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A rapid two step bacterial DNA extraction method using LasA protease of *Pseudomonas aeruginosa* MCCB 123

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A potent bacteriolytic extracellular protease producing bacterial isolate from coir retting grounds of Kerala, India was identified as *Pseudomonas aeruginosa* based on phenotypic characteristics and 16S rRNA gene sequence analysis and coded as MCCB 123 (GenBank Accession no. FJ 665510). The enzyme is biocompatible with an IC_{50} of 89.43 ± 3.11µg ml^{-1} on mammalian cell line (HeLa). LasA protease was purified to apparent homogeneity with a molecular mass of 20.5 kDa and was found to have a broad range of lytic action on the Gram-positive and Gram-negative bacterial cell walls and also on bacterial consortium. pH, temperature and incubation time for bacterial cell lysis was optimized and found to be 7.0, 35°C and 30 min, respectively with reference to *Staphylococcus aureus* subsp. *aureus*. Its broad-spectrum lytic action on wide variety of bacterial cells can be exploited in bacterial DNA extraction without the addition of detergents and chelating agents. This position the enzyme unique over the existing lytic enzymes reported in DNA extraction. This is the first report of *P. aeruginosa* LasA protease having lytic action on bacterial cell walls other than that of *Staphylococcus aureus* and its application in rapid extraction of DNA from a wide range of bacteria.

Keywords: *Pseudomonas aeruginosa*, LasA protease, lytic enzyme, *Staphylococcus aureus,* DNA extraction

Introduction

LasA protease is a staphylolytic endopeptidase secreted by *Pseudomonas aeruginosa*, an ubiquitously distributed gamma proteobacterium capable of thriving dissimilar ecological niches[1]. Some strains of *P. aeruginosa* produce two or three proteases[2-3]. Among the extracellular proteases produced by *P. aeruginosa*, LasA protease is reported to have lytic action on heat-killed cells of *Staphylococcus aureus*[4-6]. It is a 20 kDa staphylolytic endopeptidase that cleaves peptide bonds following Gly-Gly pairs[5], which enable the lysis of Gram-positive bacterial cell walls through the cleavage of the pentaglycine interpeptides that cross-link adjacent peptidoglycan chains[6, 7].

Bacteriolytic enzymes as potential agents for DNA extraction has contributed to biotechnology industry for the extraction of nucleic acids from bacteria and for cell transformation. These enzymes have potential applications in nucleic acid extraction in which bacterial cell lysis is the first step towards isolation of genomic and plasmid DNA. Many Gram-positive bacteria are resistant to lysozyme and other enzymes used for lysis due to the thick cell wall consisting of peptidoglycans[8]. Therefore, harsher DNA extraction methods such as mechanical disruption coupled with enzymatic lysis are employed for DNA extraction in Gram-positive cells[9-10]. However, they are not equally applicable to Gram-negative cells which results in DNA damage. Hence, DNA extraction from Gram-positive and Gram-negative bacteria demands distinct protocols[11]. In this context identification of an appropriate lytic enzyme with a broader spectrum of activity in both Gram-positive and Gram-negative bacteria has been considered as a desirable proposition[10].

Till date, the lytic ability of this protease on bacterial cells except *Staphylococcus aureus* is unknown. In this context, we undertook an investigation to ascertain the ability, if any, of *P. aeruginosa* MCCB 123 LasA protease to lyse other Gram-positive and Gram-negative bacterial cells, and its application in bacterial DNA extraction.

Materials and Methods

Identification of the Organism

Heterotrophic bacteria isolated from coir retting grounds of Kerala, India which later formed as part of the Microbial Culture Collection of National Centre for Aquatic Animal Health, Cochin University of

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Science and Technology, (Cochin), Kerala, was used in this study. They were screened for protease production in casein agar plates composed of (g l^{-1}): Peptone, 5; Beef extract, 5; Yeast extract, 1; NaCl, 15; agar, 20; and casein, 10. The positive cultures were detected by the presence of clear zones around the colonies due to protease production. Of the 500 isolates screened, 10 protease producing bacterial isolates were selected based on the halo zones produced around the colonies, and the segregated ones were maintained at - 80°C as glycerol stocks in nutrient broth supplemented with 60% (v/v) glycerol. The one which showed the highest protease activity was chosen for the study, and it was identified on the basis of morphological and biochemical characteristic as per Palleroni[12] followed by molecular confirmation by 16S rRNA gene sequencing[13].

Extraction of LasA Protease Enzyme

LasA protease was purified from the medium composed of (in g l^{-1}) glucose, 7.5; yeast extract, 2.5; $NH_4H_2PO_4$,10.04; Na_2HPO_4, 0.5; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.000625; $ZnCl_2$, 0.01; casein, 10.0; pH, 7.0 in a 5 l fermenter (Biostat-B-Lite, Sartorius, Germany). Fermentation was carried out at 25°C, pH 7.0 ± 0.05, 300 rpm and supplied with sterile air at the rate 2.5 l min^{-1}. For enzyme extraction the culture was centrifuged at 8260 *g* for 15 min at 4°C and the supernatant stored in 300 ml aliquots at -20°C, and used for further purification and characterization.

Partial purification of protease was carried out by precipitation of the cell-free culture supernatant with ammonium sulphate between 30 and 80% saturation. The precipitates were collected by centrifugation at 8260 *g* for 15 min at 4°C and the active fractions (staphylolytic activity) were pooled and resuspended in 20 mM Tris-HCl buffer at pH 8.5. The partially purified enzyme was dialyzed against 20 mM Tris-HCl buffer, at pH 8.5 using Amicon UF stirred cell (Millipore Corporation, USA, Model 8010), with a 10 kDa cutoff membrane (Omega, 25MM, 10K, Pall Life Sciences), and was then loaded on an AKTA prime protein purification system (GE Healthcare Biosciences, Uppsala) equipped with a C16/40 (16 mm × 40 cm) DEAE cellulose column (Sigma-Aldrich) equilibrated with 20 mM Tris HCl buffer (pH 8.5). The column was washed with the same buffer to remove the unbound proteins, and the enzyme was eluted by applying a linear gradient of NaCl from 0 - 1000 mM at a flow rate of 0.5 ml min^{-1} d, and fractions of 2 ml were collected. Active LasA enzyme fractions with staphylolytic activity were pooled and concentrated by lyophilization.

