

Growth and Morphological Characterisation of Tropical Thermophilic Bacterium *Parageobacillus caldxylosilyticus* ER4B

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Received: 14 October 2021

Accepted: 12 December 2021

Published: 31 December 2021

ABSTRACT

Parageobacillus caldxylosilyticus is a rod-shaped thermophilic bacterium that can grow optimally at high temperatures. The thermophilic features of the bacterium are expected to be largely accounted by the production of thermostable enzymes which has valuable applications in many fields. However, growth study on this species is very limited and the growth conditions at high temperatures remained unclear. Therefore, this study aimed to determine the morphological and growth characterisation of *Parageobacillus caldxylosilyticus*, including growth media preferences, optimal growth temperature, and construction of growth curve. *P. caldxylosilyticus* strain ER4B that was isolated from oil palm empty fruit bunch compost was characterised in this study. The bacterial strain was firstly identified based on 16S rRNA gene sequence, and the subsequent sequence similarity search showed that it is closest to *P. caldxylosilyticus* strain UTM6. It is found that ER4B grew best in Lennox Broth as compared to Reasoner's 2A Broth, Trypticase Soy Broth, and Nutrient Broth media. Further tests were conducted and the optimum growth temperature of the strain was determined to be at 64 °C. The bacterium forms mucoid circular punctiform colonies that are yellowish and with diameter of 2 mm to 4 mm. It is a Gram-positive rod-shaped bacterium that has a length ranging from 3 µm to 6 µm, with a diameter of around 0.5 µm.

Keywords: 16S ribosomal RNA, characterisation, fruit bunch compost, *Parageobacillus caldxylosilyticus*, thermophilic bacteria

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INTRODUCTION

Parageobacillus sp. were previously categorised together with other *Geobacillus* sp., the new genus, *Parageobacillus* was then proposed by Aliyu *et al.* (2016) as high-resolution phylogenomic analysis showed two distinct clustering among sixty-three *Geobacillus* stains, and the difference is significant to separate *Parageobacillus* from *Geobacillus* (Aliyu *et al.*, 2016; Lebre *et al.*, 2018). To date there are seven known species being classified into this genus, namely *P. caldxylosilyticus*, *P. galactosidasius*, *P. thermantarcticus*, *P. thermoglucosidasiusi*, *P. toebii*, *P. yumthangensis*, and *P. genomsp. 1* (Schoch *et al.*, 2020). Members of *Parageobacillus* are characterised by their rod-shaped morphology,

presence of Gram-positive cell wall, and most importantly, their thermophilic properties (Nazina *et al.*, 2001). Bacterial species from this genus can grow optimally at a temperature ranging from 45° C to 75 °C, while some can extend to as low as 35 °C or as high as 80 °C (Zeigler, 2014). Thermophiles such as *Parageobacillus* are expected to produce thermostable hydrolytic enzymes that are able to function at higher temperatures.

Parageobacillus caldxylosilyticus was first isolated and identified as *Saccharococcus caldxylosilyticus* from local soil in Melbourne, Australia (Ahmad *et al.*, 2000), before it was classified as *Geobacillus caldxylosilyticus* in 2001 (Fortina *et al.*, 2001) and lastly classified as *Parageobacillus caldxylosilyticus* in 2016

(Aliyu *et al.*, 2016). This species was found not only in soil (Ahmad *et al.*, 2000), chipped woody waste (Yunitsyna *et al.*, 2019), oil-contaminated soil (Adlan *et al.*, 2020), volcanic soil (Seo *et al.*, 2011), but it was also found in a liquid such as seawater (Adlan *et al.*, 2020), pond water (Abdelkader & Esawy, 2011), hot spring (Ibrahim & Ahmad, 2017; Kolcuoğlu *et al.*, 2010), central heating system water (Obojska *et al.*, 2002), and in the dairy product (Berendsen *et al.*, 2016). Besides, the species also showed high adaptability towards various environmental stress other than temperature, as it was able to tolerate glyphosate, heavy metal toxicity, oil contamination, as well as high salinity and alkalinity (Obojska *et al.*, 2002; Abdelkader & Esawy, 2011; Ibrahim & Ahmad, 2017; Yunitsyna *et al.*, 2019; Adlan *et al.*, 2020). These unique properties of *P. caldxylosilyticus* made this species a promising candidate for the production of industrially applicable thermostable enzymes such as xylanase, maltogenic amylase, and acylhomoserine lactonase, at the same time, offer an environmentally friendly solution of herbicide degradation, bioremediation of harmful chromium IV oxide, and crude oil paraffin wax degradation (Obojska *et al.*, 2002; Kolcuoğlu *et al.*, 2010; Abdelkader & Esawy, 2011; Seo *et al.*, 2011; Rahim & Ahmad, 2016; Ibrahim & Ahmad, 2017; Yunitsyna *et al.*, 2019; Adlan *et al.*, 2020).

