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Contributed Paper

Isolation and Cultivation of *Botryococcus braunii* Kützing from Northern Thailand

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ABSTRACT

Botryococcus braunii were isolated from eight natural water resources in Northern Thailand. The effect of media: modified CHU 13 medium (pH 6.7), Jaworski's medium (pH 7.0) and CA medium, on *B. braunii* isolation was compared. It was found that CA was suitable for *B. braunii* isolation. *B. braunii* HM5 isolated from the reservoir of the Hill Tribe Museum, Chiang Mai, showed the highest growth rate. The optimal condition for this strain was at ambient temperatures (26.5-32.5°C) in CA medium, supplemented with 10% CO_2, at pH 7.2 which provided 6.31 $g \cdot L^{-1}$ of biomass and 41.74% hydrocarbon accumulation.

Keywords: bio-oil, *Botryococcus braunii*, CO_2, hydrocarbon

1. INTRODUCTION

Thailand, especially the northern part, is a very suitable area for commercial cultivation of algae. This is due to suitable climate, availability of land at relatively low cost, sufficient water supply and low labor costs. In addition, many commercial algal farms have successfully operated in this area [1]. In our experience, algal strains isolated from temperate climate do not grow as well in tropical climate [2]. Therefore, it is important to isolate and characterize native algae for local cultivation.

Botryococcus braunii Kützing is a green colonial microalga. It is widespread in freshwater reservoirs, ponds and sometime can be found in brackish lakes [3]. This alga is regarded as a potential source of renewable fuel because of its ability to produce large amount of hydrocarbons. Depending on the strain and growth conditions, up to 75% of algal dry mass can be hydrocarbons [4]. The bulk of its hydrocarbons are stored in the outer walls [5]. Optimization of the nutrients and culture conditions for the improvement of growth rate and hydrocarbon productivity have been attempted [4]. Each *B. braunii* strain has individual requirement under different environmental conditions. Thus, it is necessary to isolate a native strain for mass cultivation at assigned geographical location [6]. Therefore, the focus of the present study was to isolate the Thai strains of *B. braunii* from the natural water resources in the

northern part of Thailand. The effects of isolation media were evaluated, including some parameters affecting the growth rate and hydrocarbon production. These data could be used to further optimize culture conditions. The improvement of conditions for growth and hydrocarbon accumulation of *B. braunii* will increase the opportunity for the cultivation of *B. braunii* as an alternative energy source and to compete with other sources of plant as well as petroleum fuel.

2. MATERIALS AND METHODS

2.1. Isolation of *B. braunii*

B. braunii was collected from the water resource at the Hill Tribe Museum (HM), Mae Jok Laung (MJ) reservoir, Huay Tung Tao (HT) reservoir, three water resources of Mae Hia Agricultural Research Station, Chiang Mai University (MH1, MH2 and MH3), Mae Ngud Somboonchol Dam (MN) in Chiang Mai and Bung Boraphet (BP) in Nakornsawan (Figure 1). Colonies of *B. braunii* in the water samples were detected under a microscope and a single colony was isolated with a glass micropipette [7]. Each colony was washed at least five times with sterile medium and was then cultivated in 2 mL of the following media: modified CHU 13 medium (pH 6.7) [8], Jaworski's medium (JM) (pH 7.0) [9] and CA medium (pH 7.2) [10] were used (Table 1). Ten single colonies of *B. braunii* from each media were isolated from each sampling site. The cultures were incubated at 25°C under continuous 10.8 $\mu mol.m^{-2}.s^{-1}$ illumination by florescent lamp. The percentage of successful isolation was evaluated from the number of bottles in which *B. braunii* growth could be observed. After isolation, the cultures were maintained in their isolation medium under the same described conditions.