Partial Characterization of LasA Protease

SDS-PAGE

The lyophilized active fractions of the enzyme were subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli[14] using 12% resolving gel and 5% stacking gel to determine their molecular weight.

Staphylolytic Assay

Staphylolytic activity was determined in accordance with Kessler *et al*[5] with slight modification. The activity was determined by monitoring the decrease in absorbance of a live cell suspension of *Staphylococcus aureus* subsp. *aureus* MTCC 737. The cells were harvested by centrifugation at 15,000 *g* for 15 min at 4°C and diluted to an optical density of 0.8 at Abs_{600}. The cells were suspended in 1 ml protease (1 mg ml^{-1}), pH 8.5 and incubated at 25°C for 30 min. One unit enzyme is defined as the amount that causes a decrease in the optical density by 1 unit min^{-1} at Abs_{600}. A control without enzyme was also kept.

Effect of Inhibitors on Staphylolytic Activity

In order to study the effect of protease inhibitors, LasA protease was pre-incubated for 30 min at 25°C with the specified enzyme inhibitors as the final concentration in the assay mixture. The inhibitors were 1,10 phenanthroline,1 mM; $ZnCl_2$, 0.01 and 0.1 mM; dithiothreitol, 5 and 10 mM; EDTA, 10 and 25 mM; EGTA, 25 mM; PMSF, 0.4 and 2 mM; TLCK, 5 mM; phosphoramidon, 1mM; leupeptin,10 µg ml^{-1} and soybean trypsin inhibitor, 10 and 50 µg ml^{-1}, and the assay was carried out as described above. Untreated enzyme was taken as the control (100% activity).

Elastase Assay

Elastase assay was determined using elastin congo red (Sigma-Aldrich Co.) as the substrate according Kessler *et al*[6] with slight modification. The reaction mixture consisting of 1 ml of enzyme solution, 5 mg of elastin congo red and 1 ml 50 mM Trsi HCl buffer (pH 8.0) and was incubated at 40° for 3h. Reaction was stopped by addition of 0.1 ml 100 mM EDTA, centrifuged at 8260 *g* for 15 min., and absorbance measured at 495 nm. Assays were carried out in triplicates and the mean value was expressed as elastase activity.

Cytotoxicity of LasA Protease

Cell cultures in 96 well plates (Greiner Bio-One) were developed from the mammalian cell line, HeLa, by adding 0.2 ml cell suspension (approximately 5 x 10^5 cells ml^{-1}) in minimum essential medium (MEM) supplemented with 82 mM glutamine, 1.5 g l^{-1} sodium bicarbonate and 10% fetal bovine serum and antibiotic mixture containing 100 µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin, and incubated for 12 hours at 37°C. Aliquots of purified enzyme with final concentrations of 0.5, 1.5, 2.5, 12.5, 25, 50 and 125 U/ml were added to the wells in triplicates. A control was kept without the enzyme addition. After 24 h incubation the wells were observed under phase contrast microscope (Leica, Germany) and MTT assay was performed.

MTT Assay

Briefly, the medium was replaced and 50 µl 5 mg ml^{-1} MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (Sigma-Aldrich Co.) prepared in PBS was added to each well and incubated for 5 h in dark. MTT was added to the control wells with the medium alone. After incubation, the medium was removed and formazan crystals formed were dissolved in 200 µl dimethylsulfoxide (DMSO) and the absorbance was recorded immediately at 570 nm in a microplate reader (TECAN Infinite Tm, Austria) with a reference wave length at 690 nm. The percentage of inhibited cells at each concentration was calculated followed by determining IC_{50} calculation using Probit analysis in SPSS software (SPSS 17.0).

LasA Protease in Bacterial DNA Extraction: Standardization of pH, Temperature and Incubation Time for Bacterial Cell Lysis

Optimization of DNA extraction was carried out according to the modified method of Niwa *et al*[10]. An 18 h old *Staphylococcus aureus* subsp. *aureus* MTCC 737 culture was harvested and the absorbance of cell suspension adjusted to 1.0 at Abs_{600}. The cells were suspended in 1 ml of purified LasA protease (10 mg ml^{-1}) enzyme suspended in 1 ml 50 mM sodium acetate buffer at pH 5 and 6; 10 mg enzyme suspended in 1 ml 50 mM Tris HCl buffer at pH 7 to 10) and incubated for 30 min at 25°C for pH optimization. For temperature optimization, the cells were suspended in 1 ml of LasA protease (10 mg enzyme suspended in 1 ml 50 mM Tris HCl at pH 7) and incubated for 30 min at different temperatures ranging from 25 to 75°C. To determine optimum incubation time for cell lysis, the cells were suspended in 1 ml of LasA protease (10 mg enzyme suspended in 1 ml 50 mM TrisHCl at pH 7) at 35°C and incubated up to 60 min drawing samples for DNA extraction at every 10 min interval.

After each experiment, unlysed cells were removed by centrifugation at 15,000 *g* for 15 min at 4°C. Equal volume of absolute ethanol was added to the supernatant and DNA was recovered by centrifugation at 15,000 *g* for 15 min at 4°C, and dissolved in 100 µl sterile Milli Q water. The presence of DNA was confirmed on 1% agarose gel and the DNA yield was determined from triplicate measurements at Abs_{260}. Reactions without the enzyme were included as controls. Optimum was determined based on DNA yield. The band intensity from gel image was calculated using Quantity One Software (BioRad, USA).

