PROTEASE PRODUCING *PSEUDOMONAS AERUGINOSA* STRAIN (IBC-2) FROM COAL MINES OF ORAKZAI AGENCY, PAKISTAN

AFRIDI, M. I.[1] – ALI, N.[1*] – MEMON, A. R.[2] – QASIM, M.[3] – JAMAL, Q.[3] – KHATTAK, B.[3] – ADNAN, M.[4] – KHAN, S. N.[5] – ULLAH, A.[6] – YOUNAS, F.[7] – ULLAH, F.[8]

[1]*Department of Biotechnology and Genetic Engineering, Kohat University of Science & Technology (KUST), 26000 Kohat, Pakistan*

[2]*Department of Biochemistry, Liaquat Medical University of Health Sciences Jamshoro 76090 Sindh, Pakistan*

[3]*Department of Microbiology, Kohat University of Science & Technology (KUST) 26000 Kohat, Pakistan*

[4]*Department of Botany, Kohat University of Science & Technology (KUST) 26000 Kohat, Pakistan*

[5]*Department of Zoology, Kohat University of Science & Technology (KUST) 26000 Kohat, Pakistan*

[6]*Department of Pharmacy, Forman Christian College, Lahore, Pakistan*

[7]*Sulaiman Bin Abdullah Aba Al-Khail Center for Interdisciplinary Research in Basic Sciences (SA-CIRBS), International Islamic University, Islamabad, Pakistan*

[8]*Department of Biosciences, COMSATS University (CIIT), 45550 Islamabad, Pakistan*

***Corresponding author*
e-mail: nawabali_1857@yahoo.com; phone: +92-344-303-5006; fax: +92-922-554-556

(Received 31st Oct 2018; accepted 8th Feb 2019)

Abstract. Microbial proteolytic enzymes are used to hydrolyze proteins in various bio-industrial applications but most of the enzymes are not active in extreme temperature conditions, pH and salt concentrations. This increases the demand to explore enzymes to show activity at such extreme environments. Exciting findings have been described about the extremophilic bacteria isolated first time from coal mines of Orakzai Agency, Pakistan, having protease producing properties. Among various extracted isolates, ~6 isolates were purified based on showing proteolytic activity using 2% skim milk agar media at 37 °C for 24 hrs, to identify various morphological and biochemical characteristics. The isolate, IBC-2 showed maximum zonal activity (21 mm) and was identified as *Pseudomonas aeruginosa* (JCM 5962) using 16S rRNA gene sequencing. The strain IBC-2 exhibited significantly ($P \leq 0.05$) maximum growth and protease production (335 U/ml) at 37 °C and at pH 7 after 24 hrs of incubation. The strain was also significantly ($P \leq 0.05$) more active for protease production at neutral pH (435 U/ml) than at high or low pH, reflected neutral nature of the bacteria. In conclusion, the strain IBC-2 has highest protease producing ability with distinctiveness, which can be used for wide range bio-industrial applications.
Keywords: *extremophiles, gram negative bacteria, 16s rRNA, proteolytic enzyme, natural resource*

Introduction

Microbial proteases are widely used for various physiological and biochemical purposes in a variety of industrial applications worldwide. Proteases having proteolytic

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

nature, catalyze the breakdown of peptide bonds between amino acids forming proteins (Rawlings and Barrett, 2013). The overwhelming importance of proteases is due to substrate specificity, active site, catalytic mechanism and stability (Maurer, 2004). Owing to various industrial applications, proteases have marked contribution among global enzyme market (Maurer, 2004).

Commercially, protease has many applications in leather, food and pharmaceutical industries as well as also used for bioremediation purposes (Jellouli et al., 2009; Kirk et al., 2002). Two third of the commercial proteases have been collected from microbial sources (Gupta et al., 2002). Commercial proteases are mostly obtained from bacterial origin and there are copious bacterial strains having protease producing potential, such as *Flavobacterium clostridium, Achromobacter, Actinomyces species, Staphylococcus aureas and Thermoactinomyces including Pseudomonas aurigenosa* (Prakasham et al., 2006). Bacterial proteases are usually preferred source over other sources like plant and animal on the basis of fast-growing nature as well as require minimum space for culturing. Moreover, they can be easily modified genetically for large scale purification compared to plants and animals, in order to get new enzymes with assorted characteristics (Gupta et al., 2002).

Currently, very limited (1-2%) microbes have been explored having commercial importance including extremophiles as well. Extremophiles can significantly tolerate a wide range of environmental conditions like high or low temperature (-2-121 °C), acidic and basic pH, high saline stress, a wide range of radiation as well as unusual conditions such as drought and nutrients limitations (Gomes and Steiner, 2004; Jadhav et al., 2013). In present time, researcher have focused their keen interest to investigate microbes of novel extremophile characteristics having distinctive adaptation and products that further used in stern industrial conditions. Therefore, microbes of such extreme environments need to be targeted and to be screened for novel extremozymes activities.

Coal mines are among those sources that can provides such acid-tolerant and obligate acidophilic extremophiles bacteria (Johnson and McGinness, 1991) and have greater odds to explore novel species in such hostile environments (Roohi et al., 2014). Interestingly, our lab also reported some novel halotolerant bacterial strains from salt mines of Karak (Pakistan) that have significant potential to produce both protease and amylase enzymes (Ali et al., 2016; Shah et al., 2017). Therefore, this further increased our incessant curiosity for exploring cheap microbial source of extremozymes in coal mines.

Materials and methods

Sampling and isolation of bacterial strains

The coal samples (500 g) were collected from the coal mines of Feroz Khel (33.885652°N 71.187346°E); located in Orakzai Agency, Kyber Pakhtunkhwa, Pakistan in sterilized plastic boxes and transported to laboratory (Department of Microbiology, KUST Kohat) for further processing. Before isolation, entire samples of coal were crushed using a clean & sterilized grinder and powdered samples were serially diluted to 10−3, 10−6 and 10−9 working solutions in sterilized autoclaved distilled water. The dilutions were cultured on nutrient agar plates using pour-plate technique. The colonies having contrasting culture features (n = 62) were sub cultured by frequent streaking at

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

same conditions to acquire pure colonies or isolates (Jamal et al., 2016; Roohi et al., 2012).

