

ISOLATION AND MOLECULAR CHARACTERIZATION OF PHYTASE PRODUCING BACTERIA FROM MALAYSIA HOT SPRINGS

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ABSTRACT

Potential phytase producing bacterial were isolated from different hot springs in Malaysia which were Labis (Johor), Dusun Tua (Selangor), Ulu Legong (Kedah) and Ranau (Sabah). All bacterial samples were grown in Phytase Screening Media (PSM) at temperatures of 37 °C, 45 °C, 50 °C and 55 °C for four days. Out of all samples tested, five isolates have showed clearing zone surrounding the colony which indicates presence of phytase activity. The strains were further analyzed in broth culture using sodium phytate as substrate. Among them, strain L3 was selected as the best producer (0.16 U/ml after 72 h of culture). This phytase showed optimal activity at 37 °C and pH5.5, demonstrating appropriate character for application in feed of several industrial processes. Blast homology of 16S rRNA gene sequence of isolate L3 identified 99% similarity to *Acinetobacter baumannii*.

Keywords: *Acinetobacter baumannii*; phytase; phytic acid.

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1. INTRODUCTION

Phytate is an anhydrous salt form of phytic acid which is the major storage form of phosphorus and inositol in plants seeds. Phytate capable of producing 65-80% of the total



phosphate in grains [1] and up to 80% of total phosphate in manures from monogastric animals [2]. They are strongly complexed in soils, representing a vital class of organic phosphate [3]. Phytate degradation is mediated by an enzyme, phytase to release the phosphate ions [4]. This enzyme is a type of phosphatases which hydrolyses phytate to a series of *myo*-inositol phosphate derivatives and inorganic phosphate [5]. Phytases are widespread in nature and can be found in microorganisms, plants, fungi, yeast as well as in some animal tissues [6]. Phytase producing microorganism can be found abundantly in rhizospheric soil of crop plants such as *Aspergillus ficuum* [7], *Escherichia coli* [8] and *Bacillus subtilis* [9]. According to some studies, microbial phytases are most promising for biotechnology application [10]. Based on a study by [3], although the commercial productions of phytases are more focused on the fungus *Aspergillum*, there are some studies suggested bacterial phytases can be an alternative to the fungal phytases due to their higher specificity, greater resistance to proteolysis and better catalytic efficiency. These bacteria are of interest for biotechnology application such as improving animal feed [11] and also used as soil inoculants to increase phosphate uptake by plants [12]. Currently, microbial phytases has been extensively used as feed additives in diets for swine and poultry to enhance the utilization of plant based feed [13]. Nowadays, there is no single phytase that is able to meet the diverse needs for all commercial and environmental applications [14-15]. Therefore, there is an ongoing interest in screening microorganisms, including bacteria for novel phytases and also phytases play significant role in improving the nutritive quality of food and feed that contain phytic acid.

2. RESULTS AND DISCUSSION

2.1. Isolation of Phytase Producing Bacteria

Bacterial samples were isolated from different hot spring in Malaysia such as Ranau (Sabah), Ulu Lengong (Kedah), Dusun Tua (Selangor) and Labis (Johor). The bacterial samples were screened for positive phytase producer by plating the bacteria on Phytase Screening Media (PSM), which contains sodium phytate as a source of phytate to the bacteria [16]. Bacteria strains which are capable to form a halo zone onto the surrounding medium were counted as a phytase producing bacteria. Out of all bacteria strains screened, only five strains (Strains A,

B, D3, RT and L3) forms halo zone on the surrounding media after 4 days of incubation at 37 °C showing positive results as phytase producer. During plate screening for extracellular phytase-degrading ability on PSM agar media, Strain B and Strain L3 were found to be better strains that produced approximately 32 mm clear halo zones around the colonies on fourth day of incubation (Fig. 1).

