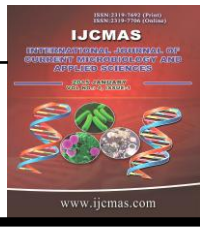
Int.J.Curr.Microbiol.App.Sci (2015) 4(1): 983-988

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 1 (2015) pp. 983-988**
http://www.ijcmas.com



Original Research Article

Studies on the potent protease producing bacteria from soil samples

M.Ravi, Krishna Rayudu, Vivek Kulkarni Subhaschandra M Gaddad, and Y.M.Jayaraj*

Department of PG Studies and Research in Microbiology Gulbarga University, Gulbarga- 585106, Karnataka, India
**Corresponding author*

Keywords

Protease,
Soil sample,
16s rRNA,
Bacillus subtilis

A B S T R A C T

A protease producing bacterial strain JRK-3 was isolated from soil sample and was identified by morphological, cultural and biochemical characters as well by 16s rRNA sequencing as *Bacillus subtilis*. On Skimmed milk agar (Screening Media), the said isolate exhibited a protease activity (a zone of 36 mm). It produced good quantity of protease in production medium (54 U/ml). The extent of protease activity was comparable to available literature. Hence the isolate has industrial potential.

Introduction

Proteases (EC 3.4.21-24 and 99; peptidyl-peptide hydrolases) are enzymes that hydrolyse proteins via the addition of water across peptide bonds and catalyze peptide synthesis in organic solvents and in solvents with low water content (Sookkheo et al.,2000; Beg et al., 2003). They constitute a very large and complex group of enzymes with different specific properties of (substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles). Proteases occupy a central position in commerce and industry, accounting for nearly 65% of the global enzyme market. They are used extensively in the detergent, leather, pharmaceutical, and food industries. Industrial proteases have application in a range of process taking advantage of the unique physical and catalytic properties of individual proteolytic enzyme types (Ward, 1991). Proteases of commercial importance are produced from microbial, animal and plant sources. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey and West, 1996). Microorganisms represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods producing an abundant, regular supply of the desired pro duct. Besides they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Rao et al., 1998, Gupta et al., 2002). Microbial

proteases, especially from *Bacillus* Sp. have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergent formulations (Beg et al., 2003).

Keeping in mind the vast application of protease in different industries, an attempt has been made to isolate protease producing novel bacterial strains from agro based wastes.

Materials and Methods

All the chemicals used were of analytical grade. Soil samples collected from different locations were analyzed for their proteolytic activity. One gram of the soil sample was weighed and added to the enrichment media at 120 rpm at 40^0 c in rotary shaker incubator for 24 hrs. Further Nutrient agar media were inoculated with 0.1 ml of enrichment culture and incubated at 40 ^{O}c. The prominent isolates were further streaked on screening media, Skimmed milk agar containing (0.5% Skimmed milk, Beef Extract 0.3%, NaCl 0.5% and 1,8% agar) . The isolates which showed maximum halo zone around the colony on the plate were selected. Isolated bacterial strains were identified by morphological, cultural and biochemical tests. Molecular level of identification (16s rRNA sequencing) was carried out at NCCS Pune.

Colony characteristics were recorded. Morphological studies were based on Gram staining, Spore staining and Phase Contrast Microscopy. Biochemical tests include Catalase, IMVIC, Nitrate Reduction, Citrate Utilization, Oxidase and Sugar fermentation tests as per standard methods using Hi Carbo Kit (KB-009). Based on these tests, tentative identification of isolate was recorded.

Molecular level of identification (16s rRna sequencing) was carried out at NCCS Pune The genomic DNA was isolated as described by (Ausubel *et al* 1987). The PCR assay was performed using Applied Bio-systems, model 9800. The sample was sequenced using a 96-well Applied Bio-systems sequencing plate as per the manufacturer's instructions. The obtained sequences of bacterial 16S rDNA (using 704 F and 907 R primers) were analyzed using Sequence Scanner (Applied Bio-systems) software. The 16 S rDNA sequence contigs were generated using Chromas Pro and then analyzed using online databases viz. NCBI-BLAST and Ribosomal Database Project (RDP) to find the closest match of the contig sequence. Phylogenetic tree was constructed using MEGA 4 software using neighbor joining method. The genomic sequences were deposited in NCBI gen bank and accession numbers were obtained.

