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COMPARATIVE STUDY ON THE PRODUCTION, PURIFICATION AND IMMOBILIZATION OF ALKALINE PROTEASE FROM *Bacillus* SPECIES PRESENT IN SOIL RHIZOSPHERE

B.S. Ramya Kumari*, V. Shashank, S. Nitesh Gowda, H.N. Sahana and M. Deepthi

Department of Biochemistry, M.S. Ramaiah College of Arts, Science and Commerce, Bengaluru – 560 054, Karnataka (India)
*****e-mail: *msrramyakumaribs@gmail.com*

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ABSTRACT

Alkaline proteases have high commercial value as these are used in a variety of industrial sectors. In present study alkaline protease was isolated from several *Bacillus* species isolated from the rhizosphere of *Poaceae* plants and then purified. On the basis of morpho-biochemical characteristics, five *Bacillus* species *viz., B. amyloliquefaciens, B. licheniformis, B. pumilis, B. cereus,* and *B. subtilis* were identified. The maximum protease activity was recorded in 1-5 dilution. *B. licheniformis* exhibited stable activity of 1.09 ± 0.09 U mg^{-1}. Dialysis and ammonium sulphate precipitation methods were used for partial purification. At each stage of purification, protease assay, protein concentration, and specific activity were determined. Among the isolated *Bacillus* species, *B. licheniformis* showed a sustained specific activity of 0.47 ± 0.02 mol^{-1} min^{-1} mg^{-1} in crude form, which rose to 0.71 ± 0.02 mol^{-1} min^{-1} mg^{-1} following salt precipitation, and reached a high level of 1.02 ± 0.08 mol^{-1} min^{-1} mg^{-1} after dialysis. The physical immobilization of proteases in sodium alginate showed satisfactory activity and stability for *B. licheniformis* (1.4 ± 0.2 U mg^{-1}). *B. licheniformis* was sequenced and its phylogenetic tree constructed. The bacterium shared common ancestor with other five distinct *Bacillus* species. The study revealed that *B. licheniformis* has potential alkaline proteases production ability.

Keywords: *Bacillus* species, extracellular protease, immobilization, phylogenetic analysis, purification

INTRODUCTION

Enzymes are biocatalysts synthesized by living cells and catalyse various biochemical reactions during metabolic activities. In the world of biotechnology, enzymes like proteases are replacing the chemical methods for producing products that are ecologically sustainable. Proteases catalyse the release of amino acids and/or peptides from hydrolytic bonds in proteins (Adinarayana *et al.*, 2003). Proteases are sub-class of hydrolytic enzymes that catalyse the hydrolysis of peptide bonds in proteinase substrates (Sony *et al.*, 2016). They are one of the major classes of enzymes which account for 60% of enzyme market (Razzaq *et al.*, 2019).

The protease enzymes are involved in various commercial processes including baking, brewing, waste treatment, leather processing, detergent formulations, meat tenderization, peptide synthesis, cheese manufacturing, protein hydrolysis, soy sauce production, pharmaceutical industry, silk industry, organic synthesis, silver recovery from damaged photographic film, analytical instruments in fundamental research, and has substantial economic value (Anwar *et al.*, 2009). The majority of world's protease are extracted from microbes, which account for over two thirds of the market

(Folasade *et al.*, 2005; Razzaq *et al.*, 2019). As compared to the animal and fungal proteases, the bacterial proteases are now in high demand due to their low cost, fast enzyme production, and vast potential for creating high yielding genetically modified strains (Souza *et al.*, 2015). Many *Bacillus* species produce extracellular alkaline proteases (Theron *et al*., 2014). *Bacillus* species are G +ve, aerobic, rod-shaped endospore-forming bacteria, commonly found in soil. They produce industrial enzymes like proteases, amylases, and other macromolecular hydrolases prolifically (Susanne *et al.*, 2010). Some *Bacillus* species have the ability to decompose and use organic debris, thereby serve as essential nutritional sources (El-Gendi *et al.*, 2022).

