



# Characterization of a Novel Recombinant $\beta$ -Lactamase from *Bacillus subtilis* R5

Amjed Ali<sup>1</sup>, Muhammad Tayyab<sup>1\*</sup>, Abu Saeed Hashmi<sup>2</sup>, Asif Nadeem<sup>3</sup>, Shumaila Hanif<sup>4</sup>, Sehrish Firyal<sup>1</sup>, Shagufta Saeed<sup>1</sup>, Ali Raza Awan<sup>1</sup> and Muhammad Wasim<sup>1</sup>

<sup>1</sup>Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Abdul Qadir Jilani Road, Lahore, Pakistan.

<sup>2</sup>Riphah International University, Raiwind Road Campus, Lahore

<sup>3</sup>Department of Biotechnology, Virtual University of Pakistan

<sup>4</sup>Department of Chemistry, University of Agriculture Faisalabad, Faisalabad

## ABSTRACT

Current study deals with the characterization of recombinant  $\beta$ -lactamase from a locally isolated strain *Bacillus subtilis* R5. The study was an initial step for the fulfillment of commercial needs of  $\beta$ -lactamase in Pakistan. The 1 kb  $\beta$ -lactamase gene was amplified by PCR using the genomic DNA of *B. subtilis* R5 as template. The purified PCR product was cloned in pTZ57R/T and sub-cloned in pET21a. Expression of recombinant protein was examined in BL21 CodonPlus (DE3) cells. SDS-PAGE confirmed the size of recombinant protein as 34 kDa. Recombinant  $\beta$ -lactamase was produced optimally when the BL21 CodonPlus (DE3) cells were induced with 0.6 mM IPTG with a post induction time of 5h at 37 °C. The characterization studies demonstrated the maximal enzyme activity at 37 °C in 50 mM sodium phosphate buffer pH 7. The presence of EDTA in the activity assay mixture reduced the  $\beta$ -lactamase activity to 91% while  $Zn^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$  enhanced the enzymatic activity to 144, 121 and 108% when used at a final concentration of 1 mM. The ionic and non-ionic detergents showed slight inhibitory impact on the recombinant  $\beta$ -lactamase activity. The enzyme exhibited the  $K_m$  and  $V_{max}$  values of 2.27 mM and 45.45  $\mu$ mol/min, respectively when benzylpenicillin was utilized as substrate. The degradative ability of recombinant  $\beta$ -lactamase to hydrolyze a variety of  $\beta$ -lactam ring containing antibiotics makes it a suitable candidate for its utilization as positive control in diagnostics and in antibiotic susceptibility testing experiments.

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## Authors' Contribution

AA performed experimental work. MT conceived the idea, provided guidance for the studies and manuscript write-up. ASH, AN, SF and SS facilitated the first author in conduction of experiments. SH, ARA and MW helped in data analysis and manuscript write-up.

## Key words

$\beta$ -lactamase, Antibiotic resistance, *Bacillus subtilis* R5, Antibiotic susceptibility, Diagnostics

## INTRODUCTION

$\beta$ -lactamases (EC 3.5.2.6) hydrolyze the amide bond between the beta carbon atom and the nitrogen atom of the four corner beta lactam ring which is core part of all beta lactam based antibiotics.  $\beta$ -lactamases render the antibiotics ineffective by breaking the beta lactam ring causing development of resistance in bacteria (Gaude and Hattiholli, 2013). These enzymes have a wide range of applications in pharmaceuticals, waste water treatment, diagnostics, food analysis, contamination control and in cancer chemotherapy (Giang *et al.*, 2014).

$\beta$ -lactamases are produced only by bacterial strains (Therrien and Levesque, 2000). Four classes of  $\beta$ -lactamases, namely A, B, C and D have been recognized based on their amino acid sequences and molecular function. While the activities of A, C and D classes of  $\beta$ -lactamases are governed by the serine based mechanism, the metallo- $\beta$ -lactamases or class B lactamases require zinc for their activity (Bush *et al.*, 1995). Uptil now more than 890  $\beta$ -lactamases have been reported from different bacterial strains (Bush and Jacoby, 2010). Bacteria have developed resistance against various antibiotics due to the presence of genes responsible for the production of  $\beta$ -lactamases, involved in catabolism of antibiotics (MacGowan and Macnaughton, 2017).

