

## Article

# Media Supplementation with Mannitol and Biotin Enhances Squalene Production of *Thraustochytrium* ATCC 26185 through Increased Glucose Uptake and Antioxidative Mechanisms

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**Abstract:** Media supplementation with exogenous chemicals is known to stimulate the accumulation of important lipids produced by microalgae and thraustochytrids. However, the roles of exogenous chemicals in promoting and preserving the terpenoids pool of thraustochytrids have been rarely investigated. Here, we realized the effects of two media supplements—mannitol and biotin—on the biomass and squalene production by a thraustochytrid strain (*Thraustochytrium* sp. ATCC 26185) and elucidated their mechanism of action. A significant change in the biomass was not evident with the exogenous addition of these supplements. However, with mannitol (1 g/L) supplementation, the ATCC 26185 culture achieved the best concentration ( $642 \pm 13.6$  mg/L) and yield ( $72.9 \pm 9.6$  mg/g) of squalene, which were 1.5-fold that of the control culture (non-supplemented). Similarly, with biotin supplementation (0.15 mg/L), the culture showed  $459 \pm 2.9$  g/L and  $55.7 \pm 3.2$  mg/g of squalene concentration and yield, respectively. The glucose uptake rate at 24 h of fermentation increased markedly with mannitol ( $0.31$  g/Lh<sup>-1</sup>) or biotin ( $0.26$  g/Lh<sup>-1</sup>) supplemented culture compared with non-supplemented culture ( $0.09$  g/Lh<sup>-1</sup>). In addition, the reactive oxygen species (ROS) level of culture supplemented with mannitol remained alleviated during the entire period of fermentation while it alleviated after 24 h with biotin supplementation. The  $\Delta$ ROS with mannitol was better compared with biotin supplementation. The total antioxidant capacity (T-AOC) of the supplemented culture was more than 50% during the late stage (72–96 h) of fermentation. Our study provides the potential of mannitol and biotin to enhance squalene yield and the first lines of experimental evidence for their protective role against oxidative stress during the culture of thraustochytrids.

**Keywords:** thraustochytrids; squalene; supplementation; mannitol; biotin; oxidative stress



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## 1. Introduction

Squalene, a natural triterpene (2,6,10,15,19,23-hexamethyltetracos-2,6,10,14,18,22-hexane; C<sub>30</sub>H<sub>50</sub>), is a crucial precursor for the biosynthesis of cholesterol, bile acids, and hormones in plants and animals [1,2]. Due to its anticancer, antioxidant, skin hydrating, and immune-stimulating properties, squalene has increasing applications in nutraceutical, pharmaceutical, and cosmetic industries [3,4], with a tremendous market value that is projected to reach USD 184 million by 2025 [5]. As of now, the shark liver is the primary source of squalene, but this has led to increased poaching and massive damage to the environment [6]. Likewise, the drawbacks of plant-based sources include long harvest time, low-production yield, and feed-food competition [7]. Therefore, sustainable production of squalene from renewable sources has been the biggest challenge for the industrial applications of squalene [4]. Interestingly, several thraustochytrid strains have been shown to have

great potential for the high-yield production of squalene because of their advantages of fast growth rate and controllable fermentation [8–11]. Thraustochytrids are a group of unicellular fungus-like marine microorganisms within the kingdom Stramenopila/Heterokonta [12]. However, low production yield remains one of the most important obstacles that limit the large-scale production of squalene using thraustochytrids [13].

To achieve high-yield production of natural products via microbial fermentation, stress-inducing strategies (e.g., nutrition starvation, salinity, temperature) have been commonly adopted [14]. Under such stress conditions, high levels of reactive oxygen species (ROS) are inevitably accumulated in the cells which usually lead to the decreased production of microbial natural products [15]. Furthermore, most microbial strains tend to accumulate free radicals that specifically attack the cell membrane, which is the prime site for squalene storage [16]. The cell membrane damage can decrease the squalene production yield, most likely through intervening regular cellular functions and overuse of cellular energy. Therefore, the reduction of ROS has been one of the most daunting challenges in the microbial production of natural products [17]. Besides, squalene produced by the cell is metabolized and used up as an important intermediate of sterol biosynthesis [2].

