Current Trends in Biotechnology and Pharmacy
Vol. 18(4) 1977-1983, October 2024, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2024.4.43

Isolation and Characterization of Protease Producing Novel *Burkholderia sp.*PS1 from Soil Sample and its Protease Production Optimization Studies

Raja Srinath R, Sanjeev Kumar K, *Pavan Kumar Pindi

Department of Microbiology, Palamuru University, Telangana – 509001, India

*Correspondent author: pavankumarpindi@gmail.com

Abstract

Extracellular proteases, due to their commercial, industrial applications have become significant and targeted for scientific research during current research. Our present report states about unfolding of potential proteases from soil sample. Even though there are diversified protease positive microorganisms was reported but still few are *Burkholderia Cenocepacia* proteases are unrevealed. Selective soil sample was collected from different places of Mahbubnagar Telangana state for screening and isolated potential protease producing microorganisms. 10^{-5} diluted sample spread over skim milk agar (High Media) and selected 108 protease positive organisms from screening. selected isolates are pushed to secondary screening, picked highest zone of hydrolysing microorganism was identified through 16s rRNA generated 1358bp amplicon was forwarded to NCBI blast, 98% homology *Burkholderia Cenocepacia* (Bks) submitted and generated accession number MH290479 with optimized Bks protease production showing maximum production at temperature 30°C, P^H -7, Inoculum size 3%, 1% glucose as carbon source,0.5% Gelatine protein and Zn metal ion was recorded.

Key words: *Burkholderia* sp, Bks, Optimization, Proteases, *Bacillus* Sp. NCBI Blast, Production.

Introduction

Microbial enzymes are core components of bio-industrial processes and they are cheaper, reliable, high yielding diversified biocatalyst. Proteases are one of the leading commercially important biocatalysts in enzymatic pool. Microbial proteases are extensively used in pharmaceutical, detergent, dairy, baking, leather and biomedical engineering for formulation of drugs, cleaning, making of edible products and reducing environmental pollution.

These enzymes profound applications in feather processes in poultry, detergent industries, pharmaceuticals, biotransformation, food processing, silk gumming, bioremediation, biosynthesis of drugs and bioconversions (1-4). The most common protease producing organisms are gram positive *Bacillus* sp. comparatively less in other species (3). Identification and screening research was active since first protease from Bacillus licheniformis was industrially commercialized as an additive in detergents in the 1960s (5) Numerous *Bacillus*-derived proteases have been isolated, purified and characterized their optimization studies to know efficiency of protease activity. Uplift of protease depends on abiotic stability with broad substrate specificity in short period of fermentation, easy downstream processing, and less cost have been

implicated (6). Many of the Bacillus-derived proteases are elevated exposure to temperatures and pH, but the majority proteases are incompatible with detergent matrices (2,5).

Apart from bacillus sp. and Pseudomonas sp. Fewer investigations was observed in protease field. Burkholderia sp. is also one of the potential protease producing gram negative bacteria it also shown efficient production observed under abiotic stress *Burkholderia cepacia, Burkholderia stabilis*. (7). Hence, proteases with optimization require for superior performance for industrial exploitations, especially for commercial utility are being sought. Although colourable screening and identification of most of the enzymes have already been found in *Bacillus thermantarcticus*, and *Bacillus mojavensis* etc. (1,8). Since investigations are continuous to find novel, efficient, ease on adoptable microbial proteases been targeted in future studies. In the present study, isolation of unexplored industrially efficient protease producing organisms from soil samples and its optimization studies relevance to commercial purposes.

Materials and Methods

Collection of soil sample

Different Soil sample was collected from various places in Mahbubnagar town and different places of Telangana state specially cattle dung landfills, poultry forms dump yard and undisturbed soils was taken around10-15cm depth keeping natural habitat without any disturb.

