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Influence of CO_2 on Growth and Hydrocarbon Production in *Botryococcus braunii*

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Abstract *Botryococcus braunii* is a green colonial fresh water microalga and it is recognized as one of the renewable resources for production of liquid hydrocarbons. CFTRI-Bb-1 and CFTRI-Bb-2 have been reported for the first time and their performance with regard to growth and biochemical profile is presented here. The present study focused on effect of carbon dioxide (CO_2) on biomass, hydrocarbon, carbohydrate production, fatty acid profile, and carotenoid content in various species of *B. braunii* (LB-572, SAG 30.81, MCRC-Bb, N-836, CFTRI-Bb-1, and CFTRI-Bb-2) at 0.5, 1.0, and 2.0% (v/v) levels using a two-tier flask. CO_2 at 2.0% (v/v) level enhanced growth of the organism, and a two-fold increase in biomass and carotenoid contents was observed in all the *B. braunii* strains studied compared with control culture (without CO_2 supplementation). At 1% and 2% (v/v) CO_2 concentrations, palmitic acid and oleic acid levels increased by 2.5 to 3 folds in one of the strains of *B. braunii* (LB-572). Hydrocarbon content was found to be above 20% at 2% CO_2 level in the *B. braunii* LB-572, CFTRI-Bb-2, CFTRI-Bb-1, and N-836 strains, whereas it was less than 20% in the SAG 30.81 and MCRC-Bb strains compared with control culture. This culture methodology will provide information on CO_2 requirement for growth of algae and metabolite production. *B. braunii* spp. can be grown at the tested levels of CO_2 concentration without much influence on culture pH.

Keywords: *Botryococcus braunii*, microalga, hydrocarbon, biomass, carotenoids, carbon dioxide, fatty acids

The unicellular photosynthetic microalga *B. braunii* is a member of the chlorophyceae, which produces algal biomass of which 70% can be hydrocarbons. It is regarded as a potential source of renewable fuel because of its ability to produce large amounts of hydrocarbons. *B. braunii* is classified into A, B, and L races depending on the type of hydrocarbons synthesized. Race-A produces C_{23} to C_{33} odd-numbered n-alkadienes, mono-, tri-, tetra-, and pentaenes, which are derived from fatty acids [15, 16]. Race B produces C_{30} to C_{37} unsaturated hydrocarbons known as botryococcenes, and small amounts of methyl branched squalenes [1, 17, 18,], whereas race L produces a single tetraterpenoid hydrocarbon known as lycopadiene [15]. Another difference among the races is the colony color in the stationary phase. Race "A" and "B" strains are known to produce exopolysaccharides up to 250 g m^3, whereas "L" race produced up to 1 kg m^3 [2]. *B. braunii* is a promising renewable resource for the production of hydrocarbons and it has been reported that on hydrocracking, the distillate yields 67% gasoline, 15% aviation turbine fuel, 15% diesel fuel, and 3% residual oil [11]. Gudin and Thomas [10] reported that *B. braunii* converts 3% of the solar energy to hydrocarbons. *B. braunii* is also known to produce large amounts of fatty acids. The quantity and composition of fatty acid varies with species and also among the races [9, 15]. Cane [2] suggested that *B. braunii* is one of the major sources of hydrocarbon in a variety of oil-rich deposits dating from the Ordovician period to the present. In the changing energy scenario, it is necessary to exploit this potential microalga for hydrocarbons. Dayananda *et al.* [5] reported optimization of media constituents for growth and hydrocarbon production. The levels of CO_2 required may vary from organism to organism [19]. Therefore, the present study focused on adaptation of various strains of *B. braunii*, especially the indigenous ones, to CO_2 concentration and its influence on biomass and hydrocarbon production, and extracellular carbohydrate, lipid, and carotenoid contents. This is very important for the outdoor cultivation of indigenous strains of *B. braunii* for use in biomass production.