Production of protease and its estimation

The potential strains selected through screening methods were tested for their protease production quantitatively in the production medium after enrichment in basal media (Tryptone- 10 g, Yeast extract 5 g, NaCl , 5 g, Glucose -5g , in 1000 ml distilled water).

The medium with 0.1 ml of fresh bacterial suspension of the selected isolate was maintained at the agitation rate 180 RPM for 24 hr at pH 7.5. From overnight grown culture in basal medium, 0.1 ml of sample was inoculated to 50 ml of production media (Tryptone 10 g, Yeast extract 5 g**,** NaCl 5 g, Glucose-0.05 g in 1000 ml distilled water, (pH 7.5)). The inoculated production medium was incubated at 40^0 C at 180 RPM for 48 hrs in a rotary shaker incubator. The 24 hr incubated production medium was centrifuged at 10,000 rpm for

15 min at 4^0 C .2 ml of the culture supernatant was used for testing the protease activity.

Further Quantification of the protease was determined by spectro-photometric method as described by (Ferrero et al, 1996). The amount of protease produced is measured by the amount of tyrosine liberated.

Enzyme assay

Alkaline protease activity was assayed by the modified method of Anson (1938) in the extracellular fraction (supernatant of 8000 rpm for 8 min at 4^0 C) using casein (500 µl) as the substrate dissolved in 300µl (Tris buffer 0.02M) pH -8.0. The above reaction mixture was incubated in hot water bath for 10 min at 40^0 C, and reaction stopped by adding 1 ml of 10% trichloroacetic acid (chilled) for 20 min. The mixture was further centrifuged at 8000 rpm for 10 min. Collect the culture filter supernatant (0.5 ml) and add 2.5 ml 0.5 M sodium bicarbonate solution and 0.5 ml threefold-diluted Folin-Ciocalteau reagent. After the reaction mixture had stood for 30 min at room temperature, the absorbance was measured at 660 nm. One protease unit was defined as the amount of enzyme required to produce 1 µg of tyrosine in 10 min at 40^0C Anson (1938) & F C R method (1929).

Calculation

$$U/ml = \frac{\text{µg of tyrosine released } \mathbf{x} \text{ Total vol of assy}}{\text{Vol of enzyme used x Time of assay } \mathbf{x} \text{ Vol used in colour development}}$$

Result and Discussion

Number of isolates were compared for their efficiency to produce protease enzyme as ordered by zone of clearance on Skimmed milk agar .Based on the higher zone of clearance, 4 isolates were selected for further investigations, (JRK-3, JRK-13, JRK-12, and JKR-1) .They were identified both by conventional and molecular methods On further testing for their protease enzyme productivity (quantitative), JRK-3 isolate was found to produce maximum quantity of protease. The details are given in Table 1 and Figure 1. The phylogenetic tree is given in Figure 2. In present study, JRK 3 strain identified as *Bacillus subtilis* strain JRMRK 9 ehibited maximum protease production. It was capable of producing 54 u/ml, which is accordance with the trend of many reports.

Table.1 Details of isolates and their protease activity

Sl No	Isolate code	Base pairs	NCBI Accession Number	Organisms identified	Zone of clearance on screening media	Maximum Protease productivity
1	JRK-3	1129	JF326509	*Bacillus subtilis*	36 mm	**54 U/ml**
2	JRK13	897	JF326519	*Acinetobacter radioresistens*	22 mm	40 U/ml
3	JRK12	346	JF326518	*Bacillus thuringiensis*	26 mm	45 U/ml
4	JKR-1	1297	HQ671068	*Bacillus cereus*	20 mm	36 U/ml

