culture broth (500 ml) was centrifuged (10,000 g, 20 min, 4°C) and filtered through a Whatman No. 1 filter paper. The filtrate was then precipitated with ammonium sulfate and dialyzed extensively against 0.1 M Tris-HCl buffer; the protein was determined in dialyzate.

The filtrate (1 ml) was loaded on Sephadex G-50 column (size 65.5 cm x 1.5 cm) (Sigma Chemicals, USA). The protein was eluted with sodium phosphate 0.05 M buffer (pH 8.1). All eluted fractions were assayed both for RNase activity as well as total protein (A_{280}). The purification steps were performed in a chromatochamber at 4°C.

The fractions showing highest RNase activity were pooled and again assayed for RNase and protein content. The specific activity of the purified enzyme was compared with that of crude enzyme and fold purification was calculated. The relative molecular mass of the purified lipase was estimated by 10 % SDS-PAGE [18]. Proteins were stained with Coomassie Brilliant Blue R-250.

Result and discussion

Screening, isolation and iidentification of RNase producing bacterium

A total of 14 different bacterial isolates were analyzed for extracellular RNase producing activity. The bacterial isolate RNS3 showed maximum enzyme production as indicated by maximum zone of halos on Nutrient agar plate (Table 1). The extracellular RNase producing bacterial isolate RNS3 showed filamentous growth (Fig.

Table 1. RNase activities analyses based on the diameter of zone of hyrolysis shown by the bacterial isolates obatined from soil sample

Bacterial isolate	Diameter of zone (cm)	Bacterial isolate	Diameter of zone (cm)
RNS 1	2.33	RNS 8	2.21
RNS 2	2.11	RNS 9	1.46
RNS 3	2.60	RNS 10	2.71
RNS 4	1.54	RNS 11	2.33
RNS 5	2.11	RNS 12	1.20
RNS 6	1.86	RNS 13	2.30
RNS 7	1.33	RNS 14	2.15

1), wavy margin and creamish rough colonies on RNA-enriched NA plate. The bacterium was found to be Gram-positive, rod-shaped, catalase and oxidase positive (Table 2). The production broth when inoculated with 10 % (v/v) of 24 hour old seed culture produced maximum RNase activity (0.22 U/ml) at 22 h while maximum growth was observed after 45 h of incubation (Fig. 2).

Optimization of medium parameters for profound enzyme activity

The bacterial isolate grown in production medium (complex) containing 0.6 % glucose as a carbon source (0.4162 U/ml; Fig. 3a & 3b), peptone as nitrogen source (0.563 U/ml; Fig. 3c) and 2.5 mM of NaCl (0.902 U/ml; Fig. 4a) showed maximum RNase production. Carbohydrates, which play key roles as structural and energy com-

Fig. 1. The filamentous growth of bacterial isolate RNS3 on a Nutrient agar Petri plate