Bacteria are a diverse group of microbes, distributed in all environments and are involved in the production of enzymes. Genus *Bacillus* is being utilized for the fulfillment of industrial demand of enzymes worldwide (Salcido *et al.*, 2013; Pasvolosky *et al.*, 2014; Liu *et al.*, 2014). Extracellular enzymes in bulk are produced industrially from the selected strains of bacteria thus it is thought that they occupy the most prominent place regarding industrial

\* Corresponding author: muhammad.tayyab@uvas.edu.pk; tayyab\_pakistan@yahoo.com  
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production of enzymes. Food and Drug Administration (FDA) has listed some *Bacillus* species to be safe for their usage in the food and drugs (Sorokulova *et al.*, 2008).

*Bacillus subtilis* R5 are Gram positive, small rod shaped, highly motile cells that show optimal growth at 30°C (Jalal *et al.*, 2009). The present study describes characterization of recombinant  $\beta$ -lactamase from *Bacillus subtilis* R5 and its utilization as a positive control for antibiotic resistance detection in diagnostic laboratories.

## MATERIALS AND METHODS

All chemicals utilized in the current study were of purified grade and procured from Sigma Aldrich (USA). The DNA extraction kit, Ins T/A cloning kit and restriction endonucleases were procured from Thermo-Fischer Scientific and Invitrogen, Life Sciences, USA.

### *Culture maintenance and isolation of genomic DNA*

The pure culture of *Bacillus subtilis* R5, a gift from School of Biological Sciences, University of the Punjab, Lahore, was maintained on LB medium and used for isolation of genomic DNA (Sambrook and Russell, 2001).

### *Cloning of $\beta$ -lactamase gene*

The  $\beta$ -lactamase gene was amplified by PCR using BL-3N: CATATGACAACTGAAGATG and BL-3C: TCACCTCTTCATTGTTTTAA as forward and reverse primers and the genomic DNA of *B. subtilis* R5 as template.  $\beta$ -lactamase gene sequence of *B. subtilis* 168 (NZ\_CP010052), the closet homolog of *B. subtilis* R5 was utilized for designing the primers. The forward primer contained the unique *NdeI* restriction site. Ligation of purified PCR product was performed in pTZ57R/T using T4 DNA ligase (Thermo-Fisher, Life Sciences, USA) at 20°C for overnight. *E. coli* DH5 $\alpha$  competent cells were transformed using the ligated mixture and blue white screening method was followed for the screening of positive clones (Sambrook and Russell, 2001). The white colonies from the plate were utilized for isolation of plasmid DNA (Jalal *et al.*, 2009). The presence of insert in the recombinant vector was examined by single and double digestion using *HindIII* and *NdeI* endonucleases. The plasmid DNA after confirmation by restriction digestion, was utilized for sequencing of  $\beta$ -lactamase gene using M13 forward and reverse primers. The obtained nucleotide sequence was utilized for deduction of amino acid sequence and for homology analysis (Naas *et al.*, 2003; Sabir *et al.*, 2017).

### *Expression analysis of $\beta$ -lactamase gene*

The  $\beta$ -lactamase gene was transferred from recombinant pTZ57R/T to pET21a already restricted with same endonucleases and this recombinant pET21a having  $\beta$ -lactamase gene was used for the transformation of BL21 Codon Plus (DE3) cells. The expression of  $\beta$ -lactamase was analyzed by growing the recombinant cells in the presence of 100  $\mu$ g/mL ampicillin. The overnight grown cells were diluted to 1% with fresh LB broth and were incubated at 37 °C until the optical density at 660 nm reached to 0.4. The cells were induced with 0.6 mM IPTG followed by further incubation of 5h at the same temperature under shaking conditions. The cell pellet after centrifugation (6000xg for 10 min) was resuspended in 10 mL sodium phosphate buffer (pH 7) and the cells were lysed by sonication. The production of recombinant  $\beta$ -lactamase was analyzed by 12% SDS-PAGE.