The stability and reusability of biocatalysts have been a major concern in the development of enzyme immobilization technologies (Homaei *et al*., 2013). Immobilization encompasses a range of cell or particle attachment or entrapment in spherical beads (Willaert, 2006). It can be used with almost any kind of biocatalyst, including enzymes, cellular organelles, and animal and plant cells. A variety of immobilization methods are employed these days in biotechnology, environmental, pharmaceutical, food, and biosensor sectors (Hassan *et al.*, 2019). The most frequent use of industrial enzymes, both in volume and monetary terms, is as detergent additive. All detergents employ the same detergent mechanisms and composed of the same ingredients. These days, one or more enzymes are routinely included in automatic dishwashing detergents and heavy-duty powder detergents to increase detergency. Immobilization is often necessary for effective performance in non-aqueous mediums. In traditional method of using enzymes as lyophilized (freeze-dried) powders, many enzyme molecules are not readily accessible to substrate molecules (Naganthran *et al.*, 2017). Enabling the use of enzymes under different conditions, such as chemical solvents, pH, temperature, and extremely high substrate concentrations, might be beneficial (Homaei *et al.*, 2013).

The traditional method of immobilization is covalent binding, which involves the making a direct chemical link between substance and enzyme. Covalent binding entails the creation of a direct chemical connection between drug and enzyme, and is a conventional immobilization. Alginate are naturally occurring polysaccharides synthesized by soil bacteria and brown seaweed (Datta *et al.*, 2013). Sodium alginate, the initial by-product of algal purification, is most frequently used in industries. In addition to segments of alternating guluronic and mannuronic acids, sodium alginate contains L-guluronic acid residues (G blocks) and ß-D-mannuronic acid residues (M blocks) [Draget *et al*., 2001; Nisha *et al*., 2012). In present study, an extracellular protease was isolated and purified from a *Bacillus* species from rhizosphere soil. Dialysis and ammonium salt precipitation were carried out throughout purification procedure. At each stage, protease activity and protein concentration have been determined, which improved the enzymes' specific activity and made them specific for commercial application. Enhancement of protease immobilisation has gained significant importance in biocatalysis. By using entrapment approach, purified protease was immobilised in sodium alginate solution, and its properties of enzyme immobilization, including activity and stability, were examined. The present study was aimed to isolate promising *Bacillus* isolate showing sustained alkaline protease enzyme production activity and construct its phylogenetic tree, so that the species could be exploited for commercial purpose.

MATERIALS AND METHODS

Isolation and screening of protease producing bacteria

The rhizosphere soil samples were taken from *Poaceae* plants from Gandhi Krishi Vigyana Kendra, Bengaluru, Karnataka (India). The samples were diluted ten times, and 0.1 mL of each dilution plated on gelatin agar. The plates were incubated at 37°C for 48 h and then flooded with mercury chloride and conc. HCl to observe the zone of precipitation (Smibert, 1994). A distinct gelatin hydrolysis zone

served as biomarker for the presence of protease-producing bacteria. The protease producers were sub-cultured on Luria-Bertani medium and stored at -80°C (De Clerck *et al.,* 2004).

Characterization and identification of selected bacterial isolates

Bacterial isolates were characterized and identified on the basis of their morpho-cultural and physio-biochemical characteristics. The tests included Gram staining, endospore formation, indole test, methyl red test, Voges Proskauer test, citrate utilization test, sugars utilization by fermentation, H_2S production and urease test. Only Gram positive rods were subjected to biochemical tests (Marathe *et al.,* 2018).

Protease production from isolates

For protease production, 24 h fresh culture of *Bacillus* species was added to a production medium containing gelatin (30 g L^{-1}), peptone (10 g L^{-1}), beef extract (3 g L^{-1}) and sodium chloride (10 g L^{-1}) at pH 7.0. The medium was incubated at 37°C for 48 h in an orbital shaker at 150 rpm. The culture was then centrifuged at 4°C for 10 min at 12000 rpm. The culture supernatant was used as the enzyme crude (Sharma *et al.,* 2017).

