

# Isolation and characterization of novel acidophilic microalgae from abandoned mining site area for carbohydrate biosynthesis and its kinetic growth study in photobioreactor

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**Abstract.** Microalgae have gained attention as an alternative feedstock for biofuel production due to its potential to accumulate carbohydrate, protein and lipid. However, current microalgae culture technology has been impeded by low biomass production, carbohydrate accumulation and contamination issue. In order to overcome this limitation, this study involves the isolation, screening and identification of acidophilic microalgae from Frog Hill. Then, the effect of different initial pH on growth and carbohydrate productivity was studied toward the selected microalgae. Further cultivation was done in indoor and outdoor using 15L fabricated PBR under optimize pH condition. At the end of experiment, the growth kinetic and carbohydrate productivity for both conditions were evaluated. Total of 4 native acidophilic microalgae were successfully isolated and was identified using 18s RNA. The *Scenedesmus parvus* was exhibited highest biomass production and carbohydrate productivity up to  $1.2640 \pm 0.001 \text{ g L}^{-1}$  and  $15.47 \pm 0.048 \text{ mg L}^{-1} \text{ d}^{-1}$  under highly acidic condition. It also showed a better performance under outdoor cultivation up to 9.54% compared to indoor cultivation. Hence, this research paper showed that the potential of novel acidophilic microalgae survives under extremely condition with high carbohydrate biosynthesis, which could be useful for industrial application.

## 1. Introduction

The rapid growing of world population required continuously increase the global demand and fuel energy. Some of the researchers also predicted that world fossil oil reserves will be exhausted in less than 50 years due to high consumption rate or demand by mankind [1]. Therefore, the renewable energy resource plays an important role to replace the exhausted fossil fuel. Microalgae becomes a promising feedstock for biofuel production due to the number of advantages, including shorter life cycle compared to terrestrial plant, carbon dioxide (CO<sub>2</sub>) bio-sequestration capability, not competing for arable lands, and accumulate chemical metabolites such as lipid, protein and carbohydrate [2]. Microalgal-carbohydrate is one of the dominant compound in microalgae biomass. It can act as the valuable chemical platform to be converted into biofuel, bioethanol, and biopolymer through biotechnological application using sustainable manner [3]. Previous study showed that *Chlorococum sp.* microalgae biomass can be used as feedstock for bioethanol production up to  $3.83 \text{ g L}^{-1}$  fermented using *Saccharomyces bayanus* under optimum condition [4].

However, the industrial production of microalgae-based products are not yet economically feasible



and not cost-effective. This situation normally due the low microalgae biomass production. One of these factor was contributed by serious contamination by unwanted microorganism which can impede the microalgae growth. Hsu and Wu [5] proved that using large scale cultivation could be resulting in lower yield due to many unknown environmental factors that can directly affect the microalgae cultivation process. Besides that, Hirooka et al. [6] study also shows that mass cultivation using open pond system was easily contaminated by undesired microorganism in which suppressing the microalgae growth and resulting in the low productivity. Then, this problem has led to increase in total cost of cultivation and resulting in difficulties of downstream purification process.

Hence, an innovation idea of large scale cultivation of microalgae using closed photobioreactor system under controlled conditions have been suggested by previous studies in which could increase the biomass productivity to ensure the feasibility of product formation [7]. Similar study also be conducted by Sánchez Mirón et al. [8] who demonstrates that *Phaeodactylum tricornutum* microalgae was achieved biomass concentration up to 4 kg m<sup>-3</sup> cultivated using 60 L of bubble column, split-cylinder airlift device and concentric draft-tube sparged airlift vessel respectively. This would be beneficial for additional product value such as omega-3 polyunsaturated fatty acids production. Besides that, Schenk et al. [9] have suggested that cultivating extremophile microalgae that can tolerate at particular condition that is hostile for other contaminants, at the meanwhile could enhance the biomass production.

Up to date, there was still scarce of information regarding the acidophilic microalgae cultivated under extreme condition for carbohydrate polymer biosynthesis. Thus, the main objective of this study was to isolate the most tolerant acidophilic microalgae and determine the carbohydrate polymer biosynthesis cultivated under acidic condition. At the end of the experiment, the suitable kinetic model was chosen based on isolated microalgae growth rate cultivated in large scale of fabricated tubular photobioreactor.