Bacterial DNA Extraction Using LasA Enzyme

Individual bacteria (Gram-positive and Gram-negative) and bacterial consortia were selected for DNA extraction. The bacterial cultures were centrifuged at 15,000 *g* at 4°C for 15 min and the absorbance of each suspension was adjusted to 1.0 at Abs_{600}, and the pellets were suspended in 1 ml of LasA protease (10 mg enzyme suspended in 1 ml 50 mM Tris-HCl, pH 7) at 35°C for 60 min. In the case of *Streptomyces* sp., cells were recovered from 1 ml of culture straightaway and DNA extraction was carried out as described above. For DNA extraction from ammonia oxidizing and nitrite oxidizing bacterial consortia[15], 1 ml of the consortium was taken, centrifuged the cells at 15,000 *g* at 4°C for 15 min, and the DNA extraction and yield determinations were carried out as described above.

Nucleic Acid Yield and Purity

The yield of nucleic acids extracted from bacterial isolates was quantified spectometrically using UV-visible spectrophotometer (UV-1601, Shaimadzu). Absorbance at 260 nm (A_{260}) was measured for each set of sample in triplicate and used to calculate the average total nucleic acid ($Abs_{260} \times 50 \times$ dilution factor) yield. The purity of extracted nucleic acid was determined by reading the absorbance at 280 nm (A_{280}) and the average ratio between Abs_{260} and Abs_{280} (Abs_{260}/Abs_{280}) was calculated for each set of samples (in triplicate). In this calculation, samples with mean Abs_{260}/Abs_{280} ratios below 1.8 were presumed to contain protein or other contaminants, whereas ratios above 2.0 were presumed to be due to the presence of RNA.

Measurement of DNA Quality by PCR Amplification of Selected Genes

To investigate the quality of DNA, PCR amplification of selected prokaryotic genes such as 16S rRNA of bacteria, along with that of β-ammonia oxidizing proteobacteria, *amoA* gene of ammonia oxidizing consortia and *nirS* gene of nitrifying bacteria were performed. The PCR programme used for 16S rRNA gene, except β-ammonia oxidizing proteobacteria was 95°C for 5 min followed by 34 cycles of 94°C for 20 s, 58°C for 30s and 68°C for 2 min[13]. However, the 16S rRNA gene from β-ammonia oxidizing proteobacteria was amplified by initial denaturation of 95°C for 3 min followed by 35 cycles of 82°C for 2.3 min, annealinag at 52.3°C for 1 min and extension of 72°C for 2.5 min[16]. The *amoA* gene of ammonia oxidizing consortia was amplified using the programme 94°C for 5 min followed by 42 cycles of 94°C for 1 min, 56.8°C for 90 s, 72°C for 90 s[17], and for the *nirS* gene of denitrifying bacteria[18], a PCR programme of 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 45°C for 40 s, 72°C for 1 min were used. All experiments were performed using the same Thermal cycler (Eppendorf) and the primers used were given in Table 1. An aliquot of 10 µl from each PCR product was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light, documented using a gel documentation system connected with Quantity One® Software (Gel Doc XR+, Bio-Rad, USA).

Statistical Analysis

Data generated from the experiments were analyzed using one-way analysis of variance (ANOVA) with post-hoc multiple comparison analysis performed using Tukey's HSD. Mean of the results was compared using SPSS 17.0 package for Windows at a significance level of $p < 0.05$. Data are presented as mean ± standard deviation.

Results and Discussion

Identification of the Protease Producing Bacterial Strain

The organism was identified as *P. aeruginosa* based on 16S rRNA gene sequencing (GenBank Accession No. FJ 665510). Comparison of gene sequence with GenBank database using BLAST algorithm available from NCBI (www.ncbi.nlm.nih.gov) showed 100% query coverage with 16S rRNA gene sequence of *Pseudomonas aeruginosa.*

Purification of LasA Protease

The LasA protease was purified by a two step process, by precipitation with ammonium sulphate followed by DEAE-cellulose chromatography. Fractions from 30-80% NaCl showed staphylolytic activity. These fractions were pooled and concentrated by ultrafiltration using a 10 kDa membrane and were then loaded into DEAE-cellulose chromatography column. The results of purification procedure are summarized in Table 2. The enzyme was purified 27.51 fold with a specific staphylolytic activity of 728.86 U/mg protein and specific elastase activity of 650.76 U/mg protein. The elution profile of the LasA protease on DEAE-cellulose column is shown in Fig. 1. The LasA protease was eluted between 0.13 M to 0.21 M NaCl (fractions, 13 to 21).

Table 1 — Primer sequences and cycle profiles used for PCR amplification

Primer	Sequence (5'-3')	Target gene	Reference
16S1 (f) 16S2 (r)	GAG TTT GAT CCT GGC TCA ACG GCT ACC TTG TTA CGA CTT	16S rRNA gene	Reddy *et al* 2000
NITA (f) NIT B (r)	CTT AAG TGG GGA ATA ACG CAT CG 16S TTA CGT GTG AAG CCC TAC CCA	rRNA gene of	Voytex and Ward β-proteo-bacterial 1995 ammonia oxidizers
nirS 1(f) nirS-6-(r)	CGA (C/T) TGG CCG CC(A/G)CAC (A/G)T CGT TGA AAC TT(A/G) CCG GT	*nirS* functional gene	Baker *et al* 1998
amoA 1(f) amoA 2(r)	GGG GTT TCT ACT GGT GGT CCC CTC (G/T) G (G/C) AAA GCC TTC TTC	*amoA* functional gene	Rotthauwe *et al* 1997

f: forward primer, r: reverse primer

Table 2 — Purification profile of LasA protease of *Pseudomonas aeruginosa* MCCB 123

Purification Step	SA (U/ml)	EA (U/ml)	Total Protein(mg)	Specific SA (U/mg)	Specific EA (U/mg)	Fold of purity
Culture filtrate	222.5	436.15	8.5	26.49	51.31	0
$(NH_4)_2SO_4$ Precipitation	221.46	374.17	4.09	54.14	91.48	2.04
DEAE-Cellulose Chromatography	218.66	195.23	0.3	728.86	650.76	27.51

SA:Staphylolytic activity EA:Elastase activity

The molecular mass of the purified enzyme was found to be 20.5 kDa by SDS-PAGE (Fig. 2).