To date, a handful of chemicals have been tested on thraustochytrids/microalgae for their antioxidative properties and potential to improve lipid yield by supplying them exogenously. Some of those chemicals include butylated hydroxyanisole [18,19], sesame oil [20,21], ascorbic acid [21,22], flaxseed oil [23], melatonin [19,21], mannitol [21], and butylhydroxytoluene [19,21]. Studies on media supplementation have proven that certain chemicals enhance lipid yield by minimizing intracellular lipid peroxidation [15]. To our knowledge, the influence of exogenous chemicals on the yield of squalene produced by thraustochytrids has been sporadically investigated [8,24–26]. More importantly, the mechanism of action of exogenous chemicals on squalene accumulation remains poorly elucidated.

In this study, we tested two exogenously added chemicals—mannitol and biotin—for their potential role as media supplements in promoting biomass and squalene production by *Thraustochytrium* ATCC 26185. Both mannitol and biotin are low-cost chemicals. Moreover, biotin is a powerful antioxidant and has been shown to improve the cell growth and increase the production of secondary metabolites [27,28]. Mannitol also provides antioxidant and hydrating properties [21,29], and is known to influence fermentation as a fermentable sugar and source of energy for many microbes [30]. Our study provides the first lines of experimental evidence for the possible mechanisms underlying the effects of mannitol and biotin supplementation on squalene yield. The major goal was to neutralize the intracellular ROS and conserve the squalene pool of the ATCC 26185 strain.