The 0.5 mL of diluted cell free supernatant was added to 5 mL of 0.65% gelatinase solution (0.65 g in 50 mM phosphate buffer, pH 7.0) and incubated at 37˚C for 10 min. Then 0.5 mL of 6.1N tricholoroacetic acid was added and incubated at 37˚C for 30 min. The mixture was then filtered and 2 mL filtrate collected before incubation in dark for 30 min with 5 mL Na_2CO_3 and 0.5 mL Folin Colin reagent (Cupp-Enyard, 2008). The enzyme activity was measured at 680 nm against a blank. by using L-tyrosine standard curve (Banik *et al.,* 2018). The enzyme units were measured using the standard tyrosine (0-1000 µg mL^{-1}) curve. One unit (U) protease enzyme activity was defined as the amount of enzyme that liberated 1 µg tyrosine mL^{-1} min^{-1} from gelatin under specified assay conditions. The enzyme activity was found using formula:

$$\text{Enzyme activity} = \frac{\mu\text{L of L}-\text{tyrosine liberated} \times \text{total volume of assay}}{\text{incubation time} \times \text{Volume of enzyme} \times \text{Volume of assay in colorimeter}}$$

Optimization of protease production

The *Bacillus* species were subjected to different culture conditions to find the optimum conditions for protease production. Protease production was estimated at various pH, temperatures, and carbon sources (Pant *et al.,* 2015).

pH: A pH range of 5 to 10 in 100 mL of gelatin production medium (g L^{-1}): gelatin (30), peptone (10), beef extract (3), sodium chloride (10) in 5 conical flasks were used to assess optimum pH for protease synthesis. The pH of medium was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 utilizing 1N HCl and 1N NaOH, respectively. The enzyme assay was done after 24 h old culture was inoculated and incubated at 37°C for 48 h.

Temperature: The production gelatin medium was inoculated with 24 h culture and incubated at different temperatures [4, 27, 37, and 42ºC] for 48 h. After incubation, the cell free culture filtrates were used for enzyme estimation.

Carbon source: Starch concentrations of 2, 4, 6, and 8% in gelatin production medium were used to evaluated as carbon source. A 24 h culture was added to the production medium, and the mixture incubated at 37°C for 48 h. Then enzyme was assayed using cell-free culture filtrate.

Purification of protease

Ammonium sulphate precipitation is widely used as the first purification step and concentration procedure. It is based on the process of salting out. Ammonium sulphate was used to precipitate protease from crude homogenate at the concentrations ranging from 40 to 60%. The mixture was centrifuged at 10000 rpm for 20 min at 4°C so as to produce pellets. The 10 mL solution of an ice-cold, pH 8.0, 0.2 M phosphate buffer was used to suspend the pellet. The suspension was subject to dialysis by using cellulose acetate membrane. Dialysed sample was incubated overnight at -4°C. The

protease and protein concentration was determined at each step to check the purity of the sample. The specific activity was estimated by using protease assay and protein concentration (Gerze *et al.,* 2005).

Determination of proteins

The protein content of enzyme preparation was determined by using Lowry's technique, with bovine serum albumin serving as a standard. The absorbance at 680 nm was used to calculate the protein concentration during purification steps (Maehre *et al.,* 2018).

Immobilization of protease

The alginate (200 mg) was dissolved in 10 mL tris-HCl buffer (0.1M pH 8.0) by heating at 30-40°C for 30 min. The liquid protease enzyme (0.5 mg mL^{-1}) was gradually swirled into the alginate solution after all the alginate granules were dissolved. For enzyme and alginate to completely homogenise, the mixture was agitated for 1 h. Then 20 mL water and 1.4 mg $CaCl_2$ were dissolved in a beaker and placed into a petri dish. The protease-alginate combination was added to $CaCl_2$ solution by using a syringe, and allowed to solidify. The beads were filtered and allowed to air dry (Gao *et al.,* 2016).

Immobilized protease enzyme assay

Gelatin solution (0.5 mL) and 1 mL Tris-HCl buffer (pH 8.1) were combined with 5.4 mg immobilized enzyme. The solution was incubated at 37°C in a water bath for 30 min. Then TCA (110 mM) was added and the mixture filtered through a filter paper. The solution was combined with 5 mL sodium carbonate and 0.5 mL Folin's reagent for 25 min, and absorbance measured at 680 nm using spectrophotometer (Rezakhani *et al.*, 2014).

Phylogenetic analysis

The immobilization assay, enzyme assay, and purification processes all yielded positive results for *Bacillus licheniformis*. Sequence analysis of 16S rDNA which occur as a conserved molecule in the bacterial domain was extracted by using HELINI Biomolecules, Chennai, India's kit and formed the basis of molecular identification of the isolate (Chaudhuri *et al.,* 2006). The 16S rDNA sequence obtained was submitted to Genbank (Accession No. OQ891379) and subjected to BLAST and phylogenetic analysis by Neighbor Joining method using Mega 7 software.

