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Research Article

CHARACTERISATION OF AN ANTARCTIC YEAST, *Glaciozyma antarctica* PI12

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ABSTRACT

Glaciozyma antarctica PI12 is a psychrophilic yeast isolated from Antarctica. It has an optimal growth in yeast peptone dextrose (YPD) and yeast mould (YM) broth media but not in potato dextrose (PD) broth medium. Early phase *G. antarctica* PI12 cells had elongated-shape and became oval-shaped as they aged. *G. antarctica* PI12 exhibited bipolar budding and formed a chain of cells during the lag and early exponential phases. The number of chains decreased as the yeast aged. It appeared mainly as a single cell at the stationary phase, and a small number of them still produced buds. Some cells at the stationary phase entered the quiescence state (G_0) as a long-term survival strategy. The *G. antarctica* PI12 cell size decreased when they entered the stationary phase. *G. antarctica* PI12 was found to produce hydrolytic enzymes, chitinase, cellulase, mannanase, and xylanase. A higher glucose concentration of 2% in the PD agar medium inhibited the activities of chitinase but not the cellulase, mananase and xylanase.

Keywords: cell morphologies, bipolar budding, yeast chain, quiescence state, hydrolytic enzyme, Antarctic yeast, *Glaciozyma* spp., enzyme activities, psychrophiles

INTRODUCTION

Psychrophiles live and grow well in extremely cold environments such as the Antarctic. Psychrophiles have attracted much attention due to their abilities to produce a variety of cold-active hydrolytic enzymes (Brenchley, 1996; Ramli et al., 2011; Ramli et al., 2013; Alias, Mazian, Salleh, Basri, & Rahman, 2014). *Glaciozyma antarctica*, previously

known as *Leucosporidium antarcticum* is a psychrophilic yeast isolated from sea-ice in Antarctica (Turchetti et al., 2011; Boo et al., 2013). The first *Glaciozyma* spp. (originally called *Leucosporidium, scottii L. capsuligenum, L. antarcticum, L. frigidum, L. gelidum, L. nivalis* and *L. stokesii*) are isolated from Antarctica and named by Fell, Statzell, Hunter, and Phaff (1969). Subsequently, researchers from Russia and Germany have isolated *L. antarcticum* from Peat bog (Golubev, Blagodatskya, Manukian, & Liss, 1981), and Willow Catkin, northeast Mecklenburg (Kockova-Kratochvilova, Wegener, & Ondrusova, 1972), respectively. In Antarctica, *G. antarctica* is isolated from Moss Cirque, Vestfold Hills, Davis Base (Turchetti et al., 2011), South Victoria Land (Connell et al., 2008), and Admiralty Bay, King George Island (Donachie, 1995).

G. antarctica strain PI12 was previously isolated from the sea ice sample collected in the vicinity of Casey station (Boo et al., 2013). It survived and grew well under extremely cold conditions making it an ideal simple eukaryotic cell for cold adaptation analysis as well as the source to clone cold-active enzymes. The cold-adaptation mechanisms of *G. antarctica* PI12 have been described by some researchers (Firdaus et al., 2018; Koh, Wong, Najimudin, & Mahadi, 2019; Wong, Boo, Voo, Zainuddin, & Najimudin, 2019). Several cold-active hydrolytic enzymes have been cloned from it and characterised (Ramli et al., 2011; Ramli et al., 2013; Alias et al., 2014). Despite being one of the most analysed Antarctic yeasts (Ramli et al., 2011; Ramli et al., 2013; Alias et al., 2014; Firdaus et al., 2018; Koh et al., 2019; Wong et al., 2019), the main general characteristics of *G. antarctica* PI12 such as cell morphologies, growth patterns and metabolic abilities remain unclear. These characteristics are important to compliment all the other scientific research on this Antarctic yeast in the future. Therefore, this study was conducted: (1) to determine the growth rate and performance, and the cell morphology, and (2) to determine its ability to degrade carboxymethyl cellulose (CMC), xylan, chitin and mannan.

