Biological Forum – An International Journal **15(6): 306-310(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Alkaline Proteases Producing *Bacillus* species Isolated from River (Aram) Side Soil

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(Received: 23 March 2023; Revised: 28 April 2023; Accepted: 14 May 2023; Published: 20 June 2023)
(Published by Research Trend)

ABSTRACT: In the current work, bacteria having the capacity to synthesise extracellular proteases were screened out. From rhizosphere soil and soil samples taken from the nearby riverfront in Satana, Dist. Nashik, Maharashtra, an effort was undertaken in order to obtain protease-producing bacterial strains. On a skim milk agar plate, ten strains showed zones of clearance around colonies. The isolates were biochemically characterised by tests such as indole, methyl red, Vogues Proskauer, citrate utilization, detection of enzyme production assays for amylase, oxidase, urease, catalase, and the capacity to hydrolyse starch. Because of protein hydrolysis, isolate RDV 01 had the greatest zone of clearance. The enzyme produced was an extracellular, alkaline protease, according to the characterization. The isolate RDV 01 was identified on the basis of morphological, biochemical characteristics, 16S rRNA sequencing, and a phylogenetic tree. After studying the morphological and biochemical characters, the isolated organism was identified as a member of the genus *Bacillus,* and NCBI blast and phylogenetic analysis confirmed that the isolate RDV 01 may be related to species JSG1 with 100% homology as revealed. Industrially important alkaline proteases production from bacterial sources has been evaluated in this study.

Keywords: Alkaline proteases, *Bacillus,* Skim milk agar, Proteolysis.

INTRODUCTION

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions in the cells (*Masi et al.,* 2021). Many different enzymes have been identified, and some of them are being used in biotechnological and industrial applications. Microorganisms are regarded as the principal source of intracellular and extracellular enzymes for industrial applications (Kocher and Mishra 2008; Pant *et al.,* 2015). Proteases are a large group of hydrolytic enzymes that catalyse the hydrolysis of the protein by cleaving the peptide bonds (Rao *et al.,* 1998; Khan *et al*., 2011; Masi *et al.,* 2014). Proteolytic enzymes are universal in occurrence, present in all living organisms, and essential for cell growth, cell signalling, metabolism, differentiation, and other physiological purposes (Rao *et al.,* 1998; Banerjee and Ray 2017). Furthermore, protease enzymes are commercially synthesised from plants, animals, and microbes (Masi *et al.,* 2014; Khan *et al*., 2011). Microorganisms are a fascinating source of alkaline proteases because they may be grown in substantial amounts in a short amount of time and have potential economic uses (Varia *et al.,* 2019; Aguilar dos *et al.* 2019; Asker *et al*., 2013; Moon *et al*., 2015; Khan *et al*., 2011; Masi *et al.,* 2021). However, most of the protease producing candidates exploited at the industrial level are *Bacillus* sp. rather than other group of bacteria (Kocher and Mishra 2008; Ferrari *et al.,* 1993; Pant 2015; Doddapaneni *et al.,* 2009; Vijayaraghavan *et al.,* 2014; Pant *et al*., 2015; Varia *et al*., 2019). Over-all, extracellular proteases have recompenses over intracellular one in term of cost and availability (Varia *et al.,* 2019; Asker *et al.,* 2013). The demand proteolytic enzymes with specificity and stability over a wide range of temperature, pH with optimum activity continues demand for the search of newer source (Sharma *et al.,* 2017; Kocher and Mishra 2008; Doddapaneni *et al.,* 2009; Rajkumar *et al.,* 2011). Proteases from microbial source have been used in various industries such as food, brewing, pharmaceutical, leather, silk and detergent etc. (Kocher and Mishra 2008; Masi *et al.,* 2014; Varia *et al.,* 2019**;** Sathyavrathan and Kavitha 2013; Das and Prasad 2010; Wajeeha *et al.,* 2021). The present investigation was conducted to isolate potential bacterial alkaline proteases from various sources. This study deals with isolation and identification of potential alkaline producer from local river (Aram) side soil of Satana of Nashik district, Maharashtra.