Despite having applicable potential in various fields, similar to the majority of *Parageobacillus* members, studies on growth characterisation and cell morphology of *P. caldxylosilyticus* are relatively less. In addition, the growth conditions appeared to be different for different strains (Ibrahim & Ahmad, 2017; Ahmad *et al.*, 2000). Without information on the optimal growth conditions it is very difficult to utilize the bacteria for downstream application (Allen & Waclaw, 2019). Therefore, this study aimed to determine the optimal growth conditions of *P. caldxylosilyticus* ER4B which was isolated from Malaysia oil palm empty fruit bunch compost. Since this is the very first study attempt on strain ER4B, the bacterium was first identified using the 16S rRNA gene sequencing and alignment prior to the any other growth characterisation. Microscopic analysis was also conducted in this study to examine the cell morphology of strain ER4B.

MATERIALS AND METHODS

Source of Bacterium

Parageobacillus caldxylosilyticus strain ER4B used for this study was previously isolated from oil palm empty fruit bunch during the composting process on 11th November 2005. It was stored in glycerol stock at -80 °C, and was recovered for the purpose of this study.

Bacterial Identification

Bacterial DNA of ER4B was isolated using an optimized protocol using Qiagen DNeasy® Blood & Tissue Kit, followed by PCR amplification of the complete 16S rRNA gene using forward primer BSF8 (5' – AGAGTTTGATCCTGGCTCAG – 3') and reverse primer BSR1541 (5' – AAGGAGGTGATCCAGCCGCA – 3'). The 16S rRNA gene was used for identification because it is by far the most common housekeeping genetic marker, and the nearly complete 16S rRNA 1.5 KB fragment sequence has been reported to allow identification up to species level (Cai *et al.*, 2003; Janda & Abbott, 2007; Johnson *et al.*, 2019; Jeong *et al.*, 2021). The amplicon size was expected to be approximately 1,500 bp. The subsequent purified 16S amplicon was then sequenced using the ABI3730XL Sanger sequencer, and the sequence was searched for similarities against NCBI databases using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed based on the 16S rRNA sequences of bacteria using Mega X. The tree was inferred using the Maximum Composite Likelihood based on 1000 resamples data set.

Determination of Optimal Culture Media

A test was conducted to determine which of the selected culture media would offer the best growth condition for the bacterium. 100 ml of Lennox Broth (LB), Trypticase Soy Broth (TSB), Reasoner's 2A (R2A) Broth, and Nutrient Broth (NB) were prepared in three replicates. Next, 10 µl of bacterial culture with OD₆₀₀ below 0.05 was inoculated into each of the freshly prepared broth media. The initial OD₆₀₀ of each culture was measured using Spectra Max 2 before they were incubated at 60°C and 180 rpm for 24 hours. The OD₆₀₀ from each replicate

were taken and recorded after 24 hours. Statistical analysis was performed using One-way Analysis of Variance (ANOVA).

Determination of Optimal Growth Temperature

The optimal growth temperature was tested after LB was determined to be the most suitable medium among the tested broth media. In order to narrow down the temperature range of ER4B, 1 µl of bacteria culture with OD₆₀₀ below 0.05, were inoculated onto LB agar plates, followed by incubation at 25 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, and 80 °C for 24 hours. After narrowing down the temperature to 60 °C and 70°C, 10 µl of bacterial culture with OD₆₀₀ below 0.05 was inoculated into freshly prepared LB broth media, before each triplicate was incubated at 60 °C, 62 °C, 64 °C, 66 °C, 68 °C, and 70 °C, at shaking speed of 180 rpm for 24 hours. The OD₆₀₀ from each replicate were taken and recorded after 24 hours.