Statistical analysis

All the experiments were performed in triplicate. The mean ± standard deviation (SD) was were calculated for protease activity, and optimization at different temperature, pH, and carbon source concentrations were tested for their significance using Microsoft Excel.

RESULTS AND DISCUSSION

Protease is an important enzyme for industry, with its application in food, waste, pharmaceutical, leather, laundry, and textile sectors. For effective enzyme production, the organism and target enzyme must be able to endure different modifications process. Based on zone formation, a preliminary search for bacterial strains that produced proteases was conducted in gelatin agar medium. In present study, *Bacillus* species were isolated from rhizosphere soil for extracellular protease synthesis. Folasade *et al.* (2005) noted that during 16 h incubation *Bacillus* species displayed their peak capacity to biosynthesize protease. Among the isolates, five species were selected based on highest zone formation (gelatin hydrolysis). The potential isolates were maintained on agar slants and stored at 4°C. Microscopic observation of isolates showed that the bacteria were Gram positive rod, grew aerobically and formed white colonies. The biochemical characteristics are presented in (Fig. 1). The potential isolates were identified as *Bacillus amyloliquefaciens, B. licheniformis, B. pumilis, B. cereus* and *B. subtilis*.

Table 1: Enzyme activity of isolated *Bacillus* species

Bacillus species	Enzyme activity [U mg^{-1}]		
	Crude sample	Ammonium salt precipitation	Dialysis
B. amyloliquefaciens	1.21 ± 0.20	0.83 ± 0.64	0.79 ± 0.18
B. licheniformis	1.26 ± 1.40	1.24 ± 0.11	0.96 ± 0.05
B. pumilus	1.29 ± 0.25	1.30 ± 0.18	1.01 ± 0.12
B. cereus	1.18 ± 0.15	1.09 ± 0.07	1.03 ± 0.06
B. subtilis	1.27 ± 0.24	1.10 ± 0.16	0.94 ± 0.05

Along with the casein hydrolysis which was reported as positive, these results were found similar to those of Folasade *et al*. (2005) and Wang *et al.* (2013). In present study, the use of gelatin as a substrate resulted in more production of proteases than casein. The crude sample from the isolates demonstrated higher protease activity in 1-5 dilutions (Table 2), where *B. licheniformis* showed stable protease activity of 1.09 ± 0.09 U mg^{-1}. Similar results were reported by Akhavan *et al.* (2011) when they utilized a screening technique on bacterial strains that were obtained from soil and employed *Bacillus* species to produce protease activity. Boominadhan *et al.* (2013) reported maximum protease activity by *Bacillus* species in 24 h of incubation at 25℃ using different concentration of nitrogen and carbon sources, respectively. Hence for higher protease production, optimization of liquid culture media was done for 3 parameters i.e., pH, temperature and carbon source. The isolates revealed that maximum protease production of 1.06 ± 0.2 U mg^{-1}at pH 7 (Fig. 2a). Similar outcomes were found for other *Bacillus* species, with pH 7.5 being the ideal level for the enzymatic activity of *B. subtilis* ITBCCB 148 (Yandri *et al.*, 2008), *Bacillus* sp. HS08 (Huang *et al.*, 2006) and *Bacillus* sp. S17110 (Jung *et al.*, 2007). According to a different research by Maal *et al.* (2009), pH 7 is optimal for the highest synthesis of alkaline protease from *Bacillus polymixa* and *B. cereus*. Other studies have shown that a pH of 7 is ideal for the maximal production of proteases (Gouda, 2006; Vidyasagar *et al.*, 2006; Fulzele *et al.*, 2011).