Accordingly, LasA protease with a broad spectrum of bacteriolytic activity was purified from an environmental isolate of *Pseudomonas aeruginosa* MCCB 123. The enzyme was purified with 27.51 fold increase in specific staphylolytic activity.

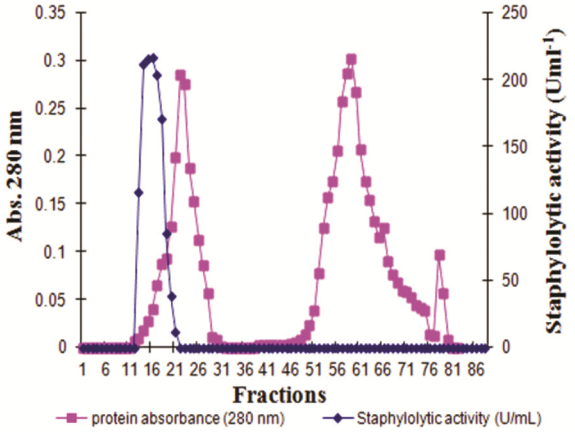

Fig. 1 — Elution profile of LasA protease on DEAE-cellulose C16/40 column.

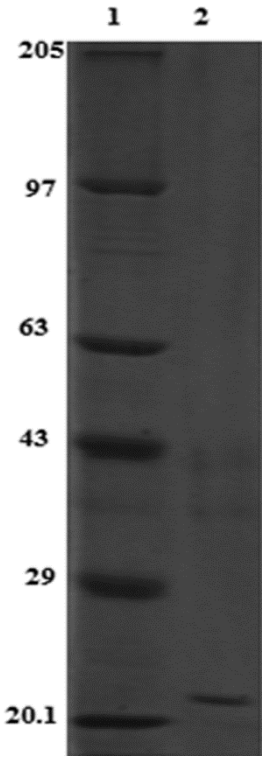

Fig. 2 — SDS-PAGE profile of purified LasA protease. Lane 1, Molecular weight markers (3 kDa, insulin; 6.5 kDa, aprotinin; 14.3 kDa, lysozyme; 20.1 kDa, trypsin soybean inhibitor; 29 kDa,carbonic anhydrase; 43 kDa, ovalbumin; 66 kDa, bovine serum albumin; 97.4 kDa, phosphorylase b; 205 kDa, myosin), lane 2, 20.5 kDa LasA protease.

Staphylolytic Activity and Effect of Inhibitors

Purified LasA protease showed 218.66 U ml^{-1} staphylolytic activity. The effects of various protease inhibitors on staphylolytic activity of LasA protease is shown in Table 3. The staphylolytic activity was markedly inhibited by metalloprotease inhibitors such as 2 mM 1, 10 phenanthroline (97.33% inhibition), 25 mM EDTA (86.16% inhibition) and 25 mM EGTA (87.72% inhibition), while other class of inhibitors such as PMSF, leupeptin and trypsin soybean inhibitor, phosphoramidon did not have a significant effect on the staphylolytic activity. The reducing agent DTT (10 mM) and metal ion $ZnCl_2$ (0.1 mM) also inhibited the enzyme activity at 97.77% and 81%, respectively.

LasA protease of *P. aeruginosa* is reported to have lytic activity on heat-killed cells of *Staphylococcus aureus*[5]. However, the action of this protease on other bacterial cell walls was hitherto unknown. Interestingly, the purified LasA protease of *P. aeruginosa* MCCB 123 could lyse the cell wall of a broad range of Gram-positive and Gram-negative bacteria. This is the first report on the lytic action of LasA protease of *P. aeruginosa* on bacteria other than on *S. aureus*.

Cytotoxicity Analysis

The preparation was found to be relatively non-toxic with an LD_{50} value of 89.43 ± 3.11 µg ml^{-1} on mammalian (HeLa) cell line (Fig.3).

Table 3 — Effect of inhibitors on staphylolytic activity

Inhibitors	Concentration of inhibitors (mM)	Inhibition (%)
Control	0	0
1,10 Phenanthroline	2	97.33
ZnCl2	0.01	25.44
	0.1	81
DTT	5	94
	10	97.77
EDTA	10	84.05
	25	86.16
EGTA	25	87.72
TLCK	5	17.22
Phosphoramidon	1	18.61
PMSF	0.4	27.50
	2	27.7
Leupeptin	10 µgml^{-1}	17.66
Trypsin soybean inhibitor	50 µgml^{-1}	21.11

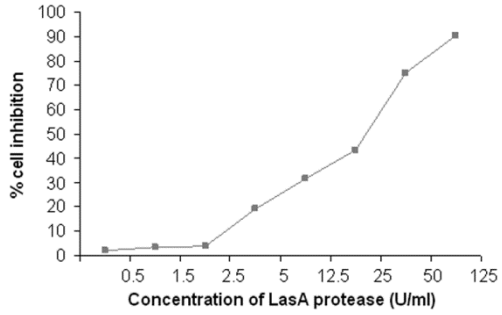

Fig. 3 — Sigmoid curve for cytotoxicity of LasA protease

Bacterial DNA Extraction Using LasA Protease and Determination of Yield and Purity

Optimization of pH for bacterial DNA extraction was determined over a range of 5 to 10 using cells of *S. aureus* subsp. *aureus*. The purified LasA protease exhibited good lytic activity on cells of *S. aureus* subsp. *aureus* from pH 5 to 10 with its optimum at 7.0 as indicated by the peaking with a DNA yield of 286.66 ± 9.46 µg ml^{-1}. Statistical analysis using One-way ANOVA revealed that there existed significant ($p < 0.05$) influence of pH between 6 to 8 on DNA yield (Table 4). The effect of temperature on lytic activity of LasA protease was investigated in a range from 25 to 75°C. The protease was found to have lytic action or cell lysis from 25 to 65°C. The optimum temperature for cell lysis was found to be 35°C with a DNA yield of 286.66 ± 9.46 µg ml^{-1}. From 45 to 75°C, there was a decreasing trend in DNA yield. Moreover, there was no significant ($p > 0.05$) difference in the DNA yield between 25 to 55°C (Table 4).