Screening and identification of proteolytic isolates

Pure isolates (n = 6) were observed for proteolytic activities using 2% skim milk (SM) agar media incubated at 37 °C for 24 hrs (Xiong et al., 2007) following Bergey's manual of systematic bacteriology for morphological and biochemical properties (Buchanan and Gibbons, 1974). Despite of producing larger proteolytic inhibitory zone and other differences like shape and color of colony, the zone of the IBC-2 was very clear comparative to IBC-5 (little turbid) which makes IBC-2 a best choice for our onward analysis (*Table 1; Figs. 1* and *S1*). Moreover, 16S rRNA sequence analysis was performed for further recognition of the chosen IBC-2 strain(s). Genomic DNA was extracted from IBC-2 strain(s) using phenol/chloroform method (Ali et al., 2016) and was amplified against 16S rRNA universal primer; 9F (5′-GA GT TT GA TC CT GG CT CA G-3′) and 1510R (5′-GG CT AC CT TG TT AC GA-3′) using DreamTaq™ Green PCR Master Mix (2X) (Thermo Scientific™) as described by Ali et al. (2016). The thermocycler (Veriti™ Applied Biosystems, Foster City, CA, USA) conditions were; a single cycle of initial denaturation (96 °C for 5 min), followed by 25 cycles (95 °C for 45 s, 52 °C for 45 s and 72 °C for 90 s), while the final elongation phase was performed at 72 °C for 10 min. The PCR amplified product of IBC-2 strain(s) was confirmed on 2% agarose gel followed by sequencing from Macrogen Lab, South Korea (http://dna.macrogen.com/eng/index.jsp). The bacterial strain (IBC-2) was BLAST on the server EZ-Biocloud (EZ-Taxon) in order to find out nomenclature (Yoon et al., 2017), whereas the phylogenetic tree was constructed using bioinformatics software MEGA 6.0 (Molecular Evolutionary Genetics Analysis version 6.0) (Tamura et al., 2013).

Partial purification and quantification of protease enzyme

For partial purification of protease enzyme, 1 ml of fresh inoculum was added to 2% SM broth media and was subjected to incubation at 37 °C for 36 hrs using shaking incubator. The cellular enzymes were partially purified by centrifugation of culture for 20 min at 4 °C for 4000g using filter injection (22 μ). Furthermore, ammonium sulphate (70% w/v) precipitation of enzymes was performed from supernatant overnight at 4 °C in a shaking incubator followed by centrifugation (4000g) at 4 °C. The pellets were dissolved in sterile water that were tested for their proteolytic activity (Nadeem et al., 2007).

Moreover, for protease quantification, a modified assay described by Nadeem et al. (2007) was used. Briefly, 1 ml (2% w/v) of the substrate casein (0.05 M glycine-NaOH pH 10) and 1 ml of crude enzyme were incubated at 40 °C for 20 min. The reaction was stopped by adding 3 ml of 10% (w/v) trichloro acetic acid (TCA) at room temperature for 30 min followed by centrifugation (100g) for 10 min at 4 °C. Afterwards, 0.5 ml supernatant was taken and dissolved in 4.5 ml of 0.05 M glycine-NaOH buffer (pH 10) and the absorbance were checked spectrophotometrically (UV-VIS 1800, Shimadzu, Kyoto, Japan) at 280 nm. The calibration curve was plotted using tyrosine (CAS number 60-18-4, Sigma Aldrich, USA) as standard on increasing concentrations of tyrosine such as 1, 2, 3, 4, 5 and 6 μg dissolved in 0.05 M glycine-NaOH buffer. One

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

proteolytic unit (1U) was defined as the quantity of the enzyme that released 1 μg of tyrosine each minute under the defined assay conditions.

Table 1. *Isolate (IBC-2) showing casein hydrolysis zone formation on skim milk agar (2%) plate*

Bacterial isolates	Colony color	Colony shape	Proteolytic zone (mm)
IBC-1	Off white	Circular	17.5
IBC-2	White	Irregular	21
IBC-5	Off white	Circular	20.5
IBC-6	Off white	Circular	18
IBC-7	Off white	Circular	18.5
IBC-10	Yellow	Circular	19

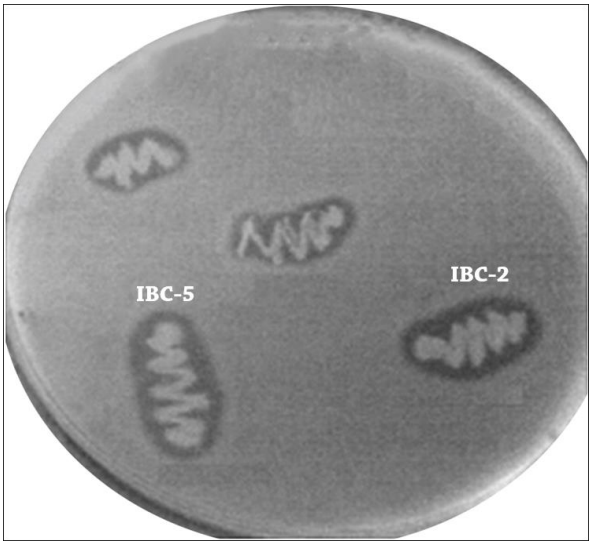

Figure 1. *Isolate (IBC-2) showing casein hydrolysis zone formation on skim milk agar (2%) plate*

