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Isolation and identification of bacteria producing extracellular protease from Port Dickson Seashore, Negeri Sembilan and Kelana Jaya Lake, Selangor

Abdullah Yahya[1], Mohammed Razip Samian[2], Aida Baharuddin[1]*

1 Department of Biotechnology, Faculty of Science, Lincoln University Collage MALAYSIA, Kelana Jaya Campus, No 2, Jalan Stadium SS7/15, Kelana Jaya, 47301, Selangor, Malaysia.
2 School of Biological Sciences, University Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia.

E-mail : envelope910@gmail.com Contact No. : Tel: +603-78063478, Fax: +603-78063479

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Abstract

This study was conducted to isolate extracellular protease producing bacteria from the Port Dickson seashore and Kelana Jaya Lake using clear zone formation assay on skim milk agar medium. The extracellular protease producing bacteria identifications were based on morphological examination, Gram staining, oxidase test, lactose fermentation test, catalase test, hemolysis test and bioMérieux's API® identification system. Nine isolates, namely S1, S19, S25, L20, L21, L30, L40, L41, L44, exhibited hydrolysis capacity (HC) between 1.5 to 2.3 when incubated at 30°C for 24, 48, and 72 hour periods. The sea isolate, namely S19, exhibited the highest HC after 24, 48, and 72 h at 30ºC. S19 was identified as *Bacillus cereus* 2 by API 50 CHB/20E. The second-best HC was represented by lake isolate L20 and identified as *Pseduomonas aeruginosa* by the API 20NE system.

Key words : Screening, Bacteria, Extracellular proteases, API® bacterial identification system

INTRODUCTION

Proteases are one of the major hydrolytic enzymes and exhibit a wide range of functions and important biotechnological applications. Proteases play a vital role in various detergents, leather, food, and pharmaceuticals industries, as well as bioremediation [1, 2]. Animals, plants, and microorganisms are protease producers. However, microbial sources are most preferred due to several advantages, such as their broad biochemical diversity and bioengineering potential [3]. Furthermore, microbes are a preferable source of protease due to their rapid growth and genetic manipulation, allowing the creation of novel enzymes with new properties desirable for their diverse applications [4].

Sixty percent of total worldwide enzyme sales are derived from microbial proteases. For most biotechnology applications, protease enzymes with higher activity and stability under acidic or alkaline pH and high temperature are the most desired because of its potentiality [3-7]. Proteases are produced by different categories of microorganisms such as yeast, moulds and bacteria [3, 7, 8]. Bacterial proteases are generally considered one of the main categories of industrial enzymes, as they constitute about a quarter of the total worldwide enzymes production [9]. About 40% of the total industrial enzymes in the world are produced by bacterial proteases [10, 11].

Extracellular proteases secreted by bacteria play important roles in biotechnology industries and products as diverse as detergent, tanning, photography, pharmaceutical, and waste treatment thus creating a need to identify new proteases with altered properties favourable to their various usage scenarios. A large number of bacterial strains isolated from seas and lakes can tolerate extreme environments such as highly acidic and alkaline conditions as well as growth in high and low temperatures, consequently the physical and chemical properties their proteases are adapted to their growth conditions. Screening of extracellular proteases from bacterial isolates from sea and lake environments may result in isolation of new proteases with unique physiochemical characteristics. Substrates such as skimmed milk agar [12] and casein-agar [13] are widely used for screening many microorganisms for protease secretion. The amount of protease produced by an organism is exhibited by the hydrolysis zone on casein agar [14]. To avoid pathogenic bacteria for biotechnological application, identification of microbial proteases is an important step that must be performed soon after the screening process.

In the present study, an effort was made to determine proteolytic activity of bacteria isolated from the Kelana Jaya Lake in Petaling Jaya, Selangor and Port Dickson seashore, Negeri Sembilan, Malaysia on skim milk agar plates using streaking and well diffusion methods. Identification of the bacteria that produced the best hydrolytic capacity was performed based on morphological observations and Gram staining, catalase test, hemolysis test, oxidase test, lactose fermentation test and bioMérieux's API® identification system.

MATERIALS AND METHODS

Sampling Site and Sampling

Two water samples were collected in January 2016, one from Port Dickson seashore in Negeri Sembilan and another from Kelana Jaya lake in Selangor. The water samples were collected in sterile containers, carried to the laboratory, and kept at 4 ºC until analysis.

Isolation and Purification of Bacteria from the Water Samples, Sea and Lake

Serial dilution was prepared for each water sample (lake and sea) by preparing 7 sterile tubes, labelled from 10^0 to 10^{-6} [15]. Two hundred microliters of the non-diluted and diluted samples were plated on nutrient agar. The spread-plate technique was used for isolating single bacterial colony. The plates were incubated for 18-24 h at 30ºC. Fifty well-separated bacterial colonies from the sea and 50 from the lake were selected at serial dilutions of 10^{-2},

10^{-3} and 10^{-4}. These bacteria were further purified by streaking on Nutrient Agar and then incubated at 30°C for 18-24 h. The selected isolated bacteria were streaked on nutrient agar three times to ensure that the selected bacterial colonies were pure, then routinely maintained at 4ºC [16].