### *Optimal production of $\beta$ -lactamase*

The production of  $\beta$ -lactamase was examined by varying the IPTG concentration from 0.1 to 1mM and with post induction time from 1 to 6h. The samples were withdrawn after every hour and were utilized for the estimation of  $\beta$ -lactamase activity.

### *Purification of recombinant $\beta$ -lactamase*

The soluble part after sonication process was applied on pre-equilibrated DEAE Sephadex A-50 column. The equilibration and washing of unbound proteins in column was done with 50 mM sodium phosphate buffer (pH 7). The bound proteins in the column were eluted by NaCl gradient prepared in the same buffer. The fractions containing  $\beta$ -lactamase activity were pooled and was applied to pre-equilibrated Sephadex G-50 gel filtration column. The purity of the fractions with  $\beta$ -lactamase activity was analyzed on SDS-PAGE. The purified recombinant  $\beta$ -lactamase was called as LAC<sub>BS</sub>.

### *Enzyme activity assay*

$\beta$ -lactamase activity was examined by following the method of Sawai and Yamagishi (1978). The assay mixture comprised of 0.1 mL enzyme solution, 2.4 mL 50 mM sodium phosphate buffer (pH 7) and 0.5 mL benzylpenicillin. The mixture was incubated at 37 °C for 10 min in a water bath and was allowed to stay at room temperature for 10 min followed by addition of 3 mL iodine solution with rapid mixing and absorbance was recorded at 540 nm. Two blanks were utilized for performing the activity assay. Blank A was comprised of 2.9 mL sodium phosphate buffer and 0.1 mL enzyme solution while Blank B was having 2.5 mL sodium phosphate buffer and 0.5 mL

benzylpenicillin. The blanks were treated in the same way as described above. One  $\beta$ -lactamase unit was the amount of enzyme which hydrolyzed 1  $\mu$ mol of substrate per min under the assay conditions.

### Characterization of $\beta$ -lactamase

#### Effect of temperature

The enzyme activity was recorded at various temperatures ranging from 15 to 45°C. Regarding the activity assay, the assay mixture was incubated at specific temperature in water bath and the absorbance was recorded as 540 nm.

#### Effect of pH

The impact of pH on enzyme activity was recorded by examining the  $\beta$ -lactamase activity in the presence of 50 mM of each of sodium acetate buffer (pH 3-5), sodium phosphate buffer (pH 5-7), Tris HCl buffer (pH 7-9) and Glycine NaOH (pH 9-10).

#### Effect of metal cations and detergents

The enzyme activity was recorded in the presence of 1mM of each of metal cations including  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{CO}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Mn}^{2+}$  as well as in the presence of 1mM EDTA. The activity was also recorded in the presence of 1% non-ionic (Triton X-100, Tween 20 and Tween 80) and ionic (SDS) detergents.

### Kinetic studies of $\beta$ -lactamase

$\beta$ -lactamase activity was examined by changing the substrate concentration from 0.1 to 1%. The attained data was used for the construction of Lineweaver Burk/ double reciprocal plot and for the calculation of kinetic parameters.

### Antibiotics degradative ability of recombinant $\beta$ -lactamase

The  $\beta$ -lactamase activity was examined against various  $\beta$ -lactam ring containing antibiotics using LB agar plates and commonly available bacterial strains.

## RESULTS

The PCR resulted in the amplification of 1 kb  $\beta$ -lactamase gene. Single digestion of recombinant pET21a with *Nde*I resulted in the linearization of the vector and double digestion with *Hind*III and *Nde*I resulted in the liberation of insert from the recombinant vector (Fig. 1). The DNA sequencing confirmed the presence of  $\beta$ -lactamase gene. The homology analysis based on deduced amino acid sequence showed the sequence identity of 100% with the non-characterized members of genus *Bacillus* whereas this lactamase did not show significant homology among the characterized counterparts.