The influence of incubation time on cell lysis with LasA protease was investigated from 10 to 60 min. The DNA yield reached almost steady after 30 min incubation (286.66 ± 9.46 µg ml^{-1}) after which it became stable, indicating that the cells were lysed within the first 30 min incubation. Incubation time was found to have a significant ($p < 0.05$) influence on DNA yield up to 30 min and after which the DNA yield reached platue and henceforth it lacked significance (Table 4).

DNA was extracted from 37 bacterial species belonging to 22 genera. The DNA yield and quality is given in Table 5. The quality of DNA was assessed by the ratio of $A_{260}/_{280}$. This ratio was found to be in the range of 1.1 to 1.3. The image of extracted DNA from various bacterial species is given in Fig. 4.

The quality of representative DNA samples were subjected to amplification of 16S rRNA gene by PCR and the expected product size of 1500 bp could be obtained (Fig. 5a). DNA was extracted from ammonia oxidizing bacterial consortia (Fig. 5b, lane 2) and nitrite-oxidizing bacterial consortia (Fig. 5c, lane 2) by the action of LasA protease attaining DNA yield of 137.5 ± 5 µg ml^{-1} and 172.5 ± 5 µg ml^{-1} respectively (Table 5). The PCR amplification of 16S rRNA gene specific for β-proteo-bacterial ammonia oxidizers from the DNA extracted from ammonia oxidizing consortia gave an amplicon of 1080 bp (Fig. 5b, lane 3) and that of *amoA* gene an amplicon of 490 bp (Fig. 5b, lane 4), and of nitrate reductase gene (*nirS*) an amplicon of 940 bp (Fig. 5c, lane 3).

Table 4 — Effect of pH, temperature and incubation time on DNA yield from *S. aureus* subsp. *Aureus*

pH	Yield (µg ml^{-1})	Temp (°C) yield (µg ml^{-1}) (min)	Time	yield	(µg ml^{-1})
5	40±2.5^{d}	25	265±6.61^{a}	10	210.83±5.20^{c}
6	44.16±3.81^{d}	35	286.66±9.46^{a}	20	237.5±5^{b}
7	286.66±9.46^{a}	45	275±10.89^{a}	30	286.66±9.46^{a}
8	216.66±3.81^{b}	55	270±9.01^{a}	40	317.5±6.61a
9	210±5^{b}	65	245.83±3.81^{ab}	50	318.33±7.21^{a}
10	185.83±5.20^{c}	75	202.5±34.73^{b}	60	319.16±5.20^{a}

Values with same superscript did not vary significantly
Standardization of pH was carried out at 25°C for 30 min.
Standardization of temperature was carried out at optimum pH 7.0 for 30 min.
Standardization of incubation time was carried out at optimum pH 7.0 and optimum temperature 35°C.

The quality of the extracted nucleic acid samples is important for further processing. Samples with mean A_{260}/A_{280} ratios below 1.8 were presumed to contain protein or other contaminants, whereas samples with ratios above 2.0 were presumed to be due to the presence of RNA[19]. However, nucleic acids preparations free of phenol should have $A_{260}/_{280}$ ratios near 1.2[20]. In case of DNA extracted using LasA protease from various Gram-positive and Gram-negative bacterial isolates, this ratio was found to be in the range of 1.1 to 1.3 and as phenol was not used in the DNA extraction hence, it could be concluded that nucleic acid preparation was of good quality for PCR and related studies.

Bacteriolytic enzymes have potential applications in DNA extraction of nucleic acids from bacteria[8]. A combination of different physical and chemical extraction methods such as chemical lysis combined with phenol-chlorofom-isoamyl alcohol extraction, sonication combined with phenol-chlorofom-isoamylalcohol extraction, freeze thaw and lysozyme SDS-lysis procedure, bead beating separation and phenol-chloroform-isoamylalcohol extraction, combination of