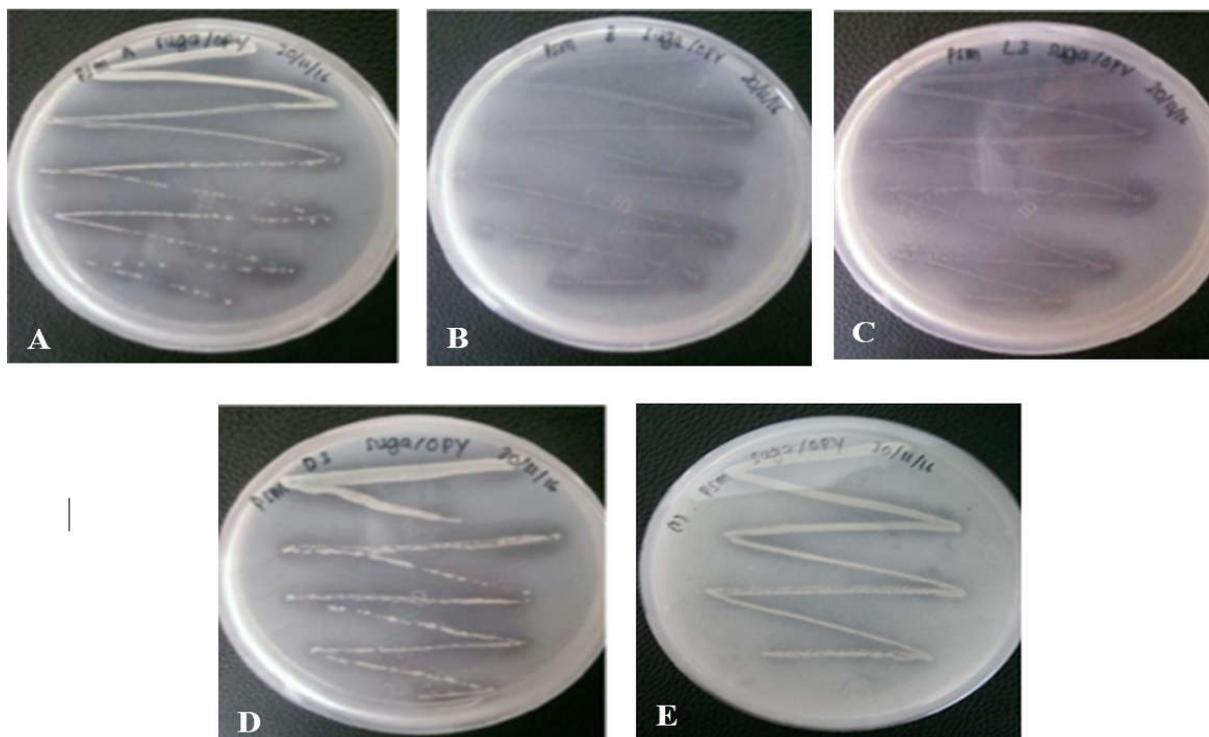


Fig.1. Five isolated strains forming halo regions after streaking on Phytase Specific Agar medium. A; Strain A, B; Strain B, C; Strain L3, D; Strain D3, E; Strain RT

Purified strains were examined under microscope for colony morphology (Table 1) and gram staining (Fig. 2). All the five strains were found to be gram negative bacteria as it retains the pink colour of safranin. Gram negative bacteria are made up of a thin peptidoglycan layer and also lipopolysachharide layer where the purple colour of crystal violet stain can be easily washed off and counter stained with pink. Colony morphology was analyzed using three important features according to Bergey's Manual of Systematic Bacteriology [17] which are form, elevation and margin. The colonies were categorized as shown in the Table 1. Based on the results from biochemical tests (Table 2), all bacteria strains showed the same results on biochemical tests with catalase positive, non motile, negative for oxidase, indole, H₂S production, methyl red, voges-proskauer and urease test. Triple sugar iron (TSI) indicates test for fermentation of lactose, glucose and sucrose. For Strain A and Strain B, TSI test were acidic

whereas Strain D3, Strain L3 and Strain RT were alkali. Strain A and Strain B shows that lactose is not fermented, but there small amount of glucose fermented and the oxygen deficient slant will turn yellow. Strain D3, Strain L3 and Strain RT were alkaline because neither lactose, glucose of sucrose was fermented nor the slants turn into deep red or purple. All strains showed positive growth on Mac Conkey Agar which indicate gram negative bacteria strains. This finding was supported by the gram stain result as showed in Fig. 2.