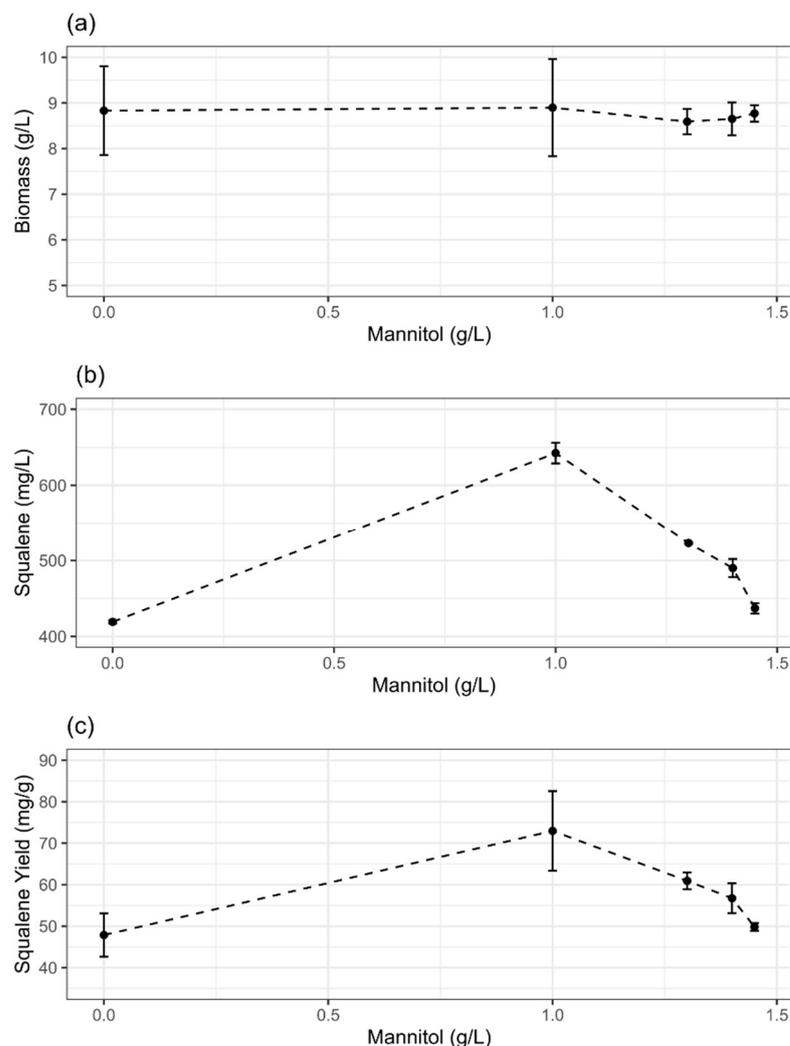
## 2. Results and Discussion

### 2.1. Effects of Mannitol and Biotin on Biomass and Squalene Production

The potential of mannitol and biotin supplementation to enhance biomass and squalene production was realized by testing various concentrations of these supplements in batch culture of ATCC 26185 strain. With the increasing concentration of mannitol (Figure 1a) or biotin (Figure 2a), biomass production did not change significantly ( $p > 0.05$ ). The range of biomass was 8.6–8.9 g/L and 7.7–8.4 g/L with mannitol and biotin supplementation, respectively. Similar results were observed when methyl jasmonate was added to the culture of *Schizochytrium mangrovei* in the concentration range of 0 mM to 0.4 mM [24]. While certain supplements (e.g., terbinafine) have been reported to result in the slight inhibition of cell growth of *Schizochytrium* strains [8,25], some other supplements such as ascorbic acid [22] and sesame oil [20] were found to improve microalgal biomass production. Overall, these findings suggest that the response, in terms of cell growth, of thraustochytrid strains to different media supplements can be inconsistent.

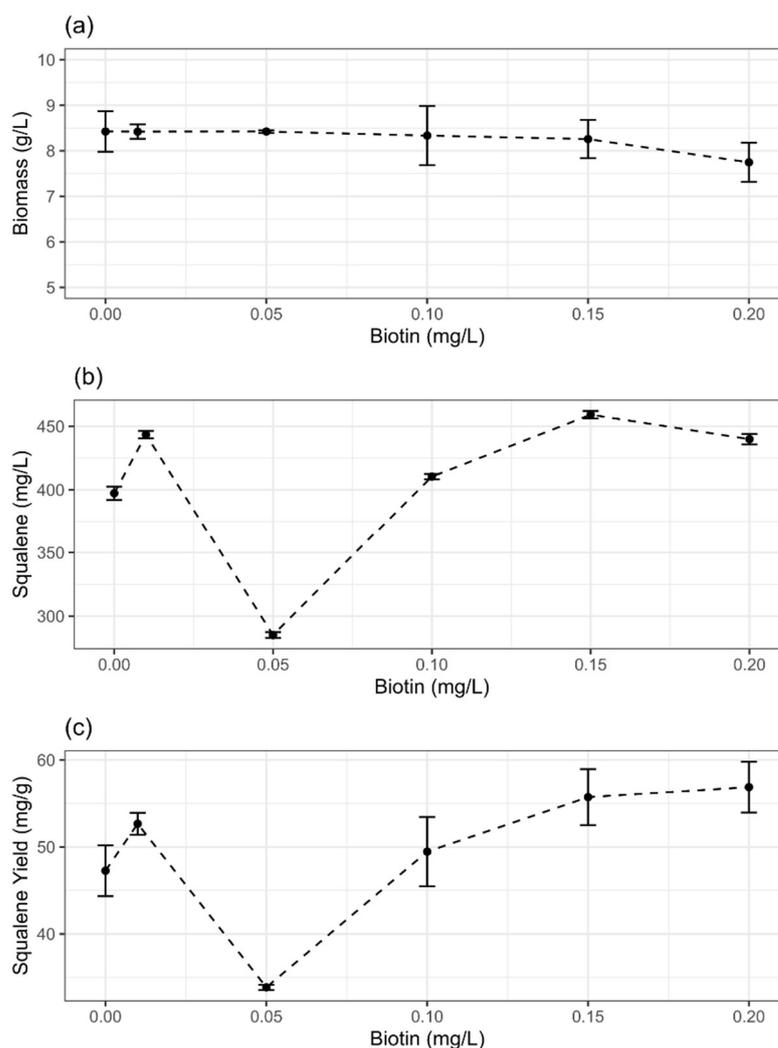
The squalene concentrations (Figures 1b and 2b) and yields (Figures 1c and 2c) varied notably along the concentration gradients of mannitol and biotin. Particularly, the responses were dose-dependent in the case of mannitol supplementation. The addition of 1 g/L mannitol or 0.15 mg/L biotin to the initial ( $t = 0$ ) culture provided the highest concentration