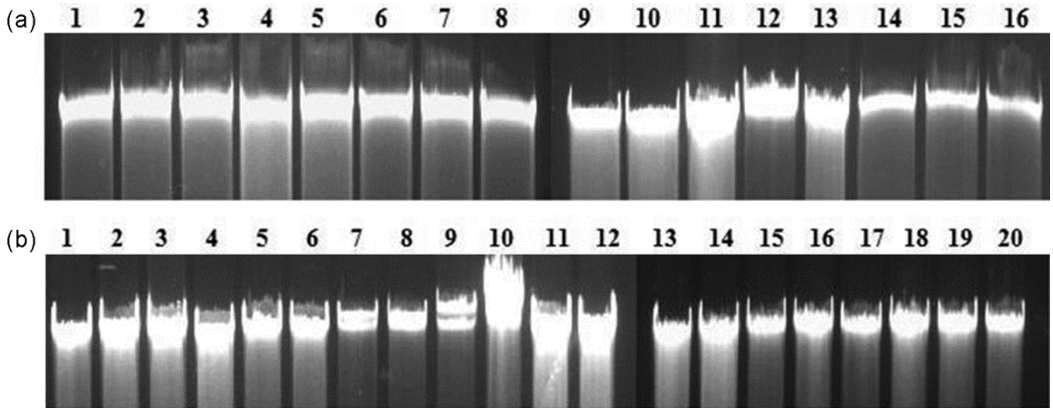

Fig. 4 — DNA extracted from Gram positive bacteria (a) and Gram negative bacteria (b) by LasA protease. (a) Lane 1, *Bacillus cereus* MTCC 1272; lane 2, *Bacillus subtilis* MTCC 2274; lane 3, *Bacillus licheniformis* MCCB 145; lane 4, *Paenibcillus polymyxa* MTCC 122; lane 5, *Lactobacillus gasseri* ATCC 4963; lane 6, *Lactobacillus* sp. ATCC 8001; lane 7, *Micrococcus lysodetikus* ATCC 4698; lane 8, *Enterococcus faecalis* NCTC 775; lane 9, *Streptococcus pyogens* MTCC 1924; lane 10, *Streptococcus cremoris* MCCB 147; lane 11, *Listeria monocytogens* MTCC 1143; lane 12, *Microbacterium* sp. MCCB 146; lane 13, *Arthrobacter nicotianae* MCCB 104; lane 14, *Streptomyces murabilis* MCCB 150; lane 15, *Streptomyces rochi* MCCB 148; lane 16, *Streptomyces tendae* MCCB 149. (b) Lane 1, *Escherichia coli* MTCC 77; lane 2, *Pseudomonas aeruginosa* MTCC 1934; lane 3, *Shigella flexneri* MTCC 1457; lane 4, *Yersenia enterocolytica* MTCC 859; lane 5, *Agrobacterium sp.* ATCC 31750; lane 6, *Vibrio chlorea* MTCC 3906; lane 7, *Vibrio parahaemolyticus* LMG 2850; lane 8, *Vibrio proteolyticus* LMG 3722; lane 9, *Vibrio harveyi* LMG 4044; lane 10, *Vibrio fluvialis* LMG 11654; lane 11, *Vibrio alginolyticus* LMG 4409; lane 12, *Vibrio mediterrenaei* LMG 11258; lane 13, *Aeromonas hydrophila* ATCC 7966; lane 14, *Aeromonas caviae* ATCC 15468; lane 15, *Aeromonas salmonicida* ATCC 27013; lane 16, *Photobacterium phospherum* ATCC 11040; lane 17, *Photobacterium leognathi* ATCC 25521; lane 18, *Plesiomonas shigelloides* ATCC 14029; lane 19, *Edwardsiella tarda* MTCC 2400; lane 20, *Marinobacter sp.* MCCB 147.

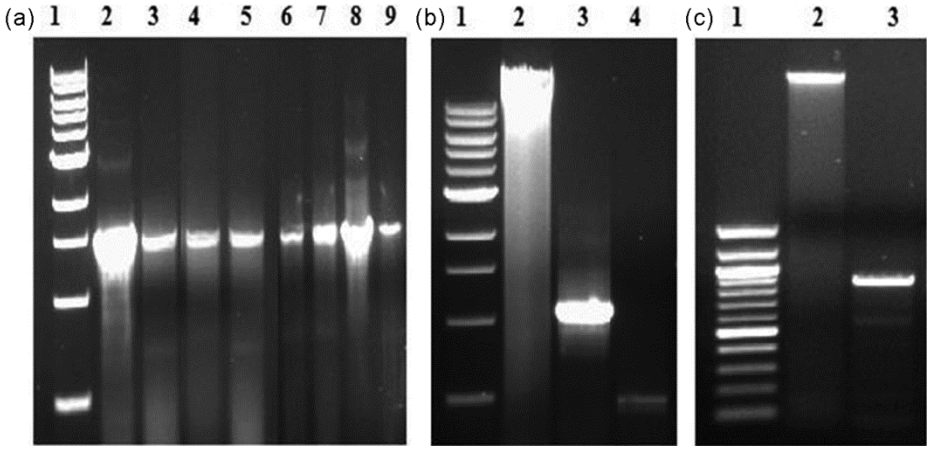

Fig. 5 — (a) PCR amplification of DNA isolated by LasA protease with universal primers for 16S rRNA gene yielding amplicons of 1500 bp. Lane 1, 1 kb DNA ladder; lane 2, *E.coli* MTCC 77; lane 3, *P.aeruginosa* MTCC 1934; lane 4, *S. flexneri* MTCC 1457; lane 5, *Y. enterocolytica* MTCC 859; lane 6, *B.cereus* MTCC 1272; lane 7, *L. gasseri* ATCC 4963; lane 8, *M. lysodetikus* ATCC 4698; lane 9, *E. faecalis* NCTC 775. b) DNA extraction from ammonia oxidizing bacterial consortium by LasA protease and detection of functional gene. Lane 1, 1 kb DNA ladder; lane 2, total DNA of the consortia; lane 3, PCR amplification of 16S rRNA gene of β-proteobacterial ammonia oxidizers yielding amplicon of 1080 bp; lane 4, 490 bp amplicon of *amoA* functional gene of the consortia. c) DNA extraction from nitrite oxidizing bacterial consortium by LasA protease and detection of functional gene. Lane 1, 100 bp DNA ladder; lane 2, total DNA of the consortium; lane 3, 940 bp amplicon of *nirS* functional gene.

lytic enzymes, detergents and chelating agents, freezing and grinding in liquid N_2 followed by phenol-chloroform extraction were reported for the extraction of bacterial DNA[21-24].

Even though the above mentioned methods proved to be suitable for bacterial DNA extraction, they have drawbacks such as laborious manipulations, prolonged incubation time, involving elution, washing and drying steps. In addition, the existing methods involve the usage of detergent such as SDS to lyse the cell wall which often remains in DNA solution and inhibits further manipulations[25]. The phenol-

chloroform extraction is time consuming and is prone to sample cross contamination and PCR inhibition from phenol/chloroform carryover[26]. Sonication results in the disruption of DNA molecules and leads to the degradation of DNA[27].

