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Microalgal production under mixotrophic conditions using cheese whey as substrate

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ABSTRACT. Microalgae are known for producing various biotechnological products. Moreover, they absorb nutrients from dairy wastewater, grow well, and accumulate valuable compounds faster. In this study, photoautotrophic and mixotrophic cultivation with different initial lactose concentrations present in cheese whey (CW) were established to investigate their effect on cell concentration (X_m, mg L^{-1}), cell productivity (P_x, mg $L^{-1}day^{-1}$), and specific cell growth (μ_{max}, day^{-1}) of *Chlorella vulgaris*, *Dunaliella tertiolecta,* and *Tetradesmus obliquus*. The biomass production of *C. vulgaris* (X_m= 1,520 ± 30.3 mg L^{-1}, Px = 147 ± 3.00 mg L^{-1}, and μ_{max} = 0.150 ± 0.00 mg L^{-1}) in mixotrophic culture with 10.0 g L^{-1} of lactose, the main constituent of CW, was notably enhanced by 55% in comparison with their photoautotrophic cultures, whereas a lower effect of these lactose concentrations on cell growth was observed in *T. obliquus* and *D. tertiolecta*. Thus, mixotrophic cultivation of *C. vulgaris* using CW as a carbon and energy source could be considered a feasible alternative to obtain high value-added biomass.

Keywords: dairy waste; by-product; lactose, Chlorophyceae.

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Introduction

Microalgae have been utilized in the formulation of feed, food, and cosmetics, as well as in bioremediation, biofertilizers, pharmaceuticals, and biofuel production, of which *Chlorella* spp. and *Dunaliella* spp. are the most widely exploited (Ahmad, Shariff, Yusoff, Goh, & Banerjee, 2018; Pourkarimi, Hallajisani, Alizadehdakhel, Nouralishahi, & Golzary, 2020; Silva et al., 2021a; Silva et al., 2021b; Moura et al., 2021; Moshood, Nawanir, & Mahmud, 2021). According to Tsolcha et al. (2016), the use of microalgae is more environmentally sustainable because it can capture CO_2 (greenhouse gas), and recycle nutrients more efficiently than terrestrial plants.

Currently, photoautotrophic cultivation (CO_2, with light) is the most common strategy for large-scale microalgae cultivation; however, this process has some drawbacks, such as longtime cultivation and low cell productivity due to cell self-shading towards the end of growth (Bezerra, Matsudo, Sato, Converti & Carvalho, 2013; Zanette, Mariano, Yukawa, Mendes, & Spier, 2019). As an alternative, mixotrophic growth overcomes these drawbacks by offering short cultivation periods with higher growth rates, utilizing photosynthesis and/or respiration metabolism pathways based on light and/or organic matter availability, reducing the irradiance requirement, and decreasing photolimitation by self-shading cells (Liu & Ma, 2009; Bezerra, Matsudo, Pérez Mora, Sato & Carvalho, 2014; Perez-Garcia & Bashan, 2015).

Melo et al. (2018) and Silva et al. (2017) showed that microalgae growth can be enhanced by utilizing free or inexpensive carbon organic substrates in mixotrophic or heterotrophic conditions. The use of wastewater in microalgae cultivation has gained considerable attention because it salvages unused nutrients, reduces freshwater demand, and avoids or reduces wastewater treatment costs, making it a green production system with simultaneous production of biomass or other value-added products (Zanette et al., 2019; Vidya et al., 2021). Agro-industrial by-products or wastewater can be used as a sustainable source to improve cell productivity and reduce production costs and pollutants discharged in the environment (Melo et al, 2018; Markou, Wang, Ye, & Unc, 2019).

For example, dairy wastewater has been used as an energy and carbon source under mixotrophic conditions for the growth of some microalgae species, such as *Chlorella vulgaris, Chlorella protothecoides, Chlorella* sp.*, Chlamydomonas polypyrenoideum, Chroococcus* sp.*, Coelastrella saipanensis, Haematococcus pluvialis, Scenedesmus obliquus, Dunaliella* sp.*,* and *Arthrospira platensis* (Abreu, Fernandes, Vicente, Teixeira & Dragone, 2012; Kothari, Prasad, Kumar, & Singh, 2013; Girard et al., 2014; Vieira Salla et al. 2016; Melo et al., 2018; Patel, Joun, Hong, & Sim, 2019; Vidya et al., 2021). Other microalgae species are obligate phototrophs owing to the lack of efficient sugar uptake mechanism or an incomplete tricarboxylic acid cycle for efficient absorption of organic carbon sources (Chen & Chen, 2006).