Table.2 Observation of researchers on protease production are as follows

Author	Organism	Zone of clearance (in mm)	Protease productivity
Devinder Kaur.Abhay Kumar Pandey 2009	*Bacillus subtilis*	ND	5.70 U/ml
Mohamed M I Halal et al 2012	*Bacillus subtilis*	14 mm	35.00 U/ml
Ferraro et al 1996	*Bacillus licheniformis MIR 29*	ND	76.37 U/ml
Vidhyasagar et al 2006	*Halogeometricum sp,s TSS 101*	ND	62 U/ml
Krishna Rayudu et al 2011	*Bacillus sp KER-17*	ND	208. 37 U/ml
Present study	*Bacillus subtilis strain JRMRK9*	36 mm	54 U/ml
	Acinetobacter radioresistens strain JRMRK 19	22 mm	40 U/ml
	Bacillus thuringiensis JRMRK18	26 mm	45 U/ml
	Bacillus cereus JRK1	20 mm	36 U/ml

Fig.1 The enzyme activity of the selected isolates

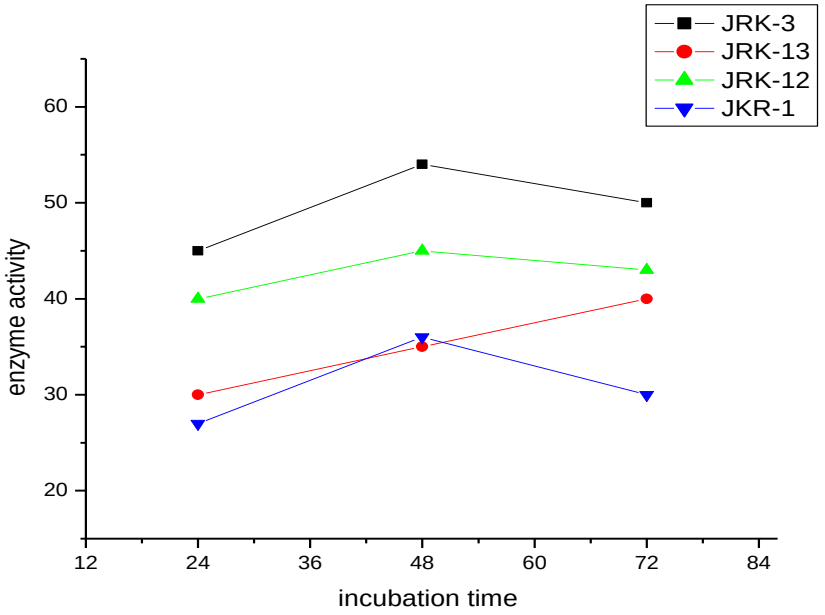
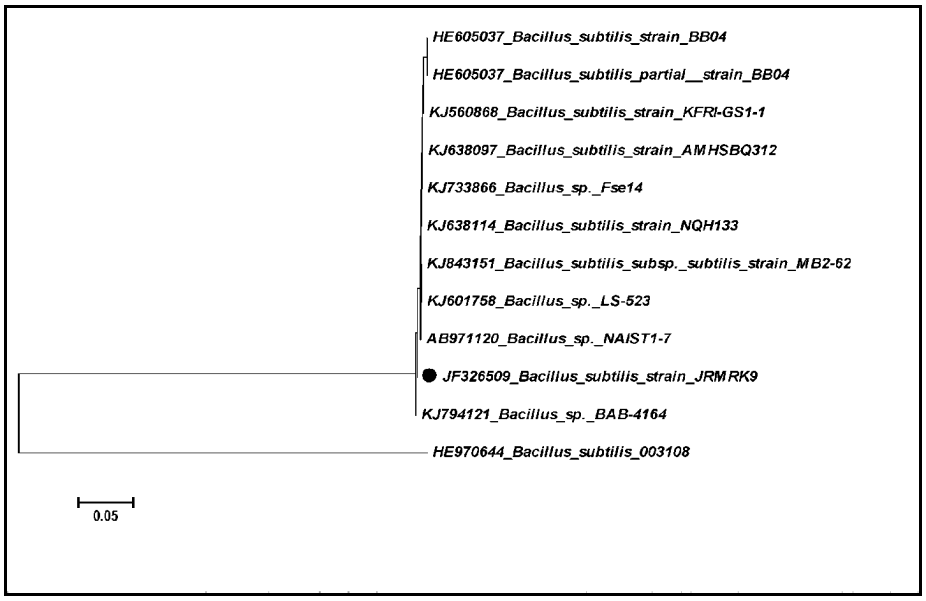