MATERIALS AND METHODS

Strain and Culture Conditions

G. antarctica strain PI12 was identified by Boo et al. (2013) based on its large subunit (LSU) rRNA and internal transcribed spacer (ITS) sequences alignment to those in the National Centre for Biotechnology Information (NCBI) GenBank. It was routinely grown in Yeast peptone dextrose (YPD) broth medium and kept in 20% glycerol stock at −80°C for long term storage.

Growth Performance in Different Media

G. antarctica PI12 was grown in YPD, yeast mould (YM), and potato dextrose (PD) broth media at 12°C with shaking at 210 rpm to compare their suitability for growth. Three culture replicates were prepared for medium. The optical density of each culture at 600 nm was measured using a spectrophotometer at an interval of 24 hours until it reached the stationary phase according to the methods described by Boo et al. (2013).

Growth Study

G. antarctica PI12 was grown in YPD broth medium at 12°C with shaking at 210 rpm. Three replicates were prepared. The optical density of each culture at 600 nm was measured from day 0 until day 53. The reading for each replicate was determined by averaging the results of five readings.

Microscopic Analysis

The morphology of *G. antarctica* PI12 was examined using a compound Olympus light microscope and Hitachi scanning electron microscope (SEM). A 10 µL of the culture was transferred to a glass slide and subjected to Gram-staining using standard microbiology protocol. It was viewed under a light microscope under a 400× magnification. In a separate analysis, 20 µL of yeast cell was fixed using 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C for 15 minutes. After fixation, the sample was centrifuged at 4,500 rpm for 1 minute. The supernatant was discarded, and the pellet was washed twice with 0.05 M phosphate buffer. The specimen was dehydrated in a series of ethanol (35%, 50%, 95%, and 99.8% (v/v)). The recovered cell pellet was resuspended in 35% (v/v) ethanol and centrifuged at 4,500 rpm for 1 minute. The supernatant was discarded. Dehydration steps were repeated two times with 50, 95, and lastly with 99.8 % (v/v) ethanol. The sample was centrifuged at 4,500 rpm for 1 minute between each dehydration step. The supernatant was discarded, and the pellet was suspended in 1 – 2 mL of hexamethyldisilazane (HMDS) for 5 minutes. HMDS was decanted after centrifugation and the pellet was dried overnight. The dried specimen was mounted onto an SEM specimen metal stub using double-sided sticky tape, coated with gold and viewed under SEM (Pueschel, 2013).

Cell Size Measurement

The length, diameter, and perimeter of *G. antarctica* PI12 measurements were carried out using Cell B software supplied together with the light microscope. Size measurements of 100, 1,800, and 4,000 lag, exponential and stationary phase cells respectively were taken. The average size of the measurements was calculated and recorded.

Metabolic Abilities

The metabolite abilities of *G. antarctica* PI12 were performed using the potato dextrose agar (PDA) and yeast mould agar (YMA) media containing 1 or 2% of glucose. Each of these media was supplemented with different carbon sources, 2.5% of hydrolysate chitin, 0.5% of carboxymethyl cellulose (CMC), 0.5% of guar, 0.2% of starch, or 0.5% of xylan. A 1 µL of yeast cell suspension grown to mid-log phase was inoculated onto the agar medium and incubated at 12°C for 14 days. The colonies were washed off from the agar medium with distilled water. The medium was stained with 1 mg/mL of congo red solution for 15 minutes and de-stained with 1 M sodium chloride (NaCl) for 15 minutes. A halo zone on the agar medium indicated that the substrate had been degraded (Carrasco et al., 2012).

RESULTS

Growth Performance in Different Media

The growth curve revealed that *G. antarctica* PI12 grew optimally in YPD and YM broth media (Figure 1) when compared to the PD broth medium. Nevertheless, YPD broth medium supported better cell growth than YM broth medium from the 72 hours of incubation and beyond.