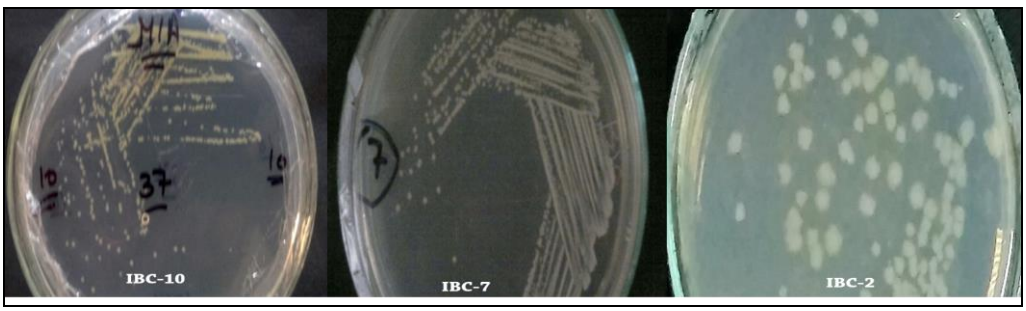

Figure S1. *Different proteolytic isolates have different shape and color of colony*

Electrophoretic protease separation

Molecular weight of the protease was determined using SDS-PAGE (12%) as depicted by Laemmli (1970). The protease enzyme (partially purified) was assorted

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

with loading dye (0.5 M Tris–HCl pH 8.0, SDS 10% (w/v), glycerol 6% (v/v), 2-β-marcaptoethanol and bromophenol blue 0.3% (v/v) after heating at 95 °C for 5 min. The crude protease was resolved with Pre-stained PageRuler™ Protein Ladder 10–180 KDa (Thermo Fisher Scientific™, Rockford, IL, USA) at 120 V for 2-2.5 hrs using Bio-Rad Mini Gel apparatus (Bio-Rad, Hercules, CA, USA). After resolving, SDS-PAGE, gel was stained by Coomassie Brilliant Blue (R-250) and proteins (protease enzyme) bands patterns were observed using white light apparatus.

Growth optimization

The isolate IBC-2 had been further optimized for its growth and protease production at varying physical factors. Pure inoculum (1 ml) of IBC-2 strain(s) was grown in Lauria-Bertani (LB) culture media (50 ml) under different incubation temperatures such as 20, 37, 50 and 60 °C using discrete media pH like 4, 5, 7, 9 and 11. After 24 hrs incubation, optimum growth of bacteria and protease production was calculated at 600 nm and the protease production was measured as per assay procedure (Ali et al., 2016).

Statistical analysis

All tests were carried out in replicates (n = 3) and statistically analyzed using SPSS v.21 (IBM SPSS Inc., Chicago, IL, USA). The results were taken in means and standard error (SE), while Tukey's honest significant difference (HSD) post hoc test were applied using ANOVAs.

Results

Identification and proteolytic potential of IBC-2 bacterial strain

In current study, a total of sixty-two (n = 62) bacterial strains were isolated, first time from coal mines of Orakzai Agency. Among them, thirty-three (n = 33) exhibit proteolytic zones (11-15 mm) but six (n = 6) out of thirty-three (n = 33) had a great potential of producing inhibitory zone with more than 15 mm (*Table 1*) (Sharmin et al., 2005). All the selected six isolates were further analyzed through biochemical and morphological identity (*Tables 1* and *2; Fig. 1*) for tentative identification. Because of largest and clear inhibitory zone, the isolate IBC-2 was with rod shaped, gram negative and showed positive tests for oxidase, catalase and gelatinase that was tentatively acknowledged as *Pseudomonas* (*Tables 1* and *2; Fig. 1*) and was selected to analysed further for their proteolytic optimization and molecular identification of the strain.

Molecular and phylogenetic analysis of isolate IBC-2

The isolate regarding IBC-2, were further evaluated at molecular level (*Fig. 2*). DNA extracted from IBC-2 isolate was amplified with 16S rRNA specific marker from EZ-Taxon server, bearing 99% sequence similarity with Pseudomonas aeruginosa JCM 5962(T) with accession number BAMA01000316. Moreover, phylogenetic tree demonstrated that IBC-2 strain is closely allied with Pseudomonas aeruginosa JCM 5962(T) as illustrated in *Figure 3*. The phylogenetic analysis involved 63 nucleotide sequences that targets 990 positions and data with gaps were removed after alignment by CLUXTAL X.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