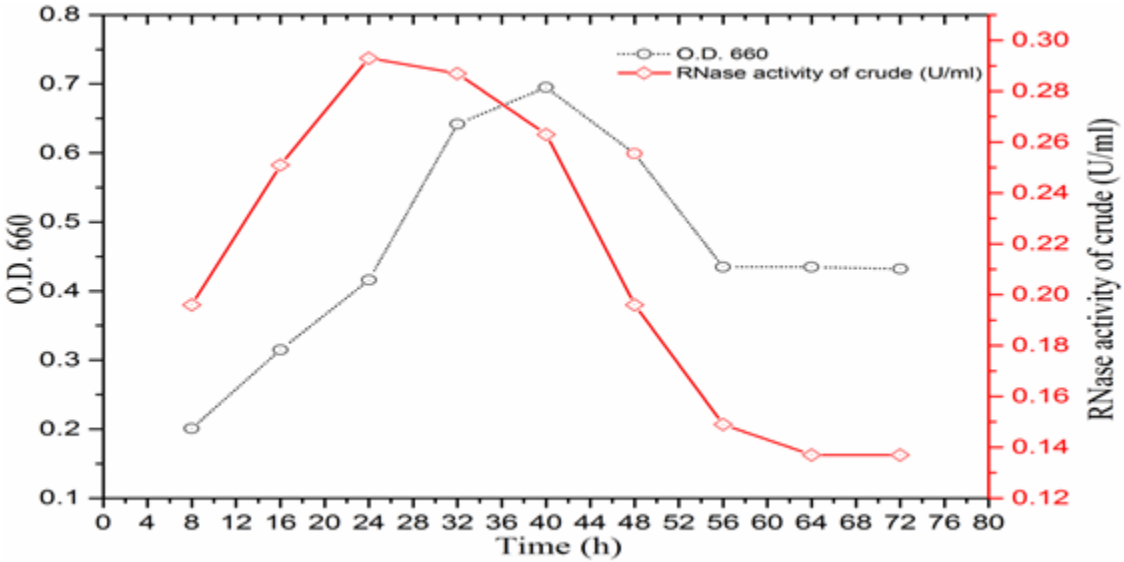

Fig. 2. Growth and RNase activity profile of bacterial isolate RNS3

Table 2. Main characteristics of the selected bacterial isolate RNS3

Characteristic	Result
Colony morphology	Creamy, Wavy and filamentous
Gram staining	Gram positive rods
Catalase and oxidase	Positive