MATERIALS AND METHODS

Screening and isolation of protease producers. The rhizosphere soil and river (Aram) side soil samples were collected from Satana in Nashik district. The collected samples were preserved in sterile containers for further study. For the screening of alkaline protease-producing organisms, serial dilution method was used. The skim milk agar (Das and Prasad 2010; Cui *et al.,* 2015; Rejisha and Murugan 2021; Masi *et al.,* 2021; Chu 2007) containing 28.0 g/L skim milk powder, 2.5

g/L of yeast extract, 5.0 g/L of tryptone, 1.0 g/L of glucose and agar 15.0 g/L at pH 8.5 was used (Masi *et al.,* 2021)**.** The samples were initially swirled in a sterile saline solution. Soil samples were diluted with sterile saline by the serial dilution method up to 10^{-9} (Masi *et al.,* 2021). Then, 0.1 mL of the final few dilutions were aseptically transferred to sterile skim milk agar plates and spread over them. All the plates were incubated at ambient temperature for 24 hours. After incubation, clear zones of hydrolysis of protein were observed on the plates. Colonies showing clear zones of hydrolysis of protein were separated and grown in skim milk broth tubes of pH 8.5 at ambient temperature for 24 hours on orbital shaker. Morphologically distinct colonies were purified and the pure cultures of the isolates numbered RDV 01 to RDV 10 were streaked on nutrient agar slants and preserved at low temperature for further study.

Identification of alkaline proteases producing isolate. Individual colonies were screened out that exhibited zones of clearance due to degradation of proteins. Isolate showing a higher zone of alkaline protease activity was characterised and identified by studying growth, morphological, and biochemical characters. The biochemical characterization of isolates was conducted by performing tests such as Indole, Methyl Red, Vogues Proskauer, citrate utilisation, and sugar utilisation tests. The detection of enzyme production such as oxidase, urease, and catalase and the ability to hydrolyse starch and gelatine (Bergey and Holt 2000; Masi *et al.,* 2021). Finally, RDV 01, an alkaline protease producing organism, was identified by 16S rRNA sequencing and phylogenetic analysis. DNA was isolated from cells of RDV 01. The 16S rRNA gene was amplified by PCR from the isolated DNA of RDV 01. The 16S forward universal AGA GTT TGA TCC TGG CTC AG and 16S reverse universal ACG GCT ACC TTG TTA CGA CTT primers were used for the amplification. The amplified PCR product was electrophoresed and visualised by staining. PCR product was purified by washing with the sodium acetate and 70% ethanol. Then forward and reverse DNA sequencing reactions of PCR amplicons were carried out on an ABI 3730xl genetic analyser to obtain the 16S rRNA sequences (Rathod and Pathak 2014). The DNA sequences were used to carry out BLAST searches with the NCBI gene bank database.

RESULTS AND DISCUSSION

Isolation and screening of bacterial strains. Individual colonies exhibiting a zone of protein hydrolysis around the colony were aseptically separated and inoculated into skim milk broth tubes individually at an alkaline pH for further enrichment. A total of ten alkaline protease producing isolates were screened out, and all were bacterial isolates. Finally, the pure cultures of isolates numbered RDV 01 to RDV 10 and were streaked on nutrient agar slants and preserved at low temperature.

Analysis of proteolytic activity. Isolates that exhibited zones of protein hydrolysis during primary screening were grown in liquid medium containing skim milk broth at pH 8 at ambient temperature for 24 hours. An aliquot of all isolated organisms was inoculated on skim milk agar plates. After 24 hours of incubation at room temperature, isolate RDV 01 had the biggest diameter of the zone of proteolysis (25 and 26 mm) (as measured in replicates), as well as an increase in zone diameter (30 and 31 mm) due to additional proteolysis after 48 hours (Fig. 1). Major reports regarding alkaline protease production from bacterial sources, principally species of the genus *Bacillus,* state their industrial suitability and advantageousness at the commercial level. A significant alkaline protease producer, the *Bacillus cereus* strain, was isolated from leather industry effluent (Masi *et al.,* 2021). It was reported that strain P5 may be *Bacillus subtilis* and produce active proteases at pH 8 when isolated from soil (Das and Prasad 2010). Alkaline proteases were produced by *Bacillus subtilis,* as reported by Pant *et al.* (2015). *Bacillus subtilis* and *Pseudomonas aeruginosa* were isolated from dairy effluents; their ability to produce alkaline proteases was also reported Masi *et al.* (2014); Aguilar Dos *et al*. (2019) stated that *B. licheniformis* LBA 46 produced proteases that exhibited optimum activity between 50 and 60°C at an alkaline pH. According to Wajeeha *et al.* (2021) alkaline proteases from *Aspergillus flavus* displayed the retention of maximum activity at pH 7 to 10.5. Zhou *et al.* (2019) successfully improved a strain of *B. licheniformis* using genetic engineering for the production of alkaline proteases. Isolate was also exhibited amylases production successfully (Vasait and Jobanputra 2015). Hence, RDV-01 was screened out and morphological, biochemical, and genetic characterization performed.

