

# Optimization and Purification of Extracellular Nuclease from *Bacillus firmus* VKPACU-1

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## ABSTRACT

A strain of *Bacillus firmus* (VKPACU-1) produced high levels of extracellular ribonuclease (RNase) when grown on tryptone, peptone and glucose media. The test strain produced a maximum amount of nuclease at the stationary phase (32 hrs). The crude enzyme was optimally active at pH 6.5 and 35°C. Tryptone and peptone are the superior nitrogen sources. The addition of Mn<sup>2+</sup> to the growth medium significantly enhanced enzyme production while glucose and maltose were excellent carbon sources for RNase production. The organism showed good growth and highest nuclease production in cultures grown with 0.5% NaCl. Peptone (0.5%), beef extract (0.5%), tryptone (0.5%), sodium chloride (0.5%) and Mn<sup>2+</sup> (2 mM) were chosen as the basal medium components as they supported the highest nuclease (RNase) production. There was higher activity with RNA and heat-denatured DNA than with native DNA. The molecular mass of the purified nuclease was about 17.1 kDa.

**Keywords:** production, carbon and nitrogen sources, growth, column chromatography

## INTRODUCTION

Nucleases are one of the hydrolytic and multifunctional enzymes that is able to catalyze the cleavage of phosphate ester bonds of both DNA and RNA (Ashokkumar *et al.* 2010). RNases are important analytical enzymes and have played a major role in the determination of RNA structure. RNases are nucleases which are produced by a diversity of organisms including bacteria, fungi, plants, sub-mammalian vertebrates, and mammalian liver, kidney, brain, placenta, pancreas, milk and semen (Guan *et al.* 2007). They are also used for the removal of RNA in single-cell protein preparations. Ribonucleases may exhibit activities other than ribonucleolytic activity, such as anti mitogenic (Ngai and Ng 2004), antibacterial, antifungal (Lam and Ng 2001), anti-proliferative, antiviral activity (Guan *et al.* 2007; Zhou and Niu 2009), HIV-I reverse transcriptase inhibitory (Wang and Ng 2000), translation inhibitory (Ng and Wang 2001) and angiogenic (Shapiro and Valle 1987) activities. Demand for RNA-free DNA and proteins samples increases with the increased production of DNA vaccines and biological drugs. They are widely used in molecular biological studies and in the food and pharmaceutical industries. As an important analytical tool, they have played a major role in studies on the structure and function of RNA. At present, the contradiction between low production and high demand is becoming acute, giving rise to the urgency of addressing the problem of screening microorganisms for the maximum production of RNase. In the present investigation, growth and nuclease production have been optimized for a newly isolated *Bacillus firmus* VKPACU-1, under various chemical and physical conditions and the enzyme has been purified to homogeneity and characterized.

## MATERIALS AND METHODS

### Isolation and screening of microorganisms

Sediment and water samples from Coovum River, situated in Chennai, Tamil Nadu, India were collected at 4°C. The isolated

strains were planted on minimal salts medium (Zhang *et al.* 2005) containing 0.2% DNA for nuclease production. All the isolated bacterial strains were further screened for extracellular nuclease production in DNA- and RNA-amended medium (Jeffries *et al.* 1957). The isolated bacterial strains were streaked on the center of Petri dishes (Borosil Glass Works Ltd., Chennai) and incubated for 48 hrs at 37°C. After incubation, the plates were flooded with 1 N HCl (Jeffries *et al.* 1957). A positive reaction indicated by the clear zone formed around the colony. The bacterial strain was adapted to selective biochemical tests (Bergey's Manual of Determinative Bacteriology 1994).

### 16SrRNA gene sequencing methodology

#### 1. DNA isolation

Genomic DNA was isolated from the selected strain by following the method of Ausubel *et al.* (1995). A single colony of the isolate was inoculated into 100 mL of the nutrient broth and incubated at 37°C for 48 hrs. The cells were harvested by centrifugation at 10000 × g for 10 min. The supernatant was discarded and the compact cell pellet was suspended in 567 µL TE buffer. To this, 30 µL of 10% SDS and 4 µL of proteinase K (25 mg/mL) were added to get a final concentration of 100 µg/mL of proteinase K. The contents were mixed thoroughly and incubated at 37°C for 1 h. To the lysing mixture, 100 µL of 5 M NaCl was added and mixed thoroughly followed by the addition of 80 µL of CTAB/NaCl solution. The contents were mixed thoroughly and incubated at 65°C for 10 min. Equal volume (0.7 to 0.8 mL) of chloroform/isoamyl alcohol (24:1 v/v) was added and centrifuged at 10000 × g for 5 min. The aqueous, viscous supernatant was transferred to another tube without disturbing the white interface. The supernatant was extracted twice with phenol: chloroform: isoamyl alcohol (49.5: 49.5: 1 v/v/v) and 0.6 vol of isopropanol was added to precipitate the nucleic acids. A stinky white DNA precipitate was clearly visible as the contents were mixed gently. The DNA was pelleted by centrifugation at 5000 × g for 10 min and washed with 70% ethanol to remove residual CTAB. The pellet was air dried and dissolved in 100 µL TE buffer and was used for the amplification of 16S rDNA and sequencing studies.