Primary screening

Autoclaved sterile water used for Serial dilution and 10^5,10^6,10^7 and 10^8 dilutions was taken for spread plate method done for collected soil on screened 1% skim milk agar and incubated it for 36hrs on 37^0C the bacterial culture which shown clear zones around the colonies was selected for progressive studies. The zone of proteolytic efficiency was determined by measuring the diameter of the colony and zone of hydrolysis. Efficient bacterial isolates *Burkholderia Cenocepacia*, Bacillus cereus, Staphylococcus aureus and Bacillus subtilis like Bacteria showed good proteolytic activity Such colonies were selected and sub cultured on nutrient agar slants and preserved at 4^0C for further study.

Assay for protease activity by plate method

To Identify effective protease producing bacteria from pre-screened, isolated positives AP1- Bacillus Sp., AP6-*Burkholderia Cenocepacia*, AP7-Bacillus cereus AP8- Staphylococcus Sp., was streaked skim milk agar (HiMedia) and left for 48hr incubated plates shown zone of hydrolysis.

Proteolytic activity

Most common substrate Azocasein (HiMedia) taken as substrate for defining the Protease catalytic activity of *Brukholderia sp* PS (9). 2ml Eppendorf reaction was prepared mixture contains 120 µL of enzyme extract and followed by added 480 µL of 10 mg /ml of 0.2M and 7.2P^H azocasein solution. Later mixture was kept under incubated for 1hr at 37 °C and later reaction was stopped by adding 600µl trichloroacetic acid (TCA) 30% (w/v). After centrifugation at 10,000 g for 5 min, 800 µl top of the later mixed with 200 µl 1·8M NaOH. The absorbance at 440 nm was measured in a UV -spectrophotometer (9). Unit for enzyme was defined as the amount that caused an increase of 0·01 in absorbance at 420 nm in the assay conditions (10).

Biochemical and molecular identification

Readymade gram staining kit was used for morphological identification performed (HiMedia grams), DNA was extracted using the genomic DNA isolation kit (HiMedia) was used a (Mo-Bio-Laboratories) and meanwhile 16S rRNA sequencing sequenced as described previously (11). Taq polymerase supplied by Bio-Rad was used for PCR, which was started

Current Trends in Biotechnology and Pharmacy
Vol. 18(4) 1977-1983, October 2024, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2024.4.43

with the primers 16S-F 5' GTTTGATCCTGGCTCAG-3' and 16SR5'-AAGGAGGTGATCCAGCCGCA-3'. The resultant partial sequence of the 16S rRNA was obtained and this sequence was performed NCBI Nucleotide BLAST to NCBI Nucleotide BLAST was showing similarity of 98.93% *Burkholderia Cenocepacia* contained 1358bp nucleotides. The molecular identificatio16S rRNA sequencing was performed at Bioserve india pvt, and identified *Burkholderia cenocenocepician*, Accesion number- MH290479. nearest taxa was with construction of Phylogenetic trees was constructed using phylogenetic tree-making algorithms, consensus sequence maximum-likelihood (ML) was selected and noted similar sequences generated on of using the PhyML program (12) and neighbour-joining (NJ) (13) using the PHYLIP package, version 3.5 (14), and the resultant tree topologies were evaluated by bootstrap analysis based on 1000 resampling. Using the SEQBOOT and CONSENSE programs in the PHYLIP package. Pairwise evolutionary distances were evaluated and computed on DNADIST program with the Kimura 2-parameter model was developed (15).

Production of protease from Burkholderia Cenocepacia

Highly efficient protease *Burkholderia Cenocepacia* colony from skim milk agar plate was inoculated in 100ml mineral medium NaCl -0·5g, K_2HPO_4-0·3g, KH_2PO_4-0.6g, $MgSo_4$ -0.1g containing 1%g casein/100ml prepared and adjusted to pH 7·0. The production of protease was placed on orbital shaker in 250 ml flasks containing 100 ml of the medium by incubation for 72 h at 37 °C. The culture was centrifuged at 10,000 g for 10 min and the supernatant was used as crude enzyme.

Optimization of protease production

Effect of inoculums size

Inoculum size increased continuous sets 1%,2%, 3%,4% and 5% Inoculum size utilise the available components that enhance the protease production. Maximum protease production was recorded with 3% inoculum size with *Brukholderia cenocepcia*.

