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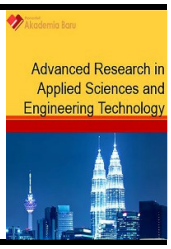

Growth Kinetics of Ethidium Bromide Mutagenized *Lipomyces starkeyi* Strains

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ABSTRACT

Yeast growth and biomass production are greatly influenced by the length of the incubation period during cultivation. Therefore, this study was conducted to investigate the growth kinetics of five *Lipomyces starkeyi* strains as determined by biomass production. The five *L. starkeyi* strains, namely *L. starkeyi* ATCC 12659, *L. starkeyi* MV-1, *L. starkeyi* MV-4, *L. starkeyi* MV-5 and *L. starkeyi* MV-8, were inoculated in sterilized Yeast Malt broth, and, incubated for 192 hr at ambient temperature. Biomass yields were assessed and calculated gravimetrically every 24 hr. Results indicated that the optimal biomass production of *L. starkeyi* ATCC 12659, *L. starkeyi* MV-1, *L. starkeyi* MV-4, *L. starkeyi* MV-5 and *L. starkeyi* MV-8 were at 120, 168, 144, 168 and 120 hr, with the concentrations of 6.64, 6.43, 9.78, 11.23 and 8.56 g/L, respectively. These results indicate that each *L. starkeyi* strain requires specific incubation period for the optimum production of fungal biomass. Therefore, by cultivating each *L. starkeyi* strain at the predetermined incubation period, biomass yields could significantly be improved for further downstream applications such as single cell protein and lipid production.

1. Introduction

Yeasts are known for their high biomass production and rapid growth rates [1]. They are capable of producing high biomass yields of between 10 to 100 g/L after 3 to 7 days of incubation period [2]. The biomass produced from these unicellular fungi is valuable because of its nutritional values, non-toxic properties and low nucleic acid content [3]. Therefore, yeasts are extensively used in various sectors of the agricultural industry as food supplements for human and animals, production of biofuels, beverages, bakery and probiotics [4-5].

Many yeasts are oleaginous. One example is *Lipomyces starkeyi*, which is reported to produce lipids up to 70% of its dry biomass under optimum cultivation conditions [6-8]. These lipids are mainly

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in the form of triacylglycerols, and, can potentially replace plant oils and animal fats in various industrial applications [9-11]. More importantly, lipids extracted from *L. starkeyi* can be converted to biodiesel via transesterification [12].

The advantages in using *L. starkeyi* for lipid production are the simple cultivation requirements and flexibilities of using low cost carbon sources as growth substrates. These substrates include glucose, a mixture of xylose and cellobiose, ethanol and sewage sludge [13]. However, the growth and biomass production rates of *L. starkeyi* are greatly affected by cultivation conditions such as carbon to nitrogen ratio, incubation period, pH and temperature [14]. These cultivation conditions have been well studied except for incubation periods, which remain poorly documented. Therefore, this study was performed to investigate the growth kinetics of several *L. starkeyi* strains, namely *L. starkeyi* ATCC 12659, *L. starkeyi* MV-1, *L. starkeyi* MV-4, *L. starkeyi* MV-5 and *L. starkeyi* MV-8 in order to determine the optimal incubation periods for optimum biomass production. The data obtained from this study can then be used to significantly improve biomass yields for further downstream applications.

2. Materials and Methods

2.1 Microorganisms and Stock Preparation

Lipomyces starkeyi ATCC 12659 was obtained from the American Type Culture Collection (Manassas, Virginia), whereas the *L. starkeyi* MV-1, *L. starkeyi* MV-2, *L. starkeyi* MV-4 and *L. starkeyi* MV-8 were selected mutant strains produced via random mutagenesis using ethidium bromide. To perform the random mutagenesis procedure, *L. starkeyi* ATCC 12659 cells (1 x 10^5 cells/mL) were inoculated in 0.15 M phosphate buffer (pH 7.0) spiked with sterile ethidium bromide, $C_{21}H_20BrN_3$ (200 µg/ml) solution. The reaction mixture was incubated for 90 min at 25 °C with shaking at 120 rpm. The treated cell pellets were then inoculated for 8 days on Yeast Malt agar plates supplemented with 10 µg/uL cerulenin for screening and selection. All yeast isolates were revived in Yeast Malt (YM) broth (consisting of dextrose, 10.0 g/L; peptic digest of animal tissue, 5.0 g/L; yeast extract, 3.0 g/L; and malt extract, 3.0 g/L) (HiMedia Laboratories, India) and incubated at 25 °C at constant shaking of 120 rpm for 3 days [15]. For long-term storage, the stock cultures were aliquoted in YM broth supplemented with 20% (v/v) of glycerol (R and M Marketing, United Kingdom) and stored at -80 °C.

