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Integrated Cultivation Technique for Microbial Lipid Production by Photosynthetic Microalgae and Locally Oleaginous Yeast

Mutiyaporn Puangbut, Ratanaporn Leesing

***Abstract*—**The objective of this research is to study of microbial lipid production by locally photosynthetic microalgae and oleaginous yeast via integrated cultivation technique using CO_2 emissions from yeast fermentation. A maximum specific growth rate of *Chlorella* sp. KKU-S2 of 0.284 (1/d) was obtained under an integrated cultivation and a maximum lipid yield of 1.339g/L was found after cultivation for 5 days, while 0.969g/L of lipid yield was obtained after day 6 of cultivation time by using CO_2 from air. A high value of volumetric lipid production rate (Q_P, 0.223 g/L/d), specific product yield ($Y_{P/X}$, 0.194), volumetric cell mass production rate (Q_X, 1.153 g/L/d) were found by using ambient air CO_2 coupled with CO_2 emissions from yeast fermentation. Overall lipid yield of 8.33 g/L was obtained (1.339 g/L of *Chlorella* sp. KKU-S2 and 7.06g/L of *T. maleeae* Y30) while low lipid yield of 0.969g/L was found using non-integrated cultivation technique. To our knowledge this is the unique report about the lipid production from locally microalgae *Chlorella* sp. KKU-S2 and yeast *T. maleeae* Y30 in an integrated technique to improve the biomass and lipid yield by using CO_2 emissions from yeast fermentation.

***Keywords*—**Microbial lipid, *Chlorella* sp. KKU-S2, *Torulaspora maleeae* Y30, oleaginous yeast, biodiesel, CO_2 emissions

I. Introduction

THE increasing demand for biofuels will create new opportunities for microorganisms and other non-food feedstocks to meet ambitious targets for renewable energy replacing fossil fuels. Microbial oils, namely single cell oil (SCO), lipid produced from oleaginous microorganisms involving yeasts, moulds, and microalgae, which have ability to accumulate lipids over 20 % of their biomass, are considered as non-food feedstock promising candidates for biodiesel production due to some advantages such as short production period, higher biomass production and faster growth compared to other energy crops, easiness to scale up [1, 2]. Microalgae have the highest oil or lipid yield among various plant oils, and the lipid content of some microalgae has up to 80% and the compositions of microalgal oils are mainly triglyceride which is the right kind of oil for producing biodiesel [3]. Microalgae may assume many types of metabolisms, such as photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic growths [4]. In photoautotrophic growth, the sole energy source for biomass production is light energy and the sole carbon source is inorganic compounds especially carbon dioxide (CO_2).

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CO_2 as a nutrient represents one of the most costly components in the cultivation of microalgae. Therefore a system that couples a waste CO_2 source with the cultivation of CO_2 fixing microalgae can not only reduce cultivation costs but also mitigate or remove CO_2, greenhouse gas (GHG) as an environmental pollution. Waste CO_2 can be provided by the flue gases from power plants or from agro-industrial plants [4, 5]. In the case of agro-industrial sector, CO_2 can be provided by using CO_2 emissions from the ethanol fermentation by yeast. The carbon credits obtained for removal of CO_2 from the ethanol plant emissions are non-taxable benefits [5]. The biofixation of CO_2 by microalgae has been proven to be an efficient and economical method, mainly due to the photosynthetic ability of these microorganisms to use this gas as a source of nutrients for their development.

The microalgae *Chlorella* sp., especially *C. protothecoides* and *C. vulgaris* are two widely available microalgae strains in the commercial applications for food and nutritional purposes. They showed great potentials as future industrial biofuel producers due to their high growth rate, and their high oil contents and they can be cultured both under photoautotrophic and heterotrophic conditions. However, the locally microalgae *Chlorella* sp. KKU-S2 isolated from freshwater taken from pond in the area of Khon Kaen province, northeastern region of Thailand, can accumulates much higher production of lipids, and the components of fatty acid from extracted lipid were palmitic acid, stearic acid, oleic acid and linoleic acid which similar to vegetable oils and suitable for biodiesel production [6].