Cheese whey (CW) is a liquid by-product of the dairy industry that contains 66-77% (w w^{-1}) lactose, 8-15% (w w^{-1}) proteins (e.g., ß-lactoglobulin and α-lactalbulmin), 7-15% (w w^{-1}) minerals (e.g., calcium and phosphorus), and vitamins (e.g., vitamins A, D, and B5) (Yadav et al., 2015; Fernández-Gutiérrez et al., 2017; Irkin, 2019). Lactose is the main component of CW and therefore results in a high chemical oxygen demand (COD) of 80-40 g L^{-1} and biochemical oxygen demand (BOD) of 30-50 g L^{-1} (Abreu et al., 2012; Malhotra & Trivedi, 2016). Their high organic content makes it difficult to biodegrade and can be of concern to the environment if disposed incorrectly. Cheese production tends to increase and requires a correct destination before being discarded in rivers (Lopes et al., 2019).

Few studies have investigated the effects of CW on microalgae cultivation under mixotrophic conditions, especially on *D. tertiolecta* and *T. obliquus*. Therefore, the aim of this study was to evaluate the growth profile and photosynthetic efficiency of microalgae *C. vulgaris*, *D. tertiolecta*, and *T. obliquus* in photoautotrophic and mixotrophic cultures supplemented with CW, providing integrated microalgae production for the dairy product industry.

Material and methods

Microorganisms and culture conditions

Chlorella vulgaris (UTEX 1803) and *D. tertiolecta* (UTEX LB999) were obtained from the University of Texas (Austin, Texas, USA), while *T.* (*Scenedesmus*) *obliquus* (A5F5402) was isolated from the Weir of Apipucos (Recife, Pernambuco, Brazil) (Silva et al., 2019). Microalgal cultivation was conducted under photoautotrophic and mixotrophic conditions. In photoautotrophic cultivation, *C. vulgaris*, *D. tertiolecta,* and *T. obliquus* were maintained and cultivated in standard basal medium (Bischoff & Bold, 1963), F/2 medium (Guillard & Ryther, 1962), and BG-11 medium (Stanier, Kunisawa, Mandel, & Cohen-Bazire, 1971), respectively. In mixotrophic cultivation, CW (g L^{-1} lactose) supplied by a cheese factory from Nazaré da Mata, Pernambuco, Brazil, was deproteinized via heat treatment (Dragone, Mussatto, Almeida e Silva, & Teixeira, 2011) and then supplemented in three different concentrations (2.5, 5.0, and 10.0 g L^{-1}) (Abreu et al., 2012) in each standard medium. All cultivations were conducted in 1 L Erlenmeyer flasks with 400 mL containing the medium and inoculum, with initial biomass concentration of 50 mg L^{-1}, and incubated at 28 ± 1°C under constant aeration pumped with air compressors. A 0.2 µm filter was used to prevent culture contamination. Light intensity, provided by cool white fluorescent lamps, were set at 52 ± 5 µmol photons m^{-2} s^{-1} for *C. vulgaris* and *D. tertiolecta* and 28 ± 1 µmol photons m^{-2} s^{-1} for *T. obliquus*. Photoautotrophic control cultures were grown under identical conditions, except for the absence of CW in the culture medium. The cultivation ended when the late exponential growth phase was reached. All experiments were performed in triplicate.

Determination of microalgal cell concentration and lactose concentration

Chlorella vulgaris (UTEX 1803), *D. tertiolecta* (UTEX LB999), and *T. obliquus* (A5F5402) cell concentrations were determined by measuring the optical density at 685 nm (Xu, Qian, Chen, Jiang, & Fu, 2010), 680 nm (Chen et al., 2011) and 650 nm (Xin, Hong-Ying, Ke, & Jia, 2010), respectively, using a previously calibrated curve relating OD to dry biomass weight.