Screening of Bacterial Producing Extracellular Protease on Skim Milk Agar

One hundred pure bacterial colonies were selected for screening of the extracellular protease production by streaking thrice on the skim milk agar plates. The plates were then incubated at 30°C and clear zones formations were monitored after 24, 48, and 72 h. The appearance of the clear zones indicated proteolysis activity via degradation of the milk protein (casein) in the agar medium [17].

Determination of Hydrolysis Capacity (HC) by Well Diffusion Assay

A hole (5 mm diameter) was punched on a skim milk agar and 10 µl of bacterial suspension inoculums was prepared by comparing to the 0.5 McFarland turbidity standards, then loaded into the hole. The plates were then incubated at 30°C and clear zones formations were monitored after 24, 48, and 72 h. The diameter of the clear zone formation was measured. Escherichia coli K12 JM109 was used as a negative control.

Proteolytic potential of positive isolates was qualitatively estimated by calculating the HC, the ratio of diameter of clearing zone and diameter of colony [18]. All HC data obtained from the well diffusion assay were confirmed by performing independent experiments in triplicate and are presented as the mean ± SD.

Identification of Bacteria Producing Extracellular Protease

The overall methodology of the work is represented in Fig.1.

Morphological Observation

The morphological identification of the isolates was performed by observing the shape, margin, texture, and opacity as well as pigments production on nutrient agar. The observations were performed via the naked eye [15].

Gram Staining

The Gram staining procedure was carried out per [19]. A thin smear of the bacteria was prepared as follows. One drop of normal saline was added to the surface of a clean glass slide. Bacterial cells were smeared on the surface of the glass slide containing a drop of normal saline with a clean sterile wire loop. The smears were then dried and fixed with heat by passing through a bunsen burner three times. Crystal violet was added to the dried smears and incubated at room temperature for 1 minute. To remove unbound crystal violet, the slide then was rinsed gently with water for a maximum of 5 seconds.