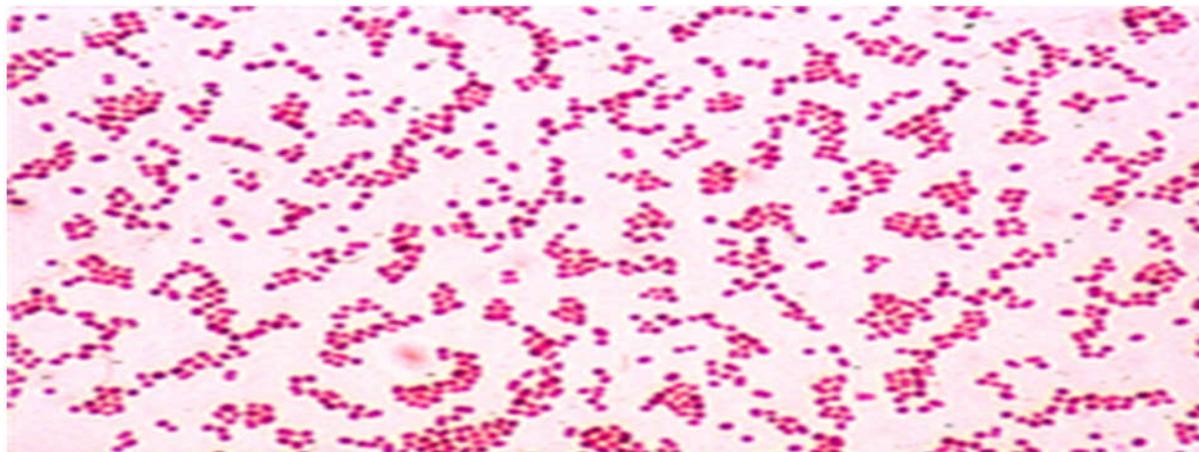


Fig.2. Gram staining results from the bacterial isolates at 1000X magnification

Table 1. Source, colony morphology and phytase production of the isolates

Bacterial Isolate	Source of Isolation	Colony Morphology*			Gram Staining	Presence of Clear Zone
		Form	Elevation	Margin		
A	Ulu Legong	Circular	Raised	Entire	Gram negative coccobacilli	+
B	Ulu Legong	Irregular	Flattened	Undulate	Gram negative coccobacilli	++
L3	Labis	Irregular	Flattened	Undulate	Gram negative coccobacilli	++
D3	Dusun Tua	Circular	Raised	Entire	Gram negative coccobacilli	+
RT	Ranau	Irregular	Raised	Undulate	Gram negative coccobacilli	+

- Characteristics are based on Bergey's Manual of Systematic Bacteriology [17].

Table 2. Biochemical characteristics of five bacteria isolates

Biochemical Tests	Bacteria Isolates				
	A	B	L3	RT	D3
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Motility	Non motile	Non motile	Non motile	Non motile	Non motile
Indole	-	-	-	-	-
H ₂ S production	-	-	-	-	-
Methyl Red	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-
Urease	-	-	-	-	-
Acid from TSI	Acid	Acid	Alkali	Alkali	Alkali
Growth on Mac Conkey agar	+	+	+	+	+

2.2. Phytase Assay and Optimum Conditions

Quantitative screening for phytase assay was done using supernatant from all isolates. The assay was carried out by growing bacterial strains in liquid medium containing sodium phytate as the substrate. Two types of liquid PSM media were prepared, one with 1% peptone and the other without 1% peptone. After three days of incubation at 37 °C, phytase assay was determined by measuring amount of liberated inorganic phosphates [16].

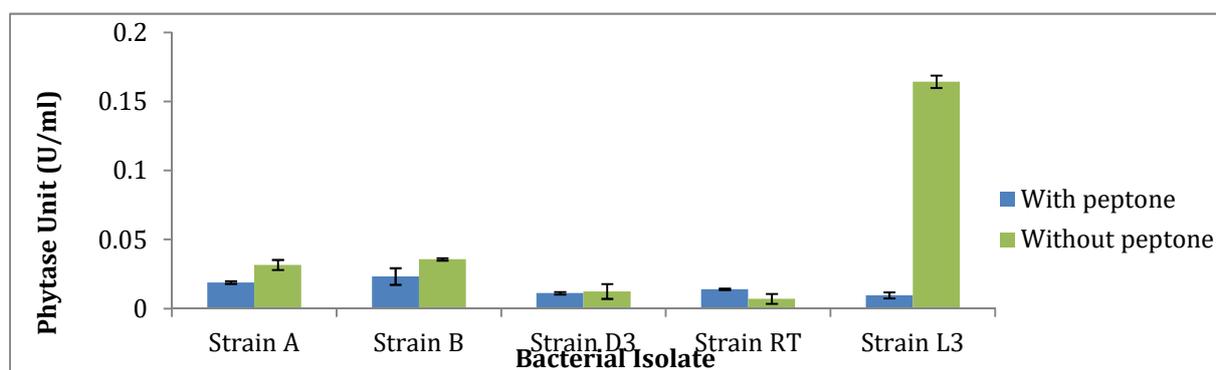


Fig.3. Phytase activity from five bacterial isolates in PSM media with peptone and without peptone