Construction of Growth Curve

The growth curve was constructed using the most suitable broth medium and optimum growth temperature for the bacterium. 10 µl of bacterial culture with OD₆₀₀ below 0.05 was inoculated into 100 ml freshly prepared LB broth media, followed by incubation at 64°C and 180 rpm. The absorbance of the cultures was measured at an interval of 3 hours, for 39 hours continuously.

Bacterial Cell and Colony Morphology

The morphology of the bacterial cell and the bacterial colony was assessed using different microscopes. For microscopic analysis of bacterial colony and Gram staining (Hucker & Conn, 1923), compound light microscope Olympus BX41 was used, whereas the bacterial cell morphology was viewed under Scanning Electron Microscope (SEM) Hitachi S3400N.

Prior to SEM examination, the cells were prepared using a modified Hexamethyldisilazane (HMDS) drying method (Murtey & Ramasamy, 2016).

RESULTS AND DISCUSSION

After sequencing and BLAST against the NCBI database, the resulting 16S rRNA sequence suggested that the bacterium has 99% similarity to *Parageobacillus caldxylosilyticus* strain UTM6, with an E-value of 0.0. The majority of the top 100 sequences producing significant alignment from BLAST were all from the species of *P. caldxylosilyticus*. The neighbor-joining phylogenetic tree was constructed based on the 16S rRNA sequence of strain ER4B against some of the sequences selected from these top 100 sequences, including those from the closely related genus. Figure 1 showed that strain ER4B of the bacterium is significantly closer to *P. caldxylosilyticus* as it formed a distinctive cluster with this species, and it is particularly close to *P. caldxylosilyticus* strain UTM6, with less than 0.01 (1%) nucleotide substitution per site. Therefore, strain ER4B is highly likely to be *P. caldxylosilyticus*. Besides, the phylogenetic relationship depicted in the tree is highly reliable, as all the bootstrap values shown on the nodes are exceptionally high (around 98).

Among the four different broth mediums, the bacterium was cultured in, LB showed the highest average OD₆₀₀ reading (0.2998) after 24 hours of incubation (Table 1). This indicated LB has significantly highest turbidity due to the highest bacterial growth rate when cultured in this broth medium ($p < 0.05$). So, LB appeared to be the most suitable growth media for ER4B as compared to the rest. Next to LB, it can be clearly seen that R2A has an average OD₆₀₀ reading of 0.1224, followed by TSB with average OD₆₀₀ reading of 0.0891. On the other hand, there was no observable growth in NB at all.



Figure 1. Phylogenetic tree based on 16S rRNA sequence similarity of ER4B and other bacterial strains selected from the BLASTn significant alignment using MEGA X. The evolutionary history was inferred using Neighbor-Joining method, whereas the distances were computed using the Maximum Composite Likelihood method, in the units of the number of base substitutions per site. *Escherichia coli* strain U 5/41 was included as outgroup. Numbers shown at the nodes represent bootstrap percentages based on 1000 resampled dataset

Table 1. Optical density (absorbance at 600 nm) at different types of broth culture media after 24 hours of incubation at 60 °C. the p-value corresponding to the F-statistic of one-way ANOVA is lower than 0.05, suggesting that results are significantly different. [F (2,6) = 97.251; p-value = 0.00003]

Types of broth media	OD ₆₀₀ readings			
	Replicate 1	Replicate 2	Replicate 3	Average
LB	0.2931	0.3009	0.3055	0.2998
R2A	0.1138	0.1354	0.1181	0.1224
TSB	0.1210	0.0890	0.0572	0.0891
NB	N/A	N/A	N/A	N/A

*N/A indicates Not Available.

