Paenibacillus dendritiformis DDS₂: A new member to alkaline-thermophilic protease producers

Vethanayagam Celesty Anpalagan, Sandrasegarampillai Balakumar and Vasanthy Arasaratnam*

Department of Biochemistry, Faculty of Medicine, University of Jaffna, Sri Lanka.
Email: arva2Garva@yahoo.com

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ABSTRACT

Aim: Most of the industrial processes require thermostable alkaline proteases. Thus, a search was initiated to isolate and characterize a bacterium which can produce thermostable alkaline protease.

Methodology and results: Best higher titer thermostable alkaline protease producing wild type organism was screened from beef, dog and fish decaying soil samples. Among the 92 bacterial strains, three strains which produced alkaline proteases having activities at pH 10.5 and above 70 °C were selected. Among the three strains, the one from the dog decaying soil (Strain DDS₁; DDS-dog decaying soil) which produced the protease showing highest activity at pH 10.5 and 73 °C and stability (half-life: 10.5 h) without additives was selected and identified. Based on the biochemical and morphological studies, strain DDS₁ could be either Paenibacillus dendritiformis or P. thiaminolyticus. From 16S rDNA sequencing, the strain DDS₁ was confirmed as P. dendritiformis.

Conclusion, significance and impact: This is the first report published to show that P. dendritiformis is a protease producer and the organism was named as P. dendritiformis DDS₁.

Keywords: Alkaline protease, thermostable, isolation, identification, Paenibacillus dendritiformis

INTRODUCTION

The use of thermostable alkaline proteases in food, textile, tanning (Sivasubramaniam et al., 2008a; Sivasubramaniam et al., 2008b; Sundararajan et al., 2011), paper pulp, chemical, pharmaceutical, detergent (Maurer, 2004) and photographic industries and waste treatment programs is increasing steadily in many parts of the world (Kumar and Takagi, 1999; Rao et al., 1998). For commercial applications and economic viability of alkaline proteases, alkaline proteases should possess high activity at alkaline pH, broad substrate specificity and the optimal temperature for activity around 60 °C (Rao et al., 1998). Thus, suitable strains need to be isolated to produce thermostable alkaline proteases to match these requirements.

Protease producing bacterial strains were isolated from hot spring (Banerjee et al., 1999), alkaline hot spring (Parawira and Zvauya, 2012), sediment sample of hot spring (Pillai et al., 2011), human skin (Deborah et al., 2000), Thai fish sauce (Reungsang et al., 2006), meat waste contaminated soil (Kalaiarasi and Parvatham, 2011), pigeon pea waste (Johnvesly et al., 2001), food waste (Mohammed, 2015), human and animal feces (Kumar and Takagi, 1999), buffalo hide (Zambare et al., 2007), composed soil sample (Arulmani et al., 2007), soil (Camila et al., 2007; Reddy et al., 2008), garden soil (Khan et al., 2011), sandy soil (Adinarayana et al., 2003), field soil (Mukhtar and Ikram-ul-Haq, 2012), marine coast (Shanmughapriya et al., 2008), alkaline soil (Emtiaz et al., 2005; Jasvir et al., 1999), laundry soil (Banik and Prakash, 2004), shore of alkaline soda lake (Gessesse and Gashe, 1997), marine water (Haddar et al., 2009), tannery waste water (Bayoudh et al., 2000; Khan et al., 2011; Kumar et al., 2008), waste dump slaughter house (Doddapaneni et al., 2007) and soap industry (Khan et al., 2011).

Proteases are produced by halophilic Bacillus (Patel et al., 2005), halophilic Bacillus clausii (Kumar et al., 2004), alkaliophilic Bacillus circulans MTCC 7942 (Patil and Chaudhari, 2013), Bacillus thermostruber (Manachini et al., 1998), Escherichia coli cloned with the gene of deep sea bacterium Alkalimonas collagenimaria AC40T (Kurata et al., 2007) and different fungi (Macchione et al., 2008). The organisms reported lack in either thermostability or alkaliophilic nature. Hence, this study was carried out to isolate a thermostable alkaline protease producer and to identify the selected strain.
MATERIALS AND METHODS

Materials

All the chemicals used were (of analytical grade) purchased from Sigma Chemical Company (USA), Oxoid (England) and BDH (England). API 50 CHB/E Medium for Bacillus identification was purchased from bioMerieux DIRECT USA.