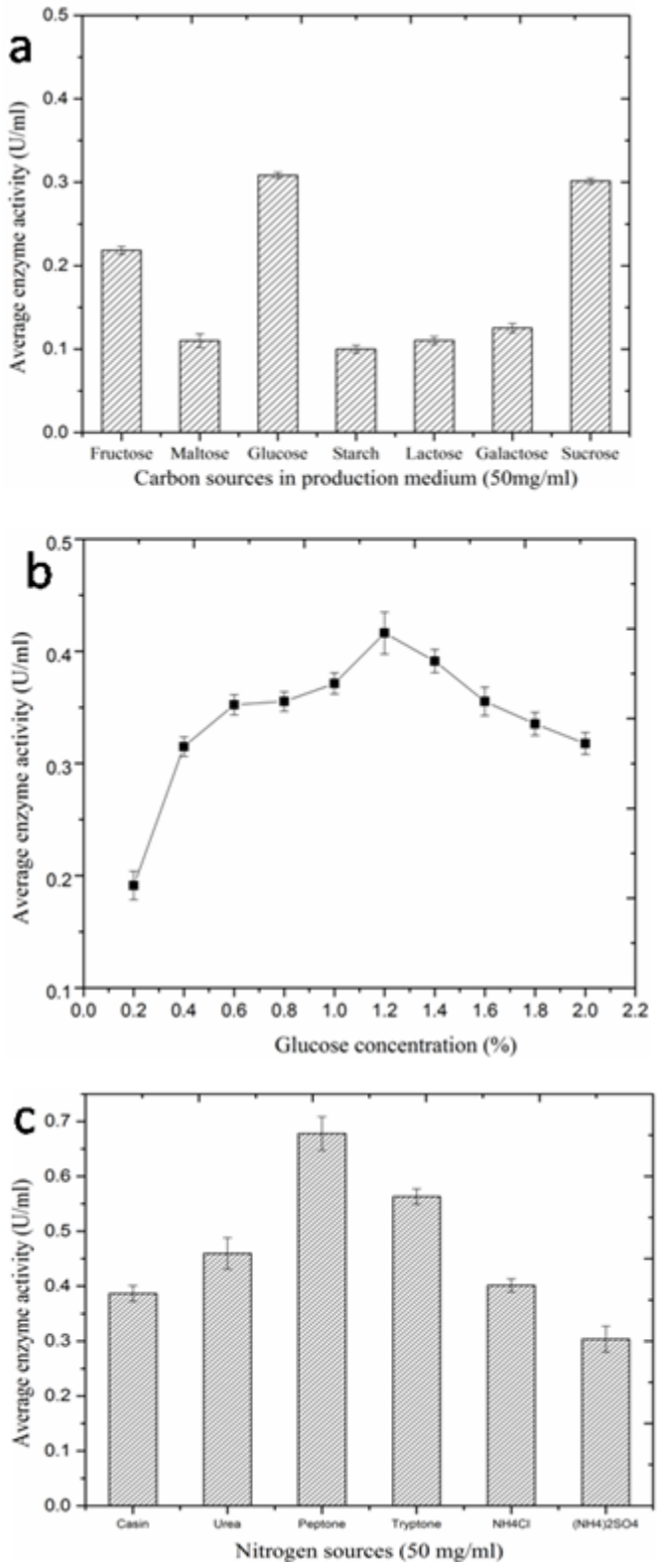

Fig. 3. Effect of different production conditions on RNase production by bacterial isolate RNS3 (a); Optimum carbon source for RNase prodution; (b); Optimum glucose concentration in broth for RNase production and (c) Optimum nitrogen source for RNase production

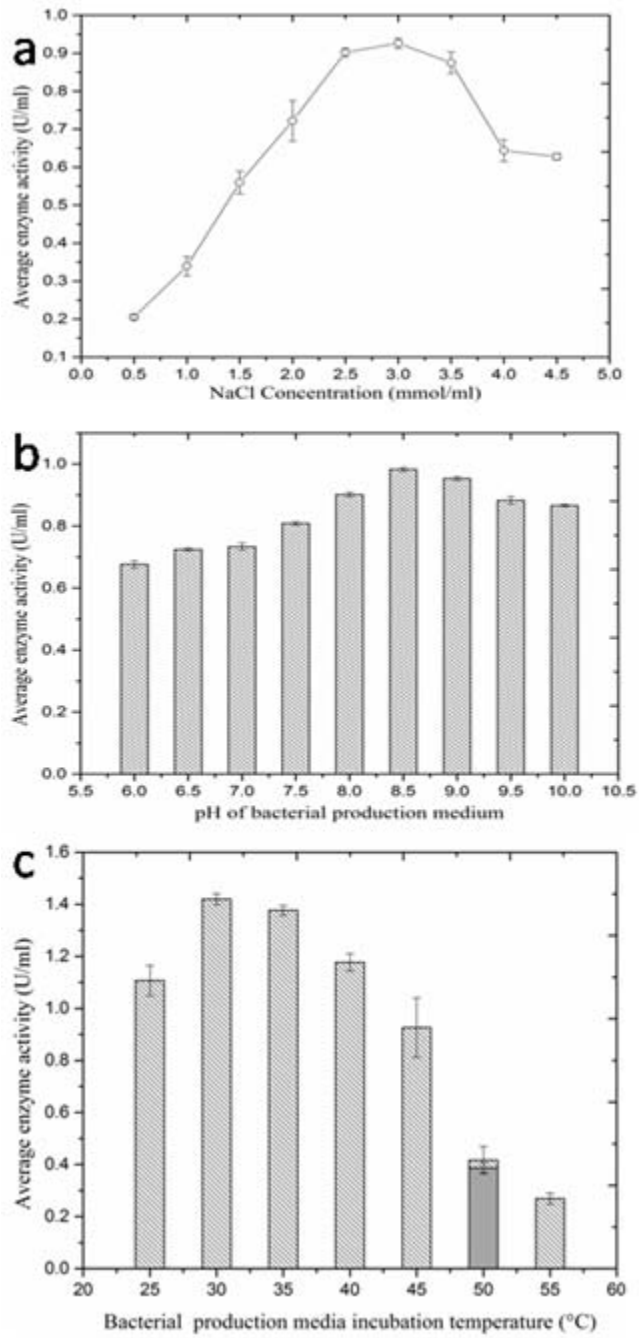

Fig. 4. Effect of different physiochemical conditions on RNase production; (a) Optimum NaCl concentration for RNase prodution; (b) Optimum pH for RNase production and (c) Optimum incubation temperature of broth for RNase prodution by bacterial isolate RNS3.