In the last decade there is a great attention on oleaginous yeasts because some of them are capable of accumulating large amounts of lipids in their cells. Oleaginous yeast can produce high amount of lipid contents with characteristics similar to vegetable oil. It also has a high growth rate and can be cultured in a single medium with low cost substrate [7, 8]. The locally oleaginous yeast *Torulaspora maleeae* Y30 has proved to accumulate lipid efficiently not only on glucose but also on sugarcane molasses and three major constituent fatty acids were palmitic acid, stearic acid, and oleic acid that are comparable to vegetable oils which can be used as biodiesel feedstock [9].

Lipid production from yeast fermentation produces CO_2 which can be provided for photosynthetic microalgae by using an integrated culture design that incorporates both CO_2 consumption and microbial oil production appear to be the best approach to enable industrial application of these new technologies for environmental benefit. Therefore, the objective of this work is to investigate the production of microbial lipid by photosynthetic microalgae *Chlorella* sp. KKU-S2 and oleaginous yeast *T. maleeae* Y30 via integrated technique of photosynthesis and fermentation.

II. Materials and Methods

A. Microalgae and Culture conditions

Chlorella sp. KKU-S2 was isolated from freshwater taken from pond in the area of Khon Kaen province, Northeastern of Thailand [6]. The seed culture was pre-cultivated onto the basal Bristol medium at room temperature for 3 days and continuous illuminated from overhead by using 80W cool-white fluorescent lamps. The basal Bristol medium was consisted of (mg/L): $NaNO_3$ 250, K_2HPO_4 75, KH_2PO_4 175, $CaCl_2$ 25, NaCl 25, $MgSO_4.7H_2O$ 75, and FeCl2 0.3, $MnSO_4.2H_2O$ 0.3, $ZnSO_4$ $7H_2O$ 0.2, H_3BO_3 0.2, $CuSO_4.5H_2O$ 0.06, and pH was adjusted to 7.0 before sterilization.

B. Yeast Strain and Culture Conditions

Torulaspora maleeae Y30 used in this study was isolated from soil samples taken from forest in the area of Chulabhorn Dam, Chaiyapoom Province Northeastern of Thailand [9]. *T. maleeae* Y30 was maintained on YM agar slant. The seed cultures were cultivated onto Lipid accumulation (LA) medium supplemented with 20g/L glucose at 30°C in an incubator shaker at a shaking speed of 150 rpm for 1 day. The LA medium was consisted of (g/L): $(NH_4)_2SO_4$ 0.1, KH_2PO_4 0.4, $MgSO_4.7H_2O$ 1.5, $ZnSO_4$ 0.0044, $CaCl_2$ 0.0025, $MnCl_2$ 0.0005, $CuSO_4$ 0.0003 and yeast extract 0.75 and pH was adjusted to 5.5 before sterilization.

C. Effect of Nitrogen Concentration on Growth and Lipid Production

Batch cultivations were performed in 4000mL Erlenmeyer flasks with a working volume of 2000mL of medium supplemented with different concentration of urea, flasks were inoculated with 10% (v/v) seed culture of microalgae and cultivated at ambient temperature (30°C) under continuous illumination by using 80W cool-white fluorescent lamps.

D. Integrated Cultivation Technique for Lipid Production

Microbial lipids production via integrated technique was performed by oleaginous yeast and microalgae. Cultivation of each strain was performed in 4000mL Erlenmeyer flask with a working volume of 2000mL. Yeast *T. maleeae* Y30 was cultivated onto LA medium (20g/L glucose) and microalgae *Chlorella* sp. KKU-S2 were cultivated onto Bristol medium with 10% (v/v) seed culture of each strain and cultivated at room temperature under continuous illumination by using 80W cool-white fluorescent lamps. The mixing of air and CO_2 from yeast fermentation was aerated during the cultivation. A schematic of a yeast fermentation flask connected to microalgae flask is shown in Fig. 1. The CO_2 produced by the yeast fermentation is split and connected directly into the surrounding microalgae flask and combined with ambient air for photosynthetic microalgae growth. To comparison of growth and lipid production, cultivation of microalgae was carried out with ambient air aerated but without the addition of CO_2 emissions from yeast fermentation.

E. Analytical Methods

The biomass concentration was determined by measuring the optical density of samples at 680 nm wavelength (OD_{680}) in a Spectrophotometer and comparing these values with prepared standard calibration curves of optical density versus dry biomass weight of microalgae strain.