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MATERIALS AND METHODS

Algal Strains

B. braunii strains were obtained from various culture collection centers such as *B. braunii* (N-836) from the National Institute for Environmental Studies, Tsukuba, Japan; *B. braunii* (SAG-30.81) from Sammlung von Algen Kulturen, Pflanzen Physiologisches Institute, Universitat Gottingen, Germany, and *B. braunii* LB-572 from University of Texas, U.S.A. Indigenous strains were collected from Kodaikanal and Mamallapuram and the strains are designated as CFTRI-Bb-1 and CFTRI-Bb-2, respectively. They are being reported for the first time. MCRC-Bb strain was procured from the Murugappa Chattier Research Institute, Chennai. The stock cultures were maintained both in agar slants and liquid cultures of modified Chu13 medium [13], which consists of (g/l) KNO_3, 0.2; K_2HPO_4, 0.04; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 6H_2O$, 0.08; Ferric citrate, 0.01; Citric acid, 0.02; pH 7.5.

Two-Tier Flask

A two-tier flask vessel consisting of two 250-ml Erlenmeyer flasks [19] was used for photoautotrophic growth of *B. braunii* spp. The lower compartment of the flask contained 100 ml of a 3 M mixture of ($KHCO_3/K_2CO_3$) at specific ratios, which generated specific CO_2 partial pressure in the two-tier flask. The concentration of buffer mixture was determined to get a CO_2 partial pressure of 0.5, 1.0, and 2.0% (v/v), respectively, as given by Usha Tripathi *et al.* [19]. The exact partial pressure of the CO_2 in the head space of the culture flask was monitored using a Protomap 2 portable gas analyzer (System Tech Instruments, U.K.) for CO_2 and O_2.

Culture Set up

The Chu 13 medium (50 ml) in the upper chamber was inoculated with a specific inoculum of algal cells, and the mouth of the upper and lower compartments were sealed tightly by cotton plug and parafilm. Two-week-old culture of *B. braunii* LB-572 grown in modified Chu 13 medium was used as inoculum at 20% (v/v). The culture flasks were incubated at 26±1°C temperature under 1.2±0.2 klux light intensity and 16:8 h light:dark cycle. The cultures were subjected to CO_2 at 0.5, 1.0, and 2% levels. The growth of culture was recorded at 5-day intervals. Similarly, SAG 30.81, MCRC-Bb, N-836, CFTRI-Bb-1, and CFTRI-Bb-2 were studied for 25 days in the presence of 2% (v/v) CO_2 concentration. Control culture flask for each strain was maintained without ($KHCO_3/K_2CO_3$) buffer in the two-tier flask. All the experiments were carried out in triplicates.

Analytical Methods

Biomass Estimation. The cultures were harvested by centrifugation at 5,000 rpm and the cells were washed twice with distilled water. Then, the pellet was freeze dried. The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight (g/l).

Chlorophyll Estimation. A known volume of *B. braunii* culture was centrifuged and the residue was extracted with methanol repeatedly. The chlorophyll content in the pooled extract was estimated spectrophotometrically by measuring the absorbance at 652 and 665 nm, respectively, and was quantified as previously described by Lichtenthaler [14].

Carotenoid Estimation. A known amount of freeze-dried algal biomass was extracted with acetone and absorbance was measured at 450 nm and the concentration of carotenoid was determined by the Davies [4] method.

Phosphate Estimation. Cell-free medium was analyzed for phosphate by the Fiske-Subba Rao method [8].

Carbohydrate Estimation. A known amount of cell-free (spent) medium was analyzed for total carbohydrate content by the phenol-sulfuric acid method [6].

Hydrocarbon Extraction. The dry biomass was homogenized in a mortar and pestle with n-hexane for 15 min and centrifuged. The extraction process was repeated twice and supernatant was transformed to a pre-weighed glass vial and evaporated under a stream of nitrogen to complete dryness. The quantity of residue was measured gravimetrically and expressed as dry weight percentage [5].

Hydrocarbon Analysis by GC. Hydrocarbon extract was purified by column chromatography on silica gel. The hydrocarbon sample was analyzed using an ELITE-5 capillary column. The conditions used were oven temperature initially at 130°C for 5 min and then increased to 200°C at the rate of 8°C min^{-1} and maintained for 2 min and then increased up to 280°C at the rate of 5°C min^{-1} and maintained for 15 min. The injector port and the detector temperatures were 240°C and 250°C, respectively [5].