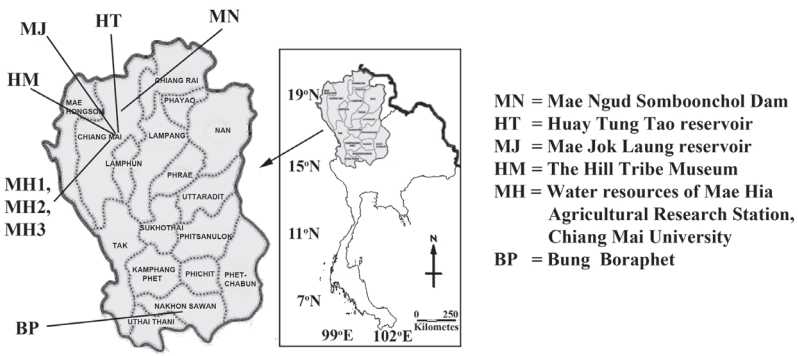

Figure 1. Location of eight water resources from which *B. braunii* was isolated.

Table 1. Composition of different media for *B. braunii* isolation.

Components ($g.L^{-1}$)	CHU 13	JM	CA
KNO_3	0.2	-	0.1
K_2HPO_4	0.04	1.24	-
$NaNO_3$	-	8.0	-
$NaHCO_3$	-	1.59	-
$Na_2HPO_4.12H_2O$	-	3.6	-
NH_4NO_3	-	-	0.05
$MgSO_4.7H_2O$	0.1	5.0	0.02
$CaCl_2.2H_2O$	0.054	-	-

Table 1. Continue.

Components ($g.L^{-1}$)	CHU 13	JM	CA
$Ca(NO_3)_2.4H_2O$	-	2.0	0.02
H_3BO_3	Trace	0.248	-
$MnCl_2$	Trace	-	-
$MnCl_2.4H_2O$	-	0.139	Trace
$ZnSO_4.7H_2O$	Trace	-	Trace
$CuSO_4.5H_2O$	Trace	-	-
$CoCl_2.6H_2O$	Trace	-	Trace
$Na_2MoO_4.2H_2O$	Trace	-	Trace
$(NH_4)_6Mo_7O_{24}.4H_2O$	-	0.1	-
$FeCl_3.6H_2O$	-	-	Trace
$Fe(NH_4)_2(SO_4)_2.6H_2O$	-	-	Trace
EDTA Fe Na	-	0.225	-
EDTA Na_2	-	0.225	-
$Na_2EDTA.2H_2O$	-	-	Trace
Citric acid	0.1	-	-
Ferric citrate	0.01	-	-
Vitamin B12	-	0.004	0.00001
Thiamine HCl (Vitamin B1)	-	0.004	0.00001
Biotin	-	0.004	-
HEPES	-	-	0.4
β-Na_2glycerophosphate.$5H_2O$	-	-	0.03

Trace is a micro element in modified CHU 13 and PIV metal in CA medium

2.2 Growth of *B. braunii*

The fastest growth strain was selected to be cultured in CA medium. The experiment was carried out in 300 mL of CA medium and incubated at 25°C with shaking at 120 rpm under continuous 10.8 $\mu mol.m^{-2}.s^{-1}$ illumination by florescent lamp. The growth of *B. braunii* in CA medium and modified CHU13 medium was validated. After that, growth at 25°C and ambient temperature were compared and the effects of pH and CO_2 concentration on the growth were also studied. *B. braunii* was cultivated using 300 mL of CA medium at pH 7.2, 8.0, 8.5, 9.0 and 9.5. For CO_2 experiments, the alga was cultivated in 300 mL of CA medium, pH 7.2 with different concentrations of CO_2. Pure CO_2 and N_2 were mixed and adjusted by gas mixer (Febix International, Chiang Mai, Thailand) to give an appropriate concentration of 1% and 10% CO_2 at the flow rate of 100 $mL \cdot min^{-1}$. Filter-sterilized air (0.04% CO_2) at the same flow rate was used as control. Samples were collected at 5-day intervals. All the experiments were carried out in triplicate.