## 2. Materials and Methods

### 2.1 Sample collection and isolation of acidophilic microalgae

The small portion of soil containing green microalgae was collected from the abandoned mining site area, Frog Hill (5.4354° N, 100.4847° E). Then, the sample containing microalgae were enriched in autoclaved acidic bold basal medium, BBM (pH 3.0) in 250mL conical flask, sparging using 15% (v v<sup>-1</sup>) CO<sub>2</sub> concentration. The chemical composition of BBM as followed: NaNO<sub>3</sub> 25 g L<sup>-1</sup>, MgSO<sub>4</sub>.7H<sub>2</sub>O 7.5 g L<sup>-1</sup>, NaCl 2.5g L<sup>-1</sup>, K<sub>2</sub>HPO<sub>4</sub> 7.5 g L<sup>-1</sup>, KH<sub>2</sub>PO<sub>4</sub> 17.5 g L<sup>-1</sup>, CaCl<sub>2</sub>.2H<sub>2</sub>O 2.5 g L<sup>-1</sup>, ZnSO<sub>4</sub>.7H<sub>2</sub>O 8.82 g L<sup>-1</sup>, MnCl<sub>2</sub>.4H<sub>2</sub>O 1.44 g L<sup>-1</sup>, MoO<sub>3</sub> 0.71 g L<sup>-1</sup>, CuSO<sub>4</sub>.5H<sub>2</sub>O 1.57 g L<sup>-1</sup>, Co(NO<sub>3</sub>)<sub>2</sub>.6H<sub>2</sub>O 0.49 g L<sup>-1</sup>, H<sub>3</sub>BO<sub>3</sub> 11.42 g L<sup>-1</sup>, EDTA 50 g L<sup>-1</sup>, KOH 31 g L<sup>-1</sup>, FeSO<sub>4</sub>.7H<sub>2</sub>O 4.98 g L<sup>-1</sup> and H<sub>2</sub>SO<sub>4</sub> 1 mL.

Next, the sample was isolated by combination method between dilution with agar streaking technique for obtaining the axenic culture. Then the single colony was inoculated into BBM medium under controlled culture room at 30 ± 2°C, illuminated light with photon intensity of 20.99 μmol m<sup>-1</sup> s<sup>-1</sup> provided by cool daylight tube with photoperiod cycle of 12 h: 12 h (light: dark).

### 2.2 Experimental set up

A total volume of 70 mL with OD 1.00 (10% v v<sup>-1</sup>) microalgae cell was inoculated into 630 mL BBM medium similar with above conditions. Then, the cell growth rate was measured in daily basis from day 0 until days 15 and the triplicate readings were taken. The microalgal cells were recovered by centrifugation and dried using oven at 60 °C. The dried microalgal pellet was subjected for further analysis. The selected strain that possess highest growth rate with carbohydrate productivity will be subjected to different pH values medium ranging from pH 3.00 to pH 11.00 in order to study the kinetic behavior and carbohydrate accumulation. The specific growth rate (d<sup>-1</sup>) and carbohydrate productivity (mg L<sup>-1</sup> d<sup>-1</sup>) were calculated according to the following equation (1) and (2):

$$k = \frac{\ln N_1 - \ln N_2}{t_1 - t_2} \quad (1)$$

where k (d<sup>-1</sup>) is the specific growth rate, N<sub>1</sub> and N<sub>2</sub> are the biomass concentration at day t<sub>1</sub> and t<sub>2</sub>

$$CP = BP \times CW \quad (2)$$

where CP is carbohydrate productivity ( $\text{mg L}^{-1} \text{d}^{-1}$ ), CW (%) is carbohydrate content (w w<sup>-1</sup>) based on dry weight.

### 2.3 Measurement and quantification of total carbohydrate and sugar component

The total carbohydrate content was determined using phenol-sulfuric acid method following the method by Nielsen [10]. Dextrorotatory (D) (+) glucose was used as a standard for this carbohydrate analysis. The microalgae that contain highest carbohydrate productivity will be selected for the subsequent experiment.

### 2.4 Morphological identification of microalgae

Identification of the microalgae species was done through conventional method and molecular approach. Isolated strains were preliminary identified using standard morphological features. Subsequently, the total genomic DNA from the cultured sample was extracted using GF-1 plant DNA extraction kit (Vivantis Technologies, Malaysia) according to the manufacturer instruction. For PCR amplification, the extracted DNA was used to amplify the 18S rRNA with two general primers, forward primer (EUF) and reverse primer (EUR). The PCR analysis was performed by Mastercycler EP384 Thermal Cycler (Eppendorf, US) with the conditions described by Nübel et al. [11].