Fatty Acid Analysis

The lipids were extracted with chloroform:methanol (2:1) and quantified gravimetrically. The lipid sample was dissolved in benzene and 5% methanolic hydrogen chloride was added and the mixture shaken well. The mixture was refluxed for 2 h and then 5% sodium chloride solution was added and the fatty acid methyl esters (FAMEs) were extracted with hexane. The hexane layer was washed with 2% potassium bicarbonate solution and dried over anhydrous sodium sulfate [4]. FAMEs were analyzed by GC-MS (PerkinElmer, Turbomass Gold, Mass spectrometer) equipped with FID using an SPB-1 (poly(dimethysiloxane)) capillary column (30 m×0.32 mm ID×0.25 μm film thickness) with a temperature programming 130°C to 280°C at a rate of 3°C min^{-1}. The FAMEs were identified by comparing their fragmentation pattern with authentic standards (Sigma) and also with the NIST library.

RESULTS

Influence of CO_2 on Growth, Hydrocarbon, Carotenoid, and Fatty Acid Profiles of *B. braunii* LB-572

B. braunii strain LB-572 was evaluated for growth and metabolite production in a two-tier flask by providing different levels of CO_2. The two-tier flask contained nutrient medium in the upper chamber and a CO_2-generating buffer mixture ($KHCO_3/K_2CO_3$) in the lower chamber. The concentration of CO_2 in the flask was found to be the same throughout the experimental period. It was found that 2.0% (v/v) CO_2 favored rapid growth, resulting in increased biomass accumulation and hydrocarbon production at the end of the experimental period. *B. braunii* LB-572 was able to grow in all the tested concentrations of CO_2 at 0.5, 1, and 2%. The biomass yields increased with increasing concentration of CO_2 and maximum biomass was achieved at 2% CO_2 concentration over the control culture (Fig. 1). It was evident from the data in Fig. 2 that the decrease in phosphate in the medium was due to its utilization by the alga.

Hydrocarbon content in *B. braunii* was found to be similar to the growth pattern at different concentrations of CO_2, as shown in (Fig. 1). Hydrocarbon content varied in the range of 14 to 28% at different CO_2 levels, and maximum hydrocarbon content was found at 2% CO_2. The hydrocarbon profile, as analyzed by GC, indicated only a marginal

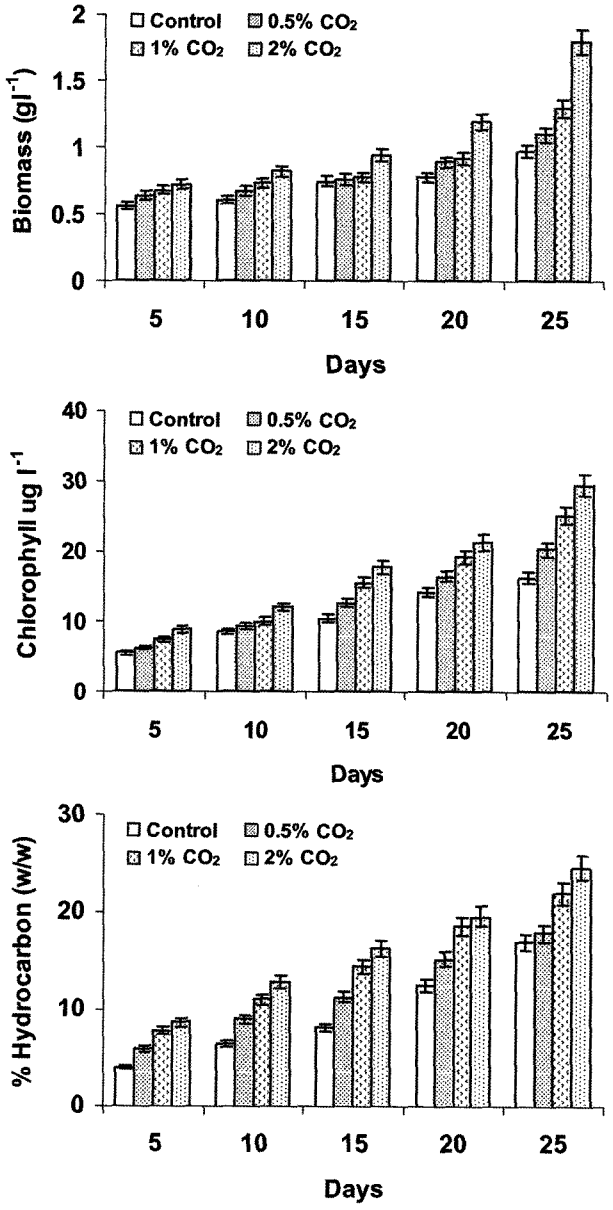

Fig. 1. Influence of CO_2 on biomass yield, and chlorophyll and hydrocarbon production in *B. braunii* LB-572.