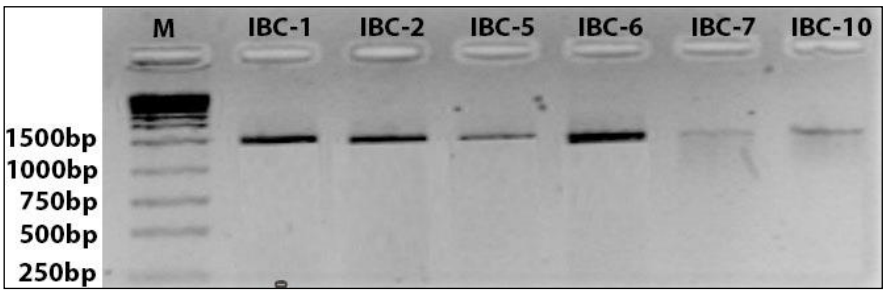

Figure 2. *PCR amplification of 16S rRNA (1.5 K bp) from IBC-2 isolate*

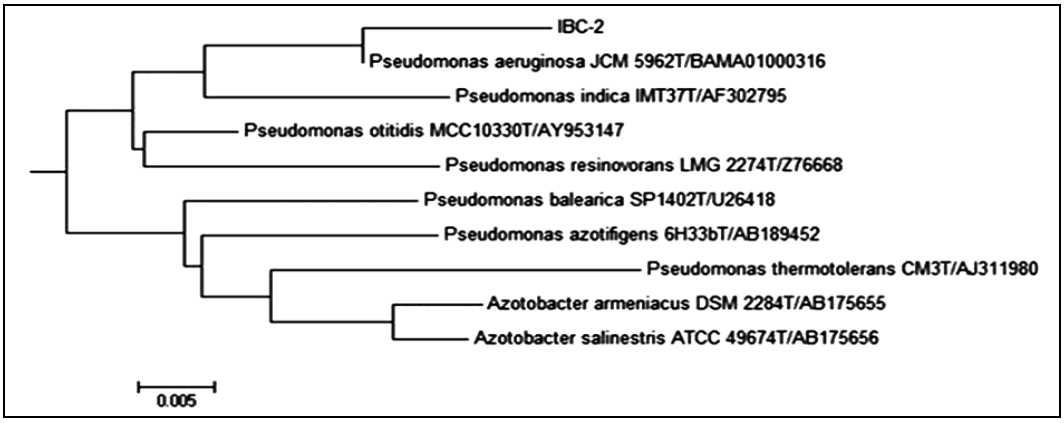

Figure 3. *Phylogenetic tree showing the interrelationships of isolate IBC-2 and its close relatives inferred from 16SrRNA gene sequence. (Evolutionary analysis was conducted in MEGA 6. Scale bar = 0.005 changes per nucleotide position)*

Table 2. *Tentative identification of the selected isolates (n = 6) on the basis of gram staining and different biochemical tests*

Bacterial isolates	Gram staining/shape	Oxidase	Catalase	Citralase	Sugar fermenter	Gas formation	H_2S formation	Motility test	Indole test	Urease test	Tentative identification (genus)
IBC-1	Gram -ve rod shape	+	+	+	+	+	-	+	-	-	*Pseudomonas spp.*
IBC-2	Gram -ve rod shape	+	+	+	+	+	-	+	-	-	*Pseudomonas spp.*
IBC-5	Gram -ve rod shape	+	+	+	+	+	-	+	-	-	*Pseudomonas spp.*
IBC-6	Gram +ve rod shape	-	+	+	-	+	-	+	-	+	*Bacillus spp.*
IBC-7	Gram -ve rod shape	+	+	+	+	+	-	+	+	+	*Proteobacteria spp.*
IBC-10	Gram +ve cocci	-	+	-	+	+	-	-	-	+	*Staphylococcus spp.*

Enzymatic activity and SDS-PAGE analysis of protease enzyme

The enzymatic activities of partially purified enzymes from all six (n = 6) bacterial isolate were spectrophotometrically performed. Among these, the isolate IBC-2 showed significantly higher proteolytic activity (300 U/ml) compared to other isolates (*Fig. 4*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

Moreover, all isolates were further resolved using polyacrylamide/bis-acrylamide (SDS-PAGE) and confirmed that the isolate IBC-2 belongs from Pseudomonas aeruginosa (30-50 KDa; *Fig. 5*) as per reported literature (Aqel et al., 2009; Dutta and Banerjee, 2006; Raj et al., 2012; Sevinc and Demirkan, 2011; Zambare et al., 2010).

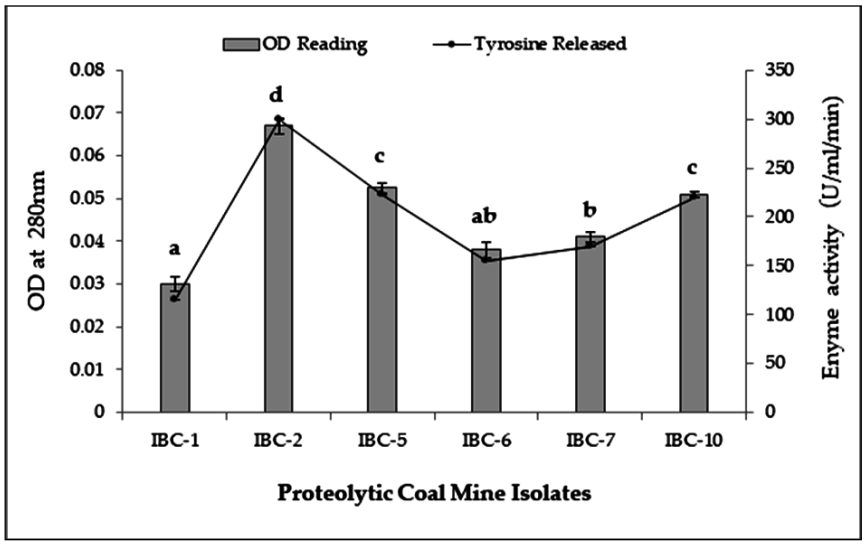

Figure 4. *Enzyme quantification of the six (IBC 1, 2, 5, 6, 7 and 10) proteolytic coal mine isolates. Data shown in bars represents mean OD of replicates (n = 3). Different alphabetical letters represent statistically significant differences (based on ANOVA) among the different isolates according to Tukey's honest significant difference (HSD) post-hoc test (P ≤ 0.05)*