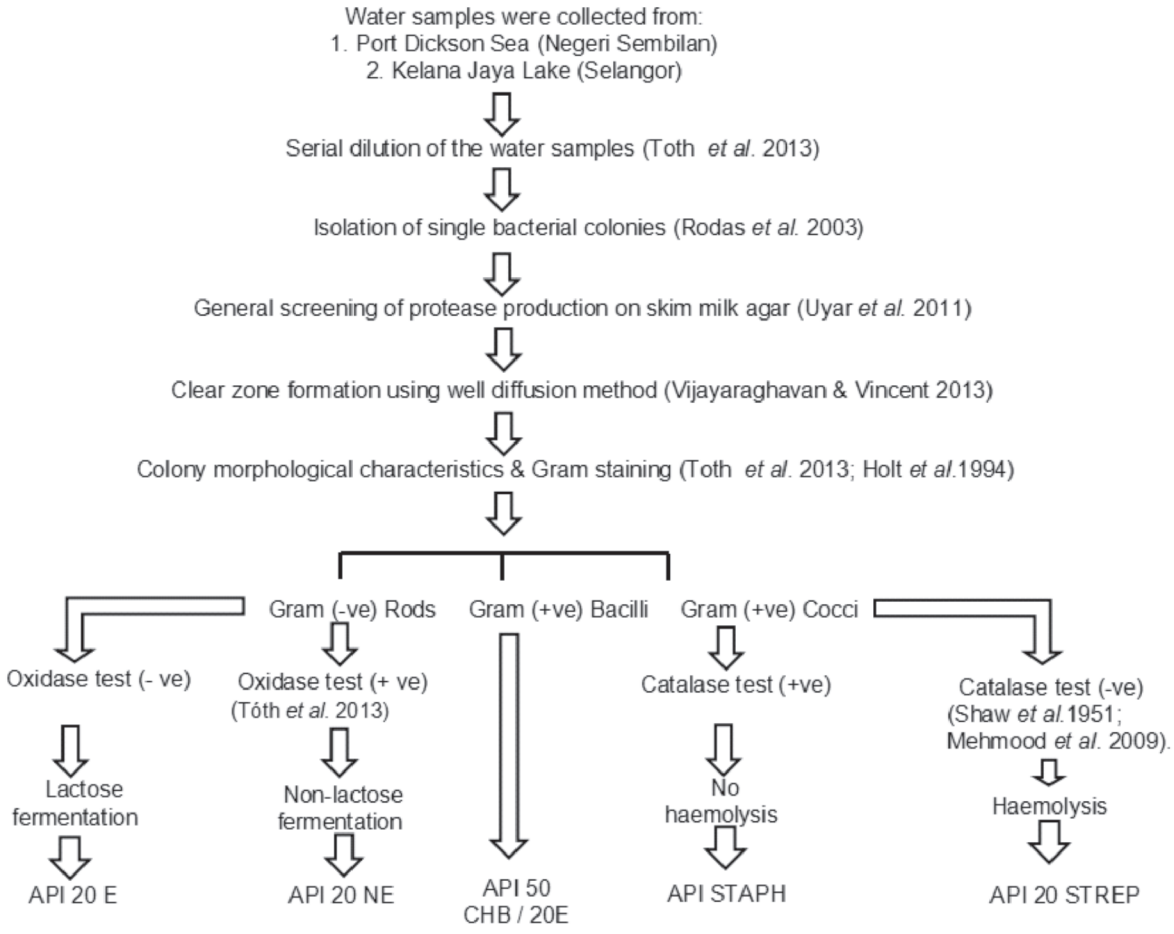

Fig. 1: Schematic representation of overall plan of work

Following to this, iodine solution was used to fix the crystal violet to the bacterial cell wall. The slide was then rinsed with ethyl alcohol (95%) for about 3 second and rinsed again with a gentle stream of water. Finally, the smeared slide was counter stained with safranin and incubated for 1 minute follow with gentle rinsing with water for a maximum of 5 seconds, then air dried at room temperature. The prepared slide was observed under oil immersion (100x) using a bright field microscope. Gram-negative bacteria stained pink/red while Gram-positive bacteria stained blue/purple.

Oxidase Test

The oxidase test was used to distinguish between oxidase negative Enterobacteriaceae and oxidase positive Pseudomadaceae, and also useful for specification and identification of many bacteria that use oxygen as the final electron acceptor in aerobic respiration [20]. Filter paper was impregnated with a drop of Oxidase reagent (1% Tetramethyle-p-phenylene diamine dihydrochloride solution). The bacterial cells were picked from fresh plate culture (24 h growth) with sterile wooden rods and placed on filter paper with the reagent. The reaction was read within 10 seconds and deep blue colour was developed which indicated positive result [15]. Pseudomonas aeruginosa was used as positive control and E. coli K12 JM109 was used as negative control.

Lactose Fermentation Test on MacConkey (MAC) Agar

The purpose of this test is to determine if a microbe can ferment the carbohydrate (sugar) lactose as a carbon source. MAC is a selective and differential culture medium. It is used to select Gram-negative and enteric bacilli and differentiate them based on lactose fermentation. The selected bacterial colonies were aseptically streaked on the MAC agar and incubated for 48 h at 35°C. The presence of creamy-pink coloured colonies indicated the growth of lactose ferments on MAC. E. coli K12 JM10 was used as a positive control and Pseudomonas aeruginosa was used as negative control.

Catalase Test

This test was used to identify organisms that produce the enzyme catalase. The function of this enzyme is to detoxify hydrogen peroxide by breaking it down into water and oxygen gas. The catalase test was performed per [21, 22]. A small amount of fresh bacterial colony (24 h growth) was transferred to a surface of clean, dry glass slide using a sterile loop. Two drops of distilled water were dropped onto the slide and mixed to make a bacterial suspension. Then, two drops of 3% H_2O_2 (bioMerieux, USA) were dropped onto the suspension. Appearance of bubbling immediately indicated a positive result while no bubbles or only a few scattered bubbles indicated a negative result. Staphylococcus

Table 1: Percentage of bacteria producing extracellular protease (positive) and not producing extracellular protease (negative).

Source of isolates	Total isolates	Positive	Percentage of positive colonies	Negative	Percentage of negative colonies
Sea	50	22	44%	28	56%
Lake	50	19	38%	31	62%