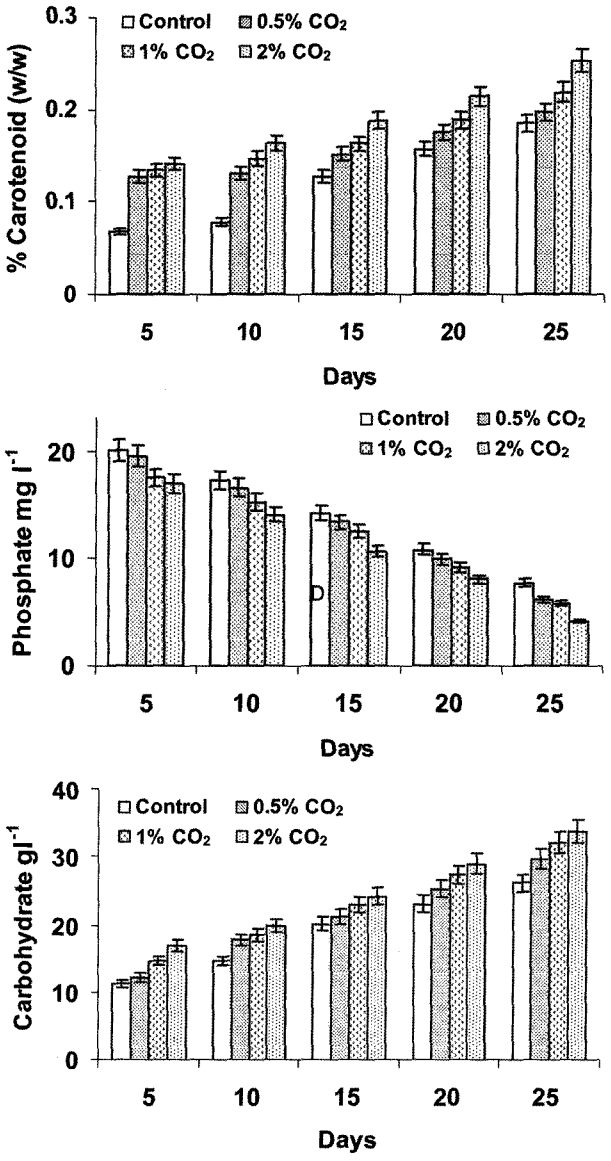

Fig. 2. Influence of CO_2 on carotenoid, phosphate, and carbohydrate in *B. braunii* LB-572.

Table 1. Influence of CO_2 concentration on production of hydrocarbons in *B. braunii* LB-572 analysed by GC.

B. braunii LB-572	Less than C_{20} %	Higher than C_{20-24} %	Less than C_{30} %	Higher than C_{30} %
Control	24.20±0.54	35.96±0.26	5.43±0.12	34.41±0.13
0.5% CO_2	15.23±0.38	30.33±0.17	8.23±0.1	46.32±0.23
1.0% CO_2	17.48±0.37	23.02±0.16	10.45±0.13	49.32±0.27
2.0% CO_2	12.99±0.23	12.79±0.08	16.74±0.04	57.58±0.38

Data represents mean±SD of three replicates. Data recorded on 25-days-old culture, represented as % of total hydrocarbon.

influence on the relative proportion of hydrocarbons lower than C_{30} and higher than C_{30} at different CO_2 levels, as shown in (Table 1).

The carotenoid content was found to increase with increase in level of CO_2 and the maximum was observed at 2% by using the spectrophotometer method (Fig. 2). The carbohydrate content in the medium, as analyzed in terms of total sugars, increased with the growth of the alga (Fig. 2). A marginal increase in carbohydrate content was observed at 0.5, 1, and 2% CO_2 cultures compared with control culture. The total fat content of the alga grown in different CO_2 levels was in the range of 28–30% (w/w) whereas in the control it was 22%. Data presented in Table 2 indicate the presence of fatty acids of C16:0, C16:1, C18:0, C18:1, C18:2, C22:0, C22:1, and C24:0, of which palmitic acid, stearic acid, and linoleic acid were found to be in higher proportion in control culture, whereas palmitic acid and oleic acid were the major fatty acids in 1% and 2% CO_2 cultures.