Table 1: Biochemical test for isolated *Bacillus* species

Tests	*Bacillus* - 1	*Bacillus* - 2	*Bacillus* - 3	*Bacillus* - 4	*Bacillus* - 5
Indole test	-	+	-	-	-
MR test	+	+	+	+	-
VP test	+	+	+	+	-
Citrate	+	-	+	+	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	-	+
Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	+	+	+	+	+
H_2S test	-	-	-	+	-
Urea test	-	-	+	-	+

Bacillus-1 = B. amyloliquefaciens, Bacillus-2 = B. licheniformis, Bacillus-3 = B. pumilis, Bacillus–4 = B. cereus, and Bacillus-5 = B. subtilis

In temperature optimization studies the highest activity of 1.06 ± 0.15 U mg^{-1} was observed at 37℃ (Fig. 2b). Similar higher protease production was observed at 37°C in *B. amovivorus*, *B. fastidiosus* and *Pseudomonas fluorescens* CM112 by Shumi *et al.* (2004) and Sharmin *et al.* (2005). Chu *et al.* (2007) reported maximum protease production at pH 8 and temperature 37℃. Vijay Anand *et al.* (2010) and Fulzele *et al.* (2011) demonstrated 37°C as ideal temperature for protease synthesis. However, Usharani and Muthuraj (2010) found 40°C as optimum temperature for protease production in *B. laterosporus*.

Nutrient source like carbon was an important factor for optimizing the protease production. Maximum yield of enzyme synthesis was noted by using several carbon sources in the production medium (Ola *et al.*, 2005). To know the optimum percentage of carbon source, the isolates were grown in varying percentage from 2 to 10. The maximum protease production of 1.1 ± 0.6 U mg^{-1} was seen at 4 and 6% (Fig. 2c). Puri *et al.* (2002) reported that *Bacillus* sp. produce more proteases in presence

of starch. The addition of starch to the culture medium increased alkaline protease enzyme production (Feng *et al.*, 2001; Chauhan and Gupta, 2004).

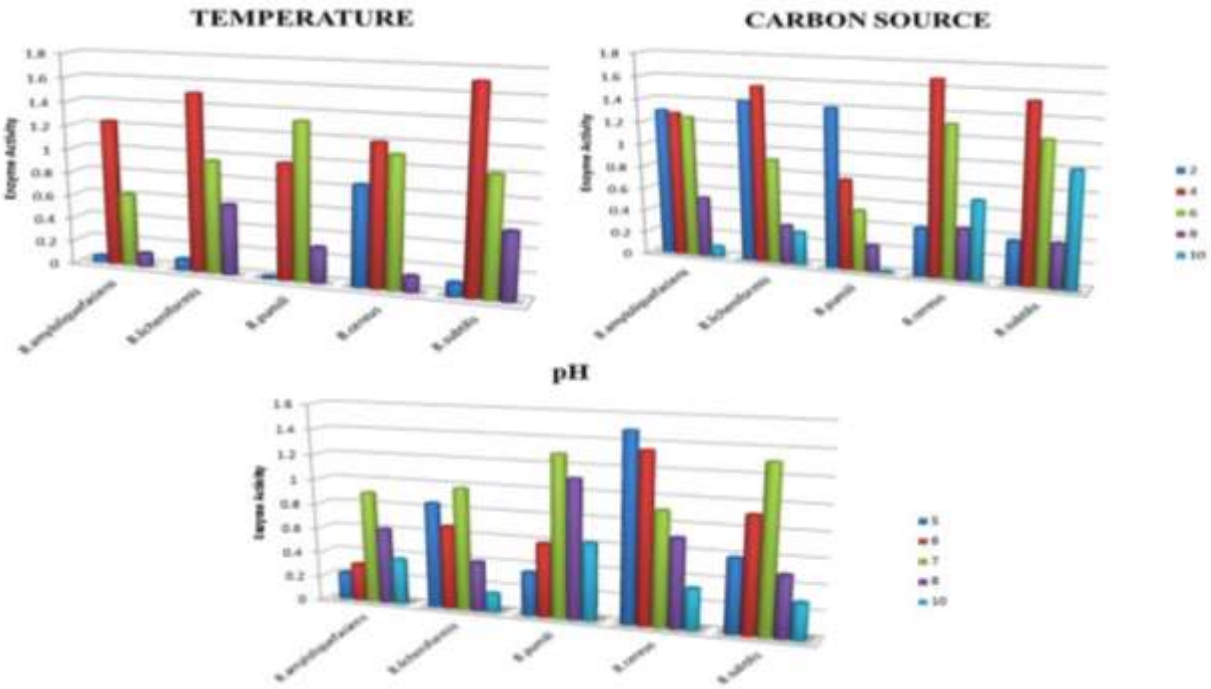

Fig. 2: Optimization of some variables affecting protease production by *B. lacinifomis* a) pH, b) temperature, and c) carbon source