EUF: 5'-GTCAGAGGTGAAATTCTTGGATTTA-3'

EUR: 5'-AGGGCAGGGACGTAATCAACG-3'

The PCR product was further analysed by 18S rRNA gene sequencing at Centre for Chemical Biology, CCB Penang using EUF and EUR primers. All sequences obtained here were blasted through NCBI GeneBank database and performed phylogenetic tree analysis.

### 2.5 Phylogenetic analysis

A search of NCBI GenBank with BLAST was used to identify the isolated microalgae species with rRNA gene sequences downloaded from the database. The sequences containing fewer than 200 nucleotides or in excess of 1000 nucleotides were removed, and sequences not belonging under microalgae species also be excluded from this study. Subsequently, all the downloaded sequences were aligned accordingly using the ClusterX 2.1 software for restricting the multiple sequences alignment within the minimum common region. The phylogenetic tree was constructed using the neighbor-joining (NJ) method and Kimura's two-parameter model implemented within Mega-X software.

### 2.6 Kinetic growth in photobioreactor

A 10% (v/v) of selected microalgae strain was further up-scaled into 15L of fabricated tubular PBR with optimum pH condition cultivated under indoor and outdoor environment. The cell growth and carbohydrate productivity were also evaluated under both cultivation condition. The basic Logistic equation and Gompertz equation from Sigmaplot version 12.5 as in equation (3) and (4) were adopted in this research to predict the kinetic behavior of microalgal growth cultivated under PBR system. Choosing a suitable model with  $R^2 > 0.95$  showed that the kinetic growth of microalgae fitted well with the suggested model.

Logistic model: 
$$C(t) = C_o e^{\mu t} \times [1 - (\frac{C_o}{C_m})(1 - e^{\mu t})]^{-1} \quad (3)$$

Gompertz model: 
$$C(t) = C_0 \times e[-e(\mu \times e^{1/C_0})] \times (C_m - t) + 1 \quad (4)$$

where  $C_0$  is the initial microalgae biomass ( $\text{g L}^{-1}$ );  $C_m$  is the maximum biomass ( $\text{g L}^{-1}$ );  $\mu$  is the specific growth rate ( $\text{day}^{-1}$ ) and  $t$  is the cultivation time (day).

### 2.7 Statistical analysis

All results were expressed in triplicate to calculate the mean  $\pm$  standard error. A value of  $P < 0.05$  was performed by using Minitab 16 software in order to consider the results were statistically significant.

## 3. Results and Discussions

### 3.1 Selection and identification of the most promising strain among the candidate strains

A total number of 4 microalgae strains namely USM\_S1, USM\_S2, USM\_S3 and USM\_S4 were successfully isolated from the soil sample. Further screening on microalgae capability to exhibit high growth rate and carbohydrate productivity under extreme conditions (low pH and high  $\text{CO}_2$  concentration) were carried out and the result was tabulated in Table 1.