Coherence between phytase production and bacterial growth on PSM medium was noticed when both parameters reached their peak on the third day at 37 °C. Bacteria strain L3 release

maximum phytase activity (> 0.16 U/ml), while bacteria strain RT showed the lowest phytase activity at 0.007 U/ml after third day of incubation in media without peptone. The highest phytase activity for media with the addition of peptone was observed by Strain B which at 0.023 U/ml of phytase activity. This activity was considered low as compare to the same bacteria in media with peptone with activity at 0.036 U/ml. We can conclude that all bacteria strains showed reduced phytase activity in media with the addition of peptone as compare to the media without peptone as showed in Fig. 3.

2.3. Molecular identification of the selected bacteria isolate

Genomic DNA of the putative bacteria strain L3 was isolated using Wizard Genomic DNA Extraction Kit (Promega) before evaluated on 1% agarose gel electrophoresis. After PCR amplification, with the help of universal primers of 16S rRNA, single discrete PCR product was observed at approximately 1.5 kb of size (Fig. 4).

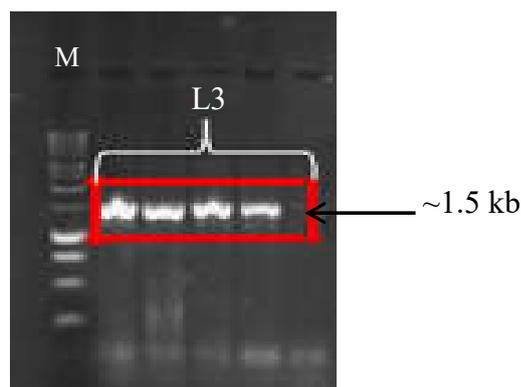


Fig.4. PCR amplification products of 16S rRNA from bacteria strain L3. PCR products with approximately 1.5 kb on 1% agarose gel. M: 1 kb DNA ladder (Promega)

BLAST homology (Fig. 5) comparison analysis of 16S rRNA region of the DNA sequences from strain L3 (Fig. 6) revealed 99% similarity to *Acinetobacter baumannii*, and thus the molecular identity of the isolate was confirmed. Bacteria strain L3 also showed the same observation for catalase positive, oxidase negative, non-motile, ability to utilize some substrates which was in agreement with [18] on the identification of *Acinetobacter baumannii* strains. Furthermore, phylogenetic tree (Fig. 7) based on 16S rRNA gene sequence using neighbour joining tree method, a powerful tool for deducing evolutionary and phylogenetic relationships among the organisms was decisive in their identification.

Alignments							
Download GenBank Graphics Distance tree of results							
	Description	Max score	Total score	Query cover	E value	Ident	Accession
<input type="checkbox"/>	Acinetobacter baumannii strain DSM 30007 16S ribosomal RNA gene, partial sequence	351	351	82%	6e-97	99%	NR_117677.1
<input type="checkbox"/>	Acinetobacter baumannii strain ATCC 19606 16S ribosomal RNA gene, complete sequence	351	351	82%	6e-97	99%	NR_117620.1
<input type="checkbox"/>	Acinetobacter baumannii strain JCM 6841 16S ribosomal RNA gene, partial sequence	351	351	82%	6e-97	99%	NR_113237.1
<input type="checkbox"/>	Acinetobacter baumannii strain CIP 70.34 16S ribosomal RNA gene, partial sequence	351	351	82%	6e-97	99%	NR_116845.1
<input type="checkbox"/>	Acinetobacter baumannii strain ATCC 19606 16S ribosomal RNA gene, partial sequence	351	351	82%	6e-97	99%	NR_119358.1
<input type="checkbox"/>	Acinetobacter baumannii strain DSM 30007 16S ribosomal RNA gene, partial sequence	351	351	82%	6e-97	99%	NR_026206.1
<input type="checkbox"/>	Acinetobacter nosocomialis strain RUH 2376 16S ribosomal RNA gene, partial sequence	333	333	82%	2e-91	98%	NR_117931.1
<input type="checkbox"/>	Acinetobacter antiviralis strain KNF2022 16S ribosomal RNA gene, partial sequence	324	324	82%	1e-88	97%	NR_115739.1
<input type="checkbox"/>	Acinetobacter Iwoffii strain DSM 2403 16S ribosomal RNA gene, partial sequence	318	318	82%	6e-87	96%	NR_026209.1
<input type="checkbox"/>	Acinetobacter Iwoffii strain JCM 6840 16S ribosomal RNA gene, partial sequence	316	316	82%	2e-86	96%	NR_113346.1