pounds in cells, are distinguished as monosaccharide and disaccharide. Various carbon sources such as glucose, fructose, maltose, sucrose, and galactose were tested to find the optimal medium for ribonuclease production from isolate RNS3 (Fig. 3a). The carbon content was maintained constant in all experiment. Among the different carbon sources, 0.6 % of glucose has shown an enhancement in the production of ribonuclease enzyme from bacterial isolate. Bacterial species uses different carbon source for maximum production of RNase, *i.e.*, glucose and lactose for better ri-

bonuclease production of *Bacillus intermedius* [27], yeast extract for *Bizionia* species [25], and glucose for *Escherichia coli* [17]. In our study, glucose was found to be better than sucrose a carbon source for ribonuclease production as reported in *Bacillus* sp. by Zhou *et al*.[38]. However some of the researchers reported that the glucose is the most effective carbon source for the ribonuclease production [9,22], which shows the positive impact of this carbohydrate on RNase production by the bacterial species. Different concentrations of glucose were tested on RNase production and found that 0.6 % is optimized concentration as shown in figure 3b. Concentration of different nitrogen sources as ammonium sulphate, casein, peptone, tryptone, urea and ammonium chloride were used to test on RNase activity produced by bacterial isolate. The maximum enzyme activity (0.5631 U/ml) was obtained when yeast peptone was used as a nitrogen source.

The effect of the other nitrogen sources on the RNase activity was determined by supplementing. Tryptone, urea, NH_4Cl, Cassin, $(NH_4)_2 SO_4$, respectively in the production broth (Fig 3c). The result was not completely consistent with the report of Demir *et al*. and Zhou *et al*. [5,37], which showed that yeast extract was a better nitrogen source needed for biosynthesis of bacterial ribonuclease.

NaCl **c**oncentration which plays an important role in membrane efflux was tested on RNase production medium and it was found that 2.5 m mol of NaCl is optimized concentration. Interestingly, 2.5 mM of NaCl showed maximum RNase activity (0.9023 U/ml) which however gradually decreased with increasing concentration (Fig 4a). Dunn 1976, reported that ionic strength of the medium effect the fidelity of Ribonuclease [6]. The results of pH studies revealed that the bacterial strain showed the highest ribonuclease activity (0.9826 U/ml)) at initial alkaline pH (8.5) of the production medium (Fig. 4b).

The effect of temperature on production of RNase by bacterial isolate was determined over the temperature between 20-60°C. The maximum RNase activity was observed at 35°C.which signified mesophilic nature of the selected bacterium RNS3. The temperature and pH have significant roles in metabolic activity of microbes. According to this result, effect of temperature was more prominent than effect of pH. The RNase activity of bacterial isolate RNS3 slightly decreased with an increasing pH (Fig. 4b) while above the temperature of 45°C, bacterial isolate showed a minor enzyme acti-vity (Fig. 4c). RNase activity gradually increased and reached a maximum at 45°C when the initial pH 8.5. These results are consistent with bacterium *Bacillus cereus* reported by Zhou et al.[38]. Some studies also agreed that bacteria required higher pH and temperature [3,5,33,37]. Similarly, optimum pH of 8.0 was recorded for RNase production when Patil optimized the production condition of the bacterium *Streptomyces thermonitrificans* [22]. Demir also observed the optimal pH 9 when optimized the RNase production condition of *Streptomyces* sp. M49-1 [5]. Mostly RNase production is lengthy by using the fungal and bacterial culture that have long lag phases [37]. This study was in agreement with the report of Demir, that reported the shortened time period for RNase production which was approximately 90 h [5]. The optimal concentration of RNA and incubation time were obtained as 10 µg/ml (1.59 U/ml RNase) and 45 min of incubation time enhanced the enzyme acti-vity. Reaction kinetics of various microbial RNases are different due to diversity in microbial RNases as well as their substrates [34].