2.2 Growth Media Preparation

The minerals broth for yeast propagation consisted of 30 g/L of glucose, 1 g/L of $(NH_4)_2SO_4$, 1.5 g/L of yeast extract, 2.5 g/L of $Na_2HPO_4.7H_2O$, 7 g/L of KH_2PO_4, 0.15 g/L of $MgSO_4•7H_2O$ and 0.1 g/L of $CaCl_2.2H_2O$ [16]. The medium was autoclaved at 121 °C for 15 min prior to use. Glucose was sterilized separately to prevent caramelization and was mixed aseptically with the other components after cooling. This method was adapted from Tapia *et al*. [17].

2.3 Growth Kinetics Experimental Setup

The experiments were conducted in 2 L modified Schott bottle (for working volumes of 1.8 L) (Duran, Germany), equipped with three silicon tubes for air inlet, air outlet and sampling purposes, as shown in Figure 1. The Schott bottles were attached to an air pump (SB2800, SOBO, China) and a peristaltic pump (Masterflex Easy Load L/S 7518-00, Cole Parmer, Malaysia) for air inlet and culture sampling, respectively. Air inlet and outlet tubes were filtered by syringe filter with nylon membrane 0.45 µM (Whatman, United Kingdom). An air diffuser was attached to the end of air inlet tube for

aeration. All apparatus were autoclaved at 121 °C for 15 min for sterilization prior to use. Three runs per culture-experiments were performed (n=3).

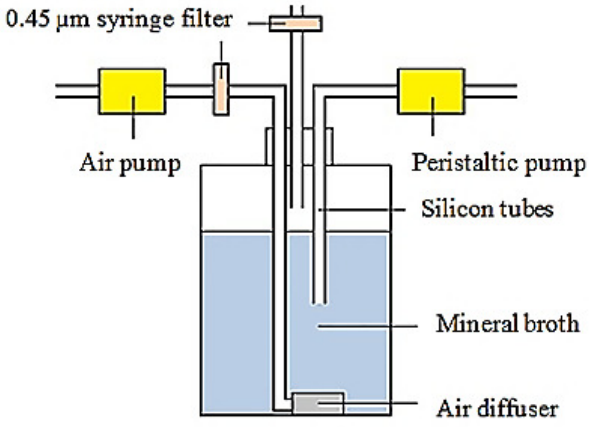

Fig. 1. Schematic diagram of the fermentation reactor for the *L. starkeyi* strains biomass production

2.4 Growth Kinetics Study and Determination of Biomass Production

Approximately 14 mL of each *L. starkeyi* strain from glycerol stocks were inoculated into sterile minerals broth for the growth kinetics experiments. All cultures were aerated and incubated at ambient temperature for 192 hr. Culture samples of 150 mL were collected at 24, 48, 72, 96, 120, 144, 168 and 192 hr, followed by centrifugation at 5,000 rpm for 10 min. The harvested yeast biomass was dried in an oven at 80 °C for 2 days and cell weight was determined gravimetrically. The growth of the *L. starkeyi* strains were expressed as the increase in dry cell mass as a function of time (hr) on a volumetric basis.

2.5 Sudan IV Staining for Lipid Detection

Approximately 1 mL of samples were collected and centrifuged at 10,000 rpm for 3 min to remove the supernatant. The cell pellets were resuspended in 1 mL of Sudan IV solution (0.5% Sudan IV in 70% ethanol) and mixed well by vortexing. The mixture was smeared on the slide and subjected to air drying for 30 min at room temperature. Counter staining was conducted using safranin for 30 sec, followed by washing with sterile distill water and air drying prior to observation under a compound microscope (Leica, DM500, China).