Ideally, DNA extraction methods should be simple, quick and efficient. Choosing an extraction method often involves a trade-off between cost (materials and labor), the optimal yield of DNA and removal of substances that could influence the PCR[21]. Through the present investigation, it is demonstrated that purified *P. aeruginasa* MCCB 123 LasA protease as such could lyse the cell wall of a variety of Gram-positive and Gram-negative bacteria, and DNA could

Table 5 — Bacterial Strains used for DNA extraction along with the DNA yield and purity

Species	code	Medium	Temp	DNA yield (µg ml^{-1})	DNA purity ($Abs_{260/280}$)
Gram positive bacteria					
Bacillus cereus	MTCC 1272	Nutrient broth	28°C	154.16±1.44	1.10±0.011
Bacillus subtilis	MTCC 2274	Nutrient broth	28°C	266.66±3.81	1.11±0.04
Bacillus licheniformis	MCCB 145	Nutrient broth	28°C	207.5±4.33	1.27±0.07
Paenibacillus polymyxa	MTCC 122	Nutrient broth	28°C	156.66±2.88	1.11±0.04
Lactobacillus gasseri	ATCC 4963	MRS broth	37°C	163.33±2.88	1.12±0.03
Lactobacillus sp.	ATCC 8001	MRS broth	37°C	132.5±5	10±0.02
Streptococcus pyogens	MTCC 1924	BHI broth	37°C	148.33±2.88	1.18±0.06
Enterococcus faecalis	NCTC 775	BHI broth	37°C	120±4.33	1.18±0.03
Micrococcus lysodetikus	ATCC 4698	Nutrient broth	37°C	140±0	1.36±0.03
Staphylococcus aureus subsp.*aureus*	MTCC 737	Nutrient broth	37°C	307.5±5	1.25±0.05
Listeria monocytogens	MTCC 1143	BHI broth	37°C	206.66±6.29	1.16±0.07
Arthrobacter nicotianae	MCCB 104	Nutrient broth	28°C	183.33±3.81	1.15±0.05
Streptomyces rochi	MCCB148	Zobell's broth	28°C	136.66±1.44	1.18±0.01
Streptomyces tendae	MCCB 149	Zobell's broth	28°C	164.16±3.81	1.18±0.07
Streptomyces murablis	MCCB 150	Zobell's broth	28°C	149.16±5.20	1.17±0.02
Microbacterium sp.	MCCB146	Zobell's broth	28°C	211.66±2.88	1.15±0.06
Gram negative bacteria					
Marinobacter sp.	MCCB 147	Zobells broth	28°C	307.5±5	1.27±0.03
Agrobacterium sp.	ATCC 31750	Nutrient broth	28°C	318.33±3.81	1.12±0.03
Shigella flexnerii	MTCC 1457	Nutrient broth	28°C	285±2.5	1.18±0.005
Yersenia enterocolytica	MTCC 859	Nutrient broth	28°C	188.33±6.29	1.15±0.01
Vibrio chlorea	MTCC 3906	Nutrient broth	28°C	264.16±7.63	1.22 ±0.05
Escherchia coli	MTCC 77	Nutrient broth	28°C	312.5±10	1.20±0.03
Pseudomonas aeruginosa	MTCC 1934	Nutrient broth	37°C	260.83 ±7.63	1.27±0.01
Aeromonas hydrophila	MTCC	Nutrient broth	28°C	326.66±2.88	1.19±0.005
Aeromonas salmonicida	ATCC 27013	Nutrient broth	28°C	334.16±5.77	1.12±0.02
Aeromonas caviae	ATCC 15468	Nutrient broth	28°C	295.83±7.63	1.25±0.04
Edwardsiella tarda	MTCC 2400	Nutrient broth	28°C	132.5±5	1.14±0.06
Vibrio harveyi	LMG 4044	Zobell's broth	28°C	282.5±6.61	1.23±0.04
Vibrio vulnificus	LMG 13545	Zobell's broth	28°C	231.66±6.29	1.23±0.003
Vibrio parahaemolyticus	LMG 2850	Zobell's broth	28°C	185.83±5.20	1.27±0.02
Vibrio mediterrenaei	LMG 11258	Zobell's broth	28°C	190.83±2.88	1.21±0.01
Vibrio proteolyticus	LMG 3722	Zobell's broth	28°C	286.66±6.29	1.19±0.007
Vibrio fluvialis	LMG 11654	Zobell's broth	28°C	369.16±6.29	1.26±0.02
Vibrio alginolyticus	LMG 4409	Zobell's broth	28°C	310.83±7.63	1.26±0.02
Plesiomonas shigelloides	ATCC 14029	Nutrient broth	28°C	142.5±4.33	1.11±0.02
Photobacterium phospherum broth	ATCC 11040	Photobacterium	28°C	158.33±6.29	1.20±0.0
Photobacterium leognathi	ATCC 25521	Photobacterium	28°C	205±4.33	1.19±0.04
Bacterial consortia	broth				
Ammonia oxidizing consortia	AMONPCU	Zobell's broth	28°C	137.5±5	1.24±0.0
Nitrite oxidizing consortia	NIOPCU	Zobell's broth	28°C	172.5±5	1.27±0.03

Abbreviations: MTCC: Microbial Type Culture Collection, Institute of Microbial Tecbnology, Chandriagh, ATCC: American Type Culture Collection, MCCB: Microbial Culture Collection of Bacteria, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, LMG: Belgian Coordinated Collections of Microorganisms.
Datas are represented as mean ± standard deviation.

be extracted without the addition of detergents or chelating agents which made this method unique over the existing methods reported in bacterial DNA extraction. The extracted DNA using *P. aeruginasa* MCCB 123 LasA protease could be directly used for PCR amplification which indicated the absence of any PCR inhibitors. This method of DNA extraction is simple, rapid and cheap and it neither requires specialized equipment, nor complicated extraction protocols with organic solvents such as phenol or chloroform or denaturing agents such as guanidium isocynate. This is the first report of a lytic enzyme being employed in DNA extraction without the addition of detergents.

DNA extraction using *P. aeruginasa* MCCB 123 LasA protease is advantageous due to the fact that several samples can be processed within a short time period of 30 min. The optimal temperature of 35ºC for DNA extraction is economical in terms of energy saving.

Conclusion

Through the present investigation *P. aeruginosa* MCCB 123 LasA protease could be demonstrated as a unique enzyme for DNA extraction from bacteria with wide range of lytic action on a variety of bacterial isolates avoiding other harsh mechanical and chemical treatments especially warranted by Gram positive bacteria.