Purification of RNase of bacterial isolate RNS3

The cell debris of the filtrate was removed by centrifugation and the supernatant was retained. The proteins were concentrated in the cell-free supernatant by addition of 60-70 % ammonium sulfate, the precipitates were collected and dialyzed against 0.5 M sodium phosphate buffer pH 7.2, extensively. The dialysate after assay for protein and RNase activity was fractionated on a gel permeation Sephadex G-50 column. Each fraction of 2 ml was monitored for protein concentration and enzyme activity (Fig. 6a). RNase purity was observed by calculating the specific activity at every purification steps (Table 3). The molecular weight of the ribonuclease was determined by SDS-PAGE gel electrophoresis. Protein band

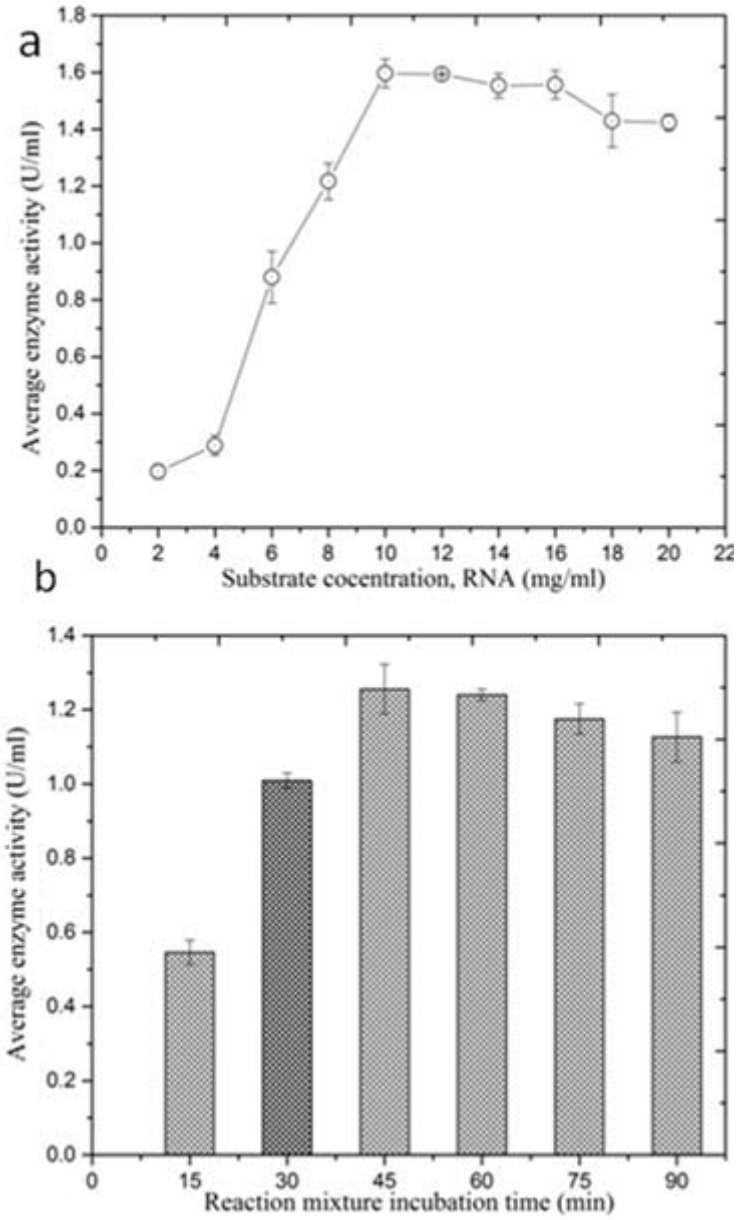

Fig. 5 (a) Optimum substrate (RNA) concentration for RNase activity; (b) Optimum incubation time of reaction mixture for Rnase activity