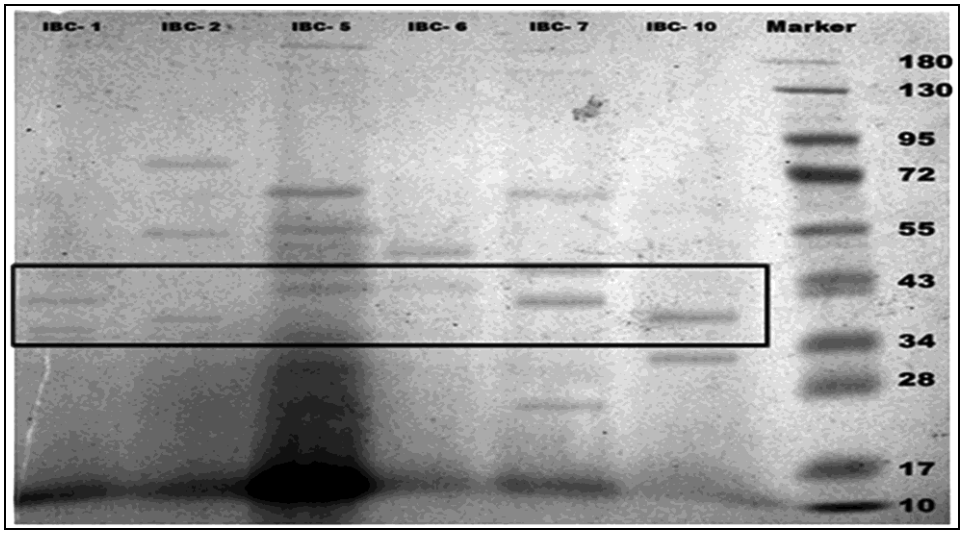

Figure 5. *SDS-PAGE gel analysis of partially purified protease enzymes extracted from different isolates of coal bacteria*

Growth optimization and protease production of selected IBC-2 isolates

The optimum growth factors (temperature and pH) were calculated for the selected IBC-2 isolates using varying degree of temperature (20, 37, 50 and 60 °C) and pH (4-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

11) in order to find out optimum bacterial growth and protease production using similar conditions in each case. Among the isolated bacterial stains, IBC-2 showed highest growth concentration at 37 °C at 600 nm after 24 hrs of incubation. Incubation at dissimilar temperatures notably affects the growth as well as protease production of the bacteria and in our study and maximum protease production (335 U/ml) was recorded at 37 °C while at temperatures lower or higher than 37 °C, low growth and lesser protease production was observed (*Fig. 6*).

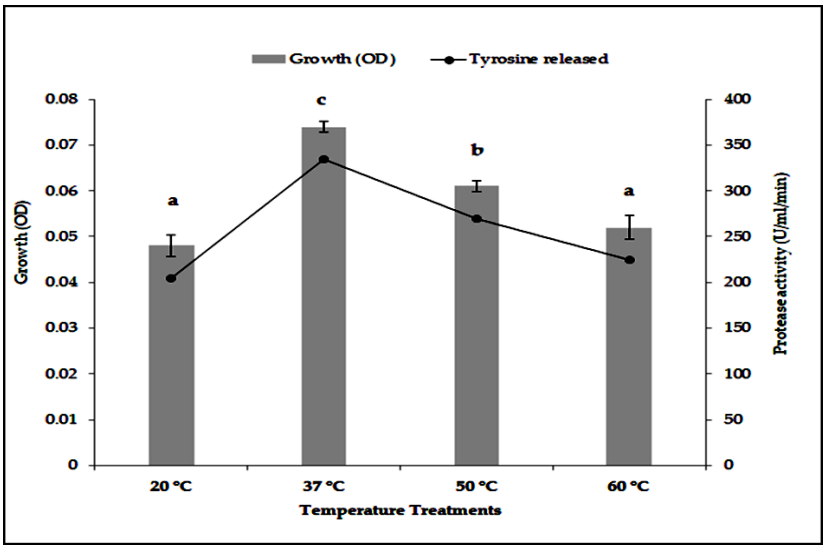

Figure 6. *Pseudomonas aeruginosa strain IBC-2 growth and protease production optimization at different incubation temperatures. Bars and line dots illustrate mean values of replicates ± standard error (n = 3). Different alphabetical letters represent statistically significant differences (based on ANOVA) at different temperature treatments according to Tukey's HSD post-hoc test (P ≤ 0.05)*

Similarly, to find out the pH effects, incubation was performed at 37 °C using different pH conditions (pH4-11). It was observed that the isolate IBC-2 favoured a pH of 7, giving optimum bacterial growth as well as significant protease production (435 U/ml). The protease production was found substantially higher than protease produced at either acidic pH (4 and 5) or alkaline pH (9 and 11) as shown in *Figure 7*. Therefore, it was concluded that IBC-2 gives best growth and maximum protease production at 37 °C while keeping the media pH neutral i.e. pH 7.

Discussion

Proteases have a wide range of commercial applications, including leather industries, food confectionaries and pharmaceuticals as well as also used in bioremediation processes (Jellouli et al., 2009; Kirk et al., 2002). Moreover, microbes, especially bacteria are among the easiest and cheapest source of proteases. On this regard, the coal mines of Orakzai Agency were screened for the isolation of commercially valuable bacteria. In our study, 33 bacterial isolates were found to have protease producing ability among total (n = 62) isolated bacterial strains. Moreover, among these 33 isolates, only 6 isolates had greater potential of casein hydrolyzing using skim milk agar

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

by producing larger zones (above 15 mm) as shown in *Tables 1* and *2*. Usually, various components such as caseins, gelatine and proteins are not utilized by bacteria, because these cannot penetrate into bacterial cell wall. This reveals the importance of extracellular proteases to solubilize the proteins and other components. The formation of zones on media containing proteins has been revealed in several scientific reports, showed that protease is usually excreted from microbial sources (Ali et al., 2016; Rajeeva et al., 2015).