## 2. Amplification of 16S rRNA gene from the isolated DNA

16S rRNA gene from the isolated DNA was amplified using the following primer sequence of forward primer (5'-AGA GAT TGA TCA TGG CTC AG-3') and Reverse primer (5'-GGT TAC CTT GTT ACG ACT T-3'). Reaction mixture containing Forward primer - 1  $\mu$ L, Reverse primer - 1  $\mu$ L, template DNA - 2  $\mu$ L, dNTP mix - 2  $\mu$ L, *Taq* polymerase - 1  $\mu$ L, *taq* buffer - 4  $\mu$ L and glass distilled water - 29  $\mu$ L. The PCR amplification of the 16S rRNA gene was carried out on MJR thermocycler with a protocol involving initial denaturation for 3 min at 93°C; 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 10 min.

### Purification of PCR products

Amplified 16S rDNA was purified for sequence determination using the QIA quick PCR purification kit (Qiagen), following the instructions of the manufacturer. Purified PCR products were eluted from the purification columns by the addition of 50  $\mu$ L Tris buffer (10 mM; pH 8.0). PCR products were purified by agarose gel (1.2%) electrophoresis. After staining with ethidium bromide, a defined band visualized under UV irradiation was excised using a sterile blade. Besides removing surplus primers, nucleotides and salts, this method possessed the advantage that incomplete (shorter) amplification fragments were also removed prior to sequencing. Subsequently, the DNA was extracted from the gel matrix, using the QIAEX gel extraction kit (Qiagen). Purified PCR products were eluted with 40  $\mu$ L TE buffer (10 mM; pH 8.0) and subjected to the T4-DNA-polymerase treatment.

### Sequencing of amplified 16S rRNA genes and identification of isolate

The amplified 16S rRNA genes were sequenced by using the ABI PRISM Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) and ABI PRISM 377 automated sequencer (Perkin-Elmer) according to the manufacturer's instructions. The resulted gene sequence was subjected for Blast-N-analysis ([www.ncbi.com](http://www.ncbi.com)) and the organism was identified based on the highest homology of the sequence with sequence available in the database.

### Determination of DNase activity by spectrophotometry

The DNase activity of the test isolate was determined according to Apte *et al.* (1993). The standard reaction mixture of 1 mL contained 100  $\mu$ g of native calf thymus DNA in 30 mM Tris-HCl buffer, pH 7.0 with 0.1 mL of cell free supernatant or purified enzyme. The reaction was initiated by the addition of dsDNA/ssDNA followed by incubation at 37  $\pm$  2°C for 1 h. Termination of reaction was achieved by the addition of 1 mL of 10% (v/v) prechilled perchloric acid and 1 mL of 0.2% (w/v) BSA. The mixture was left on ice for 10 min and then centrifuged to sediment the precipitate. The acid soluble deoxyribonucleotides in the supernatant were measured at 260 nm. One unit of nuclease activity was defined as an increase in absorbance of 1.0 at 260 nm.

### Determination of RNase activity by spectrophotometry

The RNase activity of the test isolate was determined by the method of Ho *et al.* (1998). The reaction mixture contained 500  $\mu$ L Tris-HCl (0.2 M, pH 7.0), 750  $\mu$ L H<sub>2</sub>O and 250  $\mu$ L of cell free supernatant or purified enzyme. After equilibrating the enzyme mixture at 37  $\pm$  2°C for 5 min, 625  $\mu$ L of freshly prepared RNA (1.2%, (w/v) Baker's yeast, SRL Chemical Co) was added and incubated for 15 min. After incubation, 0.1 volume of ammonium acetate (7.5 M) and 3 volume of absolute alcohol were added and the entire solution was cooled at -20°C for at least 30 min and centrifuged. The absorbance of supernatant was read at 260 nm. One unit of nuclease activity was defined as an increase of 1.0 absorbance/min at 260 nm.

## Optimization studies

The effects of culture conditions on nuclease production were assayed by growing VKPACU-1 in nutrient broth and incubated at 37°C. The test organism was grown at different temperatures ranging from 10 to 60°C, and with different amounts of NaCl: 0, 0.5, 1, 2, 3, 4, 5 and 10% (w/v). The effect of hydrogen ion concentration on growth and nuclease production was tested in the pH range 3.5–12.0. To study the effect of various carbon and organic and inorganic nitrogen sources on growth and nuclease production, VKPACU-1 was inoculated into minimal salt medium amended with glucose, lactose, sucrose, maltose, fructose, mannitol and soluble starch and various organic and inorganic nitrogen sources at 1.0% (w/v). Carbon and nitrogen sources concentration was selected based on Patil *et al.* (2005). Peptone, yeast extract, beef extract, tryptone and inorganic nitrogen sources such as ammonium sulphate, sodium nitrate and potassium nitrate were added separately. All carbon and nitrogen sources were purchased from Himedia laboratories (Mumbai, India). To determine the effect of metal ions on growth and nuclease production, the test strain was inoculated in nutrient broth medium supplemented with MnCl<sub>2</sub>, CuCl<sub>2</sub>, ZnCl<sub>2</sub>, FeSO<sub>4</sub>, CoCl<sub>2</sub>, BaCl<sub>2</sub>, CaCl<sub>2</sub>, EDTA and MgSO<sub>4</sub> at a final concentration of 2.0 mM. Growth was measured as absorbance at by UV light at  $\lambda$  = 600 nm using a UV spectrophotometer (Chemito, Mumbai, India) and nuclease activity was determined after 32 hrs of incubation. All chemicals were analytical grade reagents. All measurements were performed in triplicate.