Fig.2 The phylogenetic relationship of ***Bacillus subtilis*** **JRMRK9**- with other *bacillus* species



References

Anson M L (1938) ,Estimation of Pepsin Papain and Cathepsin with Heamoglobin J Gen Physiol 22,79-89

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987). Current protocols in Molecular Biology. Green Publishing Associates and Waley-Intersciences, New York. Loose – leaf binder.

Beg Q.K, Sahai V, Gupta R, (2003) Statistical media optimization and alkaline protease Production from *Bacillus mojavensis* in a bioreactor Process Biochem, 39, 203

Devinder Kaur Abhay kumar Pandy (2009) Partial Characterization of Bacterial Protease International Journal of Parma and Recent Research 1 (1) ,12-17

Godfrey T. and West S,(1996) Industrial Enzymology, Macmillan Press, London p. 3

Gupta R, Beg Q.K, Khan S, Chauhan B, (2002) An overview on fermentation, downstream Processing and properties of microbial alkaline proteases Appl Microbiol And Biotechnol, 60, 381

Jukes, T. H .and Cantor, C.R: (1969), Evolution of protein molecules., In: Munro HN (ed) mammalian protein metabolism, Vol 3 Academic press New York, pp 21-32

Krishna Rayudu ,Y M Jayaraj ,Ravi M , K S Shabeena Anjum (2011) Isolation Identification and Characterization of A Novel feather Degarading *Bacillus* species *Ker 17* strain Asian Journal of Biochemical and Pharmaceutical Research 4(1) 74-83

Lowry OH, Rosebrough NJ, Farr AF, Randall, RJ (1951). Protein measurement with the Folin Phenol Reagent, Journal of Biological chemistry. 193, 265-275

M A Ferrero., G R Castro., C M Abate., M D Baigori F Sineriz (1996) Thermostable alkaline protease of Bacillus Licheniformis MIR: 29 Isolation,Production and

characterization Appl Microbiol Biotechnol 45:327-332

Mohemad M I Helal ,Hasan Amer Nayera A M Abdelwahed and Madeha O I Ghobashy (2012) Physiological and Microbiological Studies on Production of Alkaline Protease from Locally Isolated Bacillus Subtilis Australian Journal of Basic and Applied Sciences 6 (3) :193-203

Rao MB, Tanksale AM, Ghatge MS, Deshpande V (1998), Molecular and biotechnology aspects of microbial proteases. Microbiol Mol Biol Rev 62: 597–635

Sookkheo B., Sinchaikul S, Phutrakul S, Chen S, (2000), Purification and characterization of the highly thermostable proteases from Bacillus stearothermophilus TLS33 Protein Expression and Purification, 20,, p 142

Tamura K Kumar S ., Nei ., M Dudley ., J (.2004) Mega 4 ; molecular evolutionary genetics analysis mega software version 4.0. Mol. Bio . Evol., 24 (8), 1596-1599

Vidyasagar, M., S. B. Prakash, and K. Sreeramulu (2006) Optimization of culture conditions for the production of haloalkaliphilicthermostable protease from an extremely halophilicarchaeon Halogeometricum sp. TSS101. Lett. Appl. Microbiol.43: 385-391.

Ward O. P, (1991), Enzyme Bioprocessing Applications In Bioprocessing (Open University Press, Buckingham, pp. 151-170

Wikstrm MB, Elwing H, Linde A (1981) Determination of proteolytic activity: a sensitive and simple assay utilizing substrates adsorbed to a plastic surface and radial diffusion in gel. Anal Biochem 118: p 240–246.