Measurement of protease activity

The activity of protease was measured by estimating the amount of tyrosine released from casein (Mao et al., 1992). One unit of protease activity is defined as the amount of enzyme that liberates 1 µg of tyrosine in one minute at pH 9.5 and 70 °C. Later based on the optimum conditions of the proteases of the different strains isolated, the activity conditions were fixed.

Media used

The nutrient-agar medium contained (g/L) nutrient broth, 10.0; peptone, 10.0; sodium chloride, 5.0; and bacteriological agar, 17.5 at pH 7.0. Both the activation and fermentation media were the same which contained (g/L) glucose, 10.0; peptone, 5.0; yeast extract, 5.0; KH₂PO₄, 10.0; MgSO₄·7H₂O, 0.2; and Na₂CO₃, 10.0 at pH 9.5.

Isolation and selection of alkaline protease producing strain

Collection of samples

Dead dog, beef and fish buried soil were selected and samples were collected in sterile containers.

Isolation of protease producers

Samples (1 g) suspended in 9 mL of sterile saline (9 g/L NaCl) were serially diluted from 10⁶ to 10⁹ dilutions and spread plated on nutrient-agar plates. Single colonies were selected on the basis of their size, shape and colour at 24 h and incubated at 40 °C and colonies diameters were measured to identify the organisms which could grow well in the nutrient agar medium at 40 °C.

Activation of the bacterial strains

Single colonies of the isolated bacterial strains were cultivated in nutrient-agar medium at 40 °C for 24 h. Two loops of bacterial cell cultures from the nutrient-agar medium were transferred into 25 mL of the activation medium, incubated in shaker water bath at 40 °C and 120 rpm for 18 h and were used as the inocula.

Selection of alkaline protease producing strains

Fermentation medium was inoculated with activated inocula (20%, v/v), incubated in shaker water bath at 40 °C and 120 rpm for 144 h. The cell free spent medium (centrifuged at 5,100 × g for 20 min) was used as the enzyme source.

Selection of alkaline and thermostable protease producing bacteria

Effect of temperature on the activities of the proteases

The effect of temperature on the activities of crude alkaline proteases from the selected isolates were determined by incubating the appropriately diluted enzymes (1 mL in 0.01 M glycine NaOH buffer, pH 9.5) for 5 min with 1 mL of casein (20 g/L, pH 9.5, 1 mL) at different temperatures (varied from 30 to 95 °C).

Effect of pH on the activities of the proteases

The effect of pH on the activities of crude alkaline proteases from the selected isolates were measured at different pH values ranging from 7.0 to 11.0 and incubated for 5 min at the respective optimized temperatures.

Thermostability of alkaline proteases

Crude alkaline proteases from selected isolates were preincubated at the respective optimum temperatures and pH values and the activities of the enzymes were monitored.

Characterization of selected thermostable higher titer alkaline protease producing strain

Morphological characters

Selected strain was grown on nutrient agar medium at 40 °C. After 24 h, the morphological characteristics, such as shape and branching pattern were observed. The strain was Gram stained (Kaiser, 2019) and motility was described by hanging drop method (Theivendrarajah, 1990). Shape of endospore was observed under oilimmersion microscope after grams staining.

Biochemical test

The strain was subjected to aerobic or anaerobic growth; production of cytochrome oxidase; acid from different sugars and sugar derivatives, urease, indole, catalase, hydrogen sulfide and acetylmethylcarbinol (Voges-Proskauer test) were tested. The reducing ability of the strain to convert nitrate to nitrite and, hydrolyze tyrosine, starch and casein were studied (Theivendrarajah, 1990). Growth of the selected strain was tested at 40, 45, 50, 55 and 60 °C, at pH 9.5 and 120 rpm in the fermentation medium by measuring the optical
density (OD, at 600 nm in Spectronic 21D). Effects of different concentrations of NaCl on the growth of the strain in the fermentation medium and at pH 5.6, 7.0, 8.0, 9.0, 10.0, 11.0 in the fermentation medium were studied (Theivendrarajah, 1990).

Determination of the species of the selected strain

Morphological characters

The strain was grown on nutrient agar medium at 40 °C and at 24 h. Morphological characteristics, such as size, colour, elevation, margin and characteristic of branch pattern (tip-splitting, chiral) were studied (Tcherpakov et al., 1999).