### Purification of extracellular nuclease from test strain VKPACU-1

100 mL of exponential phase culture of test strain VKPACU-1 was used to inoculate 1000 mL of medium (optimized) in 4 L Hoffkin's flask. After 32 hrs of incubation on a rotary shaker with 250 rpm at 35°C (optimized temperature), the cells were harvested by centrifugation at 8000  $\times$  g for 30 min at 4°C and the supernatant containing the extracellular nuclease was preserved for purification process. All the purification procedures were carried out at 4°C.

### Protein determination

Protein concentration was measured by the method of Bradford (1976) using Bovine Serum Albumin BSA as the standard. During chromatographic purification, protein concentration was estimated by observing the absorbance at 280 nm.

### Extraction and purification of extracellular nuclease from test strain VKPACU1

The test isolate VKPACU1 was grown in the optimized medium at 35°C for 32 hrs. After the incubation, the cells were harvested by centrifugation at 8000  $\times$  g for 30 min at 4°C. The cell free culture filtrate was precipitated using 80% ammonium sulfate. The resulting precipitate was centrifuged and the pellet was dissolved in 30 mM Tris-HCl (pH 7.0) and dialyzed against the same buffer. The dialyzed enzyme was used for further purification studies. In the present study, all the purification steps were carried out at 4°C.

### Phenyl sepharose chromatography

Dialyzed enzyme was loaded onto a column (1.0  $\times$  7.0 cm) of phenyl sepharose pre-equilibrated with Tris-HCl (30 mM; pH 7.0) at a flow rate of mL/min. The elution of the bound enzyme was facilitated with a linear gradient of NaCl (0–1.25 M) in Tris-HCl (30 mM; pH 7.0). Those fractions having specific activity more than 300 U/mg were pooled, concentrated by lyophilization and used for the next step.

### Gel filtration chromatography

This sample was then applied to a column (2.5  $\times$  90 cm) packed with Sephadex G-100 (Sigma, 119 USA) and pre-equilibrated with 30 mM of Tris-HCl (pH 7.0). The column was then eluted with equilibration buffer. Fractions (3 mL) were collected and assayed for protein and nuclease activity as described above.

## Reverse phase high performance liquid chromatography (RP-HPLC)

The fractions containing Nuclease activity were further checked for purity using reverse phase high performance liquid chromatography (Shimadzu, Japan) and 0.5 30 cm Vydac C8 silica-based hydrophobic column (Grace, USA).

## Homogeneity and determination of purified nuclease

Molecular weight of the purified enzyme was determined by SDS-PAGE (Laemmli 1970) using Sigma high molecular-weight electrophoretic standards. The separated protein was developed by silver staining method described by Blum *et al.* (1987).

## RESULTS

### Isolation and screening of nuclease producers

In the present study 68 bacterial strains isolated from contaminated soil, water and sediment samples collected from Coovum River, Chennai, Tamil Nadu, India were brought into pure cultures. All the 68 isolates designated as VKPACU-1 to -68 were screened for the production of extracellular nuclease and among these, one isolate from the Coovum sediment sample showed good growth and very large hydrolytic zone in the plate containing minimal salts medium amended with DNA as the sole carbon/energy source. This strain VKPACU-1 was chosen for further studies as it showed the highest hydrolytic potential against nucleic acids (DNA and RNA) during the initial screening experiments. The test organism showed higher hydrolyzing activity with RNA than with DNA as the substrate and hence further studies were focused on RNA hydrolyzing activity.

### Phenotypic and biochemical characterization of VKPACU-1

The colony of the test isolate VKPACU-1 was circular, slightly raised and opaque with rhizoidal border and showed a granular shiny surface. The cells were Gram-positive motile rods, measuring about 3-4  $\mu\text{m}$  in length and  $\sim 1.0 \mu\text{m}$  in breadth. Morphological characteristics and the responses of the isolate VKPACU-1 to different biochemical tests are presented in **Table 1**.

Strain VKPACU-1 showed a positive reaction to Catalase, Oxidase, MR test, Tween-40 hydrolysis, starch hydrolysis, nitrate reduction, gelatin hydrolysis, casein hydrolysis, acid production from glucose alone and DNase activity and a negative reaction to nitrite reduction, Tween-80 hydrolysis, urea hydrolysis, indole production, VP test, citrate utilization and growth on McConkey agar (Himedia, Mumbai, India), gas formation with glucose and to H<sub>2</sub>S production which fixed the test organism to be a species of *Bacillus* based on the keys given in Bergey's Manual of Determinative Bacteriology (1994).

### Molecular characterization of the test strain based on 16S rRNA gene sequence homology

The DNA isolated from the test strain was amplified with specific eubacterial 16S rRNA gene primers and the purified PCR product was analyzed using an automated sequencer. The partial sequence of 1192 bases of the amplified 16S rRNA gene of the test isolate VKPACU-1 showed 99% homology with a maximum score of 2158 out of the total score of 2158 to the 16S rRNA gene sequence of *Bacillus firmus* strain KSC\_SF8b in the GenBank (Accession No. DQ870688) and hence was designated as *Bacillus firmus* VKPACU-1. The 16S ribosomal RNA gene sequence of strain VKPACU-1 was deposited in GenBank under Accession number EU368002.