Fig.5. BLAST homology comparison of 16S rRNA from bacteria strain L3 against NCBI database

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1 agagtttgat catggctcag attgaacgct ggcggcaggc ttaacacatg caagtcgagc
61 gggggaaggt agcttgctac cggacctagc ggcggacggg ttagtaatgc ttaggaatct
121 gcctattagt gggggacaac atctcgaaag ggatgctaat accgcatacg tctacggga
181 gaaagcaggg gatcttcgga ccttgcgcta atagatgagc ctaagtcgga ttagctagtt
241 ggtggggtaa aggcctacca aggcgacgat ctgtagcggg tctgagagga tgatccgcca
301 cactgggact gagacacggc ccagactcct acgggaggca gcagtgggga atattggaca
361 atggggggaa cctgatcca gccatgccgc gtgtgtgaag aaggcctat ggttgtaaag
421 cacttaagc gaggaggagg ctactttagt taatacctag agatagtgga cgttactcgc
481 agaataagca ccggctaact ctgtgccagc agcccgcgta atacagaggg tgcgagcgtt
541 aatcggattt actgggcgta aagcgtgcgt aggcggctta ttaagtcgga tgtgaaatcc
601 ccgagcttaa ctgggaatt gcattcgata ctggtgagct agagtatggg agaggatggt
661 agaattccag gtgtagcggg gaaatgcgta gagatctgga ggaataccga tggcgaaggc
721 agccatctgg cctaactctg acgtgaggt acgaaagcat ggggagcaaa caggattaga
781 tacctgta gtccatgccg taaacgatgt ctactagccg ttggggcctt ttaggcttta
841 gtggcgcagc taacgcgata agtagaccgc ctggggagta cggtcgaag actaaaactc

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901 aatgaattg acgggggccc gcacaagcgg tggagcatgt ggtttaatte gatgcaacgc
961 gaagaacctt acctggcctt gacatactag aaactttcca gagatggatt ggtgccttcg
1021 ggaatctaga tacaggtgct gcatggctgt cgtcagctcg tgcctgaga tgttgggtta
1081 agtcccgcaa cgagcgcaac cctttcctt acttgcagc atttcggatg ggaacttaa
1141 ggatactgcc agtgacaaac tggaggaagg cggggacgac gtcaagtcat catggcctt
1201 acggccaggg ctacacacgt gctacaatgg tcggtacaaa gggttgctac acagcgatgt
1261 gatgctaate tcaaaaagcc gatcgtagtc cggattggag tctgcaactc gactccatga
1321 agtcggaate gctagtaate gcggatcaga atgccgagg gaatacgttc ccgggccttg
1381 tacacaccgc ccgtcacacc atgggagttt gttgcaccag aagtagctag cetaactga
1441 aagaggcggg ttaccacggt gtggccgatg actgggggtga agtcgtaaca aggtagccgt
1501 aggggaacct gcggetggat cacctcctt
    
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Fig.6. The 16S rRNA gene sequence of bacteria strain L3 at 1579 bp