2.6 Scanning Electron Microscope (SEM) Analysis

Scanning electron microscopy was conducted following a modified procedure of Hughes *et al*. [18]. Approximately 1 mL of yeast cells were harvested from the culture broth and centrifuged at 10,000 rpm for 3 min to remove residual broth. The cell pellets were then resuspended and fixed in 2.5% glutaraldehyde for 30 min at room temperature before rinsing three times with sterile distilled water to remove any traces of glutaraldehyde. Next, the cells were dehydrated in successive gradations of ethanol (25%, 50%, 70%, 95% and 100% v/v) and placed in an Eppendorf tube to air dry

overnight. Finally, the samples were then observed using a scanning electron microscope (SEM) (JSM-6390LA, JOEL, USA).

3. Results and Discussion

Growth and biomass production of yeasts vary when cultured in different cultivation conditions [19]. In this study, *L. starkeyi* ATCC 12659, and the mutants strains *L. starkeyi* MV-1, *L. starkeyi* MV-4, *L. starkeyi* MV-5 and *L. starkeyi* MV-8 were cultured in sterile mineral broth consisting of glucose, nitrogen, phosphorus, magnesium and calcium. The growth kinetics and biomass production were monitored and observed for 192 hr. Growth comparisons of the strains were further analyzed by comparing the biomass dry weight obtained at lag, exponential and stationary phases.

Figure 2 shows the biomass production of the five *L. starkeyi* strains. In general, the growth patterns are similar across all strains tested throughout the incubation period. The *L. starkeyi* strains cultured in the mineral broth exhibited three main phases of growth profile, which is the lag, exponential, and stationary phases typical in most microorganisms [20]. The length of the respective phases was different depending on the availability of nutrients in the medium and the proliferation rate of the yeast strains. The initial biomass increments of the five *L. starkeyi* strains were relatively small as the cells were adapting to the cultivation conditions [21-22]. *L. starkeyi* ATCC 12659, *L. starkeyi* MV-4 and *L. starkeyi* MV-8 adapted rapidly to new cultivation conditions, whereas *L. starkeyi* MV-1 and *L. starkeyi* MV-5 responded more slowly, resulting in longer lag phases.

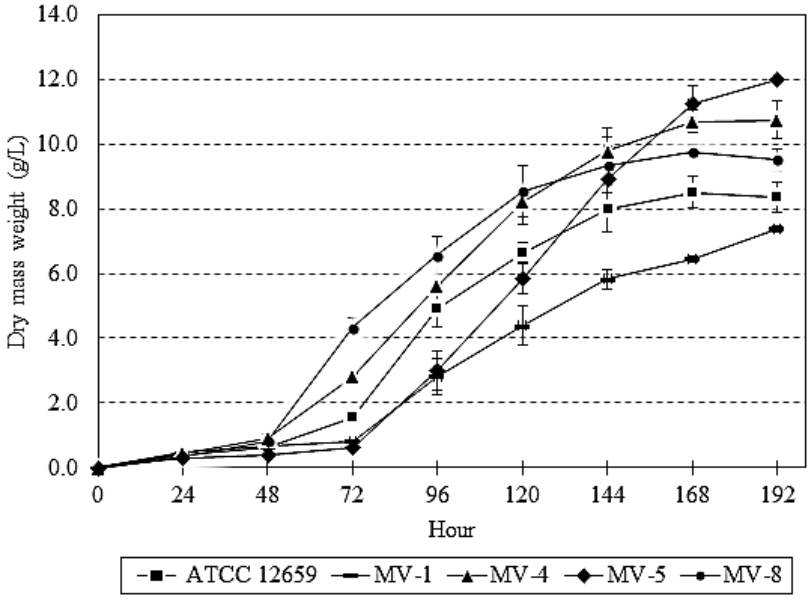

Fig. 2. Growth kinetics and biomass production of the *L. starkeyi* strains used in this study. Data shown as mean ± SD (n = 3). Error bars correspond to standard deviation of triplicate samples

The length of the lag phase duration is normally influenced by several factors, including the initial inoculum size, the physiological conditions of cells, and the culture conditions of both the original and the new growth medium [23-24]. According to Bair and Stannard, the duration of the lag phase

depends inversely on the initial inoculum size, where a small inoculum size results in longer lag phase periods [25]. In this study, the initial inoculum was standardized by inoculating 14 mL of 3 days old of stock culture (10^8 - 10^9 cells/mL) into the cultivation medium.