The protease was extracted and purified by ammonium salt precipitation and dialysis as per Sevinc *et al.* (2011). At each phase, the protease (Table 1) and protein concentrations (Table 3) were measured. Specific activity increased at each step as protein concentration decreased (Table 4). This is due to degradation of proteins by salts (Draget *et al.*, 2001; Tang *et al.*, 2004). Azhar *et al.* (2009) claimed that *Bacillus* species exhibited highest levels of pure protease activity. Among the isolates, the specific activity of *B. licheniformis* was stable (0.47 ± 0.02 μmol^{-1} min^{-1} mg^{-1}) in crude and after purification by salt precipitation it increased by 0.71 ± 0.02 μmol^{-1} min^{-1} mg^{-1} and on further purification by dialysis the highest activity of 1.02 ± 0.08 μmol^{-1} min^{-1} mg^{-1} was recorded.

Immobilization of protease onto an appropriate support material plays an essential role in food and

Table 2: Protease enzyme assay during purification steps

Test tubes	Enzyme activity [U mg^{-1}]				
	B. amyloliquefaciens	*B. licheniformis*	*B. pumilus*	*B. cereus*	*B. subtilis*
1-5	1.12 ± 0.13	1.09 ± 0.09	1.10 ± 0.10	0.99 ± 0.01	1.00 ± 0.10
1-10	1.23 ± 0.15	0.95 ± 0.14	0.97 ± 0.05	0.98 ± 0.14	1.04 ± 0.16
1-15	0.78 ± 0.17	0.69 ± 0.17	0.65 ± 0.77	0.72 ± 0.10	0.82 ± 0.10
1-20	0.51 ± 0.19	0.43 ± 0.13	0.55 ± 0.28	0.54 ± 0.15	0.64 ± 0.12

Table 3: Protein concentration observed during various purification steps

Bacillus sp.	Protein concentration [mg mL^{-1}]		
	Crude sample	Ammonium salt precipitation	Dialysis
B. amyloliquefaciens	396 ± 3.05	248 ± 3.60	97 ± 3.51
B. licheniformis	365 ± 3.05	247 ± 2.51	108 ± 3.51
B. pumilus	352 ± 3.01	179 ± 4.91	97 ± 3.01
B. cereus	363 ± 3.01	190 ± 4.50	105 ± 3.11
B. subtilis	396 ± 3.05	207 ± 3.05	114 ± 2.01

Table 4: Specific activity of protease enzyme after purification step

Bacillus sp.	Specific activity [μmol^{-1} min^{-1} mg^{-1}]		
	Crude sample	Ammonium salt precipitation	Dialysis
B. amyloliquefaciens	0.47 ± 0.03	0.69 ± 0.03	1.22 ± 0.10
B. licheniformis	0.47 ± 0.02	0.71 ± 0.02	1.02 ± 0.08
B. pumilus	0.55 ± 0.02	1.03 ± 0.06	1.21 ± 0.24
B. cereus	0.46 ± 0.02	0.77 ± 0.02	1.05 ± 0.06
B. subtilis	0.47 ± 0.02	0.82 ± 0.03	1.04 ± 0.05

detergent industries. Improvement of protease immobilization has highly been regarded due to its application as biocatalyst (Dragent *et al.,* 2001; Susanne *et al.,* 2010). Demirkan *et al.* (2017) reported a yield of 83% due to the enzyme immobilization. Jing *et al.* (2017) immobilized alkaline protease on various support materials which increased enzyme activity. Sodium alginate due to its non-toxicity, cost-effectiveness, and biodegradability is one of the immobilization matrices researched (Rao *et al.,* 2009). To obtain the best immobilization efficacy, different concentrations of sodium alginate were used, as the degree of cross-linking of gelling agent influences the pore size of the beads.