were observed when stained with Coomassie brilliant blue and it clearly indicated the purity of the protein (Fig. 6b). The molecular weight of the purified RNase was calculated to be about ~65 kDa. The optimal recovery of bacterial RNase was achieved by using salting out of protein(s) with ammonium sulfate precipitation and gel permeation chromatography. The result of RNase having ~65kDa has strong agreement with earlier reported RNases with similar molecular weight [2,4,23,31].

The optimization of production condition of ribonuclease is a valuable tool to produce an extracellular RNase quantitatively from cell-free culture broth of a bacterial isolate. The final optimized condition of production broth to produce RNAse was found to be 0.6 % (w/v) of Glucose, Peptone 5 % (w/v), 2.5 mmol of NaCl, pH 8.5 and incubation temperature 35°C. The optimal RNase activity could be assayed by using 10 µg/ml of RNA and 45 min incubation of reaction mixture at 45°C.

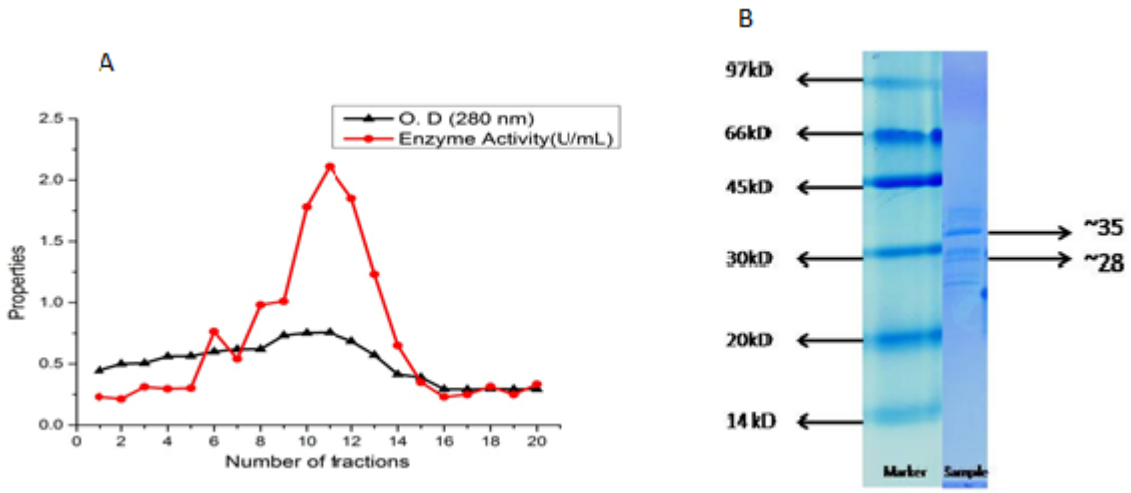

Fig. 6 (A) Sephadex G-50 column chromaography profile of RNase and (B) the SDS-PAGE (10%) profile of purified RNase

Conclusion

The bacterial isolate RNS3 isolated from soil has good efficiency to produce an extracellular RNase under the optimum condition such as 0.6 % (w/v) of Glucose, Peptone 5 % (w/v), 2.5 mmol of NaCl, pH 8.5 and an incubation temperature 35°C. The extracellular RNase was purified by using ammonium sulfate precipitation, dialysis and gel permeation column chromatography. Optimum culture condition enhanced the RNase activity by ~7-fold. Electrophoretic study by SDS-PAGE analysis revealed the enzyme to be a dimeric protein moiety having molecular weight of ~65 kDa. The purified RNase showed an enhanced specific enzyme activity of 26.3 than the earlier activity of ~2.0 U/ml.

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