Identification of Isolate. The isolated organism was mesophilic, Gram-positive, rod-shaped, and aerobic in nature. The morphological and biochemical characteristics of isolated organism RDV 01 are depicted in Tables 1 and 2, respectively. The detection of enzyme production was assessed (Fig. 2). RDV 01 exhibited the production of oxidase by the formation of a purple colour when growth was placed aseptically on an oxidase disc (Fig. 2a). The generation of oxygen bubbles when RDV 01 cells were put in a 6% H_2O_2 solution verified the production of the catalase enzyme (Fig. 2b). Amylase production was also measured by cultivating an isolate on starch agar following spot inoculation. After 24 hours of incubation at room temperature, the medium became a deep blue to brown hue after being flooded with Gram's iodine solution (Fig. 2c). A zone of clearing was seen around the growth of isolate RDV 01 owing to starch hydrolysis, revealing the synthesis of amylase enzymes. Spot inoculating RDV 01 on nutrient agar containing gelatine also resulted in a production of gelatinase enzyme. After 24 hours of room temperature incubation, the plate was inundated with an acidified mercuric chloride solution. Because of the mercuric chloride, protein gelatine precipitated, resulting in a hazy look. Because of gelatine hydrolysis, a clear zone formed around the growth (Fig. 2d). Morphological and biochemical characterization of isolate RDV 01 revealed that the characteristics studied were similar to

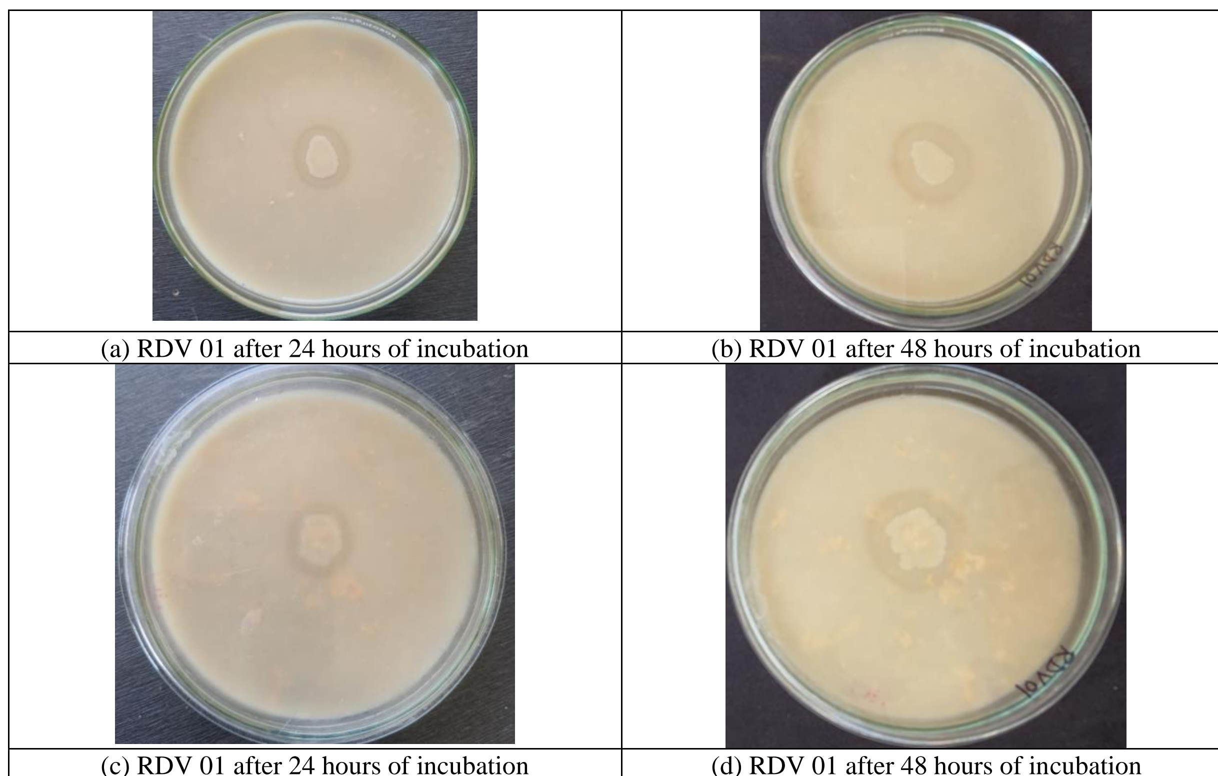
those of species in the genus *Bacillus*. Further, the isolate was identified by 16S rRNA sequencing and phylogenetic analysis. The 16S rRNA gene was amplified by PCR from the isolated DNA of RDV 01. The amplified PCR product was visualised by staining with ethidium bromide. A single discrete PCR amplicon band was observed. The 16S rRNA sequences were obtained, deposited in NCBI GenBank, and assigned the accession number OQ357876. The 16S rRNA sequences were selected based on maximum identity score; the first eight sequences for RDV 01 were selected and aligned using the multiple alignment software programme Clustal W (Doddapaneni *et al*., 2009), and the phylogenetic tree was constructed (Fig. 3). From 16S rRNA sequences, BLAST analysis showed 100% homology with *Bacillus sp.* JSG1, and phylogenetic analysis confirmed that the isolate RDV 01 might belong to the genus *Bacillus* and may be related to species JSG1 Vasait & Jobanputra (2015).