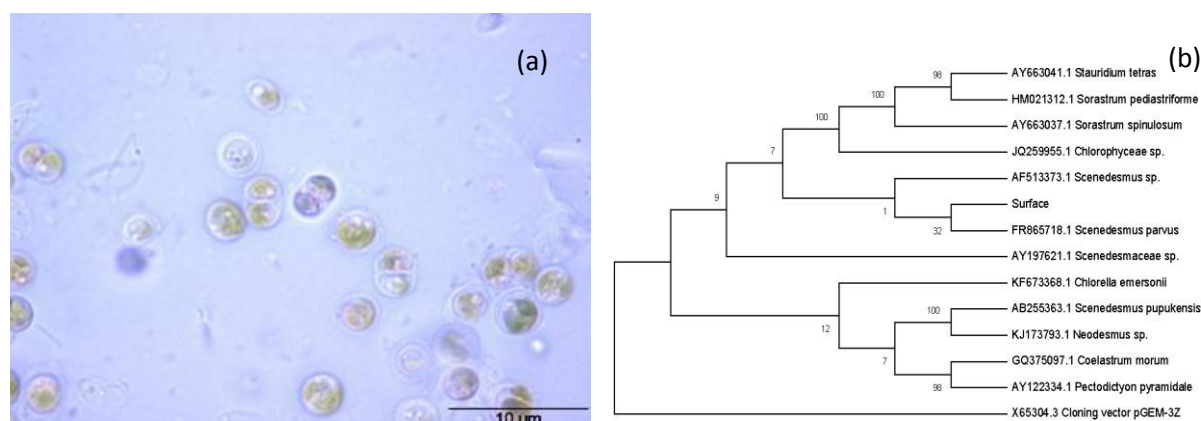
Result showed that the USM\_S1 has a great deal in their ability to produce highest cell dry weight, biomass productivity and specific growth rate which were  $1.2640 \pm 0.001 \text{ g L}^{-1}$ ,  $0.078 \pm 0.004 \text{ g L}^{-1} \text{ d}^{-1}$  and  $0.1691 \pm 0.001 \text{ d}^{-1}$  respectively under extreme condition. This result was obviously showed that strain 1 grew profusely compared to other isolates under favorable condition. Similar trend was observed by Vaquero et al. [12] who shows that *Coccomyxa onubensis* exhibits highest specific growth rate under acidic and high  $\text{CO}_2$  condition. This result was further agreed by the previous study who demonstrated that every microalgae species could exhibit different growth rate under certain environment condition [1]. Interestingly, this USM\_S1 also accumulates a moderately high amount of carbohydrate up to  $37.19 \pm 0.160\%$  with highest in carbohydrate productivity  $15.47 \pm 0.048 \text{ mg L}^{-1} \text{ d}^{-1}$  under the same cultivation condition (Table 1). Hence, it can be concluded that this specific strain of microalgae was a good candidate to be served as carbohydrate-based feedstock for biofuel and chemicals production. The strain that possess the highest growth rate and carbohydrate productivity (USM\_S1) was further subjected for molecular identification and large scale mass cultivation.

**Table 1.** Kinetic growth rate and carbohydrate productivity of microalgae cultivated under acidic condition (pH 3.00 and 15%  $\text{CO}_2$  concentration)

Microalgae	Maximum cell dry weight ( $\text{g L}^{-1}$ )	Biomass Productivity ( $\text{g L}^{-1} \text{ d}^{-1}$ )	Specific growth rate, $\mu$ ( $\text{d}^{-1}$ )	Carbohydrate content (%) (w w <sup>-1</sup> )	Carbohydrate productivity ( $\text{mg L}^{-1} \text{ d}^{-1}$ )
USM_S1	$1.2640 \pm 0.001$	$0.078 \pm 0.004$	$0.1691 \pm 0.001$	$37.19 \pm 0.160$	$15.47 \pm 0.048$
USM_S2	$0.5623 \pm 0.001$	$0.036 \pm 0.001$	$0.1110 \pm 0.001$	$22.17 \pm 1.036$	$7.98 \pm 0.037$
USM_S3	$0.1792 \pm 0.001$	$0.007 \pm 0.001$	$0.0190 \pm 0.001$	$13.12 \pm 0.618$	$0.92 \pm 0.004$
USM_S4	$0.4902 \pm 0.001$	$0.028 \pm 0.001$	$0.1042 \pm 0.001$	$22.91 \pm 0.186$	$6.42 \pm 0.005$

The identification of the selected microalgae strain (USM\_S1) was projected through morphological features and 18S rRNA sequence with the help of universal primer (EUF and EUF). Figure 1 (a) shows the morphology features of USM\_S1 under 100x oil immersion microscopic examination. Based on the figure, it shows that USM\_S1 was unicellular microorganism and appeared in form colonies. The structure of the cells was ovoid cylindrical with the dimension of 3  $\mu\text{m}$  broad and 4  $\mu\text{m}$  long. This preliminary result showed that USM\_S1 was closely related to *S. parvus* as described by Reddy and Chaturvedi [13].