Optimization of temperature and pH protease production

To find maximum production of protease various abiotic factors was adopted temperature and pH on the proteolytic activity were determined. Thermal stability was evaluated by pre incubation of the enzyme for up to 20 min at 40, 45 and 50°C then residual activity was measured as described above. The assay for optimum pH was developed using 0·1 mol/l (pH 6, 7, 8 and 9), or sodium carbonate buffer (pH 9 and 10).

Effect of carbon and nitrogen sources

Major carbon sources like glucose, Maltose, Sucrose, Lactose fructose starch was tried with 1% w/v and Nitrogen substrates like casein, soybean extract, yeast extract, beef extract, Peptone, Ammonium nitrate and potassium nitrate 0.5% of w/v was taken for protease production and thereafter 1 ml of inoculum was inoculated into the medium and then subjected to submerged showed maximum enzyme production with glucose and beef extract was noted.

Effect of metal ions

Different essential metals like Mn^{+2}, Ca^{+2}, Mg^{+2}, Zn^{+2}, Fe^{+2} of 10mM concentrations was applied for media optimization. significant change was observed in the production of protease with Ca^{+2} on *Burkholderia Cenocepacia* and observed in 2-fold *by Ca and Zn* increased production.

Result and Discussion

Screening and isolation of potential protease producing bacteria

Mixture of soil sample dilutions spreading on skim milk agar generated 108 positive

Current Trends in Biotechnology and Pharmacy
Vol. 18(4) 1977-1983, October 2024, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2024.4.43

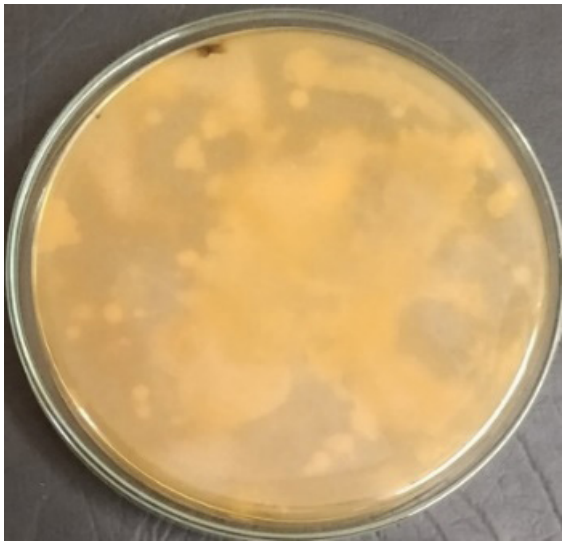

Fig.1 Primary screening on skim milk agar

Fig.2 Selective screening on skim milk agar, AP1- Bacillus subtilis, AP6-Burkholderia Cenocepacia, AP7-Bacillus cereus AP8- Staphylococcus aureus

bacterial as a result of from primary screening shown in figure.1. Selective culture showing maximum zone of hydrolysis clearly indicating that AP6 *Burkholderia sp. PS1* (Bkc-AP6) showing maximum zone hydrolysis compared to other organisms, Positive outcomes boosted to work on *Burkholderia sp.PS1* protease production and intent to know optimization of production media for futuristic industrial applications.

Fig.3 Secondary screening on skim milk agar AP6-Burkholderia Cenocepacia

Biochemical charecterization

Gram negative rod shaped structures are visualised undermicroscopic examination,resuted table.1 Cells are, motile colonies on NA (Nutrinet agar) are circular, 2 mm in diameter, White, crateri form and entire. Cells grow from 18 to 35 C with an optimum temperature of 37 ^{0} C and growth occurs in a pH range of 7 to 10 was analysed. More over biochemical charecterisation was conduted result was shown in Table .1 Catalase, oxidase, gelatinase,protease, indole and urease are positive, but vogeus proskeur , methyle red , lipase,bitrilase reductase are negative. nitrate reduction, H_2S production, Voges–Proskauer reaction and methyl red reaction are negative similarity to Burkholderia sp.