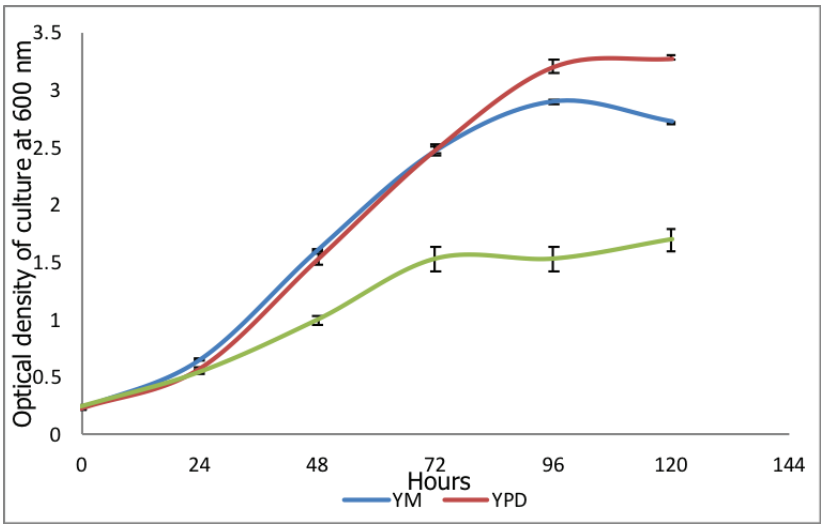

Figure 1 Growth curve of *G. antarctica* PI12 grown in YM, YPD, and PD broth media at 12°C

Growth Study

G. antarctica PI12 had a lag phase of about 3 days and entered the exponential growth phase from the 4th day until the 7th day and had a deceleration phase on the 8th day (Figure 2). The culture entered the stationary phase on the 9th day and remained at the stationary phase after that in the YPD medium.

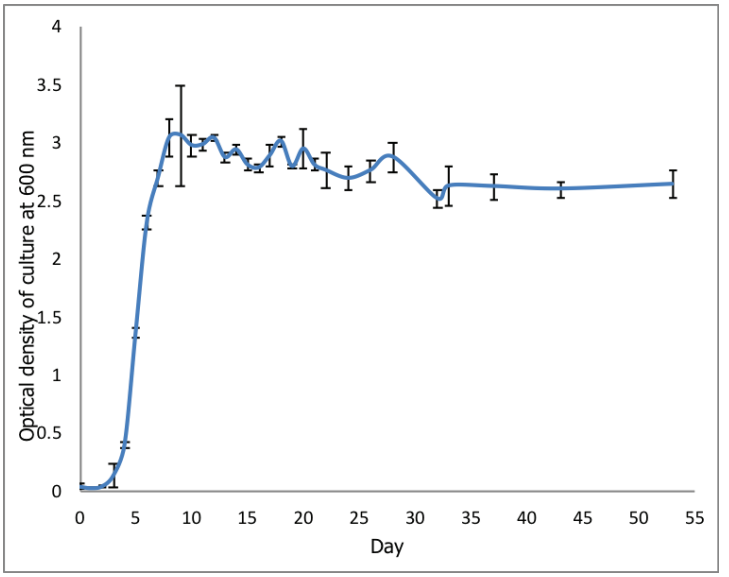

Figure 2 Growth curve of *G. antarctica* PI12 grown in YPD broth medium at 12°C

Microscopic Analysis

G. antarctica PI12 displayed different cell morphology at different growth stages. It formed elongated cells and in chains at the lag (2nd day) and early exponential phases (4th day) (Figure 3; Table 1). The number of yeast cells forming chains decreased as the cells aged. The cells had spherical, and ellipsoidal shapes with most cells having a single daughter cell at the stationary phase (19th day) (Figure 3). The cell surfaces were wrinkled and more obvious on the mother cells. The cells, especially the aged ones, were ellipsoidal (Figure 4). Bud scar was seen on the mother cells (Figure 4). Older cells or cells at the deceleration and stationary phases collapsed (Figure 5). The formation of yeast chains was observed in the SEM photos (Figure 5). *G. antarctica* PI12 exhibited a bipolar budding pattern, where buds were produced at both polar ends of a mother cell.