Maximal production of  $\beta$ -lactamase was observed at 37°C when BL21 CodonPlus (DEL) cells having recombinant pET21a were induced with 0.6 mM IPTG (Fig. 2) with a post induction period of 5h. SDS-PAGE analysis of the purified protein after DEAE Sephadex A-50 column confirmed the molecular weight of  $\beta$ -LAC<sub>BS</sub> as 34 kDa (Fig. 3).

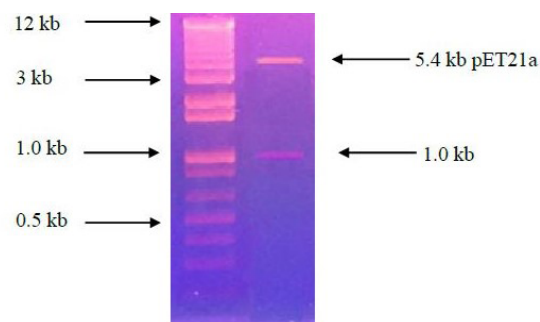


Fig. 1. Ethidium Bromide stained agarose gel electrophoresis showing the double digestion analysis of recombinant pET21a harboring  $\beta$ -lactamase gene from *Bacillus subtilis* R5. Lane 1 shows ladder 1 kb Plus (Invitrogen) while lane 2 shows the restriction digestion of recombinant pTZ57R/T with *Nde*I and *Hind*III.

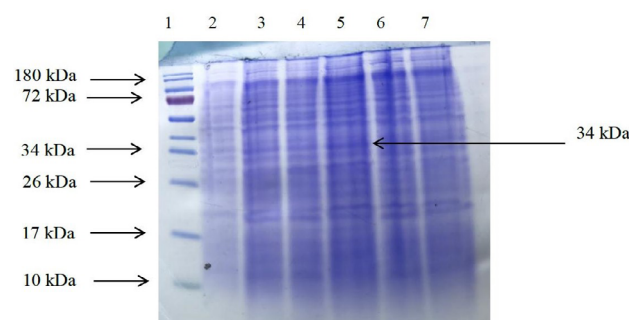


Fig. 2. Coomassie Brilliant Blue R250 stained SDS-PAGE gel demonstrating expression of  $\beta$ -lactamase gene with respect to increasing concentration of IPTG; Lane 1: Pre-stained Protein Ladder (Thermo Fisher Scientific); Lane 2: BL21 CodonPlus Cells transformed with pET21a without insert as control; Lane 3, 4, 5, 6, 7 shows the transformed BL21 CodonPlus (DE3) cells having recombinant pET21a induced with 0.2, 0.4, 0.6, 0.8 and 1mM concentration of IPTG, respectively.

LAC<sub>BS</sub> activity was linearly increased with the increase in temperature from 15 to 37°C. Further increase in temperature beyond 37°C resulted in the decreased enzymatic activity (Fig. 4). LAC<sub>BS</sub> activity was found pH dependent and was able to perform its activity between pH ranges from 3 to 10 with an optimal activity at neutral pH (Fig. 5). The presence of 1mM EDTA in the assay mixture

reduced the  $LAC_{BS}$  activity to 91%. However, the presence of  $Zn^{2+}$ ,  $Co^{2+}$  and  $Mn^{2+}$  in the activity assay enhanced the enzymatic activity to 144, 121 and 108% whereas,  $Mg^{2+}$ ,  $Ca^{2+}$  and  $Fe^{2+}$  reduced the activity to 90, 82 and 50% when used at a final concentration of 1mM. The presence of non-ionic (Triton X-100, Tween 20, Tween 80) and ionic (SDS) detergents could reduce the  $LAC_{BS}$  activity to 97, 94, 73 and 71% respectively when used at final concentration of 1% (Table I). Kinetic studies revealed the  $K_m$  and  $V_{max}$  values of 2.27 mM and 45.45  $\mu$ moles/min, respectively (Fig. 6).  $LAC_{BS}$  has degraded the  $\beta$ -lactam ring containing antibiotics and due to the degradation of antibiotics the bacterial growth was observed in the LB plates as compared to the controls.