The culture broth (5 mL) was centrifuged at 5,000 rpm for 5 min. Harvested biomass was washed twice with 5mL of distilled water. Duplicate samples of harvested biomass were analyzed for lipid yield. The total lipids were determined by the modified method of Know and Rhee (1986) with modifications [10]. Lipid content was expressed as gram lipid per gram dry biomass.

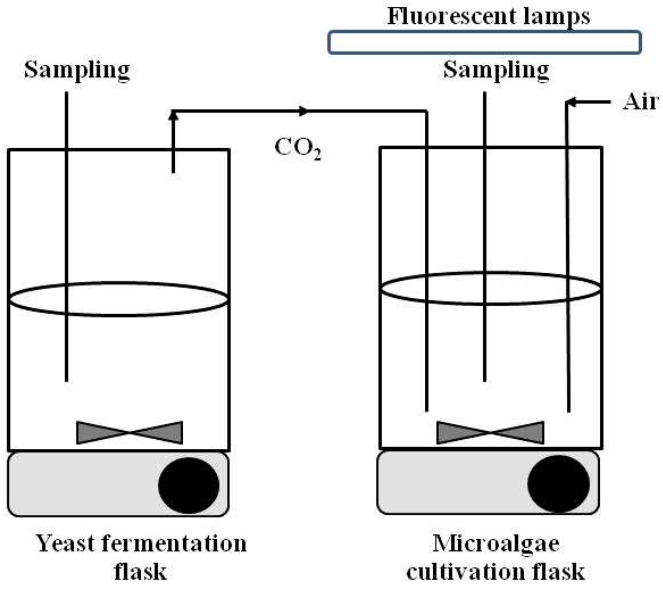

Fig. 1 Simplified schematic of yeast fermentation and photosynthetic microalgae cultivation for microbial lipid production, cultivated at ambient temperature under continuous illuminated with 80W cool-white fluorescent lamps

F. Determination of Growth Kinetic

Volumetric lipid production rate (Q_P, g/L/d) was determined from a plot between lipids (g/L) and fermentation time, specific product yield ($Y_{P/X}$, g lipid/g cell) was determined using relationship dP/dX, Volumetric cell mass production rate (Q_X, g/L/d) was determined from a plot of dry cells (g/L) versus time of fermentation (d). The specific growth rate (μ) of each strain was calculated from the slope of the linear regression of time (days) and dry biomass according to the equation: $\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$, where X_2 and X_1 are the biomass dry cell weight concentration (g/L) at time t_2 and t_1, respectively, while specific rate of lipid production (q_P, g lipid /g cells/d) was a multiple of μ and $Y_{P/X}$ [11, 12].

III. Results and Discussion

A. Effect of Nitrogen Concentration on Growth and Lipid Production

There was a correlation between the concentration of cell dry weight (g/L) and the optical density at 680nm (OD_{680}) for photoautotrophic cultured of *Chlorella* sp. KKU-S2. The following regression equation, y = 1.5343x, (R^2= 0.977) was obtained from the measurements, where y is the cell dry weight and x is OD_{680}.

As a preliminary step, photoautotrophic growth of microalgae was investigated for studying the effect of organic nitrogen concentration on growth and lipid production. When using different nitrogen concentrations, $NaNO_3$ was removed from the basal Bristol medium and replaced by organic nitrogen source urea. The urea concentrations of 5, 10 and 15 g/L were used as the initial nitrogen source to investigate the effects on cell growth and lipid yield.