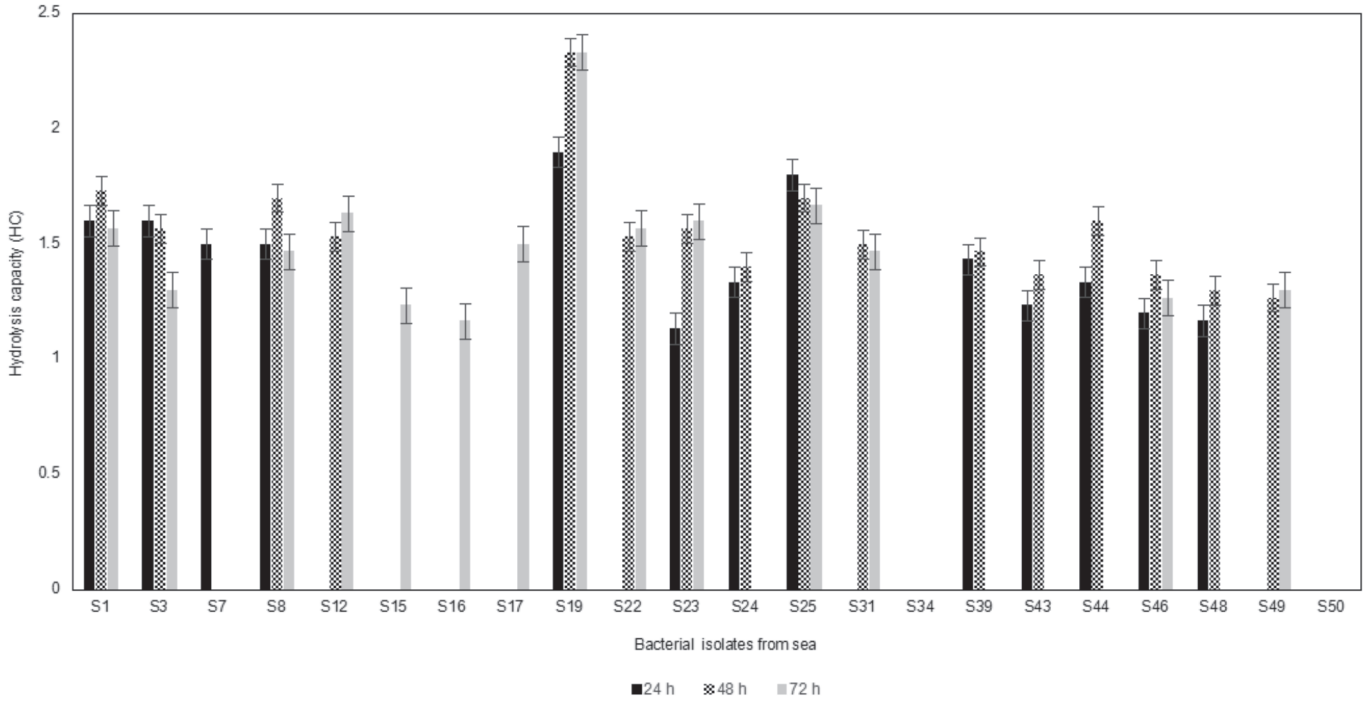

Fig. 2: Hydrolytic capacity of sea isolates after 24, 48 and 72 h incubation at 30ºC

aureus was used as the positive control and Streptococcus pyrogen was used as the negative control

Hemolysis Test on Columbia Sheep Blood Agar

A blood agar plate was used to tests the ability of an organism to produce hemolysins, enzymes that damage/lyse red blood cells (erythrocytes). The degree of haemolytic activity in these hemolysins is useful in identifying members of the genera Staphylococcus, Streptococcus and Enterococcus.

Gram-positive cocci that are catalase positive were cultured on the Columbia sheep blood agar and incubated for 24 h at 35°C. The bacterial was streaked in a long W pattern using the inoculating loop with enough of space in between the streak lines thus making it easier to observe the results. To help observed the haemolytic activity clearly; the plates were hold up to a light source. Streptococcus pyrogen was used as positive control for β-hemolysis, Streptococcus pneumonia was used as positive control for α-hemolysis and E. coli K12 JM109 was used as control for gamma on non-hemolysis.

BioMerieux's API® Bacterial Identification

BioMerieux's API® Identification kits (bioMerieux, USA) were used according to the manufacturer for identification of Gram-positive and Gram-negative bacteria. Four types of bioMerieux's API® Identification kits were used in this study. The kits used were API 50 CHB /20E, API 20NE and API STAPH (bioMerieux's API® Identification kits)

RESULTS

Screening of Bacteria Producing Extracellular Protease on Skim Milk Agar

Table 1 shows the result of screened for extracellular protease production by streaking on the skim milk agar and incubation at 30°C for 24, 48, and 72 h. Forty-one out of the 100 isolates

Table 2: Identification of bacteria producing extracelullar protease from Port Dickson Sea

		Sea bacteria isolates		
		S1	S19	S25
Colonies morphological observation	Shape/Form	Round	Irregular	Irregular
	Margin	Entire	Filiform	Filiform
	Elevation	Raised	Raised	Flat
	Opacity	Opaque	Opaque	Opaque
	Texture	Smooth	Smooth	Rough
	Pigmentation	Yellow	Pale white	Pale white
Gram staining		Positive	Positive	Positive
Shape		Cocci	Bacilli	Bacilli
Arrangement		Clusters	Single, pair and chains	Single and chains
Oxidase test		ND	ND	ND
Lactose fermentation test		ND	ND	ND
Catalase test		positive	ND	ND
Haemolysis test		negative	ND	ND
bio Merieux's API identification	T- value	could not be identified by the API STAPH system	0.76	0.75
	ID%		99.0	99.8
	Identification		Very good identification *Bacillus cereus 2*	Very good identification *Bacillus cereus 2*

ND : Not determined

exhibited proteolytic activity through the appearance of clear zones. The milk protein (casein) was hydrolyzed by extracellular protease in the agar medium thus formed the clear zones. Twenty two out of the 50 isolates were from the sea sample and 19 out of the 50 isolates were from the lake sample.