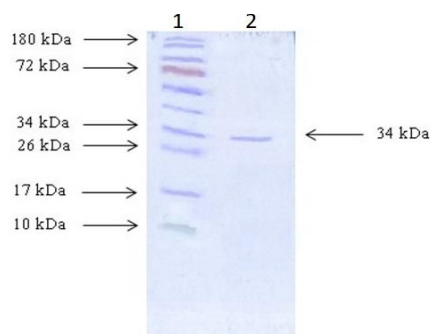


Fig. 3. Coomassie Brilliant Blue R250 stained SDS-PAGE gel demonstrates the purity and molecular weight analysis of  $\beta$ - $LAC_{BS}$ . Lane 1 is Pre-stained Protein Ladder (PageRuler™, Thermo Fisher Scientific), Lane 2 is purified  $\beta$ - $LAC_{BS}$ .

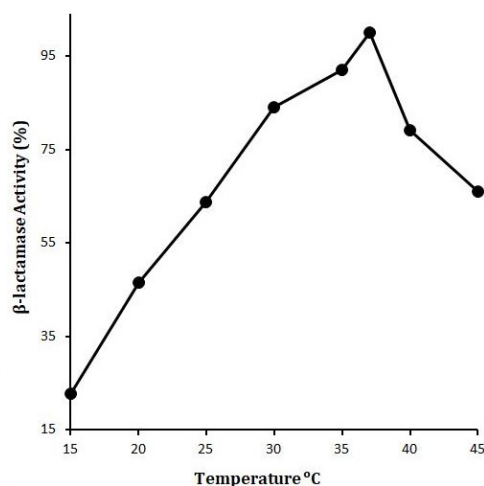


Fig. 4. Effect of temperature on  $LAC_{BS}$  activity. X-axis shows the temperature ( $^{\circ}C$ ) while Y-axis shows the  $LAC_{BS}$  activity (%). Enzyme activity was examined in 50mM sodium phosphate buffer (pH 7) and 5 $\mu$ M Benzylpenicillin as substrate.

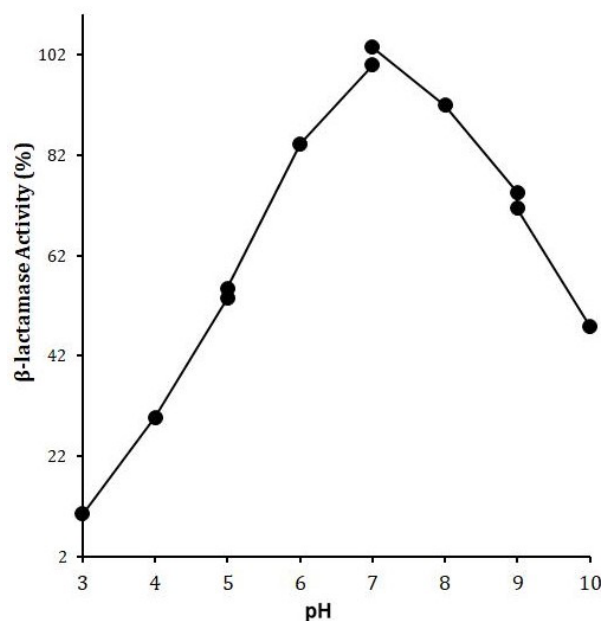


Fig. 5. Effect of pH on  $LAC_{BS}$  activity. X-axis shows the pH while Y-axis shows the  $LAC_{BS}$  activity (%). Enzyme activity was examined in 50 mM of each of sodium acetate buffer (pH 3-5), Sodium phosphate buffer (5-7), Tris-HCl buffer (7-9) and Glycine/NaOH buffer (9-10) using benzylpenicillin (5 $\mu$ M) as a substrate.