**Table 1** Physiological and biochemical characteristics of isolate VKPACU-1.

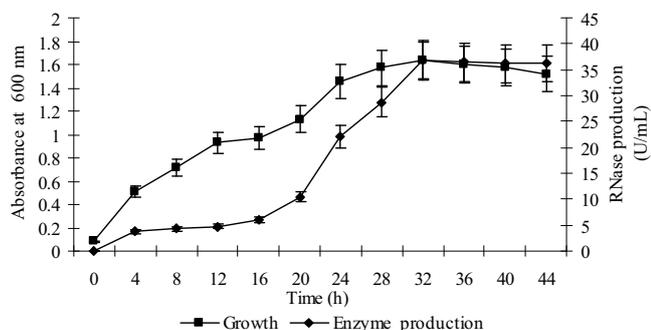
Test	Results
<b>Colony morphology</b>	
Configuration	Circular
Margin	Rhizoid
Elevation	Slightly raised
Surface	Granular Shiny
Pigment	---
Opacity	Opaque
<b>Physiological and biochemical tests</b>	
Growth at 25 - 42°C	+
Growth at pH (4 - 10)	+
Growth in NaCl (2 - 7%)	+
Growth in anaerobic agar	-
Gram's Reaction	Positive
Cell Shape	Rods
Size ( $\mu\text{m}$ )	Length: 3-4 $\mu\text{m}$ ; Width: approx. 1.0 $\mu\text{m}$
Arrangement	Short chains
Motility	+
Growth on McConkey Agar	-
Indole production	-
Methyl Red test	+
Voges Proskauer test	-
Citrate utilization	-
Acid from glucose	+
Gas from glucose	-
H <sub>2</sub> S production	-
Casein hydrolysis	+
Gelatin hydrolysis	+
Urea hydrolysis	-
Starch hydrolysis	+
Nitrate reduction	+
Catalase Test	+
Oxidase Test	+
Tween 20 hydrolysis	-
Tween 40 hydrolysis	+
Tween 80 hydrolysis	-
Acid production from carbohydrates	NIL
DNase activity	+
RNase activity	+

### Growth and extracellular nuclease (RNase) production by *Bacillus firmus* VKPACU-1

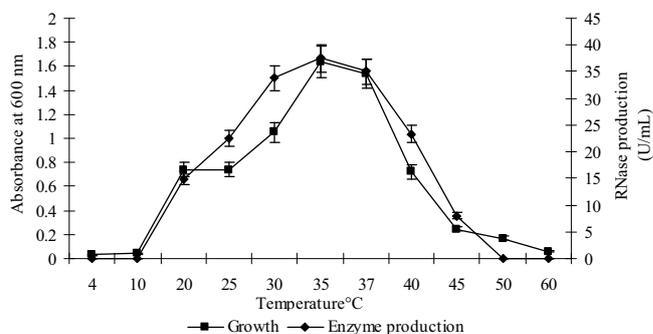
The test strain *Bacillus firmus* VKPACU-1 started growing exponentially from the time of inoculation and entered into the stationary phase at 32 hrs which was recognized by the absence of change in absorbance of the culture. Unlike the growth of the test strain, extracellular nuclease production was very low during the exponential phase and from 24 hrs onwards the nuclease production was found to be increased and reached the maximum (36.8 U/mL) at 32 hrs (**Fig. 1**). While the nuclease production was maintained at near maximum between 32 and 36 hrs, there was a slight decrease in nuclease production (36.3 U/mL) at 40 and 44 hrs which was also coinciding with a decline in growth (**Fig. 1**).

### Effect of pH on growth and nuclease (RNase) production by *Bacillus firmus* VKPACU-1

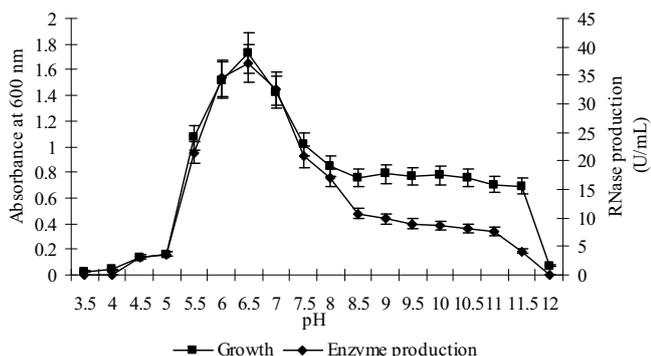
The organism exhibited growth and nuclease (RNase) production at wide pH range of 5.5-11.0. The highest growth and nuclease production was recorded at pH 6.5 and there was a fall in growth and enzyme production at pH values both below and above pH 6.5. The growth and enzyme production were inhibited completely at pH 3.5 and 12.0. However, the maximum growth was observed in the medium with pH 5.5 to 7.0. Maximum enzyme production was observed in the medium with pH 6.5 (37.1 U/mL) and the production was maintained near maximum with pH 6.0 and pH 7.0 (**Fig. 2**). The medium with pH values below 6.0 and above 7.0 did not support both growth and enzyme produc-



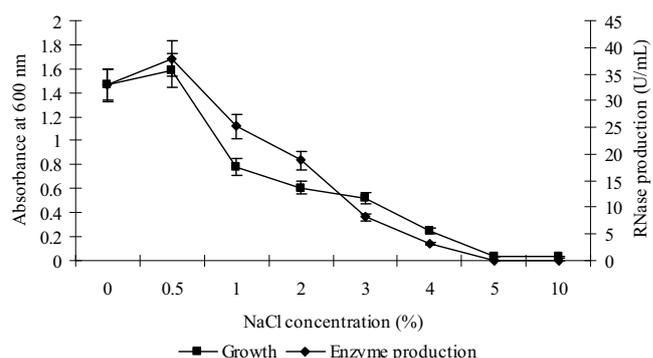
**Fig. 1** Growth and extracellular nuclease production by *Bacillus firmus* VKPACU-1.