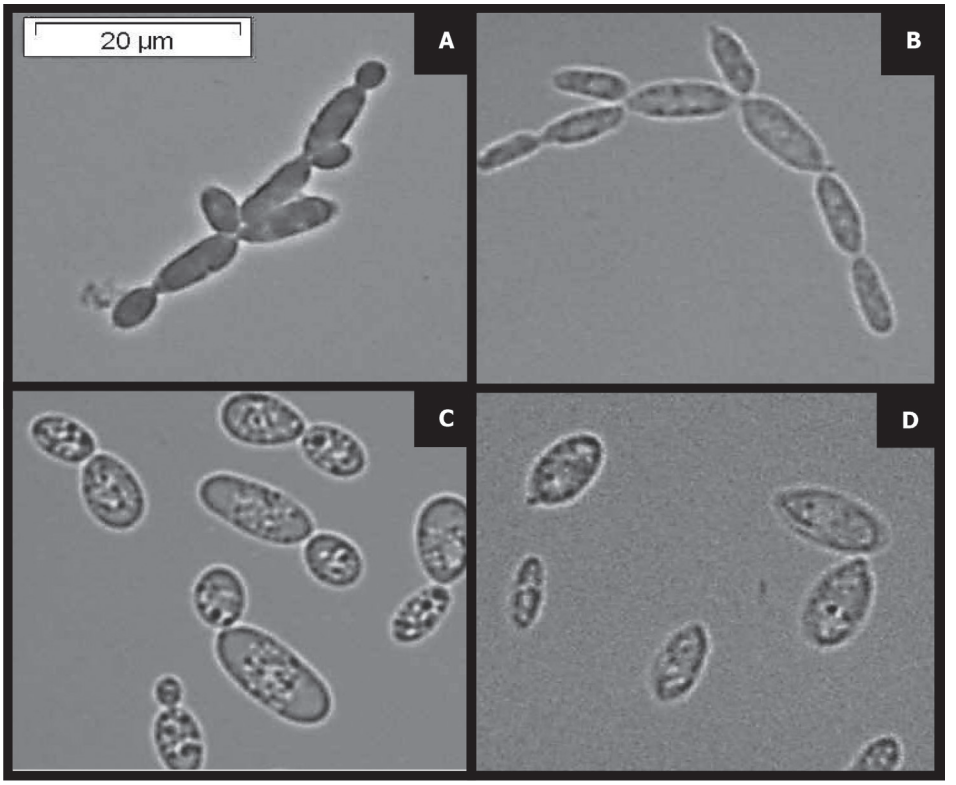

Figure 3 The morphology of *G. antarctica* PI12 was observed under a compound light microscope. Yeast cells were grown in YPD broth. Light microscope image taken at 400 times magnification. (A) Day 2 (lag phase), (B) Day 4 (exponential phase), (C) Day 8 (deceleration phase), and (D) Day 19 (stationary phase)

Table 1 Number of yeast cell chain at different growth phases

Day/ Phase	Number of yeast cell chain present per view under a light microscope (a)	Number of yeast present per view (b)	Ratio (a/b)	Comment
Lag (Day 2)	1	1	1	A small number of cells present, 8 cells yeast chain can be observed.
Exponential				
(Day 3)	5	5	1	As the culture aged, yeast chain formation decreases
(Day 4)	7	9	0.78	
(Day 5)	32	44	0.72	
(Day 6)	20	76	0.26	
(Day 7)	2	273	0.007	
Deceleration				
(Day 8)	1	252	0.004	
Stationary	–		–	No yeast chain was observed