When the nutrient content of each culture medium is compared, the most distinguishable nutrient content difference between LB and other culture media would be the presence of yeast extract at a relatively high concentration. Yeast extract is commonly added in many

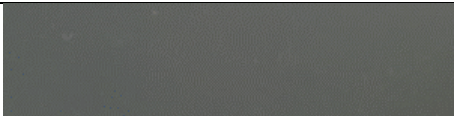





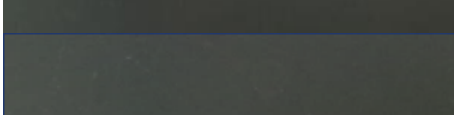
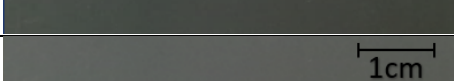
culture media as a major source of vitamins, different amino acids, as well as certain trace elements (Hakobyan *et al.*, 2012). It has been shown to serve as the best organic nitrogen source for cell growth and H₂ production by different bacteria, e.g., *Clostridium*

acetobutylicum (Kalil *et al.*, 2008) and *Rhodospseudomonas palustris* (Chen *et al.*, 2010). This suggested that high yeast extract concentration in LB is probably the main growth factor that stimulates the growth of ER4B.

As for growth temperature, no significant growth can be observed at 25 °C and 80 °C. Table 2 showed that the lowest temperature where growth of ER4B can be observed (minimum growth temperature) was between 25 °C and 30 °C, whereas the highest temperature where growth occur (maximum growth

temperature) was in between 70 °C to 80 °C (Table 2). Table 2 also showed that the growth of ER4B was very slow at 30 °C as the colonies formed were very small and transparent, but the colonies appeared to be bigger and thicker, indicating that the growth rate increased along with the temperature increment. The size of the colonies was the biggest at 60 °C, and further increase in temperature to 70 °C causes the colony size to reduce significantly, so the maximum growth temperature was expected to be just slightly above 70 °C.

Table 2. Growth of strain ER4B on LB agar plate at different temperatures

Temperature (°C)	Growth on LB agar plate
25	
30	
40	
50	
60	
70	
80	
Scale	

Further temperature tests between 60 °C to 70 °C revealed the optimum growth temperature for strain ER4B. Of the temperatures listed in Table 3, bacteria culture at 64 °C has the highest average OD₆₀₀ reading of 0.4063 after 24 hours of incubation, followed by 66 °C (0.3886), 62 °C (0.3599), 68 °C (0.3110), 60 °C (0.3000), and lastly 70 °C (0.2144). Higher OD₆₀₀ reading is equivalent to higher turbidity or higher bacterial cell density in the culture media, thus, 64 °C with the highest OD₆₀₀ reading was showing the

highest growth rate as compared to other incubation temperatures. This suggested 64 °C as the optimal growth temperature for ER4B.

The growth temperatures of *Parageobacillus caldxylosilyticus* has been briefly described by Ahmad *et al.* (2000). The study proposed that the formerly known as *Geobacillus caldxylosilyticus* has optimal growth temperature ranges between 65 °C, at the same time having a minimum temperature at 42 °C

and maximum temperature at 70 °C (Ahmad *et al.*, 2000). The reported optimal growth temperature and the maximum temperature are very similar to the temperature obtained in this study, however, the minimum growth temperature of ER4B appeared to be lower than those reported strains. However, it is still consistent with other members of *Geobacillus* genus (Zeigler, 2014). Another relatively recent growth study on *P. caldxylosilyticus* UTM6 reported an optimal

growth temperature at 55 °C (Ibrahim & Ahmad, 2017), and the reported temperature is significantly lower than the optimal growth temperature obtained from this study and Ahmad *et al.* (2000). Therefore, in terms of growth temperature, the results obtained in this study is in consistent with Ahmad *et al.* (2000), but appeared to be very different from the optimal growth temperature reported by Ibrahim & Ahmad (2017).

Table 3. Optical density (absorbance at 600 nm) at different temperature in each culture replicate after 24 hours of incubation

Temperature (°C)	OD ₆₀₀ readings			
	Replicate 1	Replicate 2	Replicate 3	Average
60	0.2978	0.2952	0.3070	0.3000
62	0.3363	0.3700	0.3734	0.3599
64	0.4204	0.3981	0.4003	0.4063
66	0.3909	0.3851	0.3898	0.3886
68	0.3270	0.3055	0.3004	0.3110
70	0.2117	0.2128	0.2186	0.2144

The growth curve of strain ER4B cultured at optimal growth temperature and broth media was constructed and depicted in Figure 2. For the first three hours, there was no significant rise in absorbance reading. This indicated that the culture was in the lag phase of growth (Walker, 1999). In this case, after ER4B was inoculated into fresh LB, it took around three hours to adjust their metabolic activities so that they can commence active cell division again.