**Fig. 3** Effect of temperature on the growth and nuclease (RNase) production by *Bacillus firmus* VKPACU-1.



**Fig. 2** Effect of pH on the growth and nuclease (RNase) production by *Bacillus firmus* VKPACU-1.



**Fig. 4** Effect of salinity on growth and nuclease (RNase) production by *Bacillus firmus* VKPACU-1.

tion. Though the organism maintained same absorbance between pH 8.0-11.0, the nuclease production was drastically reduced to below 10 U/mL. Thus the results clearly indicate the critical influence of pH on growth and nuclease production by the test isolate.

#### Effect of temperature on growth and nuclease (RNase) production by *Bacillus firmus* VKPACU-1

To elucidate the effect of temperature on the growth and nuclease production by *Bacillus firmus* VKPACU-1, the test strain was grown at different temperatures such as 4, 10, 20, 25, 30, 35, 40, 45, 50 and 60°C. Among the different incubation temperatures, the culture grown at 35°C showed highest absorbance at 600 nm indicating good growth when compared to all other incubation temperatures and the nuclease production was also maximum (37.5 U/mL) at this temperature (35°C). The test strain showed lesser optical density and lower nuclease production of 33.8 U/mL at 30°C, while the culture of the test strain at 25 and 40°C showed only 50% of the nuclease production that was recorded for 35°C (Fig. 3). Growth and nuclease production were completely inhibited at 4, 10, 50 and 60°C indicating the drastic effect of lower and higher temperatures.

#### Effect of salinity on growth and nuclease (RNase) production by *Bacillus firmus* VKPACU-1

The response of the test organism against different concentrations of NaCl was very interesting. The organism showed good growth and nuclease production even in the absence of NaCl in the medium. But the highest nuclease production of 37.9 U/mL was recorded in cultures grown with 0.5% NaCl. There was a gradual decline in growth and nuclease production with an increase in the concentration of NaCl beyond 0.5%. Although a significant level of growth and nuclease production was recorded at 1% NaCl in the medium, complete inhibition of both growth and nuclease production was observed with 5 and 10% NaCl. This result thus clearly established the high sensitivity of the test orga-

nism to salinity in the medium (Fig. 4).

#### Effect of various carbon and nitrogen sources on the growth and extracellular nuclease (RNase) production by *Bacillus firmus* VKPACU-1

Among all the carbon and nitrogen sources tested, best growth was supported by Tryptone and the nuclease production was as high as 38.2 U/mL. Peptone, beef extract and yeast extract, although supported growth well, nuclease production with these substrates was only 29.7, 31.3 and 27 U/mL, respectively. None of the inorganic nitrogen sources were effective in supporting growth as well as nuclease production. These results clearly proved the test organism's preference for complex nitrogen sources over simple inorganic nitrogen source for growth and nuclease production (Fig. 5).

Among the different carbon sources tested for their ability to support the growth and the nuclease production, only glucose and maltose supported both good growth and nuclease production by the test isolate. In the presence of glucose the test organism produced 39 U/mL and with maltose the production was 38.0 U/mL. Though sucrose and fructose supported the growth of the test strain, nuclease production in the presence of these carbon sources was only 25.4 and 17.6 U/mL respectively which was far below of the maximum produced by the test strain. Lactose, glycerol and soluble starch could influence only least extracellular enzyme production which was about 17.6, 15.5 and 15.5 U/mL, respectively (Fig. 5).

#### Effect of metal ions on growth and nuclease (RNase) production by *Bacillus firmus* VKPACU-1

Effects of various metal ions on growth and nuclease production were investigated by growing the test strain *Bacillus firmus* VKPACU-1 in nutrient broth supplemented separately with  $Mn^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$ ,  $Fe^{2+}$ ,  $Co^{2+}$ ,  $Ba^{2+}$ ,  $Ca^{2+}$ , EDTA and  $Mg^{2+}$  to a final concentration of 2 mM each.  $Mn^{2+}$  in Cl<sup>-</sup> form supported the highest nuclease production of 39.8