2.3 Determination of Growth

Cell density of *B. braunii* was determined spectrophotometrically at 680 nm (OD_{680}) [10]. Algal dry weight was obtained from the conversion using a linear correlation between OD_{680} and dry weight expressed by the following formula (Figure 2):

$$\text{Dry weight}(g \cdot L^{-1}) = 2.6839 \times OD_{680} + 0.0987$$

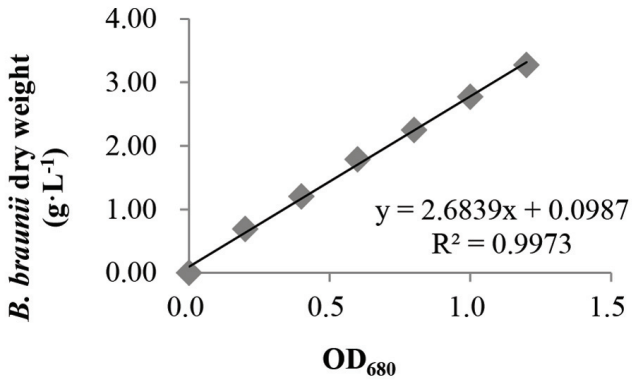

Figure 2. Correlation between OD_{680} and dry weight of *B. braunii* HM5 in CA medium.

2.4 Hydrocarbon Extraction

At the end of cultivation, all biomass was collected for hydrocarbon extraction (modified from the method of Dayananda *et al*., 2005) [11]. Algal dry weight was measured gravimetrically from freeze-dried samples. Freeze-dried algal cells were lyzed by sonication in 15 mL hexane at 10,000 Hz for 5 min and the suspension was centrifuged at 6000g for 15 min. The supernatant was evaporated to complete dryness at room temperature. Hydrocarbon content was measured gravimetrically and expressed as the dry weight percentage.

2.5 Statistical Analysis

Effect of isolation medium was analyzed by non-parametric, Kruskal-Wallis test and statistical comparison between groups was compared by Mann-Whitney U at $p < 0.05$ using SPSS version 14.0 for Windows. Growth and percentage of hydrocarbon of in each experiment was analyzed by One-Way ANOVA with post hoc Tukey's honestly significant difference (HSD) comparison of mean at $p < 0.05$ using SPSS version 14.0 for Windows.

3. RESULTS AND DISCUSSION

3.1 Isolation of *B. braunii*

The green microalga, *B. braunii*, collected from 8 water resources and some water quality parameters are presented in Table 2. It was found that HM had the highest pH (8.67), whereas MJ had the lowest pH (6.49). The temperature of each water resource was 26.0 to 31.3°C. The lowest temperature was at MJ and the highest was at BP. The alga was isolated from the three media: modified CHU 13 medium (pH 6.7), JM (pH 7.0) and CA (pH 7.2). CA was the most suitable medium for isolation as it provided the highest percentage of *B. braunii* isolates from most water samples. This might be because CA contains appropriate nutrient level for *B. braunii* from northern Thailand. The water resources in this study were low in nutrient contents which could be observed from the low conductivity value (Table 2). Although, JM is a common medium for other green algae [9], it contains high nutrient concentration (Table 1) and was not suitable for *B. braunii* isolation from those water bodies. While modified CHU13 is a common medium for *B. braunii* [11-13], it was not successful for the isolation of *B. braunii*, compared with CA. This should be because CA contains more nutrient components than CHU13, for example, it possesses two nitrogen sources: KNO_3

and NH_4NO_3. In addition, it contains vitamins which are absent in modified CHU13. Moreover, CA has 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES) which is a buffer for medium pH stabilization.

Table 2. *B. braunii* isolated from 8 water resources and some physico-chemical parameters.

Water resource	pH	Temperature (°C)	Conductivity ($\mu s.cm^{-1}$)	% *B. braunii* isolates CHU 13	JM	CA
HM	8.67	27.4	130.0	10	10	80
MJ	6.49	26.0	57.2	50	20	10
HT	7.62	26.4	54.9	20	10	30
MN	7.27	26.0	97.3	0	10	60
MH2	7.64	28.7	118.0	20	10	40
MH3	7.72	28.4	114.0	20	0	50
MH4	7.63	29.1	144.0	20	0	20
BP	6.95	31.3	221.2	20	10	30
Mean rank form Kruskal-Wallis test				124.4^b	66.3^a	184.4^c