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References

1 Kiewitz C & Tummler B, Sequence diversity of *Pseudomonas aeruginosa*: Impact on population structure and genome evolution, *J Bacteriol*, 182 (2000) 312.

2 Morihara K, Production of proteinase and elastase by *Pseudomonas aeruginosa*, *J Bacteriol*, 88 (1964) 745-757.

3 Najafi M F, Deobagkar D N, Mehrvarz M & Deobagkar D D, Enzymatic properties of a novel highly active and chelator resistant protease from a *Pseudomonas aeruginosa* PD100, *Enzy Microb Technol*, 39 (2006) 1433-1440.

4 Caballero A R, Moreau J M, Engel L S, Marquart M E, Hill J M *et al*, *Pseudomonas aeruginosa* protease IV enzyme assays and comparison to other *Pseudomonas* proteases, *Anal Biochem*, 290 (2001) 330-337.

5 Kessler E, Safrin M, Olson J C & Ohman D E, Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease, *J Biol Chem*, 268 (1993) 7503-7508.

6 Kessler E, Safrin M, Abrams W R, Rosenbloom J & Ohman D E, Inhibitors and specificity of LasA protease of *Pseudomonas aeruginosa*, *J Biol Chem*, 272 (1997) 9884-9889.

7 Spencer J, Murphy L M, Conners R, Sessions R B & Gamblin S J, Crystal structure of the LasA virulence factor from *Pseudomonas aeruginosa*: substrate specificity and mechanism of M23 metallopeptidases, *J Mol Biol*, 396 (2010) 908-923.

8 Salazar O & Asenjo A, Enzymatic lysis of bacterial cells, *Biotechnol Lett*, 29 (2007) 985-994.

9 Johnson J L, Similarity analysis of DNAs, in *Methods for General and Molecular Bacteriology*, edited by Gerhardt P, Murray R G E, Wood W E & Kreig N R (ASM Press, Washington DC) 1994, 655- 682.

10 Niwa T, Kawamura Y, Katagiri Y & Ezaki T, Lytic enzyme, labiase for a broad range of Gram-positive bacteria and its application to analyze functional DNA/RNA, *J Microbiol Methods*, 61(2005) 251-260.

11 Schneegurt M A, Dore S Y & Kulpa C F Jr, Direct extraction of DNA from soils for studies in microbial ecology, *Curr Issues Mol Biol*, 5 (2003) 1-8.

12 Palleroni N J, Genus 1, Pseudomonas, in *Bergys Manual of Systematic Bacteriology*, 1st edn, Vol I, edited by N R Krieg & J G Holt (Williams & Wilkins, Baltimore), 1984, 172-173.

13 Reddy G S N, Aggarwal R K, Matsumoto G I & Shivaji S, *Arthrobacter flavus* sp. nov., a psychrophilic bacterium isolated from a pond in McMurdo Dry Valley, Antartica, *Int J Syst Evol Microbiol*, 50 (2000) 1553-1561.

14 Laemmli U K, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, 227 (1970) 680.

15 Achuthan C, Kumar V J R, Manju N J, Philip R & Singh I S B, Development of nitrifying bacterial consortia for immobilizing in nitrifying bioreactors designed for penaeid and non-penaeid larval rearing systems in the tropics, *Indian J Mar Sci*, 35(2006) 240-248.

16 Voytex M A & Ward B B, Detection of ammonia oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with PCR, *Appl Environ Microbiol*, 61 (1995) 1444-1450.

17 Rotthauwe J H, Witzel K P & Liesack W, The ammonia monooxygenase gene *amoA* as a functional molecular marker: molecular fine scale analysis of natural ammonia-oxidising populations, *Appl Environ Microbiol*, 63 (1997) 4704-4712.

18 Barker G, Fesefeldt A & Witzel K P, Development of PCR primer systems for amplification of nitrite reductase genes (*nirk* and *nir*S) to detect denitrifying bacteria in environmental samples, *Appl Environ Microbiol*, 64 (1998) 3769-3775.

19 Sambrook J & Russell D W, Molecular cloning: a Laboratory Manual, 2nd edn, Vol III, (Cold Spring Harbor Laboratory Press, New York) 2001.

20 Lemarchand K, Berthiaumea F, Maynarda C, Harel J, Payment P *et al*, Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays, *J Microbiol Methods*, 63 (2005) 115-126.

21 Cankar K, Stebih D, Dreo T, Zel J & Gruden K, Critical points of DNA quantification by real-time PCR-effects of DNA extraction method and sample matrix on quantification

of genetically modified organisms, *BMC Biotechnol*, 6 (2006) 37-52.

22 Dauphin L A, Moser B D & Bowen M D, Evaluation of five commercial nucleic acid extraction kits for their ability to inactivate *Bacillus anthracis* spores and comparison of DNA yields from spores and spiked environmental samples, *J Microbiol Methods*, 76 (2009) 30-37.

23 Ezaki T, Saidi S M, Liu S L, Hashimoto Y, Yamamoto H *et al*, Rapid procedure to determine the DNA base composition from small amounts of Gram-positive bacteria, *FEMS Microbiol Lett*, 55 (1990) 127-130.

24 Jara C, Mateo E, Guillamón J M, Torija M J & Mas A, Analysis of several methods for the extraction of high quality DNA from acetic acid bacteria in wine and vinegar for characterization by PCR-based methods, *Int J Food Microbiol*, 128 (2008) 336-341.

25 Cheng H-R & Jiang N, Extremely rapid extraction of DNA from bacteria and yeasts, *Biotechnol Lett*, 28 (2006) 55-59.

26 Yang G, Erdman D E, Kodani M, Kools J, Bowen M D *et al*, Comparison of commercial systems for extraction of nucleic acids from DNA/RNA respiratory pathogens, *J Virol Methods*, 171 (2011) 195-199.

27 Picard C, Ponsonnet C, Paget E, Nesme X & Simonet P, Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction, *Appl Environ Microbiol*, 58 (1992) 2717-2722.