and yield of squalene. The concentration and yield of squalene with 1 g/L mannitol supplementation were  $642 \pm 13.6$  mg/L and  $72.9 \pm 9.6$  mg/g, respectively. Similarly, with 0.15 mg/L biotin supplementation, these were  $459 \pm 2.9$  g/L and  $55.7 \pm 3.2$  mg/g, respectively. The supplementation with mannitol provided better improvement (1.5-fold) in squalene production compared with biotin (1.2-fold).



**Figure 1.** Effects of mannitol supplementation on the (a) biomass, (b) squalene concentration, and (c) squalene yield of ATCC 26185 strain. The data are provided for 72 h grown culture and expressed as mean  $\pm$  SD of triplicate experiments.

Previous studies have shown that supplements such as methyl jasmonate, butanol, and terbinafine can significantly improve the squalene content of *Schizochytrium* strains. For example, the addition of methyl jasmonate (0.1 mM) to the culture of *S. mangrovei* was able to provide a 60% higher squalene yield (1.17 mg/g DCW) than that of the control [24]. Similarly, in the presence of butanol (6 g/L), a 31-fold change (from 0.65 mg/g to 20.09 mg/g) in the squalene content was reported in *S. limacinum* B4D1 [26]. With the addition of terbinafine (100  $\mu$ g/mL) to *S. mangrovei* PQ6 culture, an increase of 56.4% in squalene content (96.7 mg/g) compared to the control (61.8 mg/g) was reported [25]. Similarly, at terbinafine concentrations of 100  $\mu$ g/mL, an increase of 40% in squalene content of *Aurantiochytrium mangrovei* FB3 compared to the control has been reported [8]. Interestingly, our study for the first time showed that a low amount of mannitol (1 g/L) or biotin (0.15 mg/L) can considerably improve the squalene content without affecting the cell growth of ATCC 26186 strain.

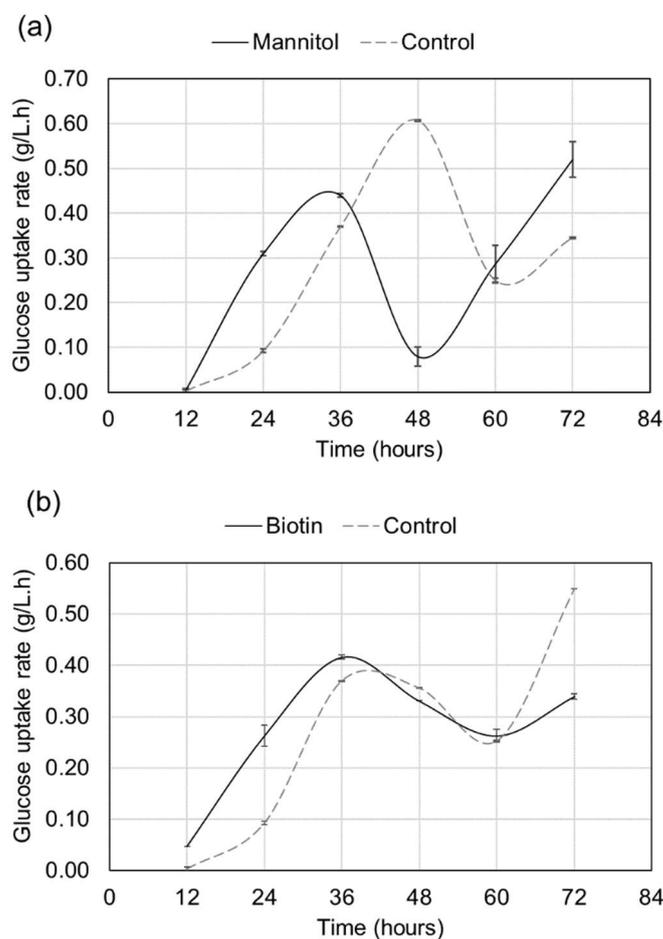


**Figure 2.** Effects of biotin supplementation on the (a) biomass, (b) squalene concentration, and (c) squalene yield of ATCC 26185 strain. The data are provided for 72 h grown culture and expressed as mean  $\pm$  SD of triplicate experiments. DMSO was added to the medium without biotin (0 g/L) as a negative control.

### 2.2. Mannitol/Biotin Increases Glucose Uptake Rate

Glucose uptake rate is considered the first rate-limiting step that drives cellular metabolism, and it depends on the extracellular glucose availability and intracellular metabolic potential [31]. To understand the effect of optimal biotin/mannitol supplementation on the glucose uptake rate, the residual glucose concentration was monitored at regular time intervals during the batch fermentation. The glucose uptake rate fluctuated markedly with fermentation time in both control and supplemented groups, and it peaked twice at different timepoints (Figure 3a,b). The first peak was observed between 36 and 48 h and the second was apparent at 72 h. With mannitol supplementation, the first peak appeared 12 h earlier than that of the control. In addition, the uptake rate at 24 h of fermentation was much greater with mannitol ( $0.31 \text{ g/Lh}^{-1}$ ) compared with that without mannitol ( $0.09 \text{ g/Lh}^{-1}$ ). Conversely, the first peak with and without biotin supplementation appeared at the same time point (36 h). Interestingly, consistent with the mannitol supplementation results, the uptake rate at 24 h of fermentation was also much higher with biotin ( $0.26 \text{ g/Lh}^{-1}$ ). These results are in agreement with a previous study that reported a significant difference in glucose uptake between non-supplemented and ascorbic acid supplemented cultures after 24 h of fermentation [22]. Taking the findings together, mannitol/biotin supplementation

might play a role in increasing the glucose uptake rate of ATCC 26185 culture during the initial phase of fermentation.



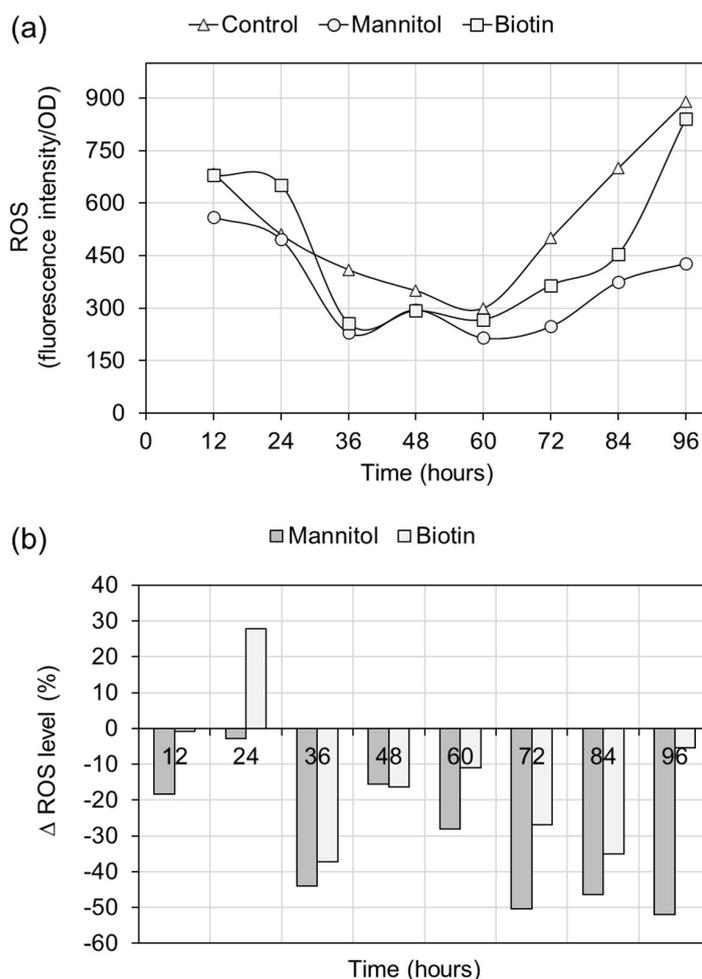
**Figure 3.** Time course of glucose consumption by ATCC 26185 culture upon (a) mannitol (1 g/L) and (b) biotin (0.15 mg/L) supplementation at 0 h of fermentation. The data are expressed as the mean  $\pm$  SD of duplicate experiments.

Previous studies on a thraustochytrid strain cultured on medium supplemented with flaxseed oil have shown that increased glucose uptake corresponds with higher biomass yields [23]. However, in our study, despite increased glucose uptake rate at 24 h of fermentation, we did not observe a significant increase in biomass yields upon mannitol/biotin supplementation (Figures 1a and 2a); instead, an increased squalene yield was evident (Figures 1c and 2c). Overall, our study suggests that an increased glucose uptake rate induced by mannitol/biotin supplementation might stimulate the metabolic flux towards the squalene biosynthetic pathway resulting in an improved squalene yield. Future research on metabolic flux distribution could provide an understanding of how glucose uptake rate regulates squalene production in thraustochytrid strains.

### 2.3. Antioxidative Properties of Mannitol and Biotin

To ascertain whether mannitol and biotin exhibit antioxidative properties, we compared the cellular levels of ROS and total antioxidant capacity (T-AOC) between control and supplemented cultures of the ATCC 26185 strain. The time courses of specific fluorescence intensity of non-supplemented and supplemented groups somewhat indicated lower ROS levels in the latter groups (Figure 4a). To further understand the extent of the alleviation in ROS levels upon mannitol/biotin supplementation, the differences in the ROS levels between the control and supplemented cultures were evaluated (Figure 4b). The results

revealed that the ROS level remained alleviated during the entire period of fermentation when the culture was supplemented with mannitol (1 g/L). Particularly, the ROS alleviation ( $\Delta$ ROS) was high (46.4–51.9%) during the late stage (72–96 h) of fermentation. Contrastingly, the  $\Delta$ ROS of culture supplemented with biotin started only after 24 h of fermentation and was lower than that with mannitol. Overall, our findings suggest that mannitol might provide better protection to the ATCC 26185 cells against oxidative damage caused by the ROS generated during the process of fermentation.

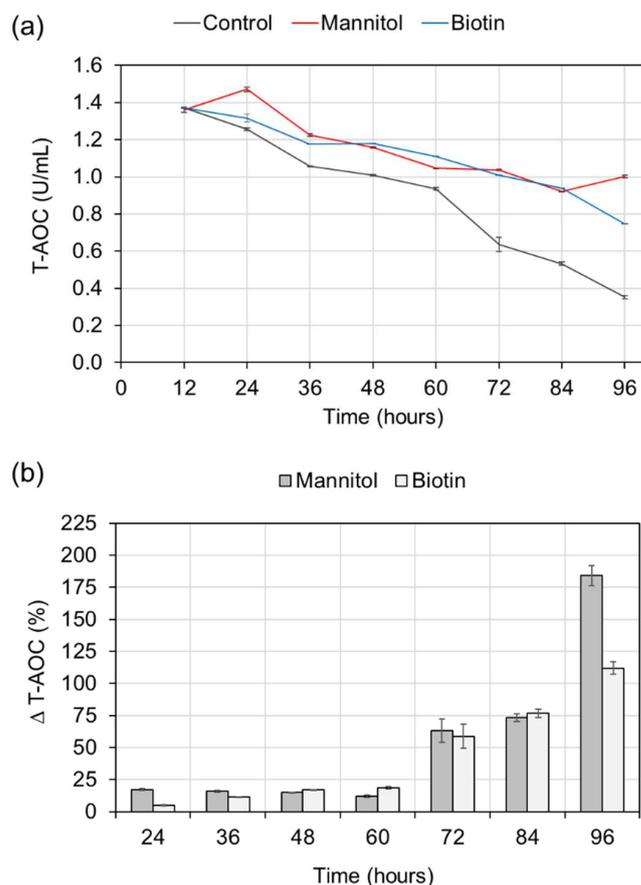


**Figure 4.** (a) Time course profiles of cellular ROS levels in control and supplemented ATCC 26185 cultures, and (b) Percentage  $\Delta$ ROS level of supplemented cultures. The culture was supplemented with 1 g/L mannitol or 0.15 mg/L biotin at 0 h of fermentation.

The T-AOC levels of control and supplemented cultures were found to decline with fermentation time. Interestingly, the supplementation with mannitol or biotin aided the slowdown of the declining trend. Furthermore, the time-course profiles of T-AOC revealed moderately increased levels in the supplemented cultures compared with the control culture (Figure 5a). Particularly, the T-AOC elevation ( $\Delta$ T-AOC) in the supplemented cultures was more than 50% during the late stage (72–96 h) of fermentation. In addition, there was a markedly higher elevation of T-AOC at 96 h with mannitol ( $\Delta$ T-AOC = 184%) than with biotin ( $\Delta$ T-AOC = 112%) supplementation.

Fundamentally, two different types of antioxidative processes are known to function in response to the cellular oxidative stress for protecting against oxidative damage to the cell [32,33]. These include the direct antioxidant scavenging activity, where antioxidants absorb ROS directly and promptly, and the expression of genes for antioxidant enzymes such as superoxide dismutase (SOD). The latter is a relatively indirect way to reduce

cellular oxidative stress. Previous studies have shown that mannitol treatment significantly enhances the SOD activity by activation of already synthesized enzyme isoforms such as Mn-SOD3, Cu/Zn-SOD3, and Cu/ZnSOD4 and by inducing the synthesis of new isoforms such as Mn-SOD2 and Cu/Zn-SOD1 [34]. Our results provide evidence for the contribution of mannitol and biotin in the enhancement of both the antioxidative processes (i.e., ROS alleviation and T-AOC elevation) within the cell.



**Figure 5.** (a) Time course profiles of T-AOC in control and supplemented ATCC 26185 cultures, and (b) Percentage  $\Delta$ T-AOC level of supplemented cultures. The culture was supplemented with 1 g/L mannitol or 0.15 mg/L biotin at 0 h of fermentation. The data are expressed as mean  $\pm$  SD of triplicate measurements.

### 3. Materials and Methods

#### 3.1. Strain and Culture Conditions

*Thraustochytrium* sp. ATCC 26185 was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The strain was maintained on agar plates containing SQU medium: 30 g glucose, 2 g yeast extract, 2 g monosodium glutamate (MSG), 0.2 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.3 g  $\text{KH}_2\text{PO}_4$ , 25 g NaCl, 1 g KCl, 5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g  $\text{NaHCO}_3$ , 0.3 g  $\text{CaCl}_2$ , 2.9 mg  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.02 mg  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.26 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.6 mg  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 8.6 mg  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , and 20 g agar in one liter of distilled water [24]. The inoculated agar plate was kept at 28 °C and sub-cultured every four weeks. The initial seed culture was prepared by transferring a single colony from the agar plate into an Erlenmeyer flask containing fresh SQU medium and then incubating the flask for 48 h at 28 °C on an orbital shaker set at 170 rpm.

#### 3.2. Batch Experiments

Various concentrations of mannitol (g/L: 0, 1.0, 1.3, 1.4, and 1.45) and biotin (mg/L: 0.01, 0.05, 0.1, 0.15, and 0.2) were tested for their effects on the biomass and squalene

production under batch conditions. The concentration ranges of mannitol and biotin were based on previous studies [29,35,36]. The stock solutions of mannitol (5 g/100 mL) and biotin (100 mg/L) were prepared in water and DMSO, respectively, and stored in the dark at  $-20\text{ }^{\circ}\text{C}$ . The seed culture was transferred to 100 mL shake flask containing 50 mL of SQUA medium: 25 g/L glucose, 5 g/L NaCl, 5 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/L MSG, 2.5 g/L yeast extract, 1 g/L KCl, 0.3 g/L  $\text{CaCl}_2$ , 0.3 g/L  $\text{KH}_2\text{PO}_4$ , 0.1 g/L  $\text{NaHCO}_3$ , and micronutrients (see Section 3.1). An appropriate volume of mannitol/biotin was added to the culture medium to achieve its desired final concentration. The addition of mannitol/biotin at 0 h of fermentation provided the optimum surge (data not shown). All experiments were carried out at  $28\text{ }^{\circ}\text{C}$  in an orbital shaker set at 170 rpm.

### 3.3. Quantification of Biomass, Squalene, and Residual Glucose

The dry cell weight (DCW) and squalene content were measured following the procedures described in our previous study [13]. To determine the concentration of residual glucose, one ml of culture broth was collected every 12 h until 72 h and centrifuged for 10 min at 10,000 rpm and  $4\text{ }^{\circ}\text{C}$ . The resulting supernatant was diluted ( $10\times$ ) with distilled water, and its glucose content was measured using the Glu KIT (Biosino Bio-Technology and Science Inc., Beijing, China) as per the manufacturer's instructions. The color intensity of the reaction mixture was measured at 505 nm in a spectrophotometer (Multiskan GO, Thermo Scientific, Waltham, MA, USA) and the concentration (g/L) was determined from the glucose standard curve. The glucose uptake rate was calculated by dividing the difference of residual glucose concentrations with the time interval between two consecutive time points.

### 3.4. Determination of Intracellular ROS and T-AOC Levels

The intracellular ROS and T-AOC levels were measured following the procedures described in our previous study [17]. The  $\Delta\text{ROS}$  level and  $\Delta\text{T-AOC}$  were calculated as a percentage of the difference between the control and supplemented groups.

### 3.5. Statistical Analysis

The group means of biomass and squalene concentration and yield were statistically analyzed at the alpha level of 0.05 using ANOVA. All statistical tests and visualization were done in R version 4.0.4 [37].

## 4. Conclusions

This study provides a practical strategy in which exogenous addition of mannitol or biotin to culture medium enhances the squalene content of *Thraustochytrium* ATCC 26185. Although these supplements did not notably improve the biomass yield, they could considerably enhance the squalene yield. The uptake rate of the medium carbon source (glucose) for the supplemented culture was significantly higher in the initial stage of fermentation. Furthermore, these supplements exhibited potential antioxidative properties; particularly, mannitol showed better ROS quenching ability than biotin. These findings suggest that possibly through the mechanism of increased glucose uptake and added protection against oxidative stress, mannitol and biotin might contribute to the enhanced squalene content of the ATCC 26185 strain.

**Author Contributions:** Conceptualization, M.K.A., G.W. and B.S.; methodology, M.K.A. and Y.H.; validation, M.K.A. and B.S.; formal analysis, M.K.A. and B.S.; investigation, M.K.A.; resources, Y.H. and M.B.; data curation, B.S. and M.K.A.; writing—original draft preparation, B.S., M.K.A. and G.W.; writing—review and editing, B.S. and G.W.; visualization, B.S.; supervision, G.W. and B.S.; project administration, Y.H. and M.B.; funding acquisition, G.W. All authors have read and agreed to the published version of the manuscript.

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**Sample Availability:** Not available.

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