Current Trends in Biotechnology and Pharmacy
Vol. 18(4) 1977-1983, October 2024, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2024.4.43

Table.1.Biochemical identification of effeciant protease producing orgonism

S. No.	Name of the test	PS1
1	Gram's staining	Negative
2	Shape	Rods
3	Motility	Motile
4	Indole test	Positive
5	VP reaction	Negative
6	MR reaction	Negative
7	iso-Citrate test	Positive
8	Catalase	Positive
9	Oxidase	Positive
10	Phosphatase	Positive
11	Lipase	Negative
12	Urease	Positive
13	Gelatinase	variable
14	Protease	Positive
15	Nitrate reduction	Negative
16	H_2S production	Negative

The partial sequece of reulsted amplified product streamed to NCBI, blast search engine generated similar queries of 1358bp nucleotides sequence was showing 100% query coverage with Zero E value result made confrimation that species belongs to Burkholderia sp.nov.PS1 was confirmed. Further submitted 1358bp sequence and generated Accention number –MH290479 named as Burkholderia cenocepicia.

Construction of phylogenitic tree

On the basis nucleotide sequence generated 1358 subjected to phylogenetic relationship of strain PS1-AP6 with previously reported species in BLAST sequence similarity search (NCBI-BLAST/EzTaxon). The results indicated that at the 16S rRNA gene sequence level, strain PS1 -AP6 was close to the phylogenetic adjacent are with 98.97% similarity *Burkholderia cenocepecia* GP3 MN240932 and Burkholderia sp. MaAL 241, (Accesion number KY810687) for1358bp with 100% blast query. Phylogenetic tree based on ML (Fig. 4). and NJ (Fig.4) trees further indicated that strain PS1-AP6 clustered with Burkholderia cenocepecia GP3 MN240932 and Burkholderia sp. MaAL 241with a phylogenetic distance of 98.97% each and distinct from the other species of the genus. Despite the high 16S rRNA gene sequence similarity. Above blast and phylogenetic analysis indicating that strain *Burkholderia sp.nov.* PS1 is could be assigned to a novel species.

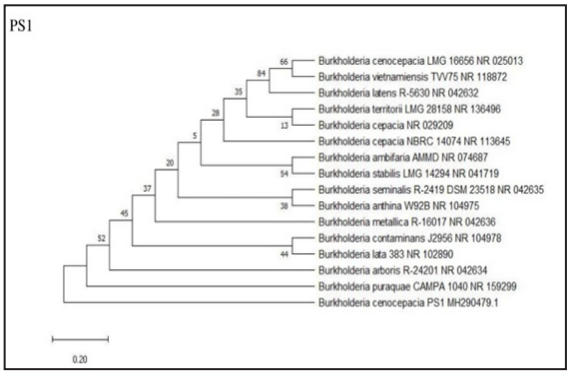

Fig.4 Phylogenitic tree constructed for *Burkholderia sp*.nov.PS1 (AC.No- MH290479)

Optimization of protease production

Globally commercialised proteases require not only isolation and identification but also industrial level utility like processeing, production and mass yeilding of an enzyme, present study optimization of production culture media revealed data of Bks protease production through submerged flask based experiment relavance to industrial applications. Size of an inoculam plays crusial role find where orgonism is enterning stationary phase, In inculum size versus production of enzyme clearly showing fig.5 inciating that maximum production has poitnted in 185 U/mL/Min at 3% later increasing inoculum also no significant difference was observed.

For Enhance mass production of enzymes, abiotic physical and chemical factors optimization is mandatory. Diffent ranges of P^H fig .7 from 4-10 and fig. 6 temperature 20-50^{o}C observations are recoreded Maximum production of Bks proease at P^H-7 (173 U/mL/Min), 35^{o}C –(165U/mL/Min) it give confirmation that Bks protease is neutral optimum nearest to

room temperature are suitable. Critical suistainability of orgnisms depends availability of carbon, nitrogen sources. where as in Bks protease production. 1% W/v of Glucose in production media 176 U/mL/Min of enzyme is produced. Compare to other carbon sources like starch (polysacharides) and disacharides (lactose, Sucrose) are less effect as carbon source than monosacharieds(Glucose Fructose,galctose) in another context Gelatine and casein like nitrogen sources leads enzymes production but ammonium nitrate as well as potasium nitrates are no significant effect was noted.