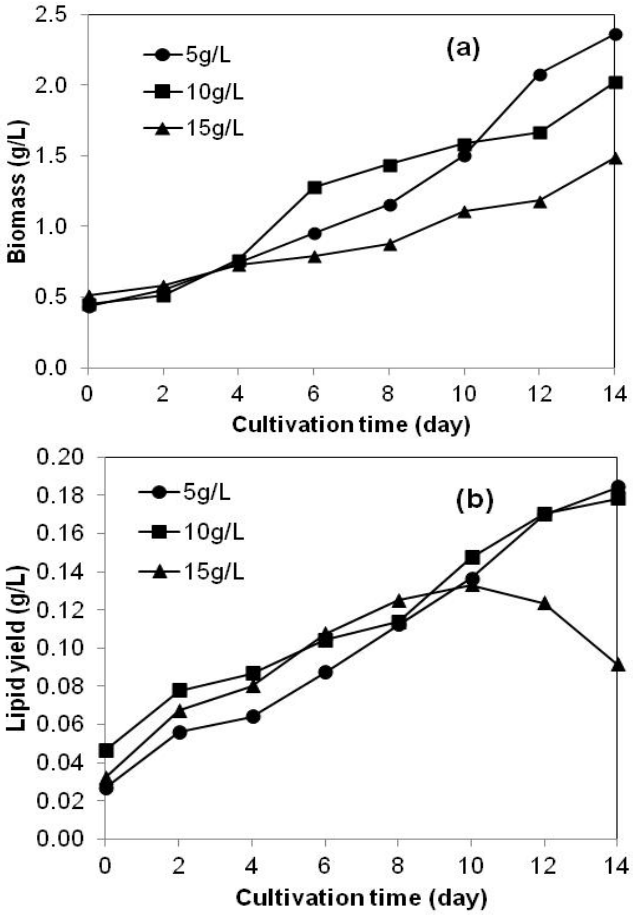

Fig. 2 Biomass concentration (a), lipid yield (b) of *Chlorella* sp. KKU-S2 on Bristol medium supplemented with different nitrogen concentration under photoautotrophic cultivation

Biomass and lipid yield of *Chlorella* sp. KKU-S2 with time in batch cultivation are presented in Fig. 2 and Table 1. Growth on different concentration of urea resulted in a significant effect on cell biomass and lipid yield. A maximum specific growth rate obtained was 0.109 (1/d) when initial urea concentration was 5g/L. A maximum biomass of 2.36g/L with lipid yield of 0.184g/L was obtained by cultivation with an initial urea concentration of 5g/L. *Chlorella* sp. KKU-S2 showed low growth when cultured with an initial urea concentration of 15g/L with a biomass of 1.489g/L with specific growth rate (μ) of 0.091 (1/d). There are no significant different of volumetric lipid production rate (Q_P) and specific rate of lipid production (q_P) by cultivation with an initial urea concentration of 5g/L and 10 g/L.

TABLE I
EFFECT OF UREA CONCENTRATION ON GROWTH KINETIC PARAMETERS OF *CHLORELLA* SP. KKU-S2 UNDER PHOTOAUTOTROPHIC CULTIVATION AT AMBIENT TEMPERATURE

Kinetic parameters	Urea concentration (g/L)		
	5	10	15
Biomass (X, g/L)	2.361	2.023	1.489
Lipid yield (P, g/L)	0.184	0.171	0.091
μ (1/d)	0.109	0.104	0.073
Q_P	0.013	0.012	0.007
Q_X	0.169	0.144	0.106
$Y_{P/X}$	0.078	0.084	0.061
q_P	0.009	0.009	0.004

B. Microbial Lipid Production by an Integrated Cultivation of yeast and microalgae

Batch cultures were investigated to improve the suitable cultivation technique for growth and lipid production from yeast *T. maleeae* Y30 and photoautotrophic microalgae *Chlorella* sp. KKU-S2 (Fig. 1). Time course of cell growth of yeast *T. maleeae* Y30 was presented in Fig. 3.

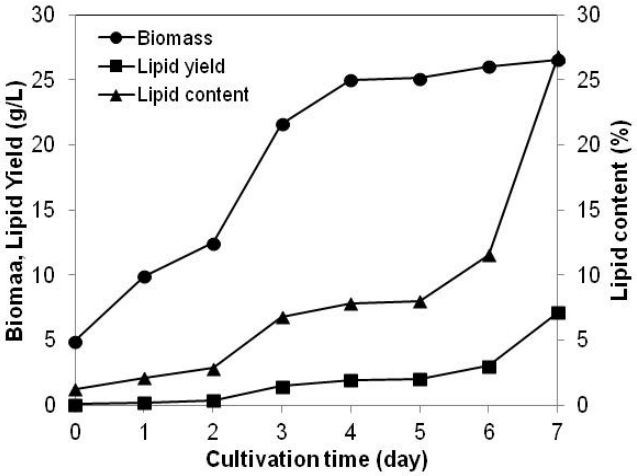

Fig. 3 Time course of cell growth of *T. maleeae* Y30 on LA medium using glucose as carbon source, cultivated at ambient temperature for 7 days