a,b and c are statistical comparison between groups by using Mann-Whitney U

3.2 Cultivation of *B. braunii*

B. braunii HM5 from the Hill Tribe Museum (HM), which grew faster than all others, was cultivated in CA (pH 7.2) and modified CHU13 (pH 6.7). It was found that the growth in CA was significantly higher than that in CHU13 ($p < 0.05$) (Figure 3a). Therefore, *B. braunii* HM5 was cultivated in CA at 25°C compared to that of room temperature. During the cultivation period, the ambient temperature ranged from 26.5 to 32.5°C. Growth at room temperature was significantly higher than that at 25°C ($p < 0.05$), with a dry weight of 4.79 $g{\cdot}L^{-1}$ and contained 37.30% hydrocarbons of its dry weight after the growth reached stationary phase at 78 days (Figure 3b). Whereas, the dry weight at 25°C was 3.98 $g{\cdot}L^{-1}$ with 29.13% hydrocarbons. A foreign strain, *B. braunii* SAG 30.81 was also cultivated at ambient temperature in modified CHU 13 as recommended by the culture collection. However, it could not survive under Thai climate without temperature control (data not shown), indicating that the Thai algal strain grew better at ambient temperature than at 25°C. This finding was somewhat expected as the water temperatures at all study sites were higher than 25°C (Table 2). Cultivation of alga at room temperature, without temperature control, would reduce the operational cost in commercial production. This suggests that the isolation and cultivation of tropical *B. braunii* should be performed at ambient temperatures.

Figure 4a shows the growth of the alga cultured at various pH levels. It was found that the alga grew better at pH 7.2 with a dry weight of 4.82 $g{\cdot}L^{-1}$ after 110 days (Table 3). The hydrocarbon content of the alga cultured at pH 7.2 was also highest at 38.08% of its dry weight (Table 3). Although it was isolated from water at the highest pH (8.67), *B. braunii* HM5 showed the highest growth at pH 7.2, which is generally recommended for green algal cultivation in CA and also contained the highest amount of hydrocarbons.

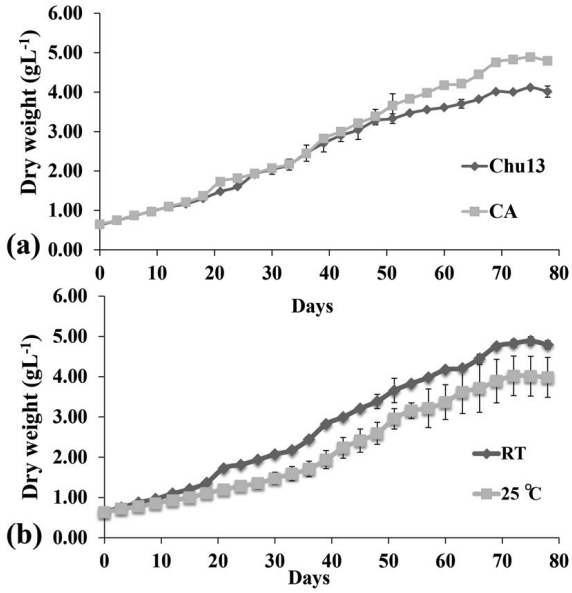

Figure 3. a: Growth of *B. braunii* HM5 in CA and modified CHU13; b: Growth of *B. braunii* HM5 in CA at ambient temperature (RT) and 25°C.