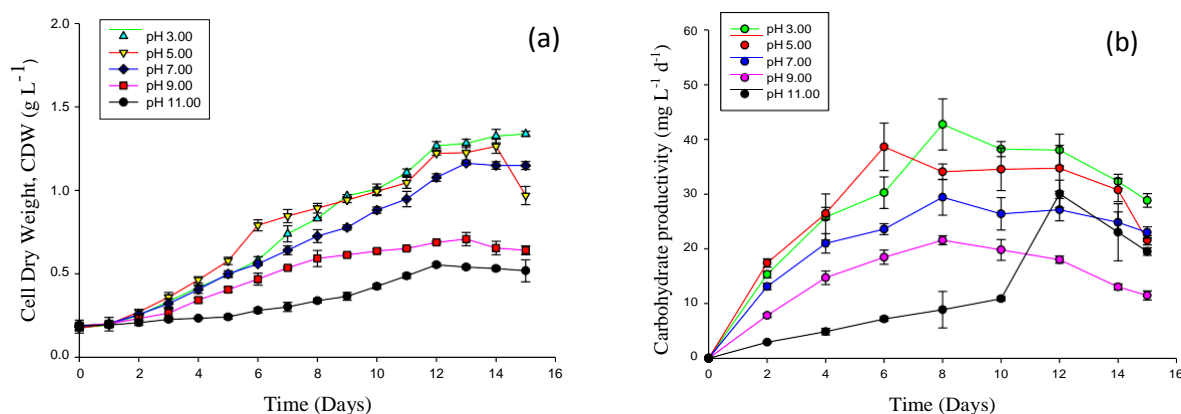
Further identification work was carried out through the 18S rRNA sequence analysis. The NCBI BLAST search was performed using the sequence as a query showed that it is most closely related to FR865718.1 *S. parvus* with 99% similarity. This strain was further confirmed through the multiple sequence alignment and phylogenetic tree analysis. The result appeared was coincided with the previous result, which was *S. parvus* (Figure 1(b)).



**Figure 1.** (a) Morphological identification of *S. parvus* under 100 X magnification oil immersion light microscope. (b) Phylogenetic trees analysis of locally isolated microalgae strain with most similar sequences retrieved from NCBI GenBank database.

### 3.2 Effect of different initial pH on *S. parvus* growth and carbohydrate productivity

The isolated microalgae was further tested in different pH cultivation medium ranging from 3.00 to pH 11.00 in order to study the extent of pH effect on *S. parvus* growth. Based on Figure 2 (a), it shows that *S. parvus* obtained highest biomass production up to  $1.339 \pm 0.001 \text{ g L}^{-1}$  under acidic condition (pH 3.00) compared to alkaline condition (pH 11.00) which showed the lowest cell dry weight  $0.5178 \pm 0.007 \text{ g L}^{-1}$  over 15 days of cultivation time. Different result has been observed by Ren *et al.* [14], who displayed a lower growth rate of *Scenedesmus* sp. strain R-16 when cultivated under acidic condition, pH 3.0. Whereas, Cortés *et al.* [15] shows that wild-type *Dictyosphaerium chlorelloides* exhibited a maximum growth rate under alkaline condition, pH 9.0. This clearly indicated that the capability of microalgae to tolerate with different pH was species-specific. Varying the initial pH of the cultivation medium also could directly affect the metabolic process in microalgae cells [16].



**Figure 2.** (a) Cell dry weight and (b) carbohydrate productivity of *S. parvus* growth over 15 days of cultivation under acidic condition (pH 3.00).

The Figure 2 (b) showed the highest carbohydrate productivity was found  $42.76 \text{ mg L}^{-1} \text{ d}^{-1}$  at day 8 when the cultivation medium was shifted to acidic condition (pH 3.00). This was due to the stress condition applied and causing the changing in metabolism of microalgae and subsequently enhance the carbohydrate productivity [2]. Al-Safaar *et al.* [17] also demonstrates that increased in carbohydrate content of *Chlorella vulgaris* and *Chroococcus minor* when applied under acidic condition. Hence, it