Biochemical test

Production of catalase and utilization of citrate were studied. The strain was inoculated with the standardized API 50 CHB/E Medium (Boyd et al., 2005). During incubation, fermentation was revealed by a colour change in the tube, caused by the anaerobic acid production was detected. During incubation, the carbohydrates are fermented to acids which produce a decrease in the pH, detected by the change in colour of the indicator. Carbohydrate utilization was determined through the extent of acid production by change in colour to yellow (Bille et al., 1992).

Conformation of the species of the selected strain by the 16S rDNA sequencing and analysis

The strain was grown on nutrient agar medium at 40 °C and at 24 h. Genomic DNA was extracted (Sambrook et al., 1989) and amplified [Universal primers corresponding to positions 8-27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492-1509R (5’-GGTTACCTTGTTACGGACTT-3’)] (Weisburg et al., 1991). The PCR products were purified and sequenced (Quillaguaman et al., 2004). The 16S rDNA sequence analysis was performed with the aid of the DNAMAN 4.03 software package by using the neighbour-joining method and the Jukes-cantor distance correction method (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Isolation and selection of alkaline protease producer

Selection of bacterial strains

The present study is aimed at isolating thermostable alkaline protease producing bacterial strains. Soil temperature is greater than atmospheric temperature and hence the bacterial strains obtained from the soils would be able to survive in hot environment and produce thermostable enzymes. Therefore, beef, dead dog and fish buried soil samples were collected. Bacterial strains were grown on nutrient agar plates and single colonies were purified. The colonies differed in any of the morphological characteristics such as sizes, shapes, elevation, colour and margin after 24 h of incubation in nutrient-agar plate were considered and 92 strains were selected. The strains which developed single colonies with different characteristics were selected (Table 1). Among the selected 92 strains, 17 (15.5%) were from beef decaying soil, 61 (66.3%) were from dog decaying soil and 15 (15.2%) were from fish decaying soil. Thus, more than 50% were from dog decayed soil.

Table 1: Different single colonies obtained with different sizes, shapes, elevation, colour and margin from beef decaying soil, dog decaying soil and fish decaying soil.

| Character | Details | Number of strains |
|-----------|---------|------------------|
| Diameter (mm) | 0-1 | 15 |
| | 2-4 | 42 |
| | 5-7 | 35 |
| | Round | 47 |
| | Irregular | 10 |
| Shape | Tip splitting | 5 |
| | Chiral morphology | 2 |
| | Filamentous | 28 |
| Elevation | Flat | 57 |
| | Low convex | 35 |
| | Pale | 45 |
| Color | White | 10 |
| | Yellow | 37 |
| | Entire | 40 |
| Margin | Irregular | 52 |

Selection of alkaline protease producers

Among the 92 bacterial strains, 37 strains gave alkaline protease activity above 4 U/mL, 20 strains produced the activity between 1-4 U/mL, while 35 strains produced the activity less than 1.0 U/mL, at 70 °C and pH 9.5. Among the 37 bacterial strains, 22 (59.5%), 9 (24.3%) and 6 (16.2%) were obtained from dog, beef and fish decaying soil, respectively (Table 2) and strains DDS2, DDS3, DDS21, DDS57 and DDS47 were selected. Even though the strains BDS1 and DDS4 produced the alkaline protease activity above 30 U/mL, they were not selected as they produced the alkaline protease activity below 32 U/mL.

Protease producing bacterial strains isolated from different sources showed varying ranges of activities. Strains isolated from meat waste contaminated soil (Kalaiarasi and Parvatham, 2011), laundry soil (Srividya and Mala, 2011), soil (Camila et al., 2007), saline soil (Mukhtar and Ikram-ul-Haq, 2012), buffalo hide (Zambare et al., 2007), sandy soil (Adinarayana et al., 2003), composed soil sample (Aruiman et al., 2007) and alkaline soil sample (Jasvir et al., 1999; Emlazzi et al., 2005) produced 0.93, 88.0, 0.5, 176.05, 500.0, 420.0, 486.0 and
Table 2: Ranges of alkaline protease activities produced by the 92 strains isolated from dog, beef and fish decaying soil samples and incubated at 40 °C and pH 9.5 and 120 rpm.