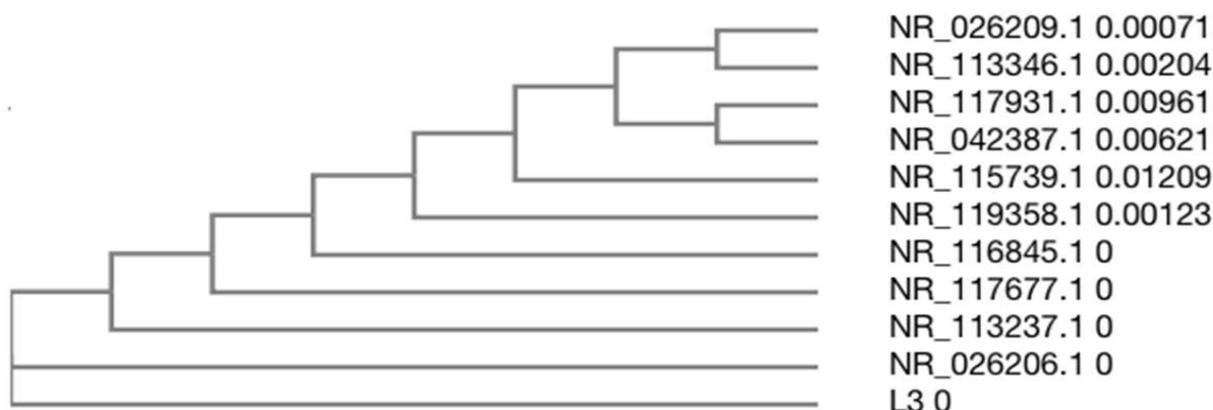


Fig.7. Phylogenetic tree based on 16S rRNA gene sequence showing relationship between bacteria strain L3 and ten related members of *Acinetobacter*. NR_026209.1; *A. lwoffii* strain DSM 2403, NR_113346.1; *A. lwoffii* strain JCM 6840, NR_117931.1; *A. nosocomialis* strain RUH 2376, NR_042387.1; *A. calcoaceticus* strain NCCB 22016, NR_115739.1; *A. antiviralis* strain KNF2022, NR_119358.1; *A. baumannii* strain ATCC 19606, NR_116845.1; *A. baumannii* strain CIP 70.34, NR_117677.1; *A. baumannii* strain DSM 30007, NR_113237.1; *A. baumannii* strain JCM 6841; NR_026206.1; *A. baumannii* strain DSM 30007

3. EXPERIMENTAL

3.1. Samples Collection

Samples were collected from four different places in hot spring in Malaysia which were Ranau (Sabah), Ulu Lengong (Kedah), Dusun Tua (Selangor) and Labis (Johor). At each site, about 1 ml of each water sample recovered by sterile syringe was transferred to each of the bottles, consisting of 10 ml media in a 30 ml sterilizes universal bottles. The sampling was conducted in triplicates. All samples were sent to the laboratory within a period of three hours before inoculation was done.

3.2. Isolation and Identification of Microorganisms from Hot Spring

Nutrient broth and nutrient agar were used for isolation of microorganism from the hot spring. One milliliter sample from of each waters was added to nutrient broth (10 ml) and incubated in water bath at 37 °C, 45 °C, 50 °C, 55 °C and 60 °C for 4 days. Growth was followed by measuring the increase in turbidity at 600 nm. Then, the culture was streaked onto a nutrient agar plate. Isolation of pure culture was done by using spread plate method and streak plate method recommended by [19]. The pure cultures were maintained on nutrient agar slants at 4 °C for further use.

3.3. Growth and Screening for Phytase Producing Bacteria

Aliquots (0.1 mL) of pure isolates of actinobacteria were cultured on Phytase Screening Medium (PSM; g/L) (g/L) Glucose, 20.0; sodium phytate, 4.0; CaCl₂.2H₂O, 2.0; NH₄NO₃, 5.0; KCl, 0.5; MgSO₄.7H₂O, 0.5; FeSO₄.7H₂O, 0.01 and agar, 15.0. The pH of PSM media was adjusted to pH 5.5 using NaOH [20-21]. All samples were incubated at 37 °C for 4 days. Colonies capable of hydrolysing Na-phytate showed clear halo zones against an opaque colour of non-hydrolyzed medium. The halo and colony diameters were measured after 4 days of incubation at 37 °C. Positive isolates were tested again for confirmation.

3.4. Phytase Assay

For phytase assay, the liquid Phytase Screening Medium (PSM) was used to grow the bacterial strains. There were two types of liquid PSM used which were with addition of peptone and without addition of peptone to compare the phytase production by bacterial strains. The bacterial strains were grown in 10 ml of liquid PSM with peptone and 10 ml of liquid PSM without peptone respectively in a 50 ml centrifuge tube. Then, they were incubated at 37°C for three days on an incubator shaker at 200 rpm. The crude enzyme was harvested by

centrifugation at 10,000 g for 10 min at 4°C and the clear supernatant was used as the source of extracellular phytases. Phytase activity was determined by measuring the amount of liberated inorganic phosphate. The reaction mixture consisted of 0.5 % sodium phytate prepared in 0.2 M sodium acetate buffer, pH 5.5 and 0.2 ml of supernatant. The mixture was then incubated at 37 °C for 30 minutes and then the reaction was stopped by adding an equal volume of 15% of trichloroacetic acid. The liberated phosphate ions were quantified by mixing 0.1 ml of assay mixture with 0.9 ml of 1.0M H₂SO₄, 10% ascorbic acid and 2.5% ammonium molybdate. After 20 minutes of incubation at 37 °C, absorbance was measured at 820 nm [16].