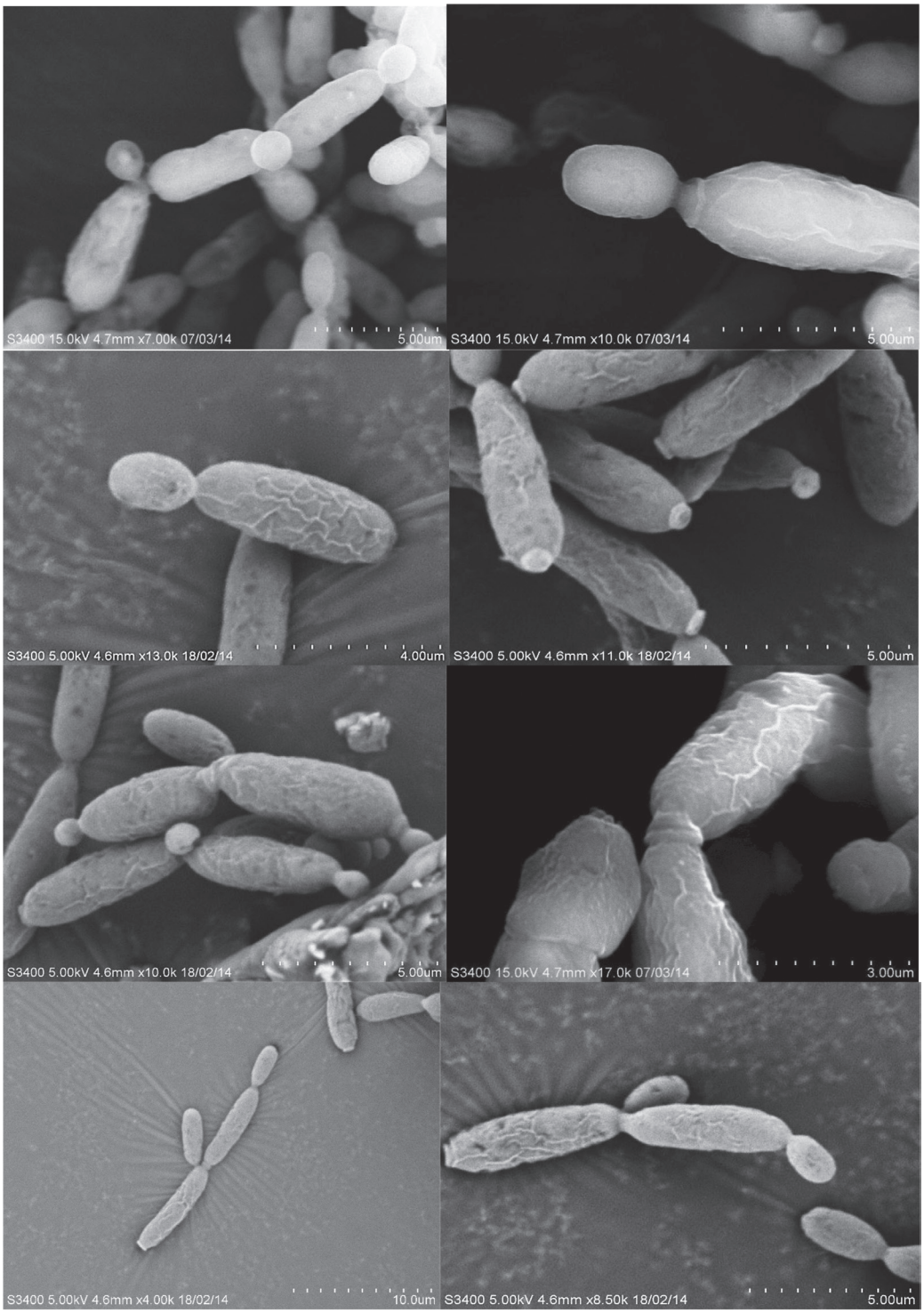

Figure 4 The morphology of *G. antarctica* PI12 at exponential phases under the SEM. *G. antarctica* PI12 was grown in YPD medium. The magnification of SEM is indicated on each photo (Days 3 and 5 yeast cells)