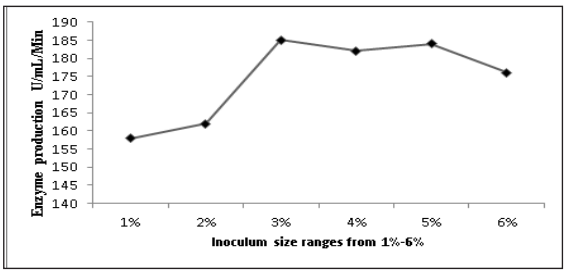

Fig.5 Effect of inoculum size on protease production

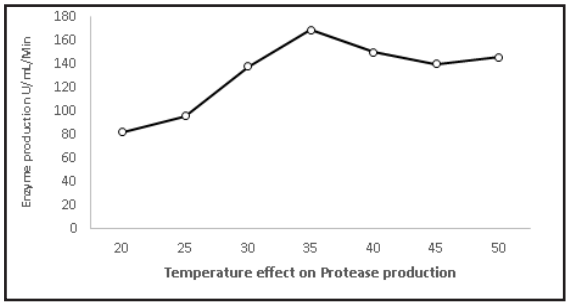

Fig.6 various temperature effect on protease production

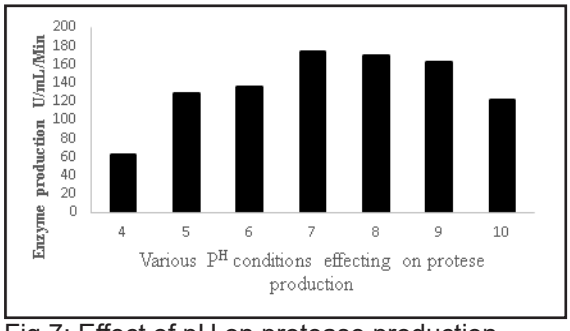

Fig.7: Effect of pH on protease production

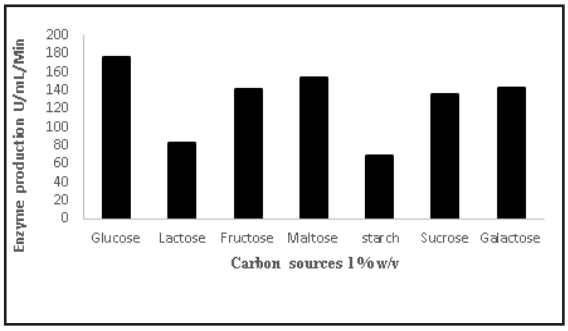

Fig.8 Effect of carbon source with 1% w/v size on protease production

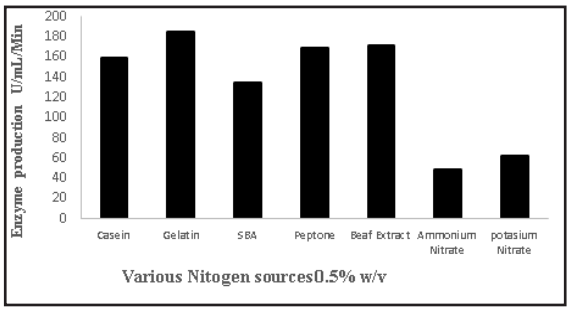

Fig.9 Effect Nitrogen sources on protease production

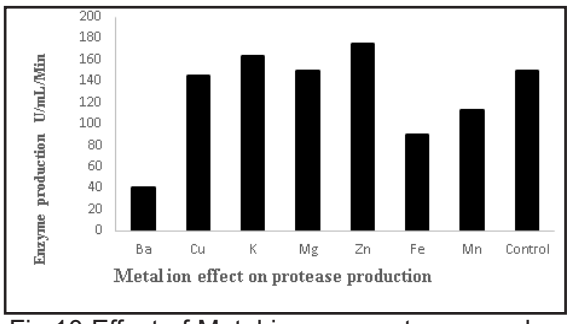

Fig.10 Effect of Metal ions on protease production

Conclusion

Isolation identification of Efficient Proteases are always plays crucial role in industrial enzymatic pool, our present study, Protease positive *Burkholderia Cenocepacia* was isolated from soil was optimised protease production temperature30oC, PH7, 3% w/v inoculum 1%v/v glucose and 0.5% gelatine is more suitable condition to produce protease. Highest

protease was recorded as 185 U/mL/Min under presence of Gelatine, 3% inoculum and metal ions like Zink and calcium. As Bks protease has more applications in industry, hence protease production from *Bks* is recommended.

Acknowledgement

The authors wish to acknowledge Department of microbiology for their laboratory support. The authors are grateful to Prof. Laxmikanth rathod, Vice-Chancellor for his encouragement and support.

References

1. N. Bhaskar, E.S. Sudeepa , H.N. Rashmi , A. Tamil Selvi (2007) "Partial purification and characterization of protease of Bacillus proteolyticus CFR3001 isolated from fish processing waste and its antibacterial activities. "Bioresource technology" Volume 98, Issue 14.

2. Gupta, R., Beg, Q.K., Lorenz, P. (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. "Appl. Microbiol. Biotechnol". 59: 15 32.

3. 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 etschnikovii J1. "J. Ind. Microbiol. Biotechnol". 36: 939-948.

4. Sareen, R., Mishra, P. (2008). Purification and characterization of organic solvent stable protease from Bacillus licheniformis RSP-09 37. "Appl. Microbiol. Biotechnol". 79: 399.