| Activity range (U/mL) | Sources of the stains and given numbers | Strain No. | Total |
|----------------------|----------------------------------------|------------|-------|
| Less than 1          | BDS, BDS14, BDS15, BDS17               | 4          |
|                      | BDS4, BDS17, BDS22, BDS26              |            |
|                      | BDS28, BDS30, BDS35, BDS38             |            |
|                      | BDS3, BDS41, BDS42, BDS43             | 26         |
|                      | BDS44, BDS45, BDS46, BDS48             |            |
|                      | BDS47, BDS50, BDS51, BDS52             | 35         |
|                      | BDS55, BDS56, BDS57, BDS58             |            |
|                      | BDS61, BDS65, BDS66, BDS67             |            |
|                      | FDS1, FDS4, FDS10, FDS12, FDS13       | 5          |
| 1-3.99               | BDS10, BDS12, BDS13, BDS16             | 4          |
|                      | BDS11, BDS22, BDS24, BDS27             |            |
|                      | BDS28, BDS31, BDS32, BDS34             | 13         |
|                      | BDS36, BDS40, BDS53, BDS56             | 20         |
|                      | BDS60, BDS62, BDS65, BDS66             |            |
|                      | FDS7, FDS8, BDS9                      | 3          |
| 4-19.9               | BDS5, BDS7, BDS8, BDS14, BDS3         | 5          |
|                      | BDS1, BDS6, BDS8, BDS9, BDS10         |            |
|                      | BDS12, BDS13, BDS14, BDS15            | 14         |
|                      | BDS18, BDS19, BDS20, BDS25            | 22         |
|                      | BDS25, BDS27                         |            |
|                      | FDS5, FDS6, FDS14, BDS17             | 3          |
| 20-29.9              | BDS2, BDS4, BDS8                      | 3          |
|                      | BDS6, BDS7, FDS2, BDS3                | 2          |
|                      | FDS11, FDS2, BDS3                     | 8          |
| 30-50                | BDS1, BDS14, BDS21, BDS23, BDS37      | 1          |
|                      | BDS16, BDS32, BDS33, BDS47            | 5          |
| 50 or above          | BDS5, BDS3                            | 2          |

DDS, dog decaying soil; BDS, beef decaying soil; FDS, fish decaying soil.

Selection of bacteria which can produce high titre of alkaline protease with better thermostability

Effect of temperature

The use of conventional enzymes is not always a straightforward approach because of the fact that many available enzymes do not withstand industrial reaction conditions, e.g. elevated temperature, extreme pH values, ionic strength, etc. The increasing industrial demands for thermostable enzymes that can cope up with industrial process conditions had led to a search for robust biocatalysts from living organisms in environments that were earlier believed to be too severe to support life. Temperature is an important factor in alkaline protease activity (Banerjee et al., 1999). The activities of crude alkaline proteases produced by DDS2, DDS3, DDS21, DDS33 and DDS47 were measured at different temperatures ranging from 30 to 95 °C and the optimum temperatures of the proteases from isolates DDS2, DDS3, DDS21, DDS33 and DDS47 were 73, 72, 70, 68 and 55 °C (Table 3). The strains DDS33 and DDS47 could be eliminated as their optimum activities were below 70 °C. Therefore, among the five strains, three strains which had temperature optima above 70 °C namely the strains DDS21, DDS2, and DDS3 were selected.

Alkaline protease obtained from strain DDS1 exhibited a temperature profile of maximum activity at 70 °C at pH 9.5 like that of Bacillus thuringiensis (Tyagi et al., 2003). Alkaline protease obtained from DDS2 showed optimum activity at 73 °C similar to Bacillus laterosporus (Manavalan et al., 2007). Alkaline protease obtained from DDS47 showed optimum activity at 55 °C and that of Bacillus cereus exhibited highest activity at 50 °C (Banik and Prakash, 2004).

Effect of pH

Reaction pH also plays an important role in the activity of enzyme (Manavalan et al., 2007). Among the selected isolates DDS2, DDS3 and DDS21 showed highest protease activity at pH 10.5 at their optimal temperatures (73, 72 and 70 °C respectively) with casein (20 g/L) and the B. thuringiensis protease showed similar pH optimum (Tyagi et al., 2003). The isolates DDS2 and DDS47 showed highest activities at pH 9.0 at their optimal temperatures (68 and 55 °C) with casein (20 g/L) and the B. laterosporus protease showed highest activity at pH 9.0 (Manavalan et al., 2007). The pH optima of DDS2, DDS3 and DDS21 also favoured their selection (Table 3).