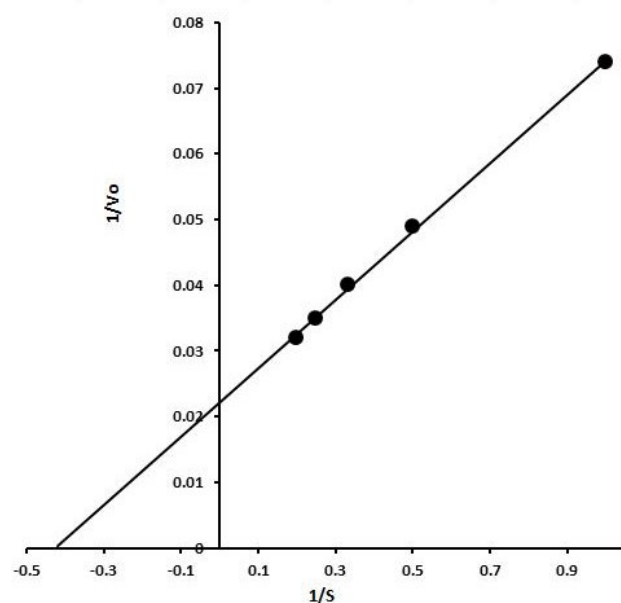


Fig. 6. Lineweaver-Burk plot for the estimation of kinetic parameters. X-axis shows 1/substrate concentration whereas Y-axis shows 1/enzyme velocity.



**Table I. Effect of metal cations and detergents on LAC<sub>BS</sub> activity.**

	Metal ions and detergents	Relative activity
Metal ions	Control	100
	EDTA	91
	Zn <sup>2+</sup>	144
	Co <sup>2+</sup>	121
	Mn <sup>2+</sup>	108
	Mg <sup>2+</sup>	90
	Ca <sup>2+</sup>	83
	Fe <sup>2+</sup>	50
Detergents	Tween 20	94
	Tween 80	73
	Triton X-100	97
	SDS	71

Metal chlorides were used in  $\beta$ -LAC<sub>BS</sub> activity assay.

## DISCUSSION

$\beta$ -lactamases are enzymes that play important role as positive control in antimicrobial susceptibility testing in clinical laboratories. These are responsible for the hydrolysis of amide bond in the  $\beta$ -lactam ring to make the antibiotic ineffective.  $\beta$ -lactamases have been classified into various classes including A, B, C and D. Classes A, C and D have serine as active site amino acid and follow the development of acyl enzyme intermediate during the hydrolysis of  $\beta$ -lactam ring. Class B lactamases are metal dependent enzymes which require Zn<sup>2+</sup> for their activity (Fisher *et al.*, 2005; Massova and Mobashery, 1998; Wilke *et al.*, 2005).

Interestingly,  $\beta$ -lactamase from current study is unique as it didn't share the basic conserved domains required for the activity of class A, C and D of  $\beta$ -lactamases and this lactamase shows its activity even in the absence of Zn<sup>2+</sup> which is the unique character of class B  $\beta$ -lactamases. The lactamase from current study have ability of showing 90% of its activity even in the presence of 1mM EDTA; however LAC<sub>BS</sub> activity is increased in the presence of Zn<sup>2+</sup>. These characteristics make this enzyme novel.

The optimal production of  $\beta$ -lactamase was observed 5h after induction of cells with 0.6 mM IPTG. The results are similar to those of lactamase produced from *Mycobacterium smegmatis* which showed optimal production 6h after induction of cells with 0.5mM IPTG (Bansal *et al.*, 2015).