5. Katsuhisa Saeki, Katsuya Ozaki, Tohru Kobayashi (2007) Susumu Its Detergent alkaline proteases: enzymatic properties, genes, and crystal structures, "Journal of biosciences and bioengineering". June;103(6)

6. Maurer, K. Detergent proteases. Current Opinion in Biotechnology, (2004) 15, 330-334.

7. Maria de Fátima Barros Leal Nörnberg, Marilene Lenz Mentges, Silvana Terra Silveira, Eduardo César Tondo and Adriano Brandelli (2011). A psychrotrophic Burkholderia cepacia strain isolated from refrigerated raw milk showing proteolytic activity and adhesion to stainless steel. Journal of Dairy Research 78 257–262.

8. Dipasquale L., Calandrelli V., Romano I., Nicolaus B., Gambacorta A., Lama L. (2008). Purification and characterisation of a highly thermostable extracellular protease from Bacillus thermantarcticus, strain M1. "Ann. Microbiol". 58 (2) 253-259.

9. Yewande Suberu a b, Idowu Akande a, Titilola Samuel a, Adekunle Lawal b, Ademola Olaniran (2003). Cloning, expression, purification and characterisation of serine alkaline protease from Bacillus subtilis RD7. "Biocatalysis and Agricultural Biotechnology" Volume 20, July 2019, 101264.Syst Biol . Oct;52(5):696-704.

10. R.C.S. Thys, F.S. Lucas, A. Riffel, P. Heeb, A. Brandelli (2004). Characterization of a protease of a feather-degrading Microbacterium species Get access Arrow. Letters in Applied Microbiology, Volume 39, Issue 2, 1 August 2004, Pages 181–186.

11. Lane, D. J. (1991). 16S/23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematics, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

12. Stéphane Guindon, Olivier Gascuel (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Bi. 2003 Oct;52(5):696-704.

13. Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4, 406–425.

14. Felsenstein, J. (1993). PHYLIP (phylogeny inference package). 3.5.1. University of Washington, Seattle, USA: Department of Genome Sciences.

15. . Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16, 111–120.