When the bacterial cells had already adapted to the new environment, they would transit from the lag phase and enter the log or exponential phase. As depicted in Figure 2, ER4B entered the acceleration phase and grew at maximum specific growth rate after the 3rd hour. At around the 9th hour, growth decelerated as essential nutrients became scarce, and eventually entered a stationary phase where the specific growth rate decreased significantly from its maximum point. The stationary phase of ER4B at 64 °C was short and it was expected to be in between 21st and

27th hour. After that, the graph exhibited a rapid drop in absorbance readings, indicating that ER4B entered the death phase very quickly after a relatively short transition period in the stationary phase (Werner-Washburne *et al.*, 1993).

When the growth curve was compared to that of strain UTM6 (Ibrahim & Ahmad, 2017), it can be seen that although strain UTM6 was cultured at 55 °C, but the pattern of the growth curve is very similar to that of ER4B. Strain UTM6 entered exponential phase around 4th hour, while the exponential phase of ER4B started after the 3rd hour. Then both strain entered stationary phase at around 21st hour. Despite having their OD₆₀₀ recorded at different time interval and different optimal growth temperature, the growth curve from this study agreed well with the reported growth curve. It is speculated that the similarities in growth is due to geographical factors, as both strains were isolated in Malaysia.

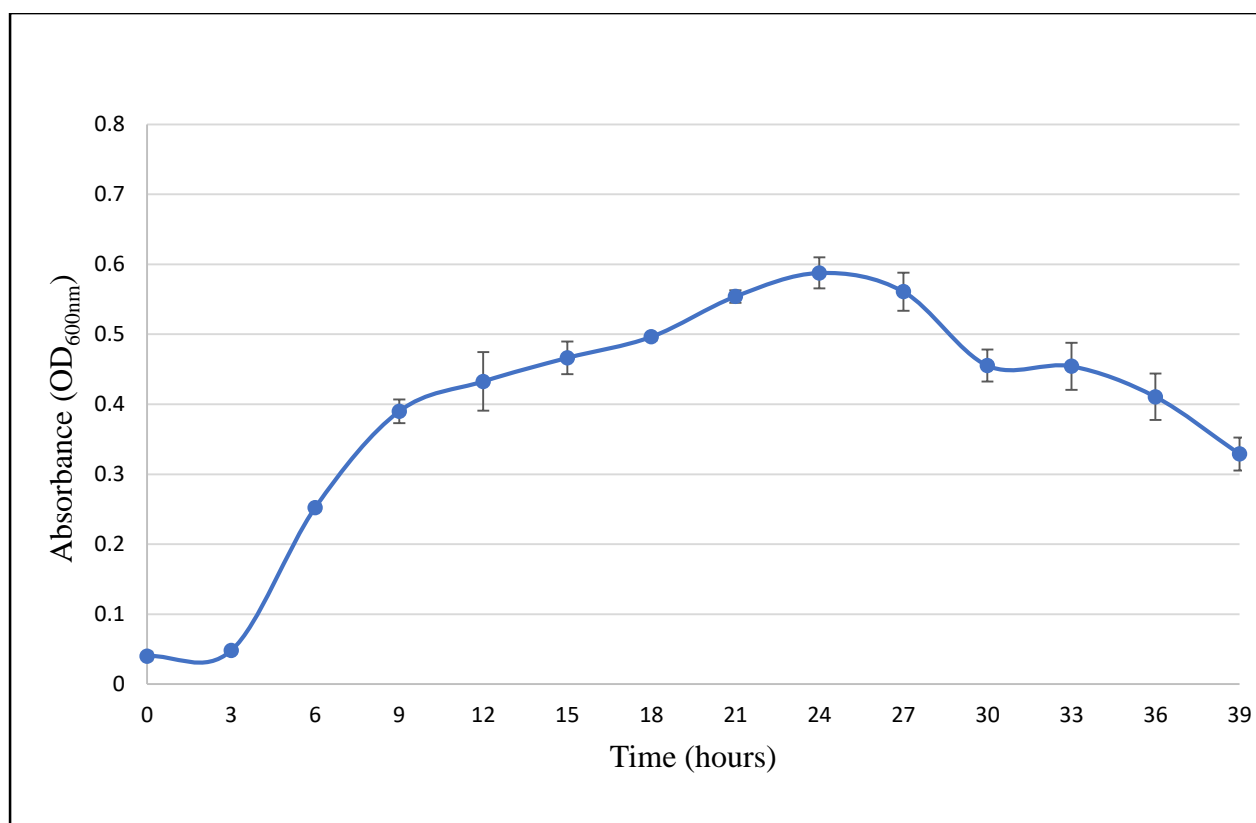