Table 1: Morphological Characters of isolate RDV 01.

Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram character	Motility
3 mm	Irregular	white	Entire	Low convex	Translucent	Sticky	Gram positive	Motile

Table 2: Biochemical characteristics of isolate RDV 01.

Parameter	Results
Capsule production	+
Enzyme Production	
Catalase	+
Protease	+
Oxidase	+
Urease	-
Starch hydrolysis	+
Gelatine hydrolysis	+
Indole Production	-
Methyl Red Test	+
Voges Proskauer	-
Citrate utilization	-
Pigment production	-
Sugar Utilization	
Glucose	+
Lactose	-
Maltose	+
Sucrose	-
Fructose	+
Xylose	-



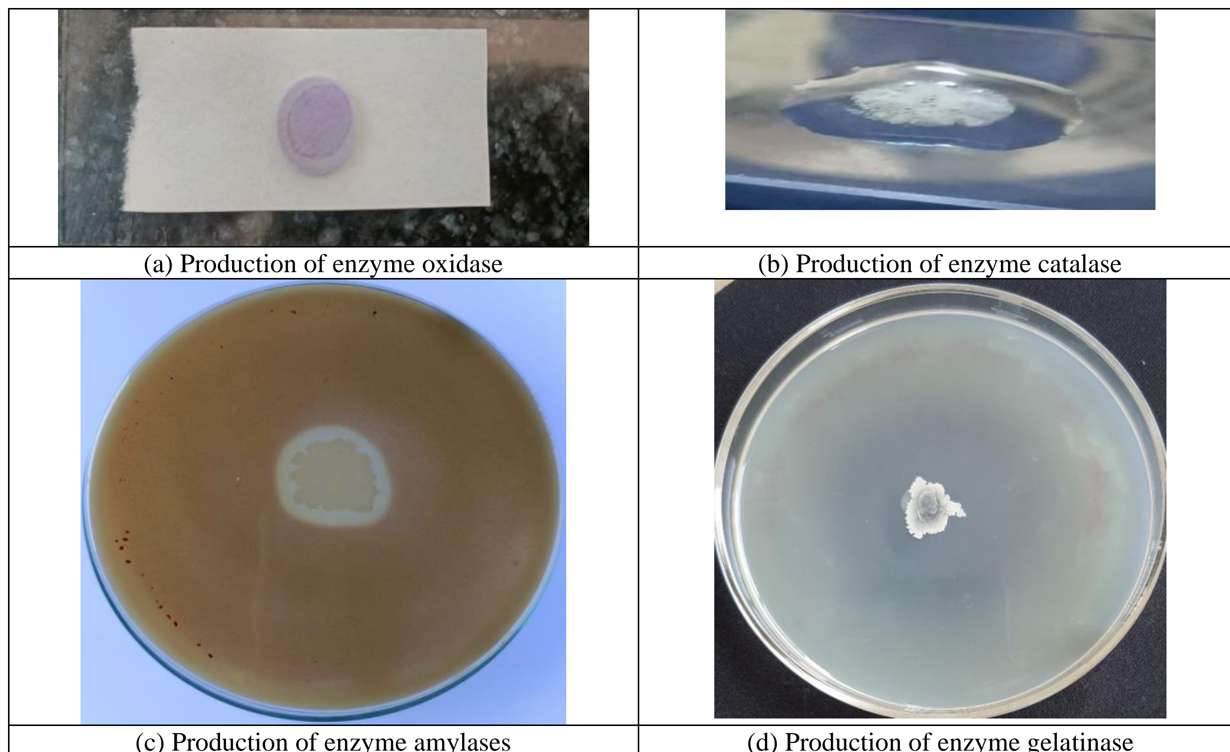
(a) RDV 01 after 24 hours of incubation

(b) RDV 01 after 48 hours of incubation

(c) RDV 01 after 24 hours of incubation

(d) RDV 01 after 48 hours of incubation

Fig. 1. Isolate RDV 01 exhibited zone of proteolysis, after (a) 24 hours and (b) 48 hours of incubation.



(a) Production of enzyme oxidase | (b) Production of enzyme catalase | (c) Production of enzyme amylases | (d) Production of enzyme gelatinase

Fig. 2. RDV 01 exhibited production of enzymes, (a) Oxidase, (b) Catalase, (c) Amylase, and (d) Gelatinase.

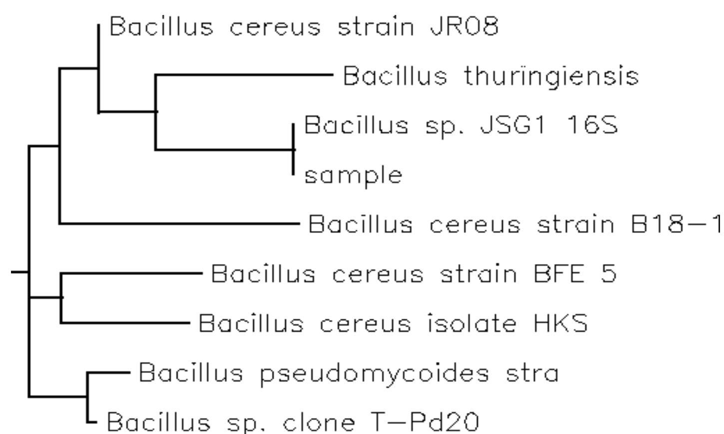


Fig. 3. Phylogenetic tree of RDV 01.

CONCLUSIONS

In this present study, 10 bacterial isolates were examined for the zone of clearance around colonies on skim milk agar plates. The bacterial isolate RDV 01 exhibited a considerably maximum zone of hydrolysis of protein. The *Bacillus* species RDV 01 was assessed for its ability to produce proteolytic enzymes and be active in an alkaline environment. In the present study, we successfully isolated and identified *Bacillus* strain RDV 01 producing alkaline proteases, which may prove their applicability for various purposes after extensive study.

FUTURE SCOPE

The potential use of the *Bacillus* strain RDV 01 as a biotechnological tool may prove excellent enzyme source for industrial relevance.

Acknowledgment. The author is thankful to the Principal and Head of the Departments of Microbiology, KAANM Sonawane ASC College, Satana, for providing laboratory facilities to carry out the of research work and for their inspiring help.

Conflict of Interest. None.

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How to cite this article: Rajendrabhai Daulatbhai Vasait (2023). Alkaline Proteases Producing *Bacillus* species isolated from River (Aram) Side Soil. *Biological Forum – An International Journal, 15*(6): 306-310.