Malaysian Journal of Microbiology, Vol 16(3) 2020, pp. 167-175
DOI: http://dx.doi.org/10.21161/mjm.190535

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Paenibacillus dendritiformis DDS_2: A new member to alkaline-thermophilic protease producers

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Received 19 July 2019; Received in revised form 7 November 2019; Accepted 26 December 2019

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 DDS_2 ; 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 DDS_2 could be either *Paenibacillus dendritiformis* or *P. thiaminolyticus*. From 16S rDNA sequencing, the strain DDS_2 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* DDS_2.

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 (Emtiazi *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), alkaliphilic *Bacillus circulons* MTTC 7942 (Patil and Chaudhari, 2013), *Bacillus thermoruber* (Manachini *et al.*, 1988), *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 thermostablility or alkalophilic nature. Hence, this study was carried out to isolate a thermostable alkaline protease producer and to identify the selected strain.

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 167-175
DOI: http://dx.doi.org/10.21161/mjm. 190535

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; KH_2PO_4, 10.0; $MgSO_4 \cdot 7H_2O$, 0.2; and Na_2CO_3, 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 10^{-6} to 10^{-9} 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 pre-incubated 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 oil-immersion microscope after grams staining.

Biochemical test

The strain was subjected to aerobic or anaerobic growth and; 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

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 167-175
DOI: http://dx.doi.org/10.21161/mjm. 190535

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 standardize 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
Shape	Round	47
	Irregular	10
	Tip splitting	5
	Chiral morphology	2
	Filamentous	28
Elevation	Flat	57
	Low convex	35
	Pale	45
Color	White	10
	Yellow	37
Margin	Entire	40
	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 samples respectively (Table 2) and strains DDS_2, DDS_3, DDS_{21}, DDS_{33} and DDS_{47} were selected. Even though the strains BDS_1 and DDS_{16} 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 (Arulmani *et al.*, 2007) and alkaline soil sample (Jasvir *et al.*, 1999; Emtiazi *et al.*, 2005) produced 0.93, 88.0, 0.5, 176.05, 500.0, 420.0, 486.0 and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 167-175
DOI: http://dx.doi.org/10.21161/mjm. 190535

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.	Strain Total
Less than 1	BDS_{11}, BDS_{14}, BDS_{15}, BDS_{17} DDS_4, DDS_{17}, DDS_{23}, DDS_{26}, DDS_{28}, DDS_{30}, DDS_{35}, DDS_{38}, DDS_{39}, DDS_{41}, DDS_{42}, DDS_{43}, DDS_{44}, DDS_{45}, DDS_{46}, DDS_{48}, DDS_{49}, DDS_{50}, DDS_{51}, DDS_{52}, DDS_{54}, DDS_{55}, DDS_{56}, DDS_{57}, DDS_{59}, DDS_{61}	26	35
	FDS_1, FDS_4, FDS_{10}, FDS_{12}, FDS_{13}	5	
1-3.99	BDS_{10}, BDS_{12}, BDS_{13}, BDS_{16} DDS_{11}, DDS_{22}, DDS_{24}, DDS_{27}, DDS_{29}, DDS_{31}, DDS_{32}, DDS_{34}, DDS_{36}, DDS_{40}, DDS_{53}, DDS_{58}, DDS_{60}	13	20
	FDS_7, FDS_8, FDS_9	3	
4-19.9	BDS_3, BDS_4, BDS_5,BDS_7, BDS_9 DDS_1, DDS_6, DDS_8, DDS_9, DDS_{10}, DDS_{12}, DDS_{13}, DDS_{14}, DDS_{15}, DDS_{18}, DDS_{19}, DDS_{20}, DDS_{25}, DDS_{37}	14	22
	FDS_5, FDS_6, FDS_{14},	3	
20-29.9	BDS_2, BDS_6, BDS_8	3	8
	DDS_5, DDS_7,	2	
	FDS_{11}, FDS_2, FDS_3	3	
30-50	BDS_1	1	5
	DDS_{16}, DDS_{21}, DDS_{33}, DDS_{47}	4	
50 or above	DDS_2, DDS_3	2	2

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

580.0 U/mL protease activities respectively at their optimum pH values and temperatures. Among the proteases produced by DDS2, DDS3, DDS21, DDS33 and DDS47, the one produced by DDS2 showed superior characters than the other proteases (Table 3).

In this study, most of the strains had been isolated from dead dog decaying soil. Only one strain from beef decaying soil produced protease activity above 30 U/mL. The strains isolated from the dog decaying soil also might have been those lived in the digestive tract of the dog when it was alive. Further the reason for higher number of organisms from the decaying dog could be because of the different proteins of the animal might have been attacked by the microbes and the volume as well as the total weight of the animal was much more than the amount of beef or fish buried. After the selection of alkaline protease producers, it was decided to select bacterial strain which can produce high titre of alkaline protease with thermostable character.

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 DDS_2, DDS_3, DDS_{21}, DDS_{33} and DDS_{47} were measured at different temperatures ranging from 30 to 95 °C and the optimum temperatures of the proteases from isolates DDS_2, DDS_3, DDS_{21}, DDS_{33} and DDS_{47} were 73, 72, 70, 68 and 55 °C (Table 3). The strains DDS_{33} and DDS_{47} 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 DDS_{21}, DDS_2, and DDS_3 were selected.

Alkaline protease obtained from strain DDS_1 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 DDS_2 showed optimum activity at 73 °C similar to *Bacillus laterosporus* (Manavalan *et al.*, 2007). Alkaline protease obtained from DDS_{47} 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 DDS_2, DDS_3 and DDS_{21} 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 DDS_{33} and DDS_{47} 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 DDS_2, DDS_3 and DDS_{21} also favoured their selection (Table 3).