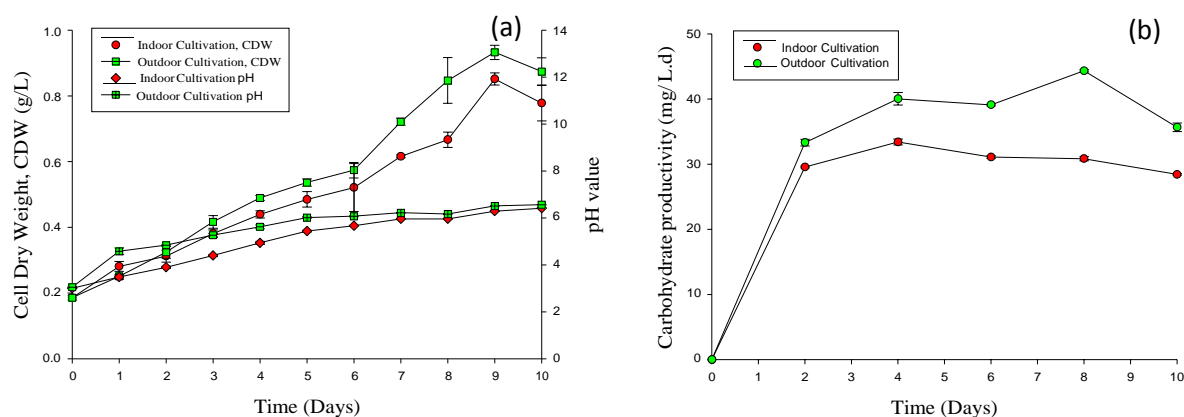
was clearly illustrated that by adjusting the pH cultivation medium could directly affect the microalgal growth rate and carbohydrate productivity.

### 3.3 The biomass production and carbohydrate productivity of *S. parvus* cultivated under indoor and outdoor condition using 15 L PBR

In order to ensure the economic feasibility of the biofuel production, therefore, selection of fast-growing microalgae strain with high carbohydrate accumulation is necessary to develop in pilot-scale cultivation to achieve and maintain the year-round harvesting capability [18]. Hence, this context has involved in identification of the selected strain to achieved in maximum cell growth rate and carbohydrate productivity by applying large scale microalgae cultivation up to 15 L closed system PBR.

*S. parvus* (Strain 1) was selected due to the highest cell density and carbohydrate productivity cultivated under highly acidic condition (Table 1). Further up-scaled cultivation was done of *S. parvus* towards two different cultivation conditions including indoor and outdoor in order to study the kinetic behavior of this selected strain. Figure 3 (a) showed that outdoor cultivation of *S. parvus* displayed a better growth result up to  $0.9326 \pm 0.083 \text{ g L}^{-1}$  compared to indoor cultivation which only exhibited  $0.8514 \pm 0.074 \text{ g L}^{-1}$  at days 9 of cultivation. The difference in cultivation growth result was due to the cultivation conditions which including temperature and light intensity. During the outdoor cultivation, higher temperature ( $33.3 \pm 1.74 \text{ }^{\circ}\text{C}$ ) and light intensity ( $20648.33 \pm 20025.90 \text{ lux}$ ) was achieved by *S. parvus* over the cultivation period (result not shown). These would favor microalgae photosynthesis rate and subsequently resulting in higher growth rate [19].

Different result has been obtained by Ritcharoen et al. [20] who showed that indoor cultivation of *Chaetoceros gracilis* was performed better compared to outdoor cultivation using flat-panel airlift photobioreactor. This phenomenon was due to the uneven light availability received during outdoor cultivation and resulting in low microalgae growth rate. Nonetheless, the feasibility of outdoor cultivation was further been agreed by previous study, Wen et al. [21] who showed the outdoor cultivation using pilot-scale raceway have a nice adaptability for *Graesiella sp.* WBG-1 compared to indoor experiment. Similar result has been observed by previous study who showed the biomass productivity of *Skeletonema costatum* microalgae was also higher up to  $28.0 \pm 0.16 \text{ mg L}^{-1} \text{ d}^{-1}$  compared to indoor cultivation [22]. Although outdoor cultivation of microalgae has encountered strong fluctuation of natural solar irradiance and temperature, however, these specific microalgae still able to perform well under harsh condition. Hence, this showed that *S. parvus* is one of the most tolerance strain that could sustain in wide range of temperature and light intensity, which could be applied for outdoor pilot-scale cultivation.



**Figure 3.** (a) Cell dry weight ( $\text{g L}^{-1}$ ) and (b) Carbohydrate productivity ( $\text{mg L}^{-1} \text{ d}^{-1}$ ) of *S. parvus* in 15 L closed photobioreactor over 10 days of cultivation time under acidic condition (pH 3.00)