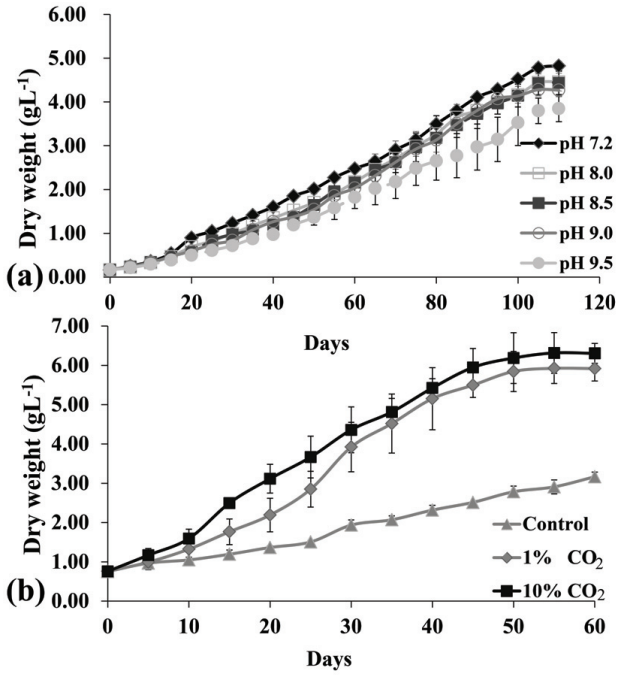

Figure 4. a: Growth of *B. braunii* HM5 cultivated in CA at ambient temperature and various pH; b: Growth of *B. braunii* HM5 in CA at ambient temperature and various CO_2 concentrations.

Table 3. Biomass productivity and hydrocarbon content of *B. braunii* HM5 in CA at various pH.

pH	Dry weight ($g \cdot L^{-1}$)	%Hydrocarbon content
7.2	4.82c	38.08%c
8.0	4.44b	33.32%bc
8.5	4.45b	33.79%bc
9.0	4.27b	28.18%ab
9.5	3.85a	24.73%a

a,b and c are statistical comparison between groups by using using analysis of variance (ANOVA) and post-hoc Tukey (HSD)

In this study, CO_2 supplementation was found to be better for growth and hydrocarbon production. The algal biomass yield increased up to1.86 and 1.99 fold when the biomass was supplemented with CO_2 at 1% and 10%, respectively (Figure 4b and Table 4). Growth of the alga with CO_2 supplementation entered the stationary phase faster than the control, in only 45 days. Even though 10% of CO_2 supplementation provided significantly higher biomass than 1%, the hydrocarbon accumulation, between 1% and 10% of CO_2 supplementation was not significantly different ($p < 0.05$). Hydrocarbon accumulation increased from 26.81% (control) to 38.59% and 41.74% with 1% and 10% CO_2 supplementation (Table 4). Typically, CO_2 concentration from industrial flue gas was around 10-20% [14,15]. Since *B. braunii* HM5 could benefit from high CO_2 concentration, it could be a promising strain for CO_2 removal in flue gas. Hence, it may be of interest in supplying CO_2 from the industrial flue gas for *B. braunii* HM5 cultivation, in order to enhance growth and reduce atmospheric CO_2 at the same time [16,17].

Table 4. Biomass productivity and hydrocarbon content of *B. braunii* HM5 in CA medium at various CO_2 concentrations.

CO_2 concentrations	Dry weight ($g \cdot L^{-1}$)	%Hydrocarbon content
Normal air (0.04% CO_2)	3.17a	28.81%a
1% CO_2	5.92b	38.59%b
10% CO_2	6.31c	41.77%b

a,b and c are statistical comparison between groups by using using analysis of variance (ANOVA) and post-hoc Tukey (HSD)

Many researches have tried to optimize culture conditions for biomass and hydrocarbon production and found that high hydrocarbon production was observed when the growth was suppressed [11, 18]. The results of the present study were different from those that have been previously reported. Supplementation of CO_2 not only promoted the growth but also induced hydrocarbon production. From previously study, N:P ratio was found to affect the biomass and hydrocarbon yield [11]. High N and low P could promote growth, while low N and high P increased hydrocarbon content. However, this study indicated that the concentration of CO_2 affected both biomass and hydrocarbon production. Therefore, there is a need to understand the optimal C, N and P ratio for growth and hydrocarbon production.

CONCLUSION

Eight *B. braunii* strains were isolated from northern Thailand. The growth rate and hydrocarbon production of *B. braunii* HM5 was highest in CA medium, pH 7.2 supplemented with 10% CO_2 at ambient temperature for cultivation. From these results, the optimization of C:N:P ratio is of great interest for further study.

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