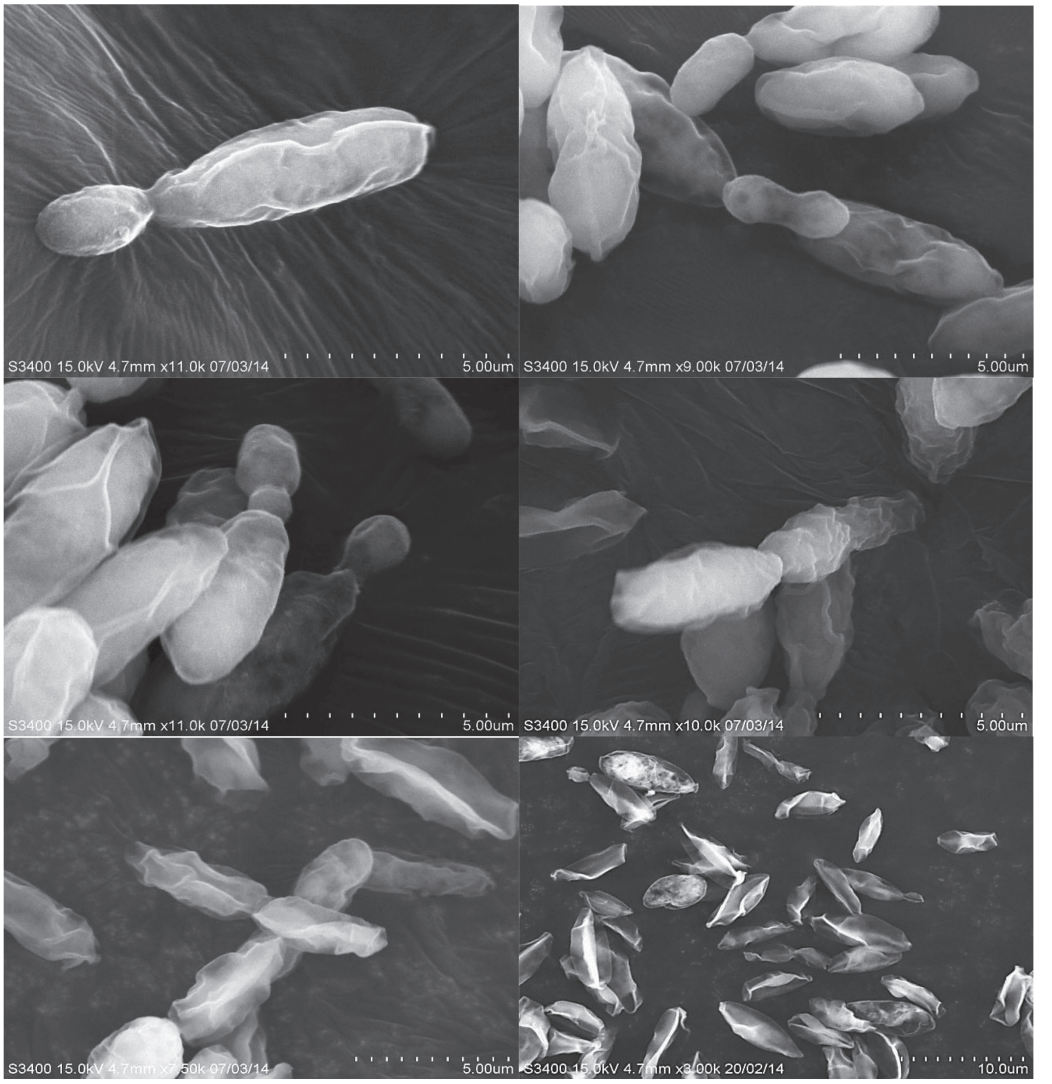

Figure 5 The morphology of *G. antarctica* PI12 at exponential phases under the SEM. *G. antarctica* PI12 was grown in YPD media. The magnification of SEM is indicated on each photo (Days 8, 11 and 32 yeast cells)

Cell Size Measurement

It was found that *G. antarctica* PI12 cell sizes gradually decreased from the lag phase to the stationary phase (Table 2). The average cell length at lag, exponential and stationary phases were 12.5, 11.2 and 10.5 µm, respectively. The average cell diameter at lag, exponential and stationary phases were 5.0, 4.8 4.7 µm respectively (Table 2).

The average bud length of lag, exponential and stationary phase cells ranged from 1.33 to 16.23, 0.99 to 14.7, 1.21 to 12.74, respectively (Table 3). The average bud diameter of lag, exponential and stationary phase cells ranged from 0.99 to 6.41, 0.87 to 7.63, 0.90 to 5.38, respectively (Table 3). Overall, the smallest bud was formed at the exponential phase while the largest bud was formed at the stationary phase (Table 3).