The concentration of lactose in CW was quantified using a high-performance liquid chromatography system: Shimadzu chromatograph model SCL-10A with a UV-VIS detector (model SPD-M10A) and a reversed-phase column (C18; 5 µm i.d., 4 × 250 mm; Supelco). Ultrapure water was used as the eluent at an isocratic flow rate of 1.0 mL $min.^{-1}$. The injection volume was 50 µL, at a column temperature of 82°C

and running time of 35 min., according to Erich, Anzmann, and Fischer (2012). Lactose was identified via the retention time and quantified via the peak area in the samples, in comparison with an external standard of lactose (Sigma Aldrich).

Biomass productivity (P_x)

P_x (mg L^{-1} day^{-1}) was calculated using Equation 1 (Patel et al., 2019):

$$P_x = \frac{(X_t - X_0)}{(t_x - t_0)} \quad (1),$$

where X_t is the biomass concentration (mg L^{-1}) at the end of the exponential growth phase (t_x), and X_0 is the initial biomass concentration (mg L^{-1}) at t_0 (day).

Determination of specific growth rate

The specific growth rate (μ_{max}, day^{-1}) was calculated using Equation 2 (Leduy & Sajic, 1973):

$$\mu = \frac{(lnN_2 - lnN_1)}{(t_2 - t_1)} \quad (2),$$

where N_1 and N_2 are the cell concentration at the beginning (t_1) and end (t_2) of the exponential growth phase, respectively.

Statistical analysis

Data represent the mean ± standard deviation (SD) of different assays. Statistical significance was determined using one-way analysis of variance, followed by Tukey's test at a 5% significance level. STATISTICA software (version 5.5, 1999 Edition; Statsoft Inc., Tulsa, OK, USA) was used for all statistical analyses.

Results and discussion

The growth of *C. vulgaris*, *D. tertiolecta*, and *T. obliquus* were evaluated under photoautotrophic and mixotrophic cultivation conditions at different initial lactose concentrations in CW. The cell growth profiles at different lactose concentrations in CW are shown in Figure 1 (A, B and C). The growth of all strains improved under mixotrophic conditions. These results are consistent with those of other studies on *C. vulgaris*, *T. obliquus*, *D. tertiolecta*, *Chlorella minutissima*, and *Nannochloropsis oculata* using dairy waste or pure lactose, which showed higher biomass production and growth rates than photoautotrophic cultures (Girard et al., 2014; Patel et al., 2019; Zanette et al., 2019).

In general, no lag phase was observed in all cultures, and the exponential growth phase was shorter in *D. tertiolecta* (5-7 days, Figure 1C) compared *C. vulgaris* and *T. obliquus* (8-10 days, Figure 1A and B). On the other hand, other microalgae, such as *C. pyrenoidosa* cultivated in pretreated whey, had a low cell growth rate in the beginning and improved after acclimatization, indicating a probable cell adaptation to the specific growth environment (Patel et al., 2019). *C. vulgaris* cultivation reached the highest cell concentration (~1500 mg L^{-1}) after 10 days, followed by *T. obliquus* (~1300 mg L^{-1}) after 8 days, and *D. tertiolecta* (~700 mg L^{-1}) after 5 days, all under mixotrophic conditions. This suggests the potential for these microalgae to be cultured in the presence of lactose as a carbon source.

All mixotrophic cultures of *C. vulgaris* showed significant differences from the photoautotrophic cultures, exhibiting rapid growth in response to an increase in lactose concentrations (Figure 1A). The mixotrophic conditions at 10 g L^{-1} of lactose resulted in a higher cell concentration (X_m of 1,520 ± 30.3 mg L^{-1}) and cell productivity (P_x of 147 ± 3.00 mg L^{-1}) ($p < 0.0001$), although the μ_{max} values were low and statistically different from the photoautotrophic conditions (Table 1).

Under mixotrophic conditions a longer cultivation time resulted in higher cell concentration values. This growth profile was similar to that reported by Abreu et al. (2012), who also used CW for *C. vulgaris* growth and observed a slightly higher X_m value when *C. vulgaris* was cultivated with 10 g L^{-1} of lactose (X_m of 1,980 ± 0.43 mg L^{-1}, P_x = 320 ± 0.13 mg $L^{-1}day^{-1}$) compared to those observed in the present study (Table 1). In addition, Patel et al. (2019) reported that both pretreated and non-pretreated (raw and hydrolyzed) whey do not contain any inhibitory component, since *C. protothecoides* yield was directly proportional to the increasing whey fraction.