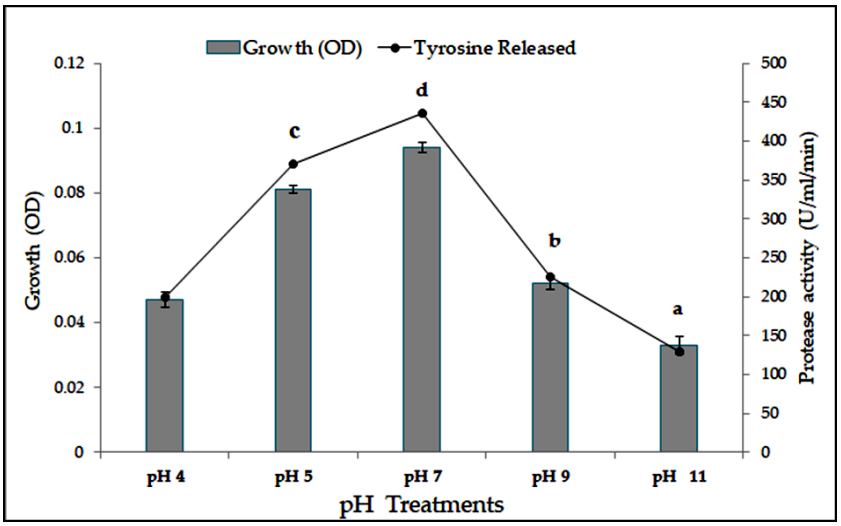

Figure 7. *Pseudomonas aeruginosa strain IBC-2 growth and protease production optimization at different pH culture conditions. Bars and line dots illustrate mean values of replicates ± standard error (n = 3). Different alphabetical letters represent statistically significant differences (based on ANOVA) at different pH treatments according to Tukey's HSD post-hoc test ($P \leq 0.05$)*

The studies for morphological characteristics of selected bacterial isolates (n = 6) showed differences in colony size, shape and color (*Table 2*) reflecting the bacterial diversity in coal mines. Furthermore, these characteristics revealed that the isolated bacteria might belong to *Bacillus, Pseudomonas, Arthrobacter, Brachybacterium* and *Staphylococcus* genus. Previously, these methods found standard for identification of various microbial species such as *Bacillus, Staphylococcus* and *Pseudomonas* spp. on the basis of morphology and different biochemical tests (Jamal et al., 2016; Jasuja et al., 2013; Roohi et al., 2014; Sethy and Behera, 2012). Although morphological and biochemical screening reveals initial microbial identification, but molecular methods such as 16S rRNA, provides precise identification of microbes (Wei Wang, 2009). In our study, the presence of *Pseudomonas aeruginosa* strain IBC-2 was first time reported as coal mine dwelling species and since very little studies have been conducted on coal mines micro flora regarding isolation of *Pseudomonas* species to be used for protease production.

Partial purification of protease from crude extract is possible by ammonium sulphate precipitation and this method is widely used for enzymes purifications (Yossan et al., 2006). As shown in *Figure 1*, all the selected six isolated strains showed clear zones on SM agar media and revealed protease activity. The protease purified from the selected isolated stain(s) were functional and effective after purification and parallel findings

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

were also revealed by Josephine et al. from different *Bacillus* sp., where the samples were collected from soil (Rajeeva et al., 2015). Among the six isolates, IBC-2 apparently revealed high protease productions and protease activity (300 U/ml/24 hrs). Analogous to our findings, maximum protease production (432 U/ml/24 hrs) was reported from *Pseudomonas aeruginosa* (Arioleand and Ilega, 2013), including *Pseudomonas aeruginosa* strain RGSS-09 as well (Rajeeva et al., 2015).

In our analysis, additional confirmation of crude purified enzymes (34-45 KDa) was performed using SDS PAGE for all selected isolates. SDS-PAGE was performed for separation of proteins and protease were found in range of 17 to 60 KDa, which is reported range for proteases (Yossan et al., 2006). Moreover, many *Pseudomonas* species showed specific molecular weights of proteases ranging from 35-50 KDa (Raj et al., 2012). In correlation to our results, a couple of study reported the molecular weight regarding protease enzymes were in the range of 30-52 KDa from various *Bacillus* sp (HS08A, HUTBS71) (Aqel et al., 2009; Dutta and Banerjee, 2006; Sevinc and Demirkan, 2011; Zambare et al., 2010).

Enzymatic activities and growth of microorganisms are significantly ($P \leq 0.05$) correlated with various incubation temperatures and has shown prominent effects on secretion of extracellular proteolytic enzymes using various microbes (Balaji et al., 2012). Similarly, IBC-2 strain significantly ($P \leq 0.05$) revealed that dense bacterial growth occurs at 37 °C when incubated for 24 hrs (*Fig. 6*). This inferred the direct relationship of bacterial growth with protease production. Different researchers worked on proteases and reported the relationship between protease production and bacterial growth under different laboratory conditions such as temperature, media and pH (Ali et al., 2016; Shah et al., 2017; Zambare et al., 2010). The optimum temperature for protease production was reported as 30 °C produced by an extremophilic *Bacillus sp* (MIG) isolated from marine water (Mohapatra et al., 2003). The specific protease producing bacteria (e.g. *Bacillus spp.)* was reported to have different range of optimum temperatures such as 35 °C (Gerze et al., 2005), 40 °C (Josephine et al., 2012), 50 °C (Anwar and Saleemuddin, 2000) and 70 °C (Sookkheo et al., 2000; Morya and Yadav, 2010). An increase in the production of protease was reported when temperature was increased up to 37 °C for *Pseudomonas sp.* from abattoir soil has been reported by Kalaiarsi and Sunitha (2009) and Akujobi et al. (2012), respectively. The decrease in protease production beyond 37 °C reported by these researchers has supported our results and it has been reported that optimum temperature has key role in the production of protease. In similar fashion, time of incubation is also important to have high protease production and it varies for different microorganisms (Mukesh Kumar et al., 2012).

Like temperature, optimum pH also contributes to protease expression and activity. In this study, protease production was observed at pH range 4-11 and bacterial growth is reported in high amount at neutral pH 7 for IBC-2 (*Fig. 7*). In similar approach, Ravishankar et al. (2012) reported high production and enzyme activity of *Bacillus subtilis* AKR3 up to pH 7 and has been found declined beyond the pH from 9-11. Supporting our work, the optimum pH 7 and temperature 37 °C was also previously reported for protease production of *Pseudomonas aeruginosa* (Raj et al., 2012). In case of *Bacillus subtilis* strain reported by Ali et al. (2016), pH 10 was the optimum pH for high protease production. These findings revealed that different bacteria need different conditions as most favourable. The bacteria under study have shown high protease

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

activity at temperature 37 °C and pH 7, which are the most suitable conditions for broad range of bio-industrial applications (Grbavcic et al., 2011; Najafi et al., 2005).