Figure 2. Growth curve of *Parageobacillus caldxylosilyticus* strain ER4B cultured in Lennox Broth at 64°C. The bacterium was in lag phase during the first three hours, immediately followed by exponential phase where the growth rate is maximum. The growth rate then slowed down at 9th hour, and entered stationary phase at 21st hour. After 27 hours of incubation, the absorbance readings were getting lower, indicating the death phase of the bacterial culture

Microscopic observation of ER4B colonies cultured on LB agar plate showed that bacterium strain ER4B formed circular punctiform colony with entire margin as shown in Figure 3. The diameter of average colonies after 24 hours of incubation is about 2 mm to 4 mm. The colony appeared to be opaque and yellowish-white in color, and it was determined as a mucoid colony as it was sticky and viscous (Mahon *et al.*, 2018). As for elevation (side view), it was flat, while the surface was smooth and shiny. As for Gram staining, the bacterial cells showed violet stain when viewed under the light microscope, suggesting that ER4B has a Gram-positive cell wall (Figure 4).

Further magnification view using a scanning electron microscope (SEM) revealed that strain ER4B has an average length of 3 μm to 6 μm , with a diameter around 0.5 μm (Figure 5). Besides, a Z ring was also observed as depicted in Figures 5(c) and (d). Z ring, also known as Filamenting temperature-sensitive mutant Z (FtsZ) ring, is a contractile ring assembled in bacterial cells that would proceed to cleave the parent cell into two daughter cells (Lutkenhaus & Addinall, 1997; Xiao & Goley, 2016). The position of the Z ring assembled in the middle of the bacterial cells indicated strain ER4B reproduced through binary fission under normal circumstances.