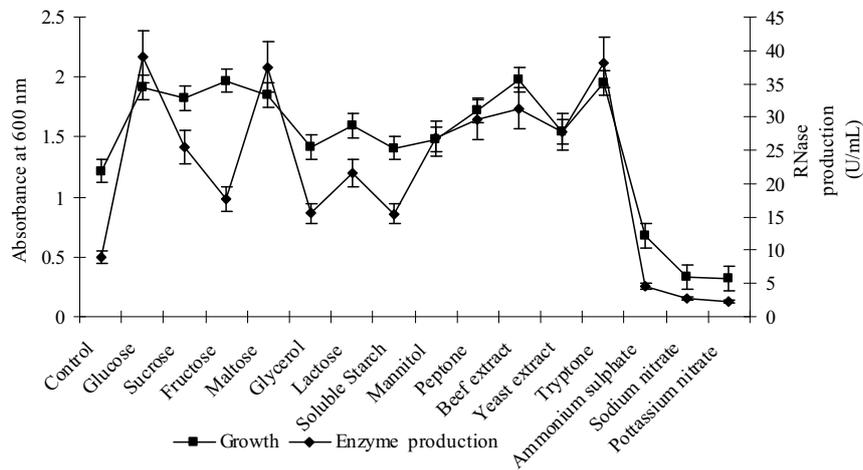


Fig. 5 Effect of carbon and nitrogen sources on growth and nuclease (RNase) production by *Bacillus firmus* VKPACU-1.

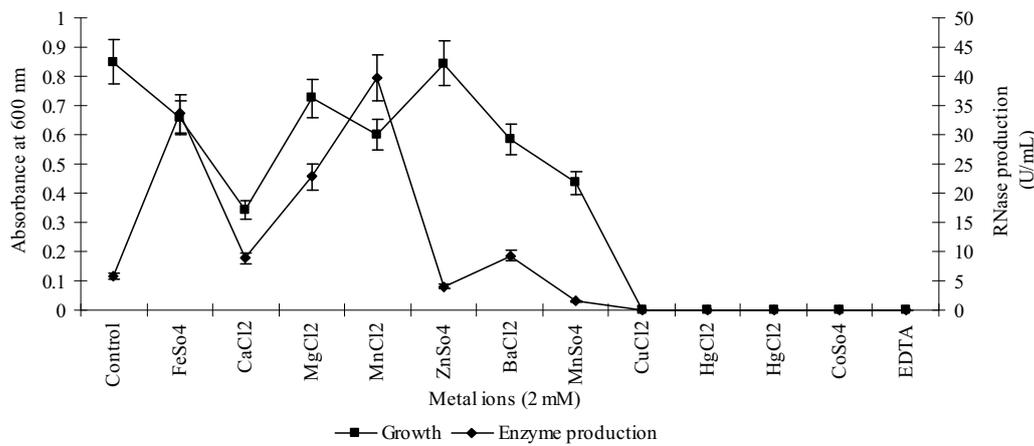


Fig. 6 Effect of metal ions on growth and nuclease (RNase) production by *Bacillus firmus* VKPACU-1.

U/mL which in sulfate form though supported moderate growth, completely inhibited nuclease production. Both growth and nuclease production were well supported by  $\text{Fe}^{2+}$  and the nuclease production was recorded as 33.6 U/mL (Fig. 6). Although the test organism was able to grow and produce the enzyme in the absence of added metal ions, higher enzyme production was recorded in the presence of certain metal ions like  $\text{Mn}^{2+}$  and  $\text{Fe}^{2+}$ . Though the growth was highest in the presence of  $\text{Zn}^{2+}$ , the nuclease production was drastically inhibited. Other divalent metal ions like  $\text{Ba}^{2+}$  and  $\text{Ca}^{2+}$  in spite of their role in supporting the growth, significantly inhibited the production of nuclease while  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Co}^{2+}$  and EDTA inhibited both the growth and nuclease production completely.

## Purification of extracellular nuclease from *Bacillus firmus* VKPACU-1

### 1. Nuclease production

After optimizing the culture conditions for the maximum production of nuclease for the test strain *Bacillus firmus* VKPACU-1, it was grown in the 4 L Hoffkin flask containing 1 L of the production medium (optimized). The extracellular nuclease produced at the end of stationary phase was obtained by centrifugation and used as the crude

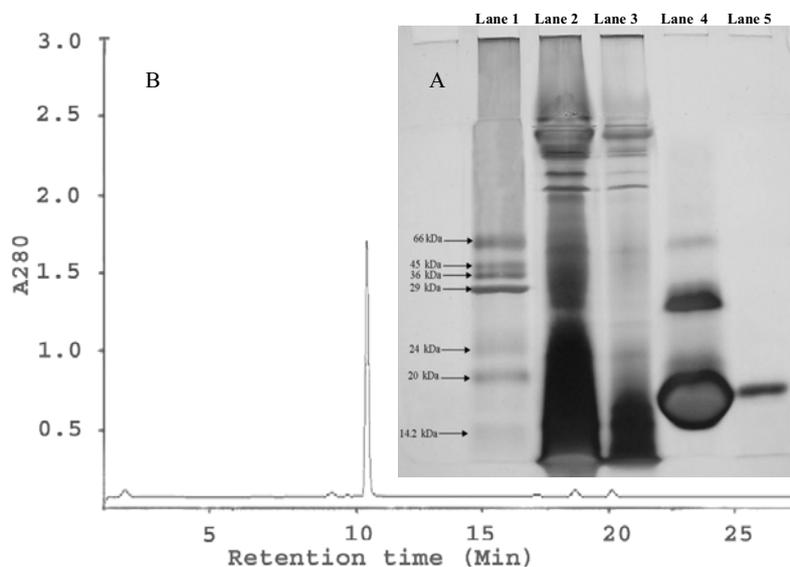
enzyme source (2 L) for further purification steps. In order to concentrate the nuclease present in culture filtrate, solid ammonium sulphate at different saturations (10, 20, 30, 40, 50, 60, 70, 80 and 90%) was added and each of the precipitate formed was dissolved in Tris-HCl buffer (30 mM; pH 7.0) and dialysed against the same buffer. Among the different ammonium sulphate saturations, the nuclease activity was recorded in 60, 70, 80 and 90% saturations which were about 21.28, 94.93, 347.56 and 125.23 U/mg protein, respectively. Hence the precipitate obtained from 70, 80 and 90% ammonium sulphate saturations were pooled and used for further purifications.