LAC<sub>BS</sub> has molecular weight of 34 kDa which is close to 36 kDa for lactamase of *Bacillus cereus* ATCC 13061 (Fenselau *et al.*, 2008) and 32.5 kDa for lactamase of *Bacteroides uniformis* (Hedberg *et al.*, 1995). LAC<sub>BS</sub> showed maximal activity at 37°C which is in agreement with  $\beta$ -lactamases from *Mycobacterium tuberculosis* (Voladri *et al.*, 1998), *Burkholderia pseudomallei* (Cheung *et al.*, 2002), *Prevotella intermedia* (Madinier *et al.*, 2001) and *Staphylococcus aureus* (East and Dyke, 1989) whereas the lactamase from *Stenotrophomonas maltophilia* (Crowder *et al.*, 1998) showed optimal activity at 35°C. In contrast to this, the lactamases from *Bacteroides fragilis* (Wang and Benkovic, 1998), *Pseudomonas aeruginosa* (Krasauskas *et al.*, 2015), *Legionella gormanii* ATCC 33297T (Mercuri *et al.*, 2001), *Yersinia ruckeri* (Mammeri, *et al.*, 2006) and *Bacillus clausii* (Girlich *et al.*, 2007) showed optimal activity between a temperature range from 25 to 30°C.

The LAC<sub>BS</sub> shows optimum activity at neutral pH which is being supported by lactamases from *Bacteroides fragilis* (Wang and Benkovic, 1998), *Pseudomonas aeruginosa* (Krasauskas *et al.*, 2015), *Stenotrophomonas maltophilia* (Mercuri *et al.*, 2001), *Prevotella intermedia* (Madinier *et al.*, 2001), *Yersinia ruckeri* (Mammeri *et al.*, 2006), *Staphylococcus aureus* (East and Dyke, 1989), *Proteus mirabilis* N29 (Sawai *et al.*, 1982) and *Bacillus clausii* (Girlich *et al.*, 2007) whereas the lactamases from *Mycobacterium tuberculosis* (Voladri *et al.*, 1998) *Legionella gormanii* (Mercuri *et al.*, 2001) and *Mycobacterium tuberculosis* (Voladri *et al.*, 1998) showed maximal activity at pH 6. However, the lactamases from *Burkholderia pseudomallei* (Cheung *et al.*, 2002) and *Pseudomonas maltophilia* GN12873 showed the optimal activity at 7.4 and pH 8 respectively (Saino *et al.*, 1982).

Metallo  $\beta$ -lactamases require Zn<sup>2+</sup> for performing their activity and these enzymes can't work in the absence of cofactor (Paton *et al.*, 1994; Concha *et al.*, 1996; Salahuddin *et al.*, 2018; Meini *et al.*, 2015). LAC<sub>BS</sub> retains 90% activity in the presence of EDTA indicating the ability of enzyme to work even in the absence of Zn<sup>2+</sup>. At the same time its activity is being enhanced with the availability of Zn<sup>2+</sup>. This might be due the stabilizing impact of Zn<sup>2+</sup> on the structure of LAC<sub>BS</sub>. The results demonstrated that LAC<sub>BS</sub> requires 0.8 mM Zn<sup>2+</sup> for performing maximal activity. In contrast to this,  $\beta$ -lactamase VIM-4 from *Pseudomonas aeruginosa* possessed maximal activity at 0.4 mM concentration of Zn<sup>2+</sup> (Lassaux *et al.*, 2011).

The presence of ionic and non-ionic detergents showed slight to moderate inhibitory impact on the LAC<sub>BS</sub> activity. Among the nonionic detergents, Tween 80 reduced the enzymatic activity to 73% as compared to Tween 20 or Triton X-100 which exhibited negligible impact and reduced the enzymatic activity to 94 or 97%, respectively.

SDS reduced the LAC<sub>BS</sub> activity to 70%.

## CONCLUSION

$\beta$ -lactamase from current study is unique as it does not show significant homology with the previously characterized lactamases. Secondly, this enzyme shows its activity similar to class A, C and D of lactamases in a metal independent way, however the activity is being increased in the presence of Zn<sup>2+</sup> that is unique characteristic of class B of lactamases. The antibiotic hydrolyzing ability of this enzyme makes it suitable for its use in diagnostic laboratory. The domestic production of  $\beta$ -lactamase will result in cost effective availability of this enzyme and will save huge foreign exchange for the import of this enzyme.

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### Statement of conflict of interest

The authors have declared no conflict of interest.

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