Hydrolytic Capacity (HC) Measurement of Sea Isolate by Well Diffusion Assay

According to the well diffusion assay, 3 out of the 22 positive isolates from the sea, namely S1, S19, and S25, exhibited the highest hydrolytic capacity based on the ratio of diameter of clearing zone and colony after 24 h of incubation at 30ºC (Fig. 2 and 3). This indicates that the bacteria isolates had the ability to degrade casein to produce nitrogen source for making proteins and nucleic acids. Among the three isolates, S19 exhibited significant increase of hydrolytic capacity as observed at 24 and 48 h, and after that it became stagnant as indicated at 72 h of incubation. Isolates S15, S16, and S17 produced the extracellular protease only at 72 h with low HC of between 1.0 - 1.5.

A spreading behaviour was observed in isolate S7, as it grew and spread rapidly over the skim milk agar surface to the edge of the agar plate at 48 h of incubation at 30 ºC. This behaviour was also observed in another isolate, namely S24; however, this pattern was only observed at 72 h of incubation at 30ºC. Due to the spreading of the colonies to the edge of the agar plate, the clear zone was diminished. Thus, the HC could not be determined at 48 h for S7 and at 72 h for S24. According to Kearns [23], this pattern of movement is called "swarming", in which bacteria arrange into large colonies with intricate and complex architectures, allowing them to expand efficiently by taking advantage of the available resources. Spreading of isolates S34 and S50 was pronounced at 24 h, with spreading seen to the edge of the petri dish. Thus, no

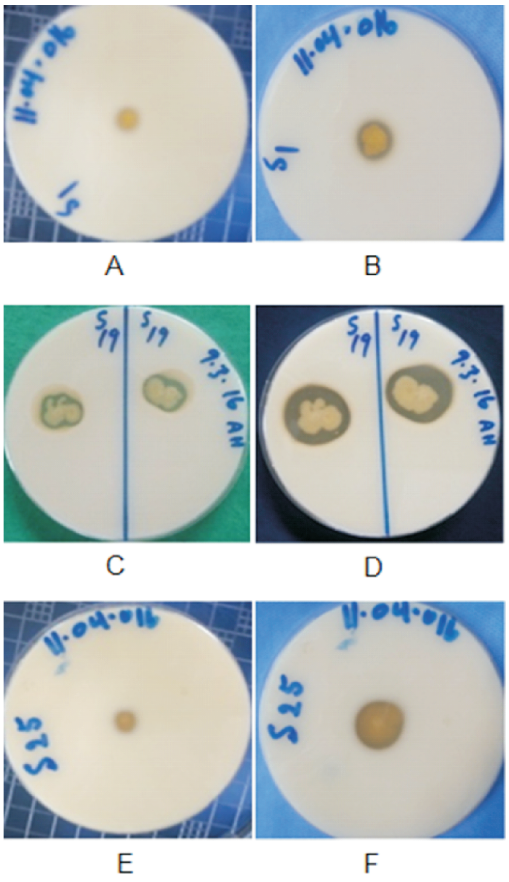

Fig. 3: Hydrolysis zone formation of sea isolates after 24 and 48 h of incubation at 30ºC. (A) S1 after 24 h. (B) S1 after 48 h. (C) S19 after 24 h. (D) S19 after 48 h. (E) S25 after 24 h. (F) S25 after 48 h.

Table 3: Identification of bacteria producing extracellular protease from Kelana Jaya Lake

		Lake bacteria isolates					
		L20	L21	L30	L40	L41	L44
Colonies morphological observation	Shape/Form	Round	Irregular	Irregular	Round	Round	Round
	Margin	Entire	Filiform	Filiform	Entire	Entire	Entire
	Elevation	Convex	Flat	Flat	Convex	Convex	Convex
	Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
	Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
	Pigmentation	Produced dark greenish/ brownish pigment on the plate	Whitish creamy	Whitish creamy	Pale white	Yellow	Pale white
Gram staining		Negative	Positive	Positive	Negative	Positive	Negative
Shape		Rods	Bacilli	Bacilli	Rods	Cocci	Rods
Arrangement		Single	Single, pair and chains	Single, pair and chains	Single	Single and chains	Single
Oxidase test		Positive	ND	ND	Positive	ND	Positive
Lactose fermentation test		Negative	ND	ND	Negative	ND	Negative
Catalase test		ND	ND	ND	ND	Positive	ND
Haemolysis test		ND	ND	ND	ND	Negative	ND
	T- value	0.78	0.43	0.63	0.50		
bio Merieux's API identification	ID%	99.4	99.7	99.4	98.7	could not be identified by the API STAPH system	ND
	Identification	Very good identification *Pseudomonas aeruginosa*	Very good identification *Bacillus cereus 1*	Very good identification *Bacillus licheniformis*	Very good identification *Stenotrophomnas maltophilia*		