Conclusion

Finally, our finding is the first inveterate report from coal mines of Orakzai Agency that investigated protease producing bacteria. Based on morphological, biochemical and 16S rRNA sequencing, and the bacteria with high protease activity; IBC-2 strain was identified as *Pseudomonas aeruginosa*. The highest protease activity was observed at temperature 37 °C and pH 7 which makes the bacteria as the better producer at conditions favoured/used in most of the bio-industrial applications. Moreover, natural resources need to be further explored in an attempt to find out novel bacterial strain(s), with an exceptional capability of surviving and maintain their hydrolytic activity within harsh condition in order to overcome the bio-industrial problems.

Acknowledgements. The authors are grateful to the Directorate of Science and Technology (DoST) Peshawar, Khyber Pakhtunkhwa-Pakistan for financial aid to implement this research work.

Conflict of interests. All authors declared no conflict of interests, and also the funding body had no role in the experiment design, methodology or data interpretation.

REFERENCES

[1] Akujobi, C. O., Odu, N. N., Okorondu, S. I., Ike, G. N. (2012): Production of protease by Pseudomonas aeruginosa and Staphylococcus aureus isolated from abattoir environment. – J. Res. Biol. 2(2): 077-082.

[2] Ali, N., Ullah, N., Qasim, M., Rahman, H., Khan, S. N., Sadiq, A., Adnan, M. (2016): Molecular characterization and growth optimization of halo-tolerant protease producing Bacillus Subtilis Strain BLK-1.5 isolated from salt mines of Karak, Pakistan. – Extremophiles 20(4): 395-402.

[3] Anwar, A., Saleemuddin, M. (2000): Alkaline protease from Spilosoma obliqua: potential applications in bio-formulations. – Biotechnol Appl Biochem 31(Pt 2): 85-9.

[4] Aqel, H., Farouk. Q., Yousef, T. K. (2009): Characterization of a purified thermostable protease from hyperthermophilic Bacillus strain HUTBS71. – European Journal of Scientific Research 31(2): 280-288.

[5] Arioleand, C. N., Ilega, E. (2013): Alkaline protease production by Pseudomonas aeruginosa isolated from the gut of pilaovate. – J. Glob. Biosci. 2(5): 126-131.

[6] Balaji, N., Rajasekaran, K. M., Kanipandian, N., Vignesh, V. Thirumurugan, R. (2012): Isolation and screening of proteolytic bacteria from freshwater fish Cyprinus carpio. – International Multidisciplinary Research Journal. 2(6): 56-59.

[7] Buchanan, R. E., Gibbons, N. E. (1974): Bergey's Manual of Determinative Bacteriology. 8th Ed. – Williams & Wilkins Co, Baltimore, USA.

[8] Dutta, J. R., Banerjee, R. (2006): Isolation and characterization of a newly isolated Pseudomonas mutant for protease production. – Braz Arch Biol Techn 49(1): 37-47.

[9] Gerze, A., Omay, D. Guvenilir, Y. (2005): Partial Purification and Characterization of Protease Enzyme from Bacillus subtilis megatherium. – Appl Biochem Biotechnol Spring(121-124): 335-345.

[10] Gomes, J., Steiner, W. (2004): The biocatalytic potential of extremophiles and extremozymes. – Food Technol. Biotechnol. 42(4): 223-235.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

[11] Grbavcic, S., Bezbradica, D., Izrael-Zivkovic, L., Avramovic, N., Milosavic, N., Karadzic, I., Knezevic-Jugovic, Z. (2011): Production of lipase and protease from an indigenous Pseudomonas aeruginosa strain and their evaluation as detergent additives: compatibility study with detergent ingredients and washing performance. – Bioresour. Technol. 102(24): 11226-33.
[12] Gupta, R., Beg, Q. K., Lorenz, P. (2002): Bacterial alkaline proteases: molecular approaches and industrial applications. – Appl Microbiol Biotechnol 59(1): 15-32.
[13] Jadhav, A. G., Jaybhaye, A. A., Musaddiq, A. A. (2013): Salt tolerant protease produced by an aerobic species belonging to the Bacillus genus isolated from saline soil. – IJSRP 3(2): 1-8.
[14] Jamal, Q., Ahmed, I., Rehman, S., Abbas, S., Yong Kim, K., Anees, M. (2016): Isolation and characterization of bacteria from coal mines of Dara Adam Khel, Pakistan. – Geomicrobiology 33(1): 1-9.
[15] Jasuja, N. D., Saxena, R., Chandra, S., Joshi, S. C. (2013): Isolation and identification of microorganism from polyhouse agriculture soil of Rajasthan. – African Journal of Microbiology Research 7(41): 4886-4891.
[16] Jellouli, K., Bougatef, A., Manni, L., Agrebi, R., Siala, R., Younes, I., Nasri, M. (2009): Molecular and biochemical characterization of an extracellular serine-protease from Vibrio metschnikovii J1. – J Ind Microbiol Biotechnol 36(7): 939-48.
[17] Johnson, D. B., Mcginness, S. (1991): A highly effecient and universal solid medium for growing mesophilic and moderately thermophilic, iron-oxidizing, acidophilic bacteria. – Journal of Microbiological Method. 13(2): 113-122.
[18] Josephine, F. S., Ramya, V. S., Devi, N., Ganapa, S. B., Siddalingeshwara, K. G., Venugopal, N., Vishwanatha, T. (2012): Isolation, production and characterization of protease from Bacillus Sp isolated from soil sample. – J. Microbiol. Biotech. Res. 2(1): 163-168.
[19] Kalaiarsi, K., Sunitha, P. U. (2009): Optimization of alkaline protease production from Pseudomonas fluorescens isolated from meat waste contaminated soil. – Afri. J. Biotechnol. 8(24): 7035-7041.
[20] Kirk, O., Borchert, T. V., Fuglsang, C. C. (2002): Industrial enzyme applications. – Curr Opin Biotechnol 13(4): 345-51.
[21] Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. – Nature 227(5259): 680-5.
[22] Maurer, K. H. (2004): Detergent proteases. – Curr Opin Biotechnol 15(4): 330-4.
[23] Mohapatra, B. R., Bapuji, M., Sree, A. (2003): Production of industrial enzymes (Amylase, Carboxymethylcellulase and Protease) by bacteria isolated from marine sedentary organisms. – Acta Biotechnol 23(1): 75-84.
[24] Morya, V. K., Yadav, D. (2010): Production and partial characterization of neutral protease by an indigenously isolated strain of Aspergillus tubingensis NIICC-08155. – Internet Journal of Microbiology. 8(1): 53-55.
[25] Mukesh Kumar, D. J., Venkatachalam, P., Govindarajan, N., Balakumaran, M. D., Kalaichelvan, P. T. (2012): Production and purification of alkaline protease from Bacillus sp. MPTK 712 Isolated from dairy sludge. – Global Veterinaria 8(5): 433-439.
[26] Nadeem, M., Qazi, J. I., Baig, S., Syed, Q. (2007): Studies on commercially important alkaline protease from Bacillus lichniformis N-2 isolated from decaying organic soil. – Turk J Biochem 32(4): 171-177.
[27] Najafi, M. F., Deobagkar, D., Deobagkar, D. (2005): Potential application of protease isolated from Pseudomonas aeruginosa PD100. – Electronic Journal of Biotechnology 8(2): 79-85.
[28] Prakasham, R. S., Rao Ch, S., Sarma, P. N. (2006): Green gram husk--an inexpensive substrate for alkaline protease production by Bacillus sp. in solid-state fermentation. – Bioresour Technol 97(13): 1449-54.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093