Stability of enzymes with temperature

The thermostability of the proteases from the strains DDS2, DDS3, DDS21, DDS47 and DDS33 were different and their half-lives were 10.5, 4.0, 2.0, 2.83 and 1.33 h respectively at their optimal temperatures and pHs. As the alkaline protease from isolate DDS2 showed best half-life among the alkaline proteases at pH 10.5 and 73 °C.
without additives (Table 3), the strain was selected and considered for identification. Previous reports on thermostability of proteases have shown half-lives of 60 and 7 h at 50 and 60 °C respectively and pH 10.5 (Bacillus brevis, Banerjee et al., 1999); 36 h, 93 min, 14 min and 6 min at 40, 50, 60 and 70 °C respectively at pH 10.0 (Bacillus licheniformis BA17, Ozturk et al., 2009) and 23 min at 70 °C (B. cereus BG1, Ghorbel et al., 2003). As the protease of the strain DDS2 showed better thermostability at alkaline pH, the strain was selected.

**Determination of the genus of the strain DDS2**

**Morphological characters**

The colonies of strain DDS2 at 24 h showed branching pattern. DDS2 cells were rod shaped, Gram-positive and motile. Spore shape was oval or round. *Paenibacillus*, *Bacillus*, *Clostridium*, *Cornebacterium*, *Lactobacillus* and *Mycobacterium* are Gram-positive rods. *Cornebacterium*, *Lactobacillus* and *Mycobacterium* do not form spores. *Bacillus* does not show branching pattern (Ash et al., 1993). Strain DDS2 showed branching pattern (Table 4). Therefore, based on these results, it can be concluded that the strain DDS2 does not belong to *Bacillus*, *Cornebacterium*, *Lactobacillus* and *Mycobacterium* and the strain DDS2 can be either *Paenibacillus* or *Clostridium* (Ash et al., 1994).

**Biochemical test**

Biochemical tests were carried out to confirm the genus of the strain. Strain DDS2 was facultative anaerobic and catalase positive. Strain DDS2 did not produce soluble pigments on nutrient agar. Strain DDS2 had the ability to produce oxidase, urease and indole. Strain DDS2 gave negative results to Voges-Proskauer test. Hydrogen sulfide is not produced by strain DDS2. It showed inability to reduce nitrite and hydrolyze tyrosine. It had the ability to hydrolyze starch and casein. It showed ability to grow at 40, 45 and 50 °C, with 5, 7, 10 % of NaCl and showed tolerance to the pH values of 7, 8, 9, 10 and 11. Growth at pH 5.6 was variable (Table 4).

Emended description of the genus *Paenibacillus*. Cells are rod shaped and Gram-positive / Gram-negative / Gram-variable and motile (Ash et al., 1993). Ellipsoidal spores are formed in swollen sporangia. No soluble pigment is produced on nutrient agar. They are facultative anaerobic or strictly aerobic. Almost all of the species are catalase producer. Production of oxidase activity is variable. The Voges-Proskauer reaction (production of acetyl methylcarbinol) is variable. Indole is produced by some species. Nitrate reduction to nitrite is variable. Hydrogen sulfide is not produced. Hydrolysis of casein, hydrolysis of starch and tyrosine is variable. Growth at pH 5.6 and growth at 50 °C are variable. Optimum growth occurs at pH 7.0. The optimum growth temperature of 19 species (all species except *Paenibacillus macquariensis*) is 28 to 30 °C. Growth is inhibited by 10% NaCl. Acid is produced from various sugars. Production of urease is variable. Some species decompose polysaccharides. Based on emended description of the genus *Paenibacillus* (Ash et al., 1994), Strain DDS2 showed most of the characteristics similar to the organism which belong to genus *Paenibacillus* (Table 4). After the identification of genus, the species of the strain DDS2 was identified.