3.5. Colony Morphology

Colony of each bacterial isolates observed under Stereomicroscope (Leica microscope). The magnification used was 1000×. Each bacterial colony was identified accordingly by margin, elevation and form. Identification of the strains primarily based on the phenotypic characters described in the Bergey's Manual of Systematic Bacteriology [17].

3.6. Gram Staining

Gram staining method was done according to [22]. For Gram Staining, a single colony from the plate were picked and placed on a slide. The sample was heat fixed on slide by carefully passing the slide through the Bunsen burner. Then, primary stain which is crystal violet was added to the sample and left for 1 minute. After that, the slide was rinsed carefully for maximum 5 seconds with running tap water. Then, a few drops of Gram's Iodine was added to the slide and left for one minute. After that, the slide was rinsed with alcohol for 3 seconds. Lastly, secondary stain which is safranin was added to the slide and left for one minute and then washed with running tap water for a maximum 5 seconds.

3.7. Biochemical Test

Biochemical tests are an analysis of enzymatic functions of the bacterial strains. There were 10 tests carried out in this biochemical characterization. Identification of the strains primarily based on the phenotypic characters described in the Bergey's Manual of Systematic Bacteriology [16-17]. The tests were catalase, oxidase, indole, motility, H₂S production, methyl red, voges-proskauer, urease, triple sugar iron (TSI) and growth on MacConkey agar.

3.8. Extraction of Genomic DNA from Bacteria Isolates

The putative bacteria strains were cultured in Luria bertani broth and incubated overnight at

37 °C in an orbital shaker at 150 rpm. Genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega). Concentration of DNA was measured using Biospectrometer and DNA was visualized using 1% agarose gel electrophoresis.

3.9. Phylogenetic Analysis of 16S rRNA Gene Sequence

Universal primers used were FD1 5' CCC AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G 3' and RD1 5' CCC GGG ATC CAA GCT TAA GCA GGT GAT CCA GCC 3' were used for amplification of 16S rRNA gene from the samples. PCR amplification was carried out in a PCR thermal cycler (Applied Biosystems). The PCR reaction mixtures contained 2 mM MgCl₂, 200 µM dNTP, 1U Taq DNA Polymerase (Promega), 1 µg template DNA and 0.5 µM forward and reverse primer. Typically, 30 cycles of amplification reactions were conducted according to the following parameters: 5 minutes for initial denaturation at 96°C; 30 cycles for 1 minute at 95°C, 2 minutes at 60 °C, 2 minutes at 72°C and a final cycle for 5 minutes at 72°C for final extension. A negative control was also loaded to eliminate the possibility of reagent contamination. PCR products stained with GelRed were analysed using 1% agarose gel and visualized under gel documentation system (Applied Biosystems).

PCR products were sent for sequencing service at First Base Laboratory Sdn. Bhd. and comparison of these sequences were carried out using available database at National Centre for Biotechnology Information (NCBI) using BLAST program (<http://www.ncbi.nlm.nih.gov>). Based on maximum identity score, first 10 sequences were selected and aligned using multiple alignment software program (Clustal Omega) to construct phylogenetic tree using Clustal W2 software. The relatedness between the species was calculated by evolutionary distance and represented in the form of phylogenetic tree.

4. CONCLUSION

From all the sampling sites; Ranau (Sabah), Ulu Lengong (Kedah), Dusun Tua (Selangor) and Labis (Johor) only five bacteria strains (A, B, D3, L3 and RT) were tested positive for phytase producers by using phytase screening media (PSM). All five isolates were found to have different ability in phytate degradation and they produces either different amount of phytate-degrading ability or their enzymes had different catalytic activity. Bacteria strain L3

release maximum phytase activity (> 0.16 U/ml) in media without peptone. Characterization of strain L3 via morphology, biochemical tests were successfully carried out. Further 16S rRNA identification has successfully identified the strain as *Acinetobacter baumannii* with 99% similarity index.

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