After cultivation for 7 days, a biomass of yeast *T. maleeae* Y30 and lipid yield reached the maximum of 23.63 g/L and 7.06 g/L were obtained, respectively. Cellular lipid content of 26.8% was obtained. Waste CO_2 produced by the fermentation of yeast *T. maleeae* Y30 during lipid production, is connected directly into the surrounding microalgae flask and combined with ambient air for photosynthetic microalgae *Chlorella* sp. KKU-S2 growth. As shown in Fig. 4, there are significant

different of optical density (OD_{680}) changes observed in the growth of microalgae during cell growth using different sources of CO_2, higher value of OD_{680} of 1.27 was obtained by cultivation of microalgae by using CO_2 from air mixing with CO_2 emissions from yeast fermentation for 7 days than that of the cultivation by using CO_2 from air. The OD_{680} of 0.913 was obtained by using CO_2 from air.

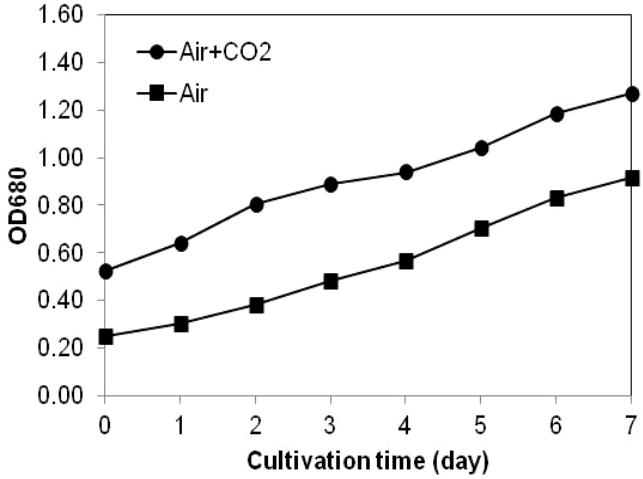

Fig. 4 Optical density (OD_{680}) of *Chlorella* sp. KKU-S2 under photoautotrophic cultivation by using CO_2 coupled with CO_2 emissions from yeast fermentation (Air +CO_2) and CO_2 from air (Air)

A maximum biomass of 8.44g/L was obtained by cultivation using CO_2 from ambient air and CO_2 emissions from yeast fermentation (Air+CO_2) after 7 days of cultivation, while a biomass of 6.34g/L was found when *Chlorella* sp. KKU-S2 was cultivated using CO_2 from air (Fig. 5 and Table 2). A maximum lipid yield of 1.339g/L was found after cultivation for 5 days by using mixing of air and CO_2 emissions from yeast fermentation, while 0.969g/L of lipid yield was obtained after day 6 of cultivation by using CO_2 from air. There are significant different of volumetric lipid production rate (Q_P), specific product yield ($Y_{P/X}$), volumetric cell mass production rate (Q_X) and specific rate of lipid production (q_P) by using different source of CO_2. A high value of all parameters was found when using CO_2 from mixing air coupled with CO_2 emissions from yeast fermentation for supported the growth and lipid production of microalgae *Chlorella* sp. KKU-S2. *Nannochloropsis oculata* exhibited increases in biomass and lipid content when the CO_2 concentration supplied was increased [13]. Similarly, *Scenedesmus obliquus* and *Chlorella kessleri* showed a particularly high potential for bio-fixation of CO_2 [14]. When oleaginous organisms are grown with an excess of carbon and limited quantity of nitrogen, they may accumulate high concentration of cellular lipid. Cultivation of oleaginous microorganisms with low nitrogen in the medium, results to the decrease of the activity of nicotinamide adenine dinucleotide isocitrate dehydrogenase (NADIDH) then the tricarboxylic acid cycle is repressed, metabolism pathway altered and protein synthesis stopped and lipid accumulation is activated [15, 16].