Carbohydrate productivity of *S. parvus* under indoor and outdoor cultivation was also evaluated in this study. Based on Figure 3 (b), it showed that highest carbohydrate productivity could be achieved by *S. parvus* up to  $44.35 \pm 0.1693 \text{ mg L}^{-1} \text{ d}^{-1}$  (days 8) under acidic outdoor cultivation. This value was

higher up to 32.74% compared to the indoor cultivation. This was due to the changes in environmental cultivation condition and subsequently affect the carbohydrate accumulation. This result was coinciding with the previous study who figured out the increasing in temperature and irradiance could enhance the carbohydrate productivity up to  $111.8 \text{ mg L}^{-1} \text{ d}^{-1}$ , which was 2.5% higher during sunny environment compared to rainy and cold condition, in Taiwan [23]. This was most probably due to biological processes within microalgae in which can contribute to turnover of enzyme, metabolic activity, growth rate and carbohydrate accumulation with the respect of temperature surrounding [23, 24]. Other study was done by Show *et al.*, [25] who also reported that at low temperature could affects the viscosity of cytoplasm, leading to inefficient of nutrient utilization, or resulting in photo-inhibition. Hence, the photosynthesis mechanism of microalgae would become not effective and subsequently lower the cell growth rate. Therefore, by selecting the robust microalgae was important in this context in order to have capability to tolerate with high temperature range and accumulate the significant amount of carbohydrate.

### 3.4 Kinetic growth model study of microalgae

The growth kinetic study should be applied to achieve a higher understanding of microalgae growth under different cultivation conditions. For this particular study, two kinetic growth models namely Logistic and Gompertz model were used to compare the suitable models that can fit well with the experimental microalgae growth data. Result shows that cultivation under indoor condition attained a higher  $R^2$  value of 0.9678 in Gompertz model, compared to 0.9677 in Logistic-type model (Table 2). This mean Gompertz model fit well with the experiment growth data. This result was further confirmed by the RMSD result shown in Table 2. A similar result was obtained in which Gompertz model shows lower RMSD compared to Logistic model. RMSD is represented a good fit to extent the correctness and the lower value specifies the finest model agreement [26]. Hence, it shows that Gompertz model is acceptable to represent the growth of *S. parvus* under indoor cultivation due to lower RMSE value and vice versa for the outdoor cultivation.

**Table 2.** Performance indicates of the nonlinear models of *S. parvus* growth under different types of cultivation

Types of cultivation	Cell growth model			
	Logistic		Gompertz	
	$R^2$	RMSD	$R^2$	RMSD <sup>a</sup>
Indoor	0.9677	0.0378	0.9678	0.0377
Outdoor	0.9771	0.0390	0.9760	0.0399

<sup>a</sup>RMSD: Root Mean Square Deviation

The suitability of Gompertz growth model was further studied by Praveen et al. [27], who showed that Gompertz model was suitable models (high  $R^2$  and low RMSE values) for describing the growth kinetic of *Chlorella sp.* under photoautotrophic, heterotrophic and photomixotrophic conditions. Other study also indicated that high  $R^2$  value which denoted by the logistic-type model showed that the suitability of this model for microalgae growth under normal condition and nitrogen-depletion condition [28]. Hence, it can be concluded that the suitability of the model was largely contributed by the differences of microalgae strains, growth medium, cultivation conditions and mode of cultivation [29]. By choosing the right model is important for predicting the microalgae growth characteristics and maximum biomass production under specific cultivation condition.

## 4. Conclusions

This present study revealed that the *S. parvus* able to achieve highest biomass prouction and carbohydrate productivity under extreme environment which were  $1.2640 \pm 0.001 \text{ g L}^{-1}$  and  $15.47 \pm 0.048 \text{ mg L}^{-1} \text{ d}^{-1}$  respectively. Then, in order to ensure the economical feasibility of the carbohydrate biosynthesis polymer, the isolated *S. parvus* was further cultivated in large scale production. The results

indicated that isolated acidophilic microalgae were suitable for carbohydrate polymer biosynthesis up to  $44.35 \pm 0.1693 \text{ mg L}^{-1} \text{ d}^{-1}$ . This is important to ensure the economic feasibility of next generation biofuel production. Lastly, this study annotates that the model is condition dependent in which different cultivation condition suitable different kinetic model. This information is useful for predicting the microalgae growth characteristics and can act as an essential platform when the cultivation is scaled-up to commercial application.

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### Acknowledgements

The authors wish to thank School of Industrial Technology, Universiti Sains Malaysia and the Ministry of Higher Education, Malaysia (USM). The project and manuscript preparation were financially supported by the RUI Grant 1001/PTEKIND/8011043 and USM Fellowship RU(1001/CIPS/AUPE001) from Universiti Sains Malaysia