### 2. Purification of nuclease by phenyl-sepharose chromatography

89 mg of protein that contained 30,932 U of nuclease activity recovered from ammonium sulphate fractions was loaded on to the phenyl sepharose column. Though five protein peaks were recorded in the eluent obtained from the phenyl sepharose column, nuclease activity was observed from the fractions 19-34 (0.475 – 0.850 M NaCl). The yield obtained was 12 mg protein which contained 15,782.5 U activity (Table 2).

Table 2 Purification chart of extracellular nuclease produced from *Bacillus firmus* VKPACU-1.

Source	Protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Culture filtrate	612	76513.91	125.02	100	1
Ammonium sulphate precipitation	89	30932.84	347.56	40.42	2.78
Phenyl Sepharose	12	15782.52	1315.21	20.628	10.52
Sephadex G-100	1.5	3133.69	2089.13	4.095	16.71



**Fig. 7 (A) Homogeneity of purified nuclease on SDS – PAGE (B) Purified fraction of RP-HPLC chromatography.** Lane 1: Marker, Albumin bovine serum 66 kDa; Ovalbumin from chicken egg, 45 kDa; Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, 36 kDa; Carbonic anhydrase from bovine erythrocytes, 29 kDa; Trypsinogen from bovine pancreas, 24 kDa; Trypsin inhibitor from soybean, 20 kDa;  $\alpha$  Lactalbumin from bovine milk, 14.2 kDa. Lane 2: 50  $\mu$ g protein (Ammonium sulphate precipitated). Lane 3: 15  $\mu$ g of protein (Ammonium sulphate precipitated). Lane 4: Protein eluted from Phenyl Sepharose chromatography (10  $\mu$ g). Lane 5: Purified protein from after Gel filtration (5  $\mu$ g).

### 3. Purification of nuclease through Sephadex G-100 gel filtration chromatography

The concentrated active fractions in Tris-HCl buffer (30 mM; pH 7.0) derived from the previous step (15,782.5 U/12 mg protein) were then loaded on to Sephadex G-100 Gel filtration column. Out of the total 80 fractions collected (each 3 mL), three protein peaks were recorded from the eluent and only a single protein peak formed by the fractions 46-64 showed nuclease activity. These active fractions were pooled and concentrated by lyophilization and further subjected to dialysis against Tris-HCl buffer (30 mM; pH 7.0). The purity of the enzyme was checked on SDS-PAGE (Fig. 7a). The number of bands was reduced with each step of purification. Based on the above purification steps, the enzyme was purified to 16.71 fold with a final recovery of 4.09% (Table 2). The specific activity of purified nuclease (RNase) was recorded to be 2089.13 U/mg protein.

#### Carbohydrate content of purified nuclease

The purified nuclease was not found to contain any sugar moiety and was thus a non glycosylated protein.

#### Homogeneity and molecular mass determination of purified nuclease from *Bacillus firmus* VKPACU-1

The purified enzyme showed only a single band in SDS-PAGE indicating its homogeneity (Fig. 7a). The purified enzyme when subjected to RPHPLC analysis exhibited a single prominent peak with the retention time of 11 minutes which strongly confirmed the homogeneity of the purified enzyme (Fig. 7b). The molecular mass of the purified extracellular nuclease was calculated through SDS-PAGE by running the standard protein molecular markers adjacent to the purified enzyme. Based on the relative mobility, the molecular mass of the purified nuclease was calculated to be 17.1 kDa.

### DISCUSSION

Nucleases are important analytical enzymes which have extensive applications in the determination of nucleic acid structure and in the preparation of mono and oligonucleotides (Curtis *et al.* 1966). Extracellular nuclease is one of

protein used in many field of biotechnology. This nucleic acid degrading enzyme can be used in several applications, particularly protein purification, by reducing the viscosity of a cell lysate and the nucleic acid contamination of the protein which, in the specific case of therapeutic proteins, is required by FDA to be less than 100  $\mu$ g per dose (Tremillon *et al.* 2010). A newly isolated *B. firmus* VKPACU-1 from sediment samples of Coovum River in Chennai, TamilNadu, India showed maximum growth and nuclease production in nutrient broth at 32 hrs (36.8 U/mL) and the production gradually decreased at 44 hrs, whereas, the double strand preferential nuclease production by *Streptomyces thermotritificans* was highest at 40 hrs with low levels of RNase activity at 24 hrs which was gradually increased to the maximum at 40 hrs (Patil *et al.* 2005). But *Serratia marcescens* produced maximum extracellular nuclease between 18 and 22 hrs while the growth was steady up to 32 hrs (Nestle and Roberts 1969).