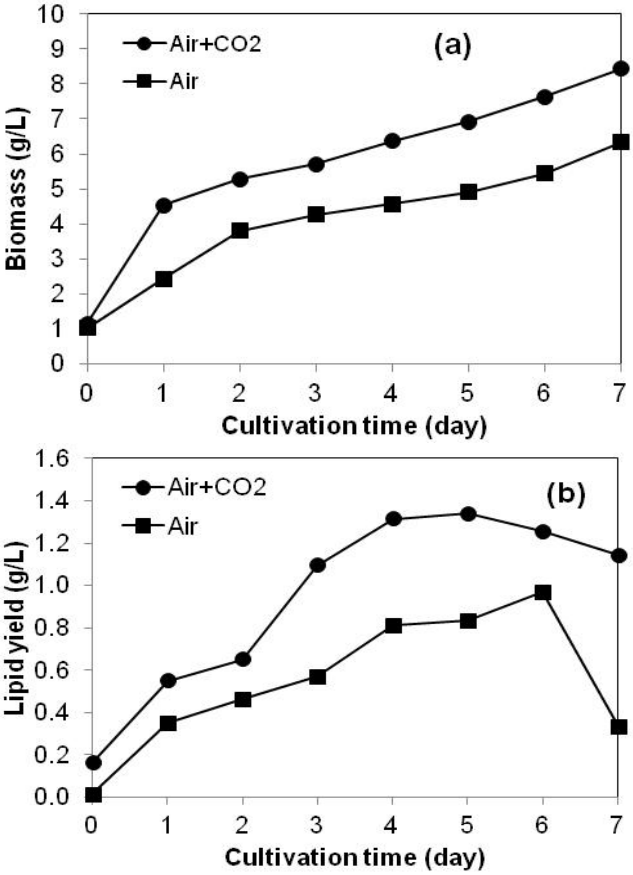

Fig. 5 Biomass (a) and lipid yield (b) of *Chlorella* sp. KKU-S2 under photoautotrophic cultivation by using CO_2 coupled with CO_2 emissions from yeast fermentation (Air +CO_2) and CO_2 from air (Air)

In case of integrated cultivation process, overall lipid yield of 8.33 g/L was obtained (1.339 g/L of *Chlorella* sp. KKU-S2 and 7.06g/L of *T. maleeae* Y30) while only 0.969g/L of lipid yield was found from *Chlorella* sp. KKU-S2 using non-integrated cultivation technique. The integration of the photoautotrophic microalgae cultivation systems into an existing yeast fermentation system is made economically feasible by the generation of two new revenue streams:

TABLE II
EFFECT OF CO_2 ON GROWTH KINETIC PARAMETERS OF *CHLORELLA* SP. KKU-S2 UNDER PHOTOAUTOTROPHIC CULTIVATION AT AMBIENT TEMPERATURE

Kinetic parameters	Culture conditions	
	Air + CO_2 emissions[1]	Air[2]
Biomass (X, g/L)	6.920	5.447
Lipid yield (P, g/L)	1.339	0.969
μ (1/d)	0.284	0.239
Q_P	0.223	0.194
Q_X	1.153	1.089
$Y_{P/X}$	0.194	0.178
q_P	0.055	0.042

[1] Cultivation time for 5 days, [2] Cultivation time for 6 days

microbial lipid from microalgae and oleaginous yeast for used as potential feedstock for biodiesel production and the capture of CO_2 emissions from the yeast fermentation stage [4, 5].

In conclusion, we present a cultivation technique for the integrated growth and lipid production of yeast and microalgae. To our knowledge this is the unique report about the microbial lipid production from locally photoautotrophic microalgae *Chlorella* sp. KKU-S2 and oleaginous yeast *T. maleeae* Y30 in an integrated technique to improve the biomass and lipid yield using CO_2 emissions from yeast fermentation resulted to reduce cultivation costs and also remove and value-added of CO_2, greenhouse gas, this process could be so called that environmental friendly process. This cultivation method will open new perspectives in the production of microbial lipid which could be used as potential feedstock for biodiesel production. In further works, increasing of microalgal biomass and lipid yield will be investigated in a 20L photobioreactor via integrated cultivation technique of photoautotrophic microalgae by using CO_2 emissions from yeast fermentation and photoautotrophic cultivation by using pure CO_2 or CO_2 from flue gases and then completed with the biodiesel production from microbial lipid via direct and indirect transesterification methods.

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