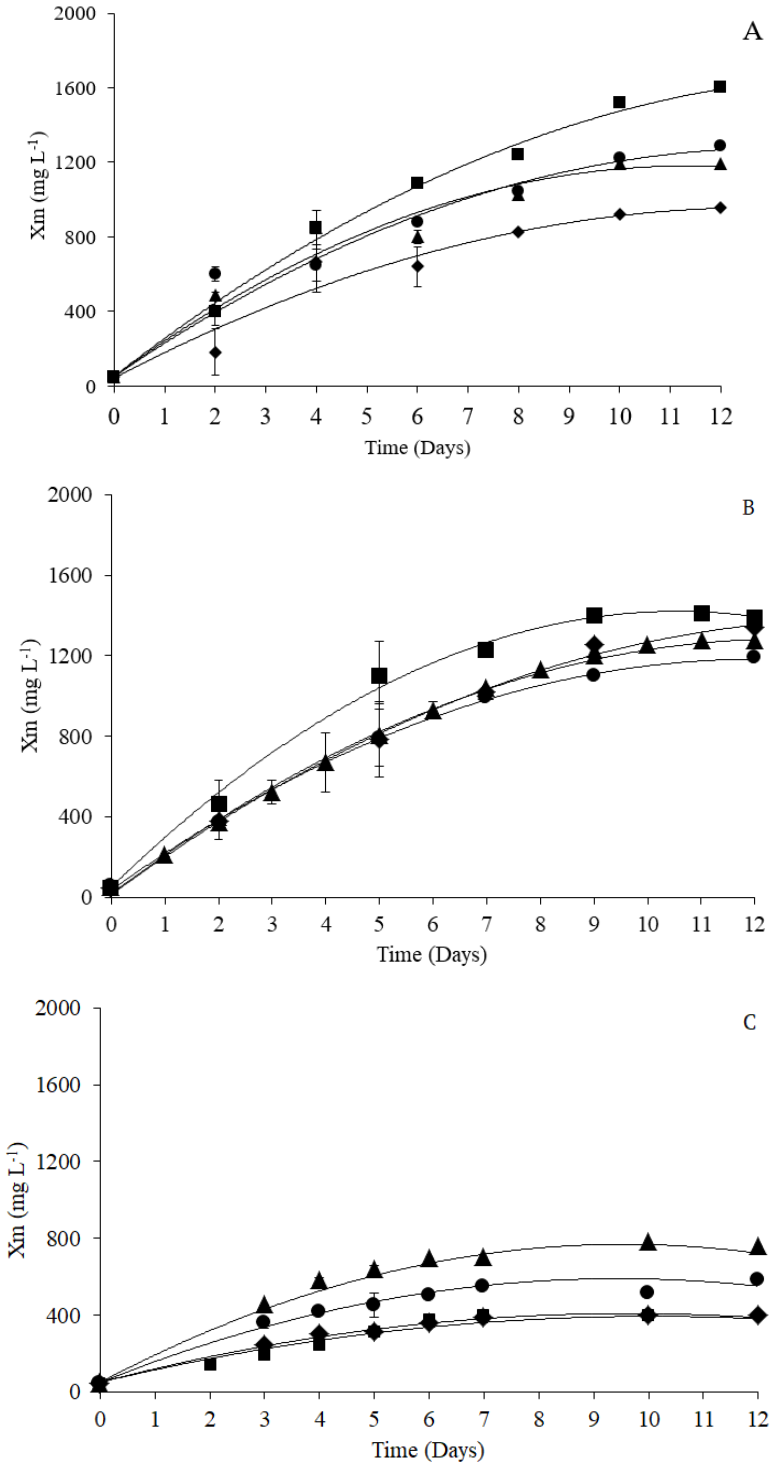

Figure 1. Growth curve of (A) *Chlorella vulgaris*, (B) *Tetradesmus obliquus*, (C) *Dunaliella tertiolecta* grown on photoautotrophic (♦) and mixotrophic conditions supplemented with cheese whey at concentrations of 2.5 g L^{-1} (▲), 5 g L^{-1}(·), 10 g L^{-1} (■). The error bars represent the standard deviations (n = 3).