Stability of enzymes with temperature

The thermostability of the proteases from the strains DDS_2, DDS_3, DDS_{21}, DDS_{47} and DDS_{33} 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 DDS_2 showed best half-life among the alkaline proteases at pH 10.5 and 73 °C

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 167-175
DOI: http://dx.doi.org/10.21161/mjm. 190535

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)
DDS_2	348	73	10.5	454	10.5
DDS_3	296	72	10.5	346	4.0
DDS_{21}	189	70	10.5	204	2.0
DDS_{33}	45	68	9.0	68	2.83
DDS_{47}	38	55	9.0	53	1.33

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); 38 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 DDS_2 showed better thermostability at alkaline pH, the strain was selected.

Determination of the genus of the strain DDS_2

Morphological characters

The colonies of strain DDS_2 at 24 h showed branching pattern. DDS_2 cells were rod shaped, Gram-positive and motile. Spore shape was oval or round. *Paenibacillus*, *Bacillus*, *Clostridiidum*, *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 DDS_2 showed branching pattern (Table 4). Therefore, based on these results, it can be concluded that the strain DDS_2 does not belong to *Bacillus*, *Cornebacterium*, *Lactobacillus* and *Mycobacterium* and the strain DDS_2 can be either *Paenibacillus* or *Clostridiidum* (Ash *et al.*, 1994).

Biochemical test

Biochemical tests were carried out to confirm the genus of the strain. Strain DDS_2 was facultative anaerobic and catalase positive. Strain DDS_2 did not produce soluble pigments on nutrient agar. Strain DDS_2 had the ability to produce oxidase, urease and indole. Strain DDS_2 gave negative results to Voges-Proscaeur test. Hydrogen sulfide is not produced by strain DDS_2. 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 DDS_2 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 DDS_2 was identified.

Determination of the species of the strain DDS2

Morphological characters

The colonies of the strain DDS_2 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.* dendritiformis is T morphotype pattern-forming. *Paenibacillus* sp showed T morphotype colony morphology and hence the strain DDS_2 could be *P. dendritiformis* (Tcherpakov, 1999).

Biochemical test

Strain DDS_2 had the ability to produce catalase and utilize citrate. The acid producing ability of the strain DDS_2 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. *pulvifaciens* do not produce catalase. Therefore, based on these results, it can be concluded that the strain DDS_2 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. dendritiformis* sp. nov. (Table 4) (Tcherpakov, 1999). Based on the biochemical tests, strain DDS_2 had the characters of either *P.*

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 167-175
DOI: http://dx.doi.org/10.21161/mjm. 190535

Table 4: Morphological and biochemical test results of the strain DDS_2.

Characters	*Paenibacillus*	Strain DDS_2
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
O_2 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-Proscaeur test	Variable	Not produce
Reduction of nitrite	Variable	Not produce
Hydrolyze tyrosine	Variable	Not produce
Hydrolyze starch	Variable	Produce
Hydrolyze casein	Variable	Produce
Growth temperature	Variable	40, 45 and 50 °C
Growth in NaCl	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 DDS_2 isolated from dog decaying soil was classified as *P. dendritiformis* with 99% of homology (Table 6). The morphological and biochemical studies, and gene sequencing, DDS_2 was confirmed to be belonging to the Kingdom: Procaryotae; Division: Bacteria; Order: Bacillales; Family: Paenibacillaceae; Genus: *Paenibacillus*; Species: *dendritiformis*. The *P. dendritiformis* DDS_2 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 BD3526 (Hang *et al.*, 2016) and *P. tezpurensis* 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 (Cornman *et al.*, 2013; Chen *et al.*, 2009; Al-Ghamdi *et al.*, 2018). *P. dendritiformis* has been used for the formation of biosurfactant (Bezza and Chirwa, 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* DDS_2 was 73 °C and 10.4 respectively. *P. tezpurensis* 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 DDS_2 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-Mannopyranoside	Positive	D-Xylose	Negative
Methyl-α-D-Glucopyranoside	Positive	L-Xylose	Negative
N-Acetyl-glucosamine	Positive	D-Fructose	Negative
Esculin	Positive	L-Rhamnose	Negative
D-Cellobiose	Positive	Methyl-ß-D-xylopyranoside	Negative
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-Lxyose	Negative
D-Raffinose	Positive	D-Arabitol	Negative
Amidon	Positive	L-Arabitol	Negative
Glycogen	Positive	Potassium-2-ketogluconate	Negative
Gentiobiose	Positive	Potassium-5-ketogluconate	Negative
D-Turanose	Positive		

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 167-175
DOI: http://dx.doi.org/10.21161/mjm. 190535

Table 6: The matching of 16S rDNA sequence obtained by PCR-amplified fragment from genomic DNA of strain DDS_2.

Description	Accession No.	Identity matching (%)
P. dendritiformis	AY359885.1	99
P. thiaminolyticus	Y16129.1	98
Paenibacillus sp. *GPTSA9*	EU330645.1	98
Bacillus sp. AT6	AB073197.1	98
Paenibacillus popilliae	FJ821592.1	98
P. popilliae strain KLN 3	AJ320490.1	98

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* DDS_2 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 DDS_2 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 DDS_2 from dog decaying soil was identified as *P. dendritiformis* and labeled as *P. dendritiformis* DDS_2. 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* DDS_2 is a useful candidate for this purpose.

ACKNOWLEDGEMENTS

Authors thank the International Program in Chemical Sciences, Sweden and Sida/ SAREC, for Financial Assistance and Mr. M. Sutharshan for technical assistance.

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