Optimal pH for the growth and nuclease production of test strain *Bacillus firmus* was recorded as 6.5 and similar observations have been made for *Bacillus subtilis* at pH 6.6 (Douglas and MacFadyen 1934) and *Streptomyces thermotritificans* also exhibited maximum nuclease production at pH 7.0 (Deshmukh and Shankar 1999). From this study, the nuclease is very close to *Bacillus* sp.

The optimum temperature for the enzyme production was 35°C which was followed by 37°C, though *Bacillus firmus* of the present study could grow and produce the enzyme between 20 and 40°C. This results has been supported by Sana *et al.* (2008) in *Bizionia* sp. and Nakamura *et al.* (1992) by *Bacillus subtilis* who have reported optimum nuclease production at 35°C where as the optimum temperature for nuclease production by *Streptomyces thermotritificans* as 45°C (Deshmukh and Shankar 2004). The RNase production by *Rhizopus stolonifer* was highest when incubated at 30°C (Chacko *et al.* 1996). The test organism showed the maximum production of nuclease with a sodium chloride concentration of 0.5% and the enzyme production decreased drastically with increase in concentration where as *Erwinia chrysanthemi* produced nuclease only in the absence of NaCl (Moulard *et al.* 1995).

The test strain produced high level of extracellular nuclease when grown in nutrient broth medium containing 1% glucose. These results were correlated when the *Streptomyces thermotritificans* in same medium (Patil *et al.* 2005). All the carbon sources supported the growth but the nuc-

lease production was decreased. In *Neurospora crassa*, it was shown that sorbose enhances extracellular DNase production (Sana *et al.* 2008). Starch though supported the growth of *Bacillus firmus* but not the nuclease production. Similar observation has been reported for *Streptomyces thermonitrificans* (Deshmukh and Shankar 1999) while in contrast to the above, enhanced extracellular DNase production by *S. levoris* (Bezborodov *et al.* 1967).

Among the various organic nitrogen sources tested, maximum growth and enzyme production were recorded only with tryptone which was followed by beef extract and none of the inorganic nitrogen sources could support the growth of *Bacillus firmus* VKPACU-1. In earlier report of (Apte *et al.* 1993), studied the influence of various organic nitrogen sources on extracellular DNase production by *Rhizopus stolonifer* noted that peptone supported the highest growth and nuclease production. The failure of inorganic sources to support the growth, but beef extract supported the growth and nuclease production in *Streptomyces thermonitrificans* (Deshmukh and Shankar 1999).

The influence of metal ions,  $Mn^{2+}$  and  $Fe^{2+}$  at 2 mM enhanced the production of nuclease of the test organism *Bacillus firmus* VKPACU-1, while other metal ions namely,  $Co^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$ ,  $Hg^{2+}$  and  $Zn^{2+}$  completely inhibited the enzyme production. Similarly  $Mn^{2+}$  induced DNase production has been recognized in *Bacillus subtilis* (Akrigg and Mandelstam 1978). *Rhizopus stolonifer* was reported to produce higher level of RNase in the presence of  $Mg^{2+}$  (12 mM),  $Mn^{2+}$  and  $Fe^{2+}$  (2 mM each) while the production was inhibited by  $Zn^{2+}$  (Chacko *et al.* 1996). Patil *et al.* (2005) observed higher level of nuclease production by *Streptomyces thermonitrificans* in the presence of 50 mM  $Mn^{2+}$ . Thus the literature showed that the majority of DNase production was enhanced by  $Mn^{2+}$ , but in the present study  $Mn^{2+}$  enhanced the production of a nuclease which is a single strand specific enzyme.

In the present study the purification of an extracellular nuclease was carried out by the test strain grown in the optimized medium. The enzyme was concentrated by ammonium sulphate precipitation and purified by hydrophobic and gel filtration chromatography. The present study employed phenyl sepharose and sephadex G-100 for purification of nuclease and found 10.52-fold with enzyme recovery of 20.6%. Similarly, the single strand specific nucleases such as S1 nuclease (Oleson and Sasakuma 1980) and *Ustilago maydis* nuclease (Holloman *et al.* 1981) have been purified using hydrophobic matrices, such as phenyl and octyl-sepharose. Many other nucleases were purified by gel filtration column which include the nucleases from *Streptomyces antibioticus* (Nicieza *et al.* 1999) and some other nucleases. Molecular weight of the nuclease is comparable to the nucleases from *Streptomyces antibioticus* (Nicieza *et al.* 1999) and *S. aureus* (Taniuchi *et al.* 1967) which are all comparatively low Mr proteins in the range of 14-20 kDa, most of the single strand specific nucleases are low molecular weight. Though many of the well studied single strand specific nucleases like P1 (Fujimoto *et al.* 1975), S1 (Oleson and Sasakuma 1980) and barely nuclease (Brown and Ho 1987) are glycoproteins, the present nuclease differs from all these enzymes by being non glycosylated.

## CONCLUSION

In this present study, an efficient nuclease producing bacterial strain isolated from coovum sediments was characterized based on biochemical and 16S rRNA gene sequence homology as *Bacillus firmus* VKPACU-1. The nuclease production was optimized by using one factor at a time approach. Optimal conditions for nuclease production were obtained by determining media composition along with temperature and pH. The nuclease secreted extracellularly by the test strain *B. firmus* VKPACU-1 was a multifunctional enzyme which showed more hydrolyzing activity on RNA than the ssDNA and dsDNA. Extracellular nuclease

secreted by the test strain was purified to homogeneity.

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