All of the *L. starkeyi* strains exhibited progressive increase in biomass weight during the exponential phase. Biomass production of *L. starkeyi* ATCC 12659, *L. starkeyi* MV-4 and *L. starkeyi* MV-8 gradually increased throughout the incubation period, reaching their optimal levels at 120, 144 and 120 hr, with values of 6.64, 9.78 and 8.56 g/L, respectively. On the other hand, *L. starkeyi* MV-1 and *L. starkeyi* MV-5 reached their optimal biomass production at 168 hr, with values of 6.43 and 11.23 g/L, respectively. Overall, the time taken to achieve maximum biomass ranged from 120 to 168 hr, corresponding to the late-exponential phase for all the strains tested.

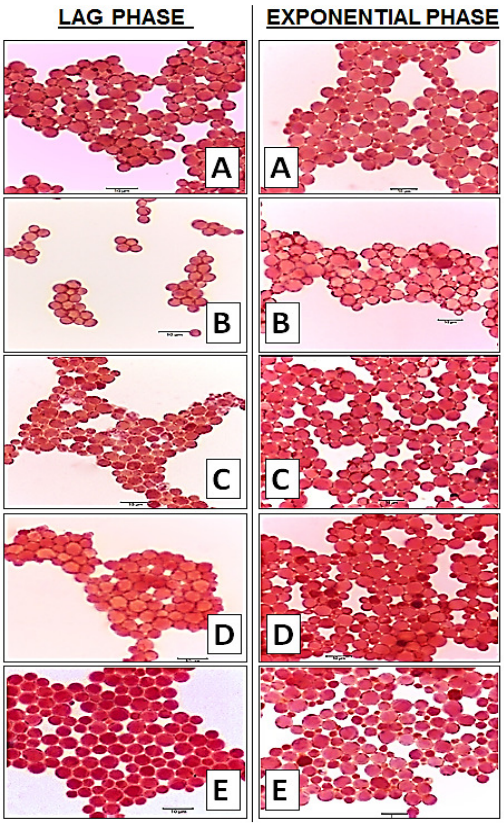

Fig. 3. Sudan IV staining microscopic images of (A) *L. starkeyi* ATCC 12659, (B) *L. starkeyi* MV-1, (C) *L. starkeyi* MV-4, (D) *L. starkeyi* MV-5 and (E) *L. starkeyi* MV-8 cells at 1000X magnification during the lag and exponential phases. (Bar - 10 µm)

Extended period of incubation resulted in the decrease of biomass production. This was probably due to the depletion of nutrients in the medium at the stationary phase [26-27]. *L. starkeyi* ATCC 12659, *L. starkeyi* MV-4 and *L. starkeyi* MV-8 reached the stationary phase within 168 hr, whereas *L. starkeyi* MV-1 and *L. starkeyi* MV-5 reached the stationary phase within 192 hr. Interestingly, *L. starkeyi* MV-5 and *L. starkeyi* MV-1 showed a longer life span compared to other strains. One possible

explanation is the ability of the cells to undergo less robust Gap 1 (G1) phase arrest, and therefore exhibit longer lifespan which is in agreement with Jimenez *et al*. [28]. However, their growths were slower compared to *L. starkeyi* ATCC 12659, *L. starkeyi* MV-4 and *L. starkeyi* MV-8 that exhibited longer lag phases. Among the strains tested, the highest dry biomass weight was obtained from *L. starkeyi* MV-5, whereas the lowest was obtained from *L. starkeyi* MV-1, indicating that the latter had poorer growth performance.

As shown in Figure 3, the morphological changes of the five strains of *L. starkeyi* cells during lag and exponential phases were observed under a light microscope at 1,000× magnification. Overall, there was no obvious difference between the shapes of the cells, with either globose or ellipsoidal shapes being more predominant [29]. It was also observed that the cell size increased in relation with the incubation time due to increase in RNA and protein content of cells [30]. The sizes of the cells increased from 5 µm at lag phase to approximately 6 to 8 µm at the exponential phase. In addition, cells at exponential phase were denser than that of lag phase, possibly due to the rapid proliferation of cells during exponential phase.