**Table 3: Characters of the proteases produced by the selected strains.**

| Strain | Activities at pH 9.5 and 70 °C (U/mL) | Optimum temperature (°C) | Optimum pH | Activities under respective optimum conditions (U/mL) | Half-life (h) |
|--------|-------------------------------------|--------------------------|------------|--------------------------------------------------------|--------------|
| DDS2   | 348                                 | 73                       | 10.5       | 454                                                    | 10.5         |
| DDS3   | 296                                 | 72                       | 10.5       | 346                                                    | 4.0          |
| DDS21  | 189                                 | 70                       | 10.5       | 204                                                    | 2.0          |
| DDS33  | 45                                  | 68                       | 9.0        | 68                                                     | 2.83         |
| DDS27  | 38                                  | 55                       | 9.0        | 53                                                     | 1.33         |

**Determination of the species of the strain DDS2**

**Morphological characters**

The colonies of the strain DDS2 at 24 h had pale colour, with irregular margin, 1.4 ± 0.3 mm in diameter, low convex elevation, moist and shiny surface. The most distinguishing characteristic among the genus *Paenibacillus*, *P. denticristiformis* is T morphotype pattern forming. *Paenibacillus* sp showed T morphotype colony morphology and hence the strain DDS2 could be *P. denticristiformis* (Tcherpakov, 1999).

**Biochemical test**

Strain DDS2 had the ability to produce catalase and utilize citrate. The acid producing ability of the strain DDS2 was confirmed by the studies of API 50 CH is a standardized system (Table 5). *Paenibacillus brasilensis*, *P. azotofixans*, *P. borealis* and *P. graminis* do not produce acid from glycerol. *Paenibacillus polymyxa* and *P. peoriae* produce acid from L-arabinose. *Paenibacillus macerans* and *P. odorifer* produce acid from inulin (Weid et al., 2002). *Paenibacillus larvae* subsp. *larvae* and *P. larvae* subsp. *pulvificans* do not produce catalase. Therefore, based on these results, it can be concluded that the strain DDS2 does not belong to *P. brasilensis*, *P. azotofixans*, *P. borealis*, *P. graminis*, *P. polymyxa*, *P. peoriae*, *P. macerans*, *P. odorifer* and *P. larvae*. These biochemical tests were compared with *P. denticristiformis* sp. nov. (Table 4) (Tcherpakov, 1999). Based on the biochemical tests, strain DDS2 had the characters of either *P.
Table 4: Morphological and biochemical test results of the strain DDS₂.

| Characters                  | Paenibacillus | Strain DDS₂ |
|-----------------------------|---------------|-------------|
| Shape                       | Rod           | Rod         |
| Spore Shape                 | Ellipsoidal / oval / round | Oval or round |
| Motility                    | Motile        | Motile      |
| Gram staining               | Gram-positive / Gram-negative / Gram variable | Gram-positive |
| Colony morphology           | T morpho type / chiral | T morpho type |
| O₂ requirement              | Facultative anaerobic or strictly aerobic | Facultative anaerobic |
| Hydrogen sulfide            | Not produce   | Not produce |
| Catalase                    | Generally produce | Produce |
| Oxidase                     | Variable      | Produce     |
| Urease                      | Variable      | Produce     |
| Indole                      | Some species produce | Produce |
| Vogues-Proscacuer test      | Variable      | Not produce |
| Reduction of nitrite        | Variable      | Not produce |
| Hydrolyze tyrosine          | Variable      | Not produce |
| Hydrolyze starch            | Variable      | Produce     |
| Hydrolyze casein            | Variable      | Produce     |
| Growth                      | Variable      | 40, 45 and 50 °C |
| temperature                 | Variable      | 5, 7, 10% of NaCl |

...dendritiformis or P. thiaminolyticus (Tcherpakov. 1999). To further confirm the species of the strain, the 16S rDNA was analysed.

16S rDNA sequencing and analysis

Almost complete sequence (1452 bps) of the 16S rDNA was obtained and deposited in the Gene bank data base under accession no. AY359885.1. Based on the gene sequencing studies, the strain DDS₂ isolated from dog decaying soil was classified as P. dendritiformis with 99% of homology (Table 6). The morphological and biochemical studies, and gene sequencing, DDS₂ was confirmed to be belonging to the Kingdom: Prokaryota; Division: Bacteria; Order: Bacillales; Family: Paenibacillaceae; Genus: Paenibacillus; Species: dendritiformis. The P. dendritiformis DDS₂ organism produced protease, which is active at pH 10.5 and 73 °C. Further the protease of this strain had the half-life of 10.5 hours (Table 3).