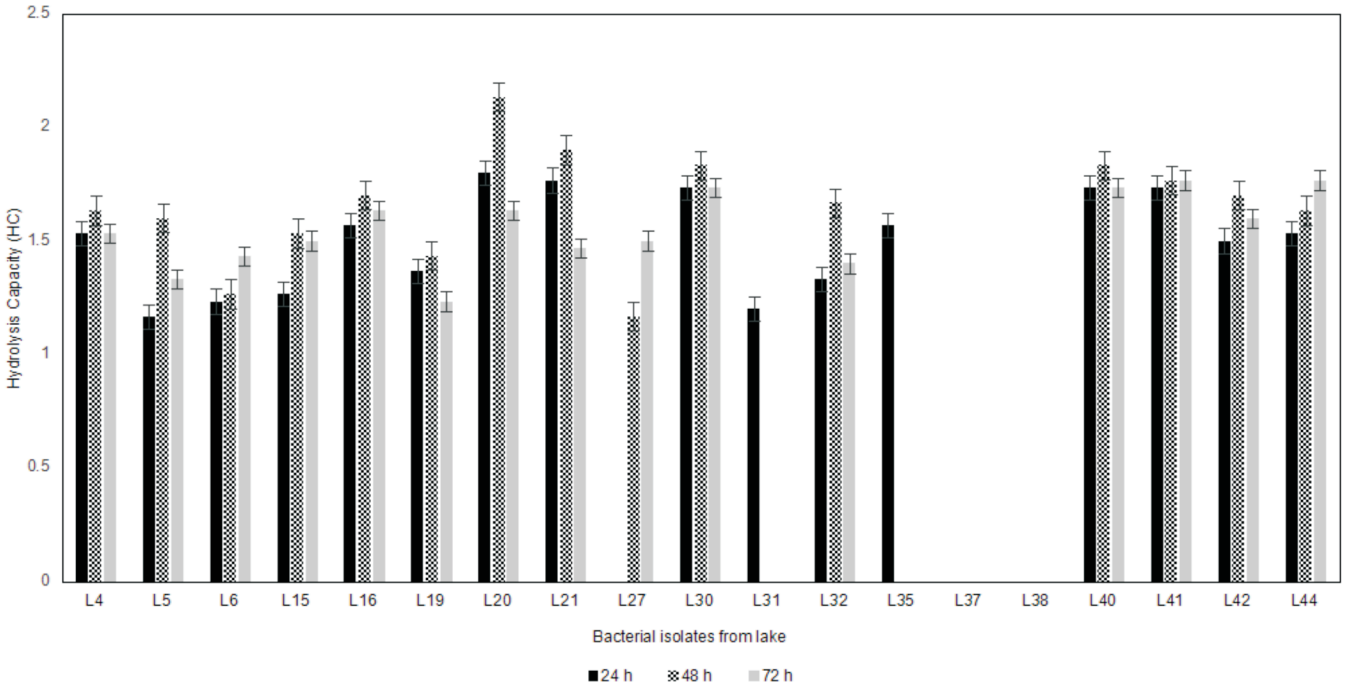

Fig. 4: Hydrolytic capacity of lake isolates after 24, 48 and 72 h incubation at 30ºC

clear zone was observed. Interestingly, this spreading behaviour was not observed when the streaking technique was used in the first screening.

Hydrolytic Capacity (HC) Measurement of Lake Isolate by Well Diffusion Assay

As observed in Fig. 4 there are 3 patterns of HC exhibited by L20, L21, L30, L40, L41 and L44 isolates. These 6 bacteria exhibited the highest HC for lake isolates. The L20 isolate exhibited the second best HC after the S19. The L20 and L21 have the same patterns, where both exhibited increasing hydrolytic capacity from 24 h and reached the highest HC at 48 h, and then the HC decrease as observed at 72 h. The second pattern of hydrolytic capacity exhibited by bacteria L30, L40 and L41 where the HC was almost similar at 24, 48, and 72 h of incubation time. Although the HC was slightly increased from 24 to 48 h, it was not significant. The last pattern of HC showed by L44 where there is a steady increasing of the hydrolytic capacity from 24 to 72 h of incubation.

Similar to the sea isolates, few bacterial colonies namely L31, L35, L37 and L38 grew and spread rapidly over the skim milk agar surface to the edge of the agar plate. Due to this phenomenon, the HC could not be determined (Fig. 4).