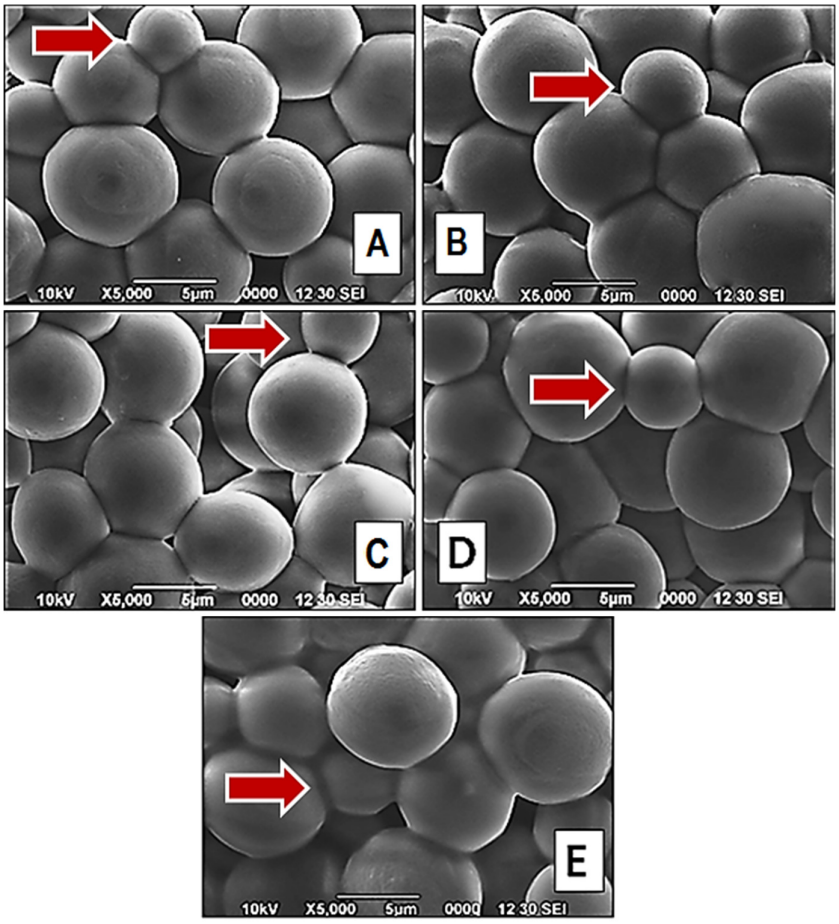

Fig. 4. Scanning electron micrographs of *L. starkeyi* ATCC 12659 (A), L. *starkeyi* MV-1 (B), *L. starkeyi* MV-4 (C), *L. starkeyi* MV-5 (D) and *L. starkeyi* MV-8 (E) cells 5,000X magnifications. Note: Arrows indicate daughter cells

Figure 4 shows the scanning electron micrographs (SEM) of the five *L. starkeyi* strains during the exponential phase. The budding cells were found to be dormant and thickened at this phase. At this point, the mother cells form daughter cells by multilateral budding. From Figure 4, the initial size of daughter cells were observed to be smaller than the mother cells before increasing in sizes, followed by separation from the mother cells. Scanning electron microscope (SEM) allows better observation of cell morphology at higher magnification (5,000X) compared to the light microscope (1,000X) [31]. However, this approach is challenging because requires it complex fixation protocols to immobilize the non-adherent cells that may lead to changes in the structure, morphology, and physical-chemical properties of the cells [32].

4. Conclusion

Optimization of incubation period for obtaining the highest biomass yields in five *L. starkeyi* strains were accomplished in this study. *L. starkeyi* ATCC 12659, *L. starkeyi* MV-1, *L. starkeyi* MV-4, *L. starkeyi* MV-5 and *L. starkeyi* MV-8 require 120, 168, 144, 168 and 120 hr of incubation period to produce maximum biomass dry weight with values of 6.64, 6.43, 9.78, 11.23 and 8.56 g/L, respectively. These results showed that each strain requires specific incubation periods for peak biomass production. Cultivating the *L. starkeyi* strains at the optimized incubation period could significantly improve yeast biomass yields for further downstream applications such as lipids production.

Acknowledgements

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