Table 2 Average cell size at different phases

Average mean ± SD / Phase	Length (µm)	Diameter (µm)	Area (µm²)	Perimeter (µm)
Lag phase	12.47±5.04	5.00±1.43	57.12±32.14	31.99±11.03
Exponential phase	11.21±4.65	4.78±1.73	48.54±28.98	28.96±10.14
Stationary phase	10.57±3.36	4.72±1.56	47.94±23.74	28.52±7.96

Table 3 Average range of bud size at different phases

Average range of bud size (µm) / Phase	Length	Diameter	Area
Lag Phase	1.33 – 16.23	0.99 – 6.41	3.84 – 76.82
Exponential Phase	0.99 – 14.72	0.87 – 7.63	1.11 – 79.55
Stationary Phase	1.21 – 12.74	0.90 – 5.38	1.67 – 54.15

Metabolic Abilities

G. antarctica PI12 exhibited chitinase, carboxymethyl cellulase (cellulase), mannanase, and xylanase activities by the formation of halos on chitin, CMC, guar gum and xylan media, respectively (Table 4). Similar results were observed in yeast grown on PDA containing 1% and 2% glucose and the assay substrates, except that mannanase activity was not detected. Also, the chitinase activity was suppressed when 2% of glucose was present in the PDA medium.

Table 4 Summary of the chitinase, CMCase (cellulase), mannanase, and xylanase activity of *G. antarctica* PI12. (+) = with activity; (–) = no activity; (±) = mild to low activity

Polysaccharides / Media	Chitin	CMC	Guar	Xylan
PDA (+1% glucose)	+	±	–	+
PDA (+2% glucose)	±	±	–	+
YMA (+1% glucose)	+	±	+	±
YMA (+2% glucose)	+	±	+	+

DISCUSSION

It is important to ensure that the yeast is growing in the most suitable medium when conducting experiments. According to Lodish et al. (2000), yeast can grow in a minimal broth medium containing glucose or dextrose as a carbon source, nitrogen, phosphorus, and trace metals. This was observed in the current study whereby *G. antarctica* PI12 was able to grow on PD, YPD and YM media (Figure 1). Nevertheless, it grew better in YPD and YM media when compared to the PD medium (Figure 1). YPD and YM media contained yeast extract and bacto-peptone that are richer in nutrient contents when compared to the potato dextrose medium ingredients. Overall, YPD is still a better growth medium for *G. antarctica* PI12 when compared to YM medium. Hence, YPD was used in several subsequent experiments to grow *G. antarctica* PI12.

G. antarctica PI12 is a relatively slow-growing yeast despite being incubated at its optimal growth temperature of 12°C (Boo et al., 2013), requiring 3 days exit lag phase. The overall slower growth rate was most probably due to the lower chemical reaction rates to maintain adequate metabolic fluxes at 12°C (Feller & Gerday, 1997). After the exponential stage, yeast cells usually enter the death phase, which is indicated by a decline in the OD readings. However, the OD_{600nm} readings of *G. antarctica* PI12 at the stationary phase remained almost constant until day 53 (Fig. 2). The extended stationary phase of *G. antarctica* PI12 suggested that the cells remained viable and intact. According to Gray et al. (2004), a long period of stationary phase suggests that *Saccharomyces cerevisiae* has probably entered an alternative (G_0) or resting state, where the cells entered non-proliferating state or exited from the normal cell cycle. This resting state is known as quiescence or G_0. Yeast is reported to enter quiescence or G_0 when growth conditions are unfavourable such as during starvation due to depleting nutrients (Wei, Nurse, & Broek, 1993) and the accumulation of toxic metabolites (Galdieri, Mehrotra, Yu, & Vancura, 2010). This implied that the long stationary phase exhibited by *G. antarctica* is very similar to other yeasts such as *Saccharomyces cerevisiae*.

Although *G. antarctica* PI12 shared some of the model yeast, *S. cerevisiae* growth properties, and produced off-springs through budding, their morphologies differed slightly. Unlike *S. cerevisiae* that is usually ellipsoidal and spherical, *G. antarctica* PI12 formed elongated cell, apart from the ellipsoidal and spherical cell. The production of daughter cells was almost always at the polar ends in *G. Antarctica* PI12 while *S. cerevisiae* budding sites are axial, polar and random (Saito et al., 2004; Vopálenská, Hůlková, Janderová, & Palková, 2015). *G. antarctica* PI12 exhibited bipolar budding, whereby the daughter cells arose at both ends of the elongated cell. According to Walker (1998), bipolar budding gives rise to the buds which are restricted at the ends or poles of the elongated cells along the longitudinal axis of the mother cells. Also, bipolar budding is not restricted to one cell at the end, which means that two or more buds may arise from the same pole at the same time.