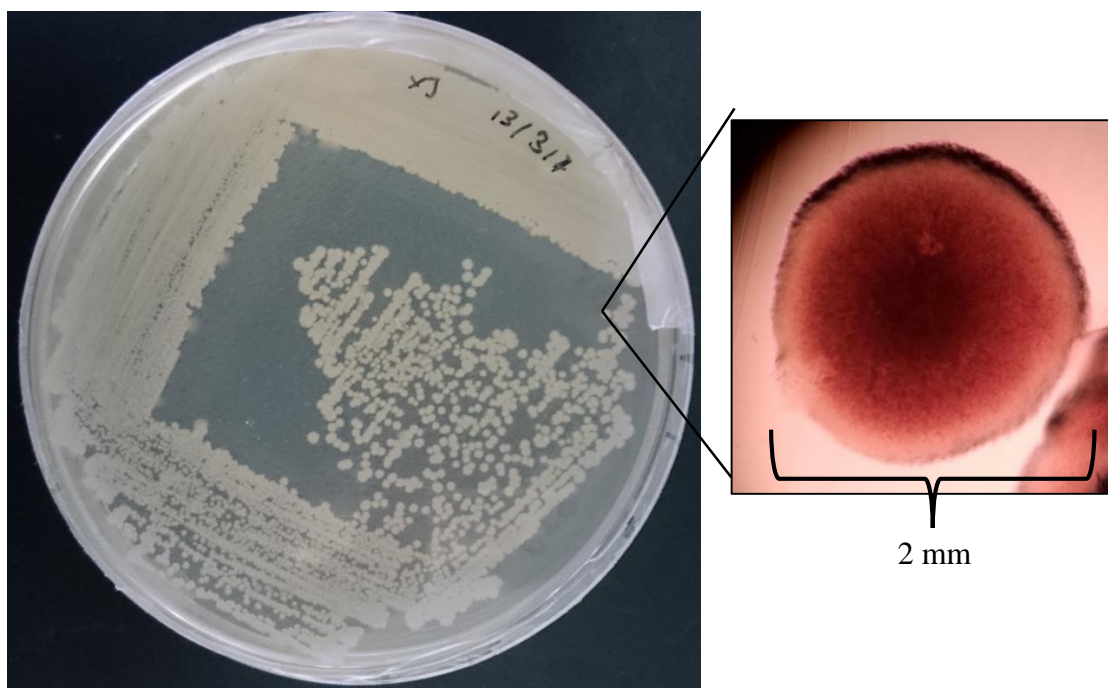


Figure 3. Microscopic view of ER4B single colony for colony morphology characterisation. ER4B forms circular punctiform colony with entire margin that is about 2 mm to 4 mm in diameter

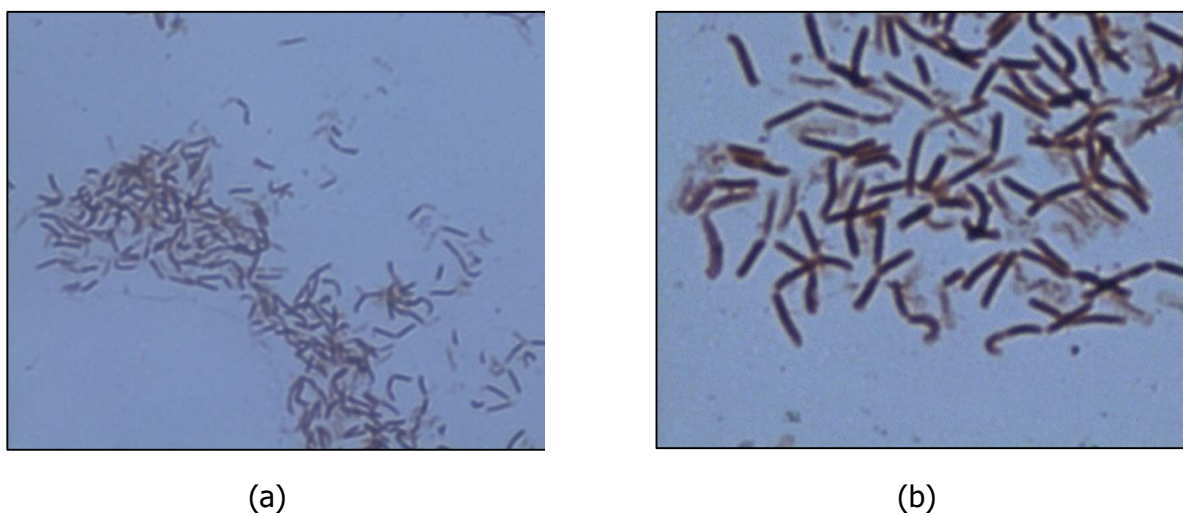


Figure 4. Microscopic view of Gram-stained ER4B under (a) 400x and (b) 1,000x magnification using light microscope. Violet stains remained in the cell wall of the bacterial cells, suggesting that ER4B is a Gram-positive bacterium.