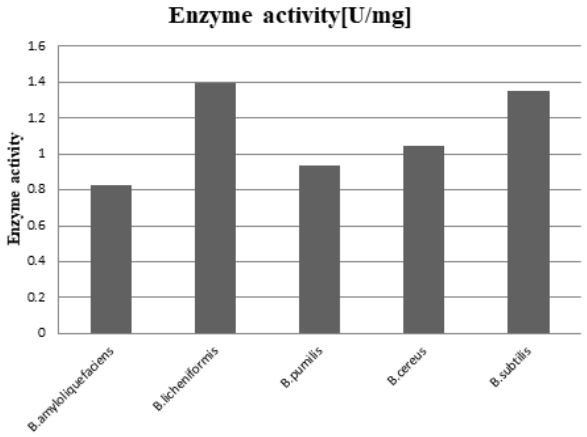

Fig. 4: Immobilized enzyme assay of isolated *Bacillus* species

The alginate gel matrix's substrate and product should freely flow in and out of the pores of beads while yet preserving the enzyme in the micro-environment of beads. The pore size of beads increases with increase in sodium alginate concentration, thus enhancing the amount of enzyme leakage from beads (Riyaz *et al.,* 2009). In this study, the protease enzyme was enclosed in alginate beads, and a spectro-photometric technique was used to measure protease assay (Ertesvag *et al.,* 1998). The study showed that protease enzyme displays adequate stability and good activity of 1.4 ± 0.2 U mg^{-1} after being immobilized in sodium alginate beads from *B. licheniformis* (Fig. 4).

Phylogenetic analysis was performed using the Mega 7 tool with 5 different FASTA sequencing data obtained from NCBI nucleotide BLAST. The study revealed that among the several *Bacillus* species *viz., B. aerius, B. paralicheniformis, B. piscis, B. glycinifermentans* and *B. haynesii*, only *B. aerius* showed a close relationship with *B. licheniformis* having common ancestor (Fig. 5).

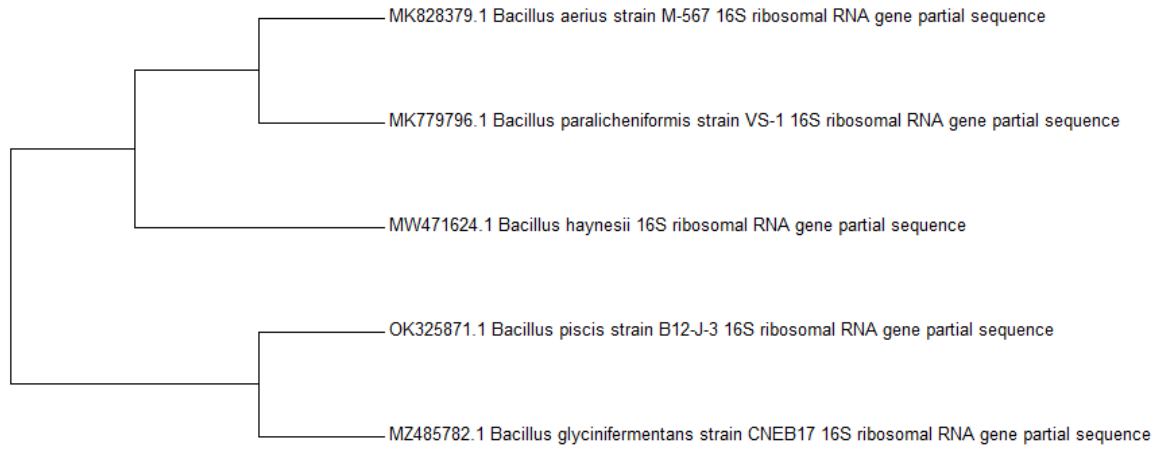

Fig. 5: Phylogenetic tree of *Bacillus licheniformis* constructed using Mega 7

Conclusions: Amongst the identified *Bacillus* species isolated from rhizosphere soil, *B. licheniformis* possessed higher protease activity. The present study standardised the optimum growth parameters for maximum enzyme production, which can be effectively used in large-scale protease production for commercial use. The optimum temperature and pH were 37°C and 7.0, respectively, and the best carbon sources were starch @ 4 or 6%. This information has enabled the formulation of media compositions for maximum protease production. The protease was purified by ammonium sulphate precipitation and dialysis methods. The purified proteases were immobilized using sodium alginate and protease activity was determined. Purified protease can be used for a variety of purposes in detergent, food and pharmaceutical industries.

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Conflict of interests: The authors declare that they have no conflict of interest in the publication.

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