Chain *G. antarctica* PI12 cell formation was observed at both lag and exponential phases. But the frequency gradually decreased as the culture aged in the late exponential phase from day 6 onwards. The formation of chain *G. antarctica* PI12 cells was not observed in the stationary phase (Figure 2; Table 1). According to Powell, Zandcyke, Quain, and Smart (2000), chain formation is a characteristic phenotype of some brewing yeast strain and the formation occurred due to ineffective separation between mother and daughter cells. Besides, complete separation between the mother-daughter cells is not necessary to continue reproduction (Powell et al., 2000). Chain of *G. antarctica* PI12 cells formation frequency decreased as the cells aged. This was due to the culture cell density increment, and the availability of the nutrient deceased. As a result, it was not able to support the cells to reproduce or divide. Additionally, cells do not actively reproduce or divide when culture entered stationary phase. The SEM electron micrograph showed similar structures to those taken using a light microscope but with more details. Wrinkled mother cells were observed under the SEM and this could be caused by the chemical used during the sample preparation steps, or it could be a normal morphology of the cell, but the latter possibility is higher. Figure 4 shows that the buds did not wrinkle even though they were exposed to the same sample preparation steps. According to Powell et al. (2000), as the cells age, the wrinkling on the cell surface increases to compensate for the decrease in the surface area.

The *G. antarctica* PI12 cell sizes were larger at lag and exponential phases than those at the stationary phase (Table 2 and Table 3). According to Turner, Ewald, and Skotheim (2012), budding and fission yeasts are coordinated to reach a certain size threshold during the cell-cycle before the cells can move to the next phase. In *Schizosaccharomyces pombe,* cell cycle arrest takes place at the G2-M phase transition while in *S. cerevisiae,* cell cycle arrest takes place at G1-S phase (Johnston, Pringle, & Hartwell, 1977). As a result, the arrested cell size increased to achieve the size threshold. Hence, the larger *G. antarctica* PI12 cell size at the lag phase was likely to be controlled similarly. Generally, cells grow rapidly on rich media divide at a larger

size when compared to cells growing slowly under poor nutrient conditions (Turner et al., 2012) such as *G. antarctica* PI12 which had larger cells initially at the lag and exponential phases. When nutrients in the medium were depleted at the stationary phase, smaller cells were formed.

G. antarctica PI12 lives in an environment with limited nutrient and carbon source. Hence, an organism with the ability to use a wider source of carbon will have an advantage to survive and grow when compared to others that do not. Interestingly, *G. antarctica* PI12 produced hydrolytic enzymes that degraded chitin, CMC, guar gum and xylan, indicating that it can utilize those substrates as carbon sources. However, the production of mannanase by *G. antarctica* PI12 occurred in the YMA medium but not in the PDA medium (Table 3). This indicated the starch in PDA medium suppressed mannanase but not the chitinase, carboxymethyl cellulase (cellulose), and xylanase production in *G. antarctica* PI12. Stoll, Stalbrand, and Warren (1999) reported the production of proteins with endo mannanase activity of *Cellulomonas fimi* decreases when glucose is present. However, this did not happen to *G. antarctica* PI12, although the inclusion of 2% of glucose into the PDA medium did reduce chitinase enzyme production. The overall results show that *G. antarctica* PI12 produced several hydrolytic enzymes to allow the cells to utilize complex types of the substrates, and glucose was not necessary the only primary source of carbon for its metabolism and energy.

CONCLUSION

Overall, we found that *G. antarctica* PI12 shared some of the characteristics of other yeasts, and at the same time has its unique properties such as its cell morphology and sizes at different phases of growth. These are probably the reasons why *Glaciozyma* spp. and *G. antarctica* PI12 specifically are so successful in inhibiting various low nutrient habitats such as Antarctica.

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