Identification of Bacteria Producing Extracellular Protease

Morphological observation, Gram-staining, oxidase test, lactose fermentation test, catalase test, hemolysis test and API® identification results of the nine isolates namely S1, S19, S25, L20, L21, L30, L40, L41 and L44 that exhibited the best HC (between 1.5 to 2.3) when incubated between 24 to 72 h at 30°C are shown as in Table 2 and Table 3.

From the Gram staining experiment and shape observation, 4 bacteria isolate namely S19, S25, L21 and L30 were identified as Gram-positive with bacilli shape thus were subjected to the API

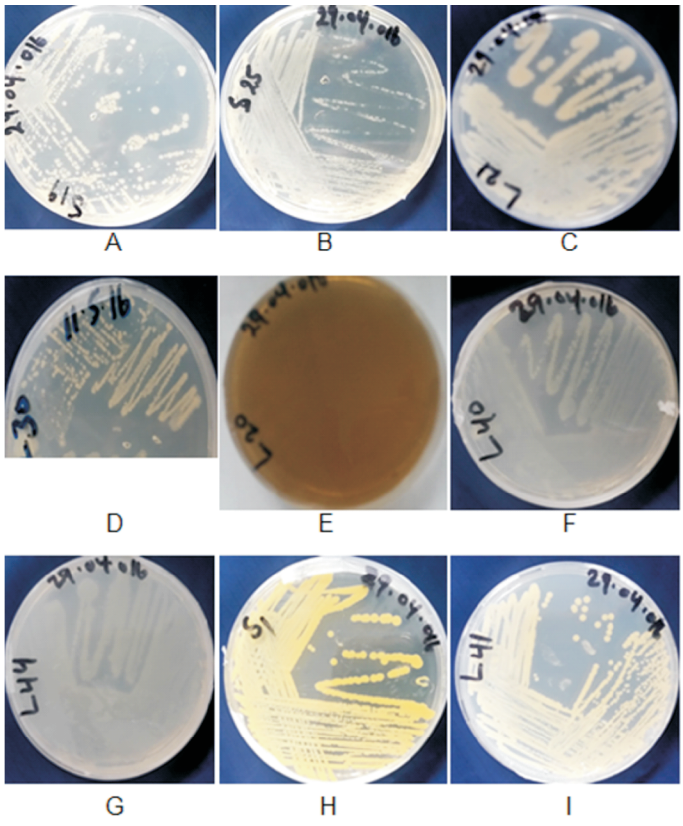

Fig. 5: HBacteria producing extracellular protease from Port Dickson Sea, and Kelana Jaya Lake. (A) S19 - *Bacillus cereus* 2. (B) S25 - *Bacillus cereus* 2. (C) L21 - *Bacillus cereus* 1. (D) L30 - *Bacillus licheniformis.* (E) L20 - *Pseudomonas aeruginosa.* (F) L40 - *Stenotrophomonas maltophilia.* (G) L44 not determined. (H) S1 could not be identified by API STAPH. (I) L41 could not be identified by API STAPH

50 CHB / 20E bacteria identification system. The sea isolate S19 exhibited 99.0% identification with Bacillus cereus 2 with a high confident level of (T-value) 0.76. The S19 isolate did not produce pigment but it exhibited pale white colour (Fig. 5A). The S25 sea isolate was also identified as a Bacillus cereus 2 with a very good identification percentage of 99.8% and a T-value of 0.75%. Similar to S19, the S25 colonies possessed pale white colour and did not produced pigments (Fig. 5B). Although S19 and S25 were identified as Bacillus cereus 2, the patterns of HC of both bacteria were somehow different. Unlike S19, the HC of the S25 were almost similar (HC ~ 1.8, 1.7, 1.7) at 24, 48, and 72 h of incubation whereas S19 at 24 h was 1.9 and exhibited significant increased as observed at 48 h (HC = 2.3) and remain the same at 72 h. The lake isolates, L21 and L30 were identified as Bacillus cereus 1 and Bacillus licheniformis, respectively. These isolates did not produce pigment and exhibit whitish creamy colonies (Fig. 5C and 5D).

The Gram-negative rod isolates that showed oxidase positive and non-lactose fermentation, L20 and L40 were subjected to the API 20NE bacteria identification system. The L20 isolates was identified as Pseudomonas aeruginosa with a very good identification (ID % = 99.4%) and confident level, T-value of 0.78. Furthermore, the L20 isolate exhibited transparent brownish colonies and produced dark greenish/brownish pigment on the agar surface (Fig. 5E). The dark greenish diffused pigment on the agar surface was one of the Pseudomonas aeruginosa characteristics. The L40 isolate was identified as Stenotrophomonas maltophilia with a very good identification of 98.7% and high confident level, T-value of 0.5. The L40 did not produce pigment and exhibited pale creamy colonies (Fig. 5F). Stenotrophomonas maltophilia is an aerobic Gram-negative bacillus that is found in various aquatic environments. The identification of L44 was not determined but it exhibited pale creamy colonies similar to L40 (Fig. 5G). Based on the morphological observations, L44 might be classified as Stenotrophomonas sp, similar to L40.