[29] Raj, A., Khess, N., Pujari, N., Bhattacharya, S., Das, A., Sundara Rajan, S. (2012): Enhancement of protease production by Pseudomonas aeruginosa isolated from dairy effluent sludge and determination of its fibrinolytic potential. – Asian Pacific Journal of Tropical Biomedicine 2(3): S1845-S1851.
[30] Rajeeva, G., Soni, T., Sheffalika, S. (2015): Production and characterization of thermotolerant-organic solvent resistant acidic protease by Pseudomonas aeruginosa RGSS-09 isolated from dairy sludge. – Asian J. Biochem. 10(2): 52-66.
[31] Ravishankar, K., Kumar, M. A., Saravanan, K. (2012): Isolation of alkaline protease from Bacillus subtilis AKRS3. – Afri J. Biotechnol. 11(69): 13415-13427.
[32] Rawlings, N. D., Barrett, A. J. (2013): Handbook of Proteolytic Enzymes. 3rd Ed. – Academic Press, New York.
[33] Roohi, A., Ahmed, I., Iqbal, M., Jamil, M. (2012): Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, Pakistan. – Pak. J. Bot. 44(Special Issue March): 365-370.
[34] Roohi, A., Ahmed, I., Paek, J., Sin, Y., Abbas, S., Jamil, M., Chang, Y. H. (2014): Bacillus pakistanensis sp. nov., a halotolerant bacterium isolated from salt mines of the Karak Area in Pakistan. – Antonie Van Leeuwenhoek 105(6): 1163-72.
[35] Sethy, K., Behera, N. (2012): Antimicrobial activity of thermotolerant bacterial isolate from coal mine spoil. – AJMR 6(26): 5459-5463.
[36] Sevinc, N., Demirkan, E. (2011): Production of protease by Bacillus sp. N-40 isolated from soil and its enzymatic properties. – J. Biol. Environ. Sci. 5(14): 95-103.
[37] Shah, I., Azam, N., Din, G., Ali, N., Ullah, W., Qasim, M., Shehzad, A., Muhammad, N. (2017): Isolation and characterization of protease producing bacteria from soil samples of District Kohat, Pakistan. – JBMS 2(1): 1-5.
[38] Sharmin, S., Hossain, M. T., Anwar, M. N. (2005): Isolation and characterization of a protease producing bacteria Bacillus amovivorusand optimization of some factors of culture condition for protease production. – J. Biol. Sci. 5(3): 358-362.
[39] Sookkheo, B., Sinchaikul, S., Phutrakul, S., Chen, S. T. (2000): Purification and characterization of the highly thermostable proteases from Bacillus stearothermophilus TLS33. – Protein Expr Purif. 20(2): 142-51.
[40] Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013): MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. – Mol Biol Evol 30(12): 2725-9.
[41] Wei Wang, M. S. (2009): Phylogenetic relationships between Bacillus species and related genera inferred from 16s rDNA sequences. – Braz J Microbiol 40(3): 505-21.
[42] Xiong, H., Song, L., Xu, Y., Tsoi, M. Y., Dobretsov, S., Qian, P. Y. (2007): Characterization of proteolytic bacteria from the Aleutian deep-sea and their proteases. – J Ind Microbiol Biotechnol 34(1): 63-71.
[43] Yoon, S. H., Ha, S., M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J. (2017): Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. – Int J Syst Evol Microbiol 67(5): 1613-1617.
[44] Yossan, S., Reungsang, A., Yasuda, M. (2006): Purification and characterization of alkaline protease from Bacillus megaterium isolated from Thai fish sauce fermentation process. – Science Asia 32(2006): 377-383.
[45] Zambare, V., Nilegaonkar, S., Kanekar, P. (2010): A novel extracellular protease from Pseudomonas aeruginosa MCM B-327: enzyme production and its partial characterization. – N Biotechnol. 28(2): 173-81.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1703_60816093