Studies on Growth and Hydrocarbon Production in Various Strains of *B. braunii*

Based on the data obtained for *B. braunii* strain LB-572 presented in Figs. 1–2 and Tables 1–2, it was clear that 2% CO_2 was suitable for enhanced growth and metabolite production. Therefore, all the stains were studied at the 2% CO_2 (v/v) level. A significant increase in biomass, chlorophyll, carotenoid, hydrocarbon, and fat contents in the *B. braunii* strains (Table 3) was observed. A 2.5-fold increase in the biomass yield was observed in CFTRI-Bb-2 and N-836 spp., when compared with control cultures.

Table 2. Influence of CO_2 concentration on lipid profile in *B. braunii* LB-572.

Fatty acids	Control %	1% CO_2 %	2% CO_2 %
16:0	13.14±0.04	28.52±0.15	35.87±0.16
16:1	11.65± 0.08	13.45±0.07	2.97±0.09
18:0	26.19±0.11	6.21±0.01	4.28±0.05
18:1	12.35±0.07	27.23±0.13	32.71±0.13
18:2	20.26±0.11	14.26±0.10	6.06±0.05
22:0	4.31±0.09	4.87±0.05	1.05±0.14
22:1	11.42±0.06	2.27±0.09	Trace
24:0	Trace	2.69±0.01	16.92±0.12

Data represents mean±SD of 3 replicates. Data recorded on 25-days-old culture. Represented as relative % of total fatty asics.

SAG-30.81, MCRC-Bb, and CFTRI-Bb-1 showed 2–3 folds increases in chlorophyll and 1–1.5-fold in carotenoid contents. The carotenoid content was higher in the CFTRI-Bb-2 strain than the others. Carbohydrate was analyzed in terms of total sugars, which increased with growth of alga (data not shown). Phosphate content decreased in *B. braunii* strains because of its utilization by growing alga. The fat content in the CFTRI-Bb-2 and CFTRI-Bb-1 strains was found to be more than 30%, whereas in SAG 30.81, MCRC-Bb and N-836 it was around 25% (Table 3). Hydrocarbon content was observed to be more than 20% in the CFTRI-Bb-2, CFTRI-Bb-1, and N-836 strains, whereas it was less than 20% in the SAG 30.81 and MCRC-Bb strains. In general, a 1.5 to 2-fold increase in hydrocarbon content was obtained in CO_2 supplemented cultures than the control cultures. As analyzed by GC, CO_2 concentration had only a marginal influence on the hydrocarbon profile (Table 4). The relative proportion of hydrocarbons lower than C_{20} decreased, whereas higher than C_{30} increased at 2% CO_2 compared with control cultures.

DISCUSSION

B. braunii is well known for hydrocarbon production, which can be used as a renewable fuel resource. However, its slow growth as reported [12] was the limiting step for its successful exploitation. Usha Tripathi *et al.* [19] reported higher growth and carotenoid production in different microalgae under 0.5, 1.0, and 2.0% CO_2 concentrations It was also reported that different algae require different levels of CO_2 for their photoautotrophic adaptability [19]. Therefore, different levels of CO_2 were studied for enhancing the biomass and other metabolites production in *B. braunii*. The increase in biomass yields and other metabolites in different strains of *B. braunii* in response to a CO_2-enriched atmosphere, when compared with control cultures in the present study, suggests the improved growth and metabolite production of the organism to the tested levels of CO_2. Bubbling of CO_2 at higher levels (>2%) or continuous bubbling at 2% level resulted in significant decrease in the pH of the culture medium. This may be possibly due to a poor buffering capacity of the medium as the salt strength was low (medium constituents are given in Materials and Methods). The variation in the

Table 3. Influence of 2% CO_2 concentration on biomass, chlorophyll, hydrocarbon, fat, and carotenoid content in various *B. braunii* strains.