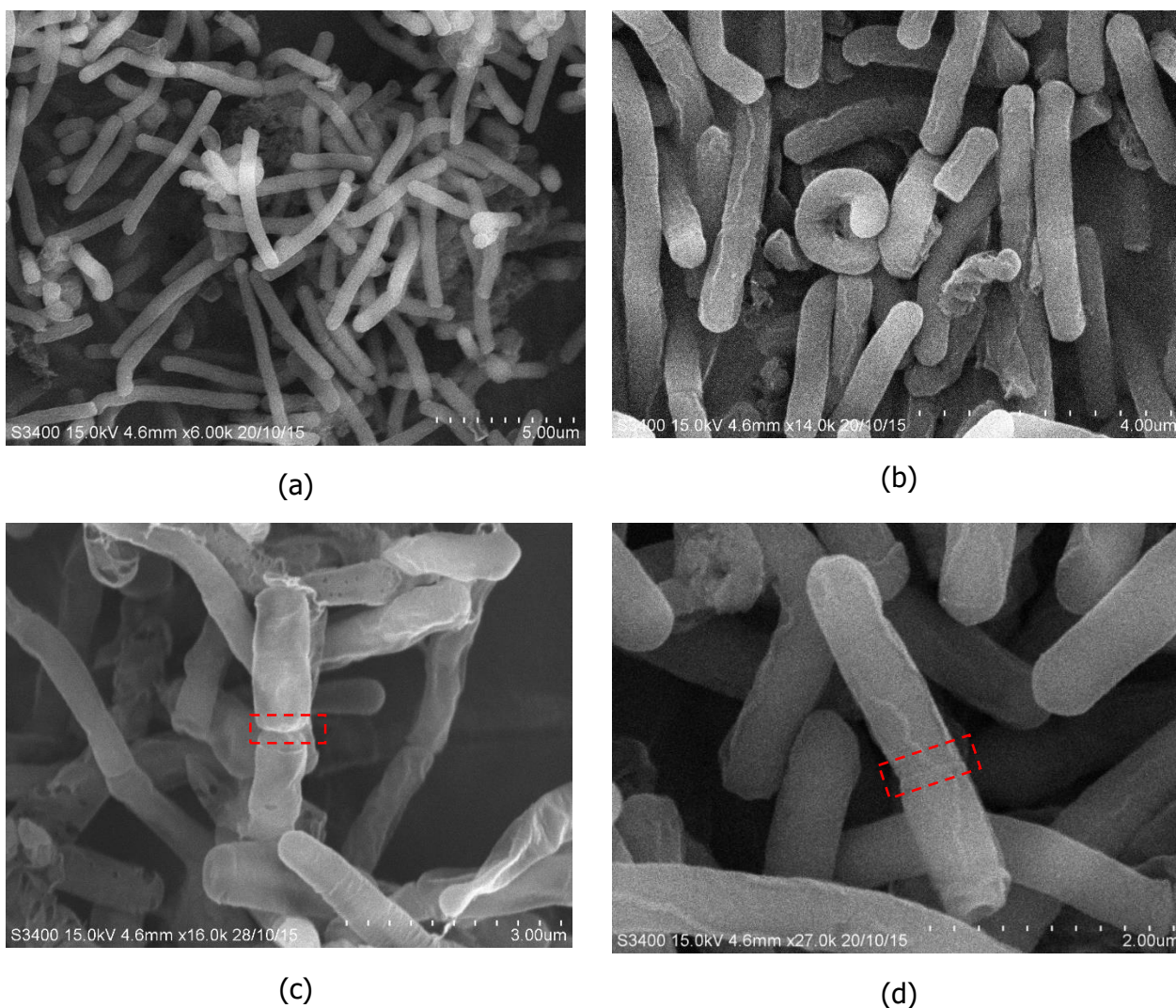


Figure 5. SEM view of ER4B under different magnification. (a) 6,000x; (b) 14,000x; (c) 16,000x; and (d) 27,000x magnification. Z ring was observed as highlighted in red

CONCLUSION

ER4B isolated from tropical oil palm empty fruit bunch compost was preliminarily identified as *Parageobacillus caldxylosilyticus* based on the high similarity of its' 16S rRNA sequence to *P. caldxylosilyticus* strain UTM6 (KR867680.1). Growth characterisation showed that the bacterium is a facultative thermophile as it can grow at a temperature as low as 30 °C while having a maximum growth temperature of 70 °C. Besides, strain ER4B has an optimal growth temperature at 64 °C in LB media, and the growth curves were also constructed in order to determine the growth pattern. Lastly, strain ER4B was exhibited as a Gram-positive, rod-shaped bacterium through microscopic analysis, while forming mucoid circular punctiform

colonies which were opaque and yellowish-white in color when growing on LB agar plate.

ACKNOWLEDGEMENTS

The funding support from the Ministry of Science, Technology, and Innovation (MOSTI), Malaysia, under the Antarctica Flagship Programme (Sub-Project 1: Grant no. FP1213E036) is gratefully acknowledged.

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