In *T. obliquus* cultivation, no significant differences in the Xm, Px, and µmax values were observed between the photoautotrophic and lactose-supplemented cultures at 2.5 and 5.0 g L-1 (Table 1). Moreover, these results clearly show that the presence of 10 g L-1 lactose induced rapid *T. obliquus* growth until day 7, after which it considerably slowed down, similar to a stationary growth phase (Figure 1B, Table 1). In these conditions, *T. obliquus* obtained the highest values of Xm (1,315±18.5 mg L-1), Px (158.0 ± 1.8 mg L-1day-1), and μmax (0.182 ± 0.05 day-1) (Table 1). These results are consistent with other results found on *T. obliquus* in mixotrophic conditions, wherein 40% (v v-1) of the culture medium was substituted with CW permeate, and the highest biomass yield was obtained in mixotrophic conditions (3.6 ± 0.4 mg L-1 versus 2.7 ± 0.2 mg

L-1 for heterotrophic cultures) after 13 days (Girard et al., 2014). Furthermore, the use of 40 g L-1 of pure lactose in *T. obliquus* cultures showed higher specific productivity when compared to heterotrophic conditions at day 8 (Bentahar, Doyen, Beaulien & Deschênes, 2018).

Table 1. Growth parameters of *Chlorella vulgaris*, *Dunaliella tertiolecta* and *Tetradesmus obliquus* cultivated under photoautotrophic and mixotrophic conditions.

Species	Growth parameters				
	Culture Medium	X_m (mg L^{-1})	Tc (days)	μ_{max} (day^{-1})	P_x (mg $L^{-1}day^{-1}$)
C. vulgaris	Photoautotrophic	827.9 ± 163.3^{a,A}	8	0.154 ± 0.001^{a,A}	97.4 ± 16.3^{a,A}
	Mixotrophic (2.5 g L^{-1})	1,195 ± 43.77^{b,A}	10	0.174 ± 0.001^{b,A}	115 ± 2.79^{a,A}
	Mixotrophic (5 g L^{-1})	1,225 ± 131.3^{b,A}	10	0.174 ± 0.002^{b,A}	124 ± 5.93^{b,A}
	Mixotrophic (10 g L^{-1})	1,520 ± 30.3^{c,A}	10	0.150 ± 0.001^{c,A}	147 ± 3.00^{c,A}
T. obliquus	Photoautotrophic	1,255 ± 92.2 ^{a,B}	9	0.159 ± 0.007^{a,B}	131.0 ± 7.6^{a,B}
	Mixotrophic (2.5 g L^{-1})	1,224 ± 146.2^{a,A}	9	0.153 ± 0.005^{a,B}	127.9 ± 3.21 ^{a,B}
	Mixotrophic (5 g L^{-1})	1,104 ± 187.9 ^{a,B}	9	0.148 ± 0.008 ^{a,B}	116.6 ± 14.4 ^{a,A}
	Mixotrophic (10 g L^{-1})	1,315 ± 18.5^{b,B}	8	0.182 ± 0.005^{b,B}	158.0 ± 1.8^{b,B}
D. tertiolecta	Photoautotrophic	318.0 ± 19.2^{a,C}	5	0.161 ± 0.001^{a,B}	53. 6 ± 2.70^{a,C}
	Mixotrophic (2.5 g L^{-1})	700.0 ± 1.92^{b,B}	7	0.164 ± 0.004^{a,C}	92.8 ± 2.40^{b,C}
	Mixotrophic (5 g L^{-1})	549.0 ± 9.64^{c,C}	7	0.167 ± 0.002^{b,C}	71.4 ± 3.42^{c,C}
	Mixotrophic (10 g L^{-1})	311.6 ± 21.a,^{C}	5	0.159 ± 0.002^{a,C}	52.3 ± 2.14^{a,C}

Data are expressed as mean ± SD. Tukey test was performed. Means in the same column followed by different letters represent significant differences ($p < 0.05$). Lowercase letter compares among treatments and uppercase letter compares among species. * X_m = Maximum biomass concentration. TC = Cultivation time. ** μ = Specific growth rate during exponential growth phase. *** Px = Biomass productivity.

An increase in *D. tertiolecta* growth was observed with the addition of CW of up to 2.5 g L^{-1} of lactose (Table 1), leading to important growth stimulation and reaching the stationary phase more rