

Screening of Medium Constituents for the Cultivation of *Scenedesmus dimorphus* UTEX 1237 using 2^k Factorial Design Approach

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Abstract. Freshwater microalga, *Scenedesmus dimorphus* is a beneficial natural resource for producing biofuel and novel bioproducts. *S. dimorphus* growth greatly depends on physical culture conditions as well as medium constituents. A study was conducted to select the suitable medium components that could promote high biomass production in *S. dimorphus* UTEX 1237. Screening of growth medium constituents was performed using the two-level factorial design (2^k FD) method. *S. dimorphus* was cultivated in TAPS aquaculture medium employing two types of carbon source (sodium bicarbonate and acetic acid) and three types of nitrogen sources (ammonium chloride, sodium nitrate, and urea). Consequently, medium components that significantly influenced biomass production comprised of acetic acid, ammonium chloride, and urea. Two weeks of *S. dimorphus* cultivation in shake flask system consisting of 1.5 g/L of acetic acid, 10 g/L of ammonium chloride and 0.4 g/L of urea was found to affect a specific growth rate of 0.0049 h⁻¹ and biomass productivity of 0.05 g/L/day, thus affecting 66.7% improvement over normal TAP formulation.

1. Introduction

The immense diversity of microalgae has traditionally drawn the attention of scientific communities into manipulating them for human consumptions. Given the increasing demand leading to the continuous spike in petroleum pricing and the alarming impact of global warming due to the burning of fossil fuels, numerous reports were published extolling the advantages of shifting to microalgal biofuel on the basis of low energy requirement for production, non-toxicity, biodegradability and low carbon dioxide emissions into the atmosphere [1]. Microalgae biomass is composed of major macromolecules such as lipids, carbohydrates, and proteins which can be selectively converted into liquid biodiesel, bioethanol, biomethane, biohydrogen, and bio-oil by subjecting them to different biological conditions [2]. Microalgae proved a promissory source of biofuel since it results in higher yields with respect to other lignocellulosic biomass, being capable of producing 100 times more



energy per hectare compared to the well-known terrestrial crops, i.e., corn, soybean, rapeseed, oil palm and jatropha [3].

Some of the biotechnologically relevant microalgae species are *Chlorella*, *Spirulina*, *Dunaliella*, *Haematococcus*, *Isochrysis*, and *Scenedesmus* [4]. *Scenedesmus* spp. are appropriate to be used for laboratory-scale studies and mass intensive cultivation since they have a high tolerance to differences in light intensity, pH, temperature, and high concentration of carbon dioxide [5]. The utilization of *Scenedesmus* biomass has advances beyond the field of biofuel as they are now being incorporated into plastic formulation or used as cell factory to produce PLA or PHA bioplastics [6], biogenic synthesis of silver nanoparticles [7], source of biocement or bioconcrete [8], and natural bioactive compounds (NBC) for treatment and prevention of human diseases [9].

Large scale microalgae cultivation for biofuel or any of the specialized chemicals might not be economically viable as much of the total costs are concentrated in the production of microalgae biomass (50%) whereas another 20%-30% is allocated to the biomass recovery process (harvesting and drying) [10]. Low biomass concentration will imply greater labor and energy requirement for dewatering effort. The ability of microalgae to increase their biomass density greatly depends on the cultivation conditions and growth medium. Natural medium such as soil medium is quite cheap to produce, but it does not necessarily contain all the nutrients required for the growth of microalgae. Thus, an artificial formulation is introduced whereby the nutrient composition and concentration are manipulated according to the species of interest. Improvement of medium composition can be achieved through proper screening and optimization exercise.

Based on the number of factors involved, sometimes the total experimental runs to be performed can be quite daunting if the traditional varying one-factor-at-a-time (OFAT) method is practiced. Therefore, statistical-based experimental design such as the two-level factorial design (2^k FD) or Plackett-Burman is usually employed to simplify the process. These techniques are efficient for the screening of significant factors from multiple dependent variables and to determine the existence of potential interactions between them, a boon which traditional OFAT experiments were unable to demonstrate precisely [11]. In this study, the formulation of enhanced TAP growth medium specifically for freshwater microalga, *Scenedesmus dimorphus* UTEX 1237 was focused on different types and concentrations of carbon and nitrogen sources. Screening of significant medium constituents affecting specific growth rate and final *S. dimorphus* biomass concentration is reported using two-level factorial design.

2. Materials and Methods

2.1. Microalga Species

Scenedesmus dimorphus UTEX 1237 was originally sourced from the UTEX Culture Collection, University of Texas, United States. The stock culture was maintained and sub-cultured monthly in TAP medium slant agar and kept at the Bioprocessing and Biomanufacturing Research Centre (BBRC), Universiti Putra Malaysia under 4°C and exposed to light and dark photoperiod of 16h: 8h.

2.2. Inoculum and Seed Culture Preparation

For uses as inoculum, a loop-full of microalga colony from slant agar was transferred into 50 mL Falcon tube containing 10 mL of sterilized TAP medium. The inoculum was incubated at 25 °C in an orbital incubator shaker (Model IS-971 Lab Companion), agitated at 120 rpm for 14 days. Illumination was provided by T5 fluorescent lamps with light and dark photoperiod of 16h: 8h as well. For subsequent seed culture preparation, 10 mL of microalgae culture was then transferred into 250 mL Erlenmeyer flasks containing 90 mL of TAP medium and incubated for another 14 days.

2.3. Medium for Microalga Growth

Tris-Acetate-Phosphate (TAP) medium [12] was used as a basal medium to cultivate *S. dimorphus*. Two types of carbon sources (acetic acid and sodium bicarbonate) and three types of nitrogen sources

(sodium nitrate, urea, and ammonium chloride) were tested. All microbiological-grade carbon and nitrogen sources used were stable when sterilized at 121°C, 15 psi for 15 min. Some of the components were autoclaved separately to prevent any Maillard reaction. All cultivations were performed in 100 mL medium with 10% (v/v) inoculum from seed culture prepared earlier. The cultures were grown in an orbital incubator with all conditions maintained and incubation period set for 21 days.

2.4. Experimental Design

Carbon and nitrogen sources in the original TAP medium comprised of acetic acid and ammonium chloride. This study assessed the effectiveness of other alternatives to medium formulation. Two-level factorial design (2^k FD) was used to screen for the most significant factors influencing the *S. dimorphus* biomass yield. As illustrated in Table 1, five variables factors were chosen, which were sodium bicarbonate (X_1), acetic acid (X_2), ammonium chloride (X_3), sodium nitrate (X_4) and urea (X_5), with each factor having two levels of concentration: -1 (low level) and +1 (high level). Each component's concentration at these two levels was taken from the most minimum and maximum values utilized by other investigators in literature for *Scenedesmus* species. The main response variable was biomass concentration (g/L). Design-Expert (Version 7.0.0, Stat-Ease Inc, Minneapolis, USA) software was used for designing the experiment as well as analysis of results. Variables which imparted a major effect on elevating *S. dimorphus* biomass concentration were identified based on confidence level above 95% ($P < 0.05$). The significant factors and possible interactions identified from half-normal plot analysis were chosen for generation of the first-order model for the response as per Equation (1) following evaluation of effects and interaction by analysis of variance (ANOVA):

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_5X_5 \quad (1)$$

Where Y is response; B_0 is modal constant, and B_i is the linear coefficient. The variable, X_i is the non-coded independent variables. The coefficient of determination, R^2 , and adjusted R^2 was used for evaluation of the fitness of the model.

Table 1. Experimental range and levels of independent variables

Variables (g/L)	Symbol	Actual levels of coded factors	
		-1	+1
Sodium bicarbonate	<i>A</i>	0.40	10.0
Acetic acid	<i>B</i>	0.50	1.50
Ammonium chloride	<i>C</i>	10.0	16.0
Sodium nitrate	<i>D</i>	0.20	5.00
Urea	<i>E</i>	0.40	9.00

2.5. Microalga Growth Analysis

Biomass measurement of *S. dimorphus* UTEX 1237 was obtained through the gravimetric method. 2 mL of culture samples taken at every two-day interval were transferred into pre-weight clean centrifuge tubes and centrifuged at 10 000 rpm for 10 minutes (Eppendorf 5415 D mini centrifuge, Germany). Upon supernatant removal, individual cell pellet together with centrifuge tube would be allowed to dry at 95°C in the oven for several days and weighed until constant dried cell weight was achieved. The biomass measurement was calculated in terms of g/L.

2.6. Growth Kinetics Calculation

The time-specific measurements of *S. dimorphus* UTEX 1237 dried cell weight were fitted to the Baranyi-Roberts microbial propagation model [13]. The dynamic model calculation of growth kinetic parameters was made possible via online curve-fitting DMFit software (www.combase.cc), in which

the lag phase duration (λ , h) and maximum specific growth rate during exponential phase (μ , h^{-1}) were estimated.

3. Results and Discussion

3.1. Screening of Medium Constituents Affecting *S. dimorphus* Biomass Concentration

Screening is a crucial step in eliminating components that do not influence the response. Thus, this procedure ensures that the medium composition provided is utmostly favorable for the growth of microalgae and to accumulate the product of interest in desired amounts. In this study, two-level factorial (2^k FD) is preferred over Plackett-Burman (PB) design as 2^k FD is a more comprehensive tool in investigating each complete trial or replication of the experiment with all possible combination of factors' levels and the interaction between each factor. Screening by PB design is more economical to identify a few main factors affecting the outcome, only when the interaction between factors are not significant. Hence, if there are significant interactions, PB design could provide misleading results.

Besides acetic acid and ammonium chloride, three more commonly employed medium components for aquaculture purposes were identified from previous research in the literature. The five-variable experimental design using 2^k FD had created a total of 32 cultivation runs ($N = 2^5$), as shown in Table 2.

Table 2. Two-level factorial design showing actual levels and responses

Std order run	A	B	C	D	E	Dried Cell Weight (g/L)	
						Actual	Predicted
1	0.40	0.5	10.0	0.2	0.4	0.554	0.57
2	10.0	0.5	10.0	0.2	0.4	0.585	0.57
3	0.40	1.5	10.0	0.2	0.4	0.632	0.66
4	10.0	1.5	10.0	0.2	0.4	0.706	0.68
5	0.40	0.5	16.0	0.2	0.4	0.319	0.38
6	10.0	0.5	16.0	0.2	0.4	0.342	0.38
7	0.40	1.5	16.0	0.2	0.4	0.530	0.46
8	10.0	1.5	16.0	0.2	0.4	0.522	0.46
9	0.40	0.5	10.0	5.0	0.4	0.617	0.57
10	10.0	0.5	10.0	5.0	0.4	0.595	0.57
11	0.40	1.5	10.0	5.0	0.4	0.595	0.66
12	10.0	1.5	10.0	5.0	0.4	0.638	0.66
13	0.40	0.5	16.0	5.0	0.4	0.339	0.38
14	10.0	0.5	16.0	5.0	0.4	0.350	0.38
15	0.40	1.5	16.0	5.0	0.4	0.520	0.46
16	10.0	1.5	16.0	5.0	9.0	0.459	0.46
17	0.40	0.5	10.0	0.2	9.0	0.402	0.34
18	10.0	0.5	10.0	0.2	9.0	0.373	0.34
19	0.40	1.5	10.0	0.2	9.0	0.499	0.42
20	10.0	1.5	10.0	0.2	9.0	0.355	0.42
21	0.40	0.5	16.0	0.2	9.0	0.309	0.29
22	10.0	0.5	16.0	0.2	9.0	0.328	0.29
23	0.40	1.5	16.0	0.2	9.0	0.359	0.37
24	10.0	1.5	16.0	0.2	9.0	0.292	0.37
25	0.40	0.5	10.0	5.0	9.0	0.398	0.34
26	10.0	0.5	10.0	5.0	9.0	0.295	0.34
27	0.40	1.5	10.0	5.0	9.0	0.399	0.42
28	10.0	1.5	10.0	5.0	9.0	0.312	0.42

29	0.40	0.5	16.0	5.0	9.0	0.279	0.29
30	10.0	0.5	16.0	5.0	9.0	0.239	0.29
31	0.40	1.5	16.0	5.0	9.0	0.502	0.38
32	10.0	1.5	16.0	5.0	9.0	0.310	0.37

Choosing which of the five variables deemed significant to *S. dimorphus* growth requires observation of the half-normal plot and ANOVA table. Upon fitting to a multiple regression model as in Equation (1), p-value generated by ANOVA table will determine whether the model, as a whole, is significant. A backward elimination procedure was carried out during variable selection to reduce the number of unnecessary model terms (usually by removing variables or interaction terms one at a time, beginning with the term having the highest p-value). Continuous subtraction of these terms would consequently update the overall model's p-value. Elimination was stopped when only terms with p-values below the acceptable threshold of 0.05 were left in the model, resulting in a reduced model as in Table 3.

Table 3. ANOVA table of the reduced factorial model following backward elimination

Source	Sum of Squares	Degrees of freedom	Mean Square	F-value	P > F
Model	0.46	6	0.077	30.44	<0.0001
B- Acetic acid	0.053	1	0.053	21.01	0.0001
C- Ammonium chloride	0.12	1	0.12	47.23	<0.0001
E-Urea	0.22	1	0.22	86.64	<0.0001
CE	0.040	1	0.040	15.62	0.0023
Residual	0.094	27	3.493E-003		
Cor Total	0.53	31			

The resulting relationship of all significant factors toward *S. dimorphus* growth was represented by a linear regression Equation (2). The fitness of the model, as expressed by R^2 , is 0.8211, indicating that 82.11% of the variability in the response can be explained by this linear model. In addition, the goodness of fit of the regression equation is also tested by looking at adjusted R-squared. R^2_{Adj} is 0.7946, and the predicted R-squared is 0.7487. The R^2_{Adj} which is 0.7946, indicates a still high degree of agreement between the observed and predicted values for the response studied, suggesting that the proposed model equation can provide satisfactory results.

$$\text{Biomass concentration (g/L)} = 0.8770 + 0.0816B - 0.0332C - 0.0548E + 2.729 \times 10^{-3} CE \quad (2)$$

The half-normal plot determines the significant factors affecting the response. Graphically, these factors are represented by the points that deviate far to the right-side from the linear line in the plot (Figure 1). Urea (*E* factor) is the furthest, indicating the strongest signal, then followed by ammonium chloride (*C* factor), acetic acid (*B* factor) and interaction between ammonium chloride and urea (*CE* factor). This interpretation concurs with F-values ranking in the ANOVA table. Contribution-wise, it was calculated by Design Expert that the effect of urea was 41.72%, ammonium chloride was 22.74%, acetic acid was 10.12%, and the *CE* interaction term was 7.52% towards the response.

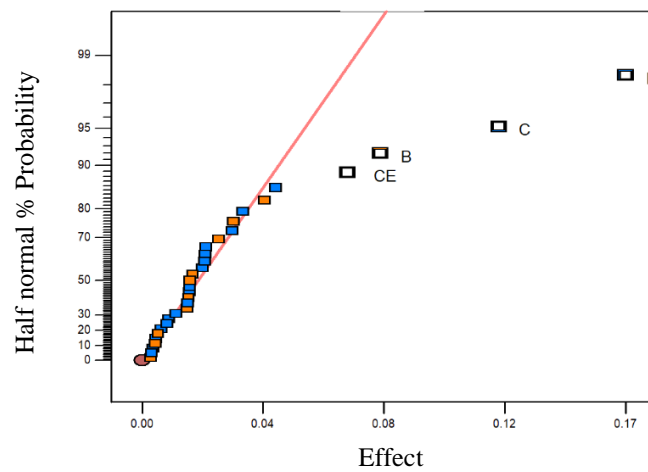


Figure 1. Half-normal plot depicting the selection of the main effect of acetic acid (*B*), ammonium chloride (*C*) and urea (*E*).

3.2. Response Analysis of *S. dimorphus* UTEX 1237 Biomass Concentration

The effect of each major factor, as well as any interaction among factors towards influencing *S. dimorphus* biomass propagation, were statistically investigated. As mentioned previously, one carbon source; acetic acid (*B* factor) and two nitrogen sources; ammonium chloride (*C* factor) and urea (*E* factor) have a significant statistical effect whereas sodium bicarbonate (*A* factor) and sodium nitrate (*D* factor) supplemented at given concentration range did not noticeably affect the cell growth. Moreover, the positive and negative effects of these three important variables are presented in Figure 2.

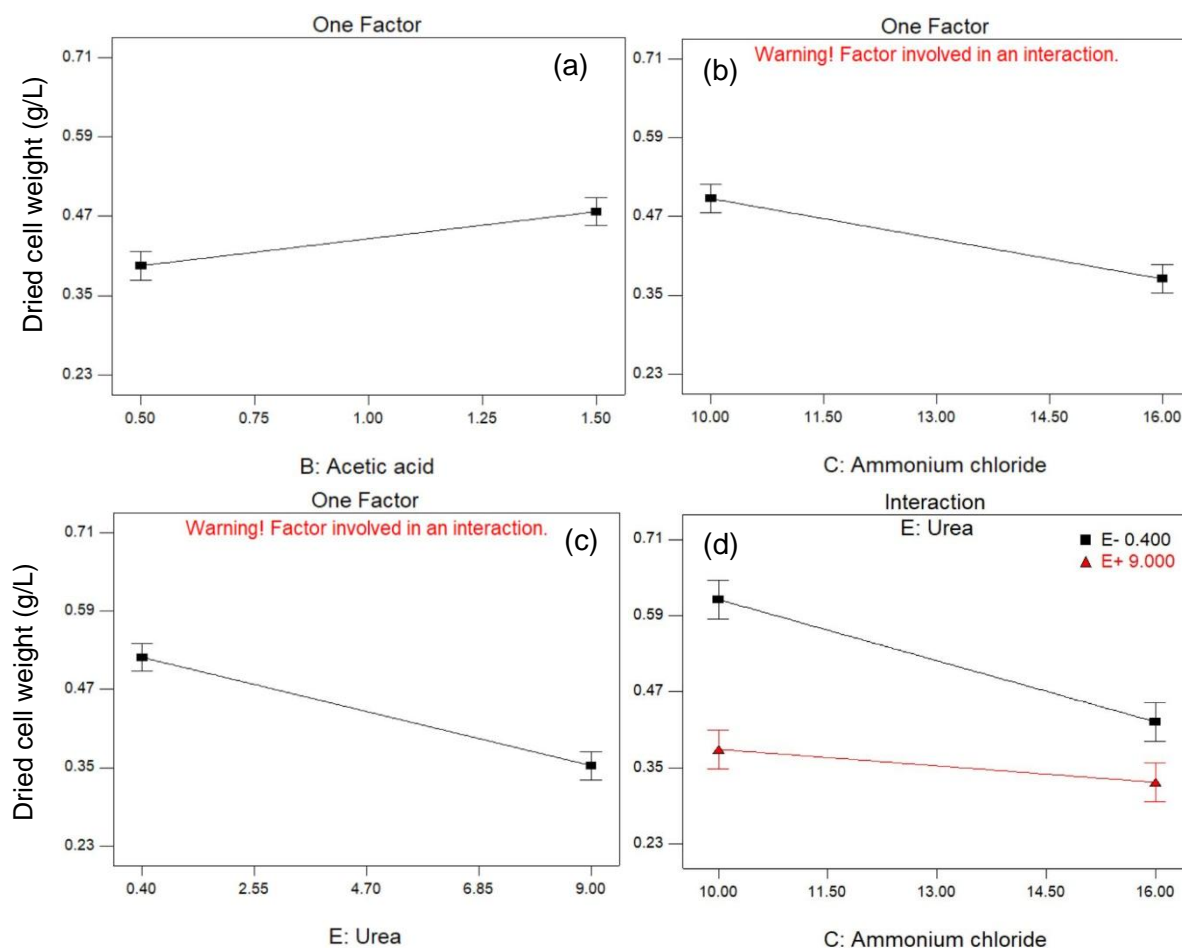


Figure 2. The plot of significant factors and interaction at 95% confident interval

As for major factors, acetic acid in Figure 2(a) posed a positive effect with an increased in concentration level, improving biomass concentration from 0.395 g/L to 0.477 g/L. Nonetheless, both ammonium chloride and urea imparted negative. Interestingly, significant mutual effect occurred between both nitrogen sources when *S. dimorphus* biomass was found higher at low urea concentration of 0.4 g/L coupled with low level of ammonium chloride of 10 g/L compared to a high point of 16 g/L, affecting substantial dried cell weight of 0.615 g/L against individual effect of low level ammonium chloride (0.497 g/L) and urea (0.512 g/L). The biomass yield reduced with an increase of both medium constituents. Thus, the optimal TAP medium composition from 2^k FD screening was registered by standard order run no. 4 in Table 2 which comprised of 1.5 g/L of acetic acid, 10 g/L of ammonium chloride and 0.4 g/L of urea giving a final yield of biomass at 0.706 g/L.

3.3. Comparison of Microalga Growth between 2^k FD and Original TAP Formulation

The time course data of *S. dimorphus* UTEX 1237 growth for cultivation that had employed formulation based on the outcome of 2^k FD screening together with a culture grown in normal TAP medium is shown in Figure 3.

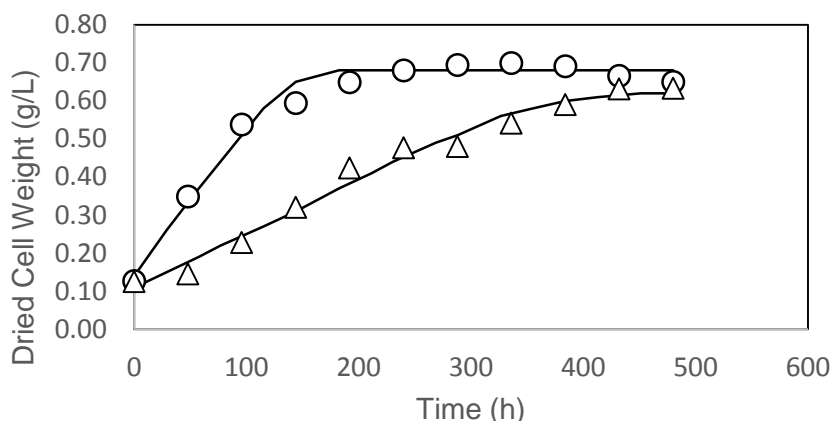


Figure 3. Growth profile of *S. dimorphus* UTEX 1237 on the modified medium from 2^k FD screening (circle) and original TAP (triangle). The solid line represents growth as simulated by Baranyi-Robert kinetic model

No apparent lag phase was observed in both cultures, indicating rapid adapting of *S. dimorphus* cells from seed to production medium. There are distinct regions defining the exponential to stationary growth phase around Day 6 in culture tested with the newly modified medium. The original formulation, however, shows a much gradual exponential growth and seemed to decelerate around Day 16. From kinetic model calculation, this translates to improved maximum specific growth, μ_{\max} of 0.0049 h⁻¹ for the modified medium against 0.00141 h⁻¹ in normal TAP. Improvement of biomass saw the highest density was achieved on Day 14 at 0.706 g/L (productivity at 0.050 g/L/day) whereas in the original TAP it was 0.633 g/L gained at the end of 21 days run (productivity at 0.030 g/L/day).

Acetic acid content was 1.05 g/L in the original in TAP medium. By adding 0.45 g/L more to the medium, it significantly improved the mixotrophic growth of *S. dimorphus*. However, any more addition of substrate concentration should be done carefully, as the nature of this organic acid or bicarbonate, by algal consumption would release carbon dioxide in the water to form carbonic acid that can decrease the culture pH [14]. *S. dimorphus* affinity towards acetic acid is encouraging since this substrate can be synthesized inexpensively and in great quantities from petroleum resources [15].

The preference towards ammonium ion instead of nitrate might due to the strains having low nitrate reductase activity for reducing nitrate via nitrite to ammonium. The highest contribution to response is shown by urea at 0.4 g/L. According to Sakamoto *et al.* [16], urea is a better nitrogen source for the cultivation of microalgae than nitrates and ammonia due to less energy is required for the assimilation process and having the ability to pass through the plasma membrane of microalgae cells. Most fast growing microalgae preferred ammonia as their nitrogen source, while urea, a reduced form of nitrogen source can also be readily hydrolyzed into ammonia, lending to the synergistic effect toward the cell growth by having two nitrogenous-rich compounds in the medium.

Conclusion

Two-level factorial design on five TAP medium constituents has shown that *S. dimorphus* UTEX 1237 biomass concentration was significantly affected by acetic acid, urea and ammonium chloride. Additionally, a significant interaction existed between the two nitrogen sources. The proposed medium composition for enhancing yield was as follow; 1.5 g/L of acetic acid, 10 g/L of ammonium chloride and 0.4 g/L of urea, which achieved final biomass productivity of 0.050 g/L/day, translating to 66.7% improvement over normal TAP formulation.

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