The importance of proteases produced by Paenibacillus in industry was first described by Rai et al., (2010). However, only few data concerning the production of these enzymes are available. Paenibacillus sp. strain BD2526 (Hang et al., 2016) and P. tezepurensis sp. nov. AS-S24-II (Rai et al., 2010) were the two Paenibacillus strains which were reported to produce proteases. Protease produced by Paenibacillus may represent new sources for biotechnological use (Alvarez et al., 2006). Paenibacillus dendritiformis was reported to produce a lethal protein that kills at the interface of the encroaching colonies (Beer et al., 2011). P. dendritiformis infects honeybee colonies (Cormnan et al., 2013; Chen et al., 2009; Al-Ghamdi et al., 2018). P. dendritiformis has been used for the formation of biosurfactant (Bezza and Chiwa, 2015), for the production of chitosanase (Sun et al., 2018) and for the disease protection and growth promotion of potatoes (Lapidot et al., 2015).

Thus, this is the first study reporting P. dendritiformis as a protease producer. The optimum temperature and pH for the protease produced by the P. dendritiformis DDS₂ was 73 °C and 10.4 respectively. P. tezepurensis sp. nov. AS-S24-II protease showed the temperature and pH optima as 45-50 °C and 9.5 respectively (Rai et al., 2010).

Table 5: API 50 CH test results for strain DDS₂ to form acid from different sources.

| Sources             | Results       | Sources             | Results       |
|---------------------|---------------|---------------------|---------------|
| Glycerol            | Positive      | D-Fucose            | Positive      |
| D-Ribose            | Positive      | L-Fucose            | Positive      |
| D-Adonitol          | Positive      | Potassium gluconate | Positive      |
| D-Galactose         | Positive      | Erythritol          | Negative      |
| D-Glucose           | Positive      | L-Arabinose         | Negative      |
| D-Mannose           | Positive      | D-Arabinose         | Negative      |
| Methyl-α-D-          | Positive      | D-Xylose            | Negative      |
| Mannopyranoside     |               |                     |               |
| Methyl-α-D-          | Positive      | L-Xylose            | Negative      |
| Glucopyranoside     |               |                     |               |
| N-Acetyl-glucosamine|               |                     |               |
| Esculin              | Positive      | L-Rhamnose          | Negative      |
| D-Cellobiose        | Positive      | Methyl-β-D-         | Negative      |
|                     |               | xylopyranoside      |               |
| D-Maltose           | Positive      | L-Sorbose           | Negative      |
| Amygdalin           | Positive      | Dulcitol            | Negative      |
| Arbutin             | Positive      | Inositol            | Negative      |
| Salicin             | Positive      | D-Mannitol          | Negative      |
| D-Lactose           | Positive      | D-Sorbitol          | Negative      |
| D-Melibiose         | Positive      | Inulin              | Negative      |
| D-Sucrose           | Positive      | Xylitol             | Negative      |
| D-Trehalose         | Positive      | D-Tagatose          | Negative      |
| D-Melezitose        | Positive      | D-Lyxose            | Negative      |
| D-Raffinose         | Positive      | D-Arabinol          | Negative      |
| Amidon              | Positive      | L-Arabinol          | Negative      |
| Glycogen            | Positive      | Potassium-2-ketogluconate | Negative |
| Gentiobiose         | Positive      | Potassium-5-ketoglucanate | Negative |
| D-Turanose          | Positive      |                     |               |

Table 6: Description of the strain DDS₂.

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while that of Paenibacillus sp. strain BD3526 was an acid protease having the temperature optimum at 60 °C (Hang et al., 2016). Hence, the protease produced by P. dendritiformis was superior to those already reported in the pH and temperature optima. Further the enzyme also showed very high temperature stability at the optimum reaction conditions (half-life 10.5 h). These properties can make protease from P. dendritiformis DDS2 an ideal candidate for detergent industry.

CONCLUSION

Among the 92 bacterial strains isolated, 37 strains which gave alkaline protease activity above 4 U/mL were selected. Among the rest 37 strains, strain DDS2 produced highest alkaline protease activity and showed optimum activity at 73 °C and at pH 10.5 with a good thermostability without additives at the optimum conditions. The strain DDS2 from dog decaying soil was identified as P. dendritiformis and labeled as P. dendritiformis DDS2. For the applications in detergent industries alkaline proteases should possess high activity at alkaline pH, broad substrate specificity and the temperature optima around 60 °C and P. dendritiformis DDS2 is a useful candidate for this purpose.

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