The S1 and L41 were identified as Gram-positive coccus showed catalase positive and hemolysis negative were subjected to the API STAPH bacteria identification system. The S1 exhibited yellow colonies (Fig. 5H) and L41 (Fig. 5I) possessed pale yellow colonies. Both colonies showed Staphylococcus characteristics; Gram-positive coccus, catalase positive and no haemolysin on blood agar. However, both isolates could not be identified by the API STAPH system. It seems that S1 and L41 might belong to the Micrococcus sp. family.

DISCUSSION

The motivation of conducting this project is to discover new unique properties of extracellular protease which is biotechnological important. Though plants and animals also produce extracellular proteases, microorganisms are preferred source of proteases because of their rapid growth, limited space required for their cultivation, longer shelf life and the ease with which they can be genetically manipulated to generate improved enzymes. Proteases with high activity and stability in high alkaline range and high temperatures are interesting for bioengineering and biotechnological applications. According to Cui et al. [24] alkaline protease produced by marine bacteria had significant activity and stability at high pH and temperatures. In addition, this study also explored the ability of bacteria isolated from the freshwater lake located in the Kelana Jaya city to produce extracellular protease with unique properties.

Skim milk was used as the proteolytic screening medium due to its simplicity and cost effective. The bacteria were grouped according to their efficiency of producing extracellular protease by calculating the hydrolysis capacity (HC), i.e. the ratio of diameter of clearing zone and colony. The S1 and L41 could not be identified by the API STAPH identification system although these bacteria exhibited Staphylococcus characteristic. The S1 produced yellow pigment with L41 produced more lightly yellow pigment, Gram-positive coccus, catalase positive and no haemolysin was detected on blood agar. These bacteria might belong to the Micrococcus sp. The 16S ribosomal RNA gene sequencing can be used for the confirmation. .

The Bacillus family are represented by the S19 and S25. Both were identified as Bacillus cereus 2. L30 is identified as Bacillus licheniformis, and L21 is identified as Bacillus cereus 1. Although these bacteria belongs to the same family, they exhibited different patterns of HC value that were observed at 24, 48 and 72 hours. Multitudes of Bacillus species producing extracellular protease had been discovered since 1970 [25]. As of July 15, 2017, Google Scholar search on "extracellular protease production by Bacillus licheniformis" and "extracellular protease production by Bacillus cereus" produced about 14,900 and 26,100 results, respectively. Vijayaraghavan et al. [26] showed Bacillus cereus strain AT produced a high level of alkaline protease using cow dung substrate (4813 ± 62 U g−1) under solid-state fermentation. From the industrial point of view, cow dung is an ideal substrate for enzyme bioprocess due to its cheap cost and availability. Current study on alkaline protease by B. licheniformis can be found in [27-29].

Extracellular protease from P. aeruginosa had been studied extensively as indicated by the 46,100 results as of July 15, 2017. For example, a novel extracellular protease from Pseudomonas aeruginosa MCM B-327 was identified that may have application in dehairing for environment-friendly leather processing [30]. Optimization of fermentation conditions, extracellular protease characterization and cloning of the extracellular protease gene from Stenotrophomonas maltophilia had been studied by [31-33]. According to Xiuqin et al. [32] S. maltophilia strain D2 produce an extremely high optimal pH and moderate thermal tolerance of the alkaline serine protease, suggested it may be potential for use as a biocatalyst in the industry. Interesting feature exhibited by the S. maltophilia KB13 where it could metabolize 10 g/l chicken feathers as a sole source of carbon and nitrogen [33]. This strain produces protease activity and reached its maximum level (103.26 ± 7.09 U/ml) on the 4th day of incubation.

CONCLUSION

In conclusion, 22 out of the 50 bacteria from the sea sample and 19 out of the 50 bacteria from the lake sample were successfully isolated to produce extracellular protease, as indicated by the clear zone formation on skim milk agar. Nine isolates, namely S1, S19, S25, L20, L21, L30, L40, L41, L44 exhibited the best HC (between 1.5 to 2.3) when incubated between 24 to 72 h at 30°C. Sea isolate S19 exhibited the fastest as well as the highest extracellular protease producer after 24, 48, and 72 h at 30ºC. The S19 was identified as Bacillus cereus 2 by API 50 CHB/20E. Further investigation on the growth kinetics of the extracellular protease, type of substrate source and type of protease produced by S19 (Bacillus cereus 2), S25 (Bacillus cereus 2), L20 (Pseudomonas aeruginosa), L30 (Bacillus licheniformis), L21 (Bacillus cereus 1) and L40 (Stenotrophomonas maltophilia) need to be evaluated in order to

characterize their extracellular protease properties.

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