B. braunii strains	Biomass g/l	Chlorophyll mg/ml	Hydrocarbon % (w/w)	Fat % (w/w)	Carotenoid % (w/w)
SAG-30.81	0.75±0.01	5.65±0.18	7.75±0.17	12.42±0.08	0.115±0.01
SAG-30.81*	1.34±0.10	15.54±0.11	17.88±0.04	26.12±0.04	0.273±0.03
MCRC-Bb	0.62±0.02	8.21±0.18	8.51±0.16	13.5±0.07	0.137±0.07
MCRC-Bb*	1.52±0.04	20.11±0.10	19.53±0.09	25.71±0.10	0.281±0.12
N-836	0.43±0.01	9.75±0.05	9.21±0.056	15.23±0.13	0.201±0.13
N-836*	1.18±0.11	25.79±0.13	21.91±0.08	28.45±0.08	0.352±0.18
CFTRI-Bb-1	0.827±0.02	11.15±0.07	13.68±0.11	14.87±0.02	0.235±0.09
CFTRI-Bb-1*	1.82±0.03	28.91±0.06	22.13±0.19	31.52±0.21	0.457±0.16
CFTRI-Bb-2	0.915±0.06	14.27±0.14	11.85±0.26	16.01±0.13	0.357±0.11
CFTRI-Bb-2*	1.91±0.10	31.13±0.16	24.63±0.19	33.48±0.09	0.514±0.15

Data represents mean±SD of three replicates. Data recorded on 25-days-old culture.
*Fed with 2% CO_2 concentration.

hydrocarbon content and profile to different concentrations of CO_2 (Tables 1 and 4) observed in the present study ("A" race) are in contrast to the results of Wolf *et al.* [20], who reported that 0.3% CO_2 ("B" race) enriched air favors the formation of lower botryococcenes (C_{30}-C_{32}), whereas cultures sparged with ambient air accumulated higher botryococcenes (C_{33}-C_{34}) and also increased the doubling time of the culture to 6 days in comparison with 40 h. These differences could be attributed to the differences of the strains as they belong to A race and B race and produce hydrocarbons by different metabolic routes. Similarly, Metzger *et al.* [16] reported increase in hydrocarbon content from 5% in unaerated conditions to 20–61% when air mixed with 1% carbon dioxide was supplied, depending on the origin of the strain. CO_2 enrichment favored the formation of palmitic and oleic acids as the major fatty acids when compared with control. Fang *et al.* [7] also reported palmitic acid and oleic acids as the major components in *B. braunii*. Like many microalgae, *B. braunii* strains showed enhancement in biomass, fat, and carotenoid contents (2–3-fold) in CO_2-enriched atmosphere compared with control culture without CO_2, showing the metabolic efficiency through photoautotrophic growth. The performance of the indigenous strains of *B. braunii* was comparable to the other cultures used in this study. Therefore, the indigenous strains can be exploited for production of the hydrocarbons and fats in outdoor conditions.

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Table 4. Influence of 2% CO_2 on hydrocarbon production in various *B. braunii* strains.

B. braunii strains	Less than C_{20} %	Higher than $C_{20\text{-}24}$ %	Less than C_{30} %	Higher than C_{30} %
SAG-30.81	17.38±0.30	40.92±0.22	12.07±0.12	29.63±0.24
SAG-30.81*	12.98±0.29	30.64±0.07	11.54±0.09	44.53±0.29
MCRC-Bb	22.75±0.13	31.17±0.09	15.99±0.06	30.23±0.23
MCRC-Bb*	10.23±0.32	23.66±0.24	10.52±0.11	55.59±0.32
N-836	21.53±0.25	25.64±0.21	18.31±0.16	34.52±0.30
N-836*	10.13±0.27	28.36±0.18	5.27±0.07	56.24±0.36
CFTRI-Bb-1	18.76±0.10	29.27±0.23	12.93±0.14	38.95±0.15
CFTRI-Bb-1*	6.42±0.09	25.79±0.06	9.41±0.05	58.38±0.37
CFTRI-Bb-2	13.90±0.10	30.31±0.18	14.54±0.11	41.25±0.27
CFTRI-Bb-2*	7.69±0.17	19.43±0.12	5.43±0.13	67.45±0.43

Data represents mean±SD of three replicates. Data recorded on 25-days-old culture.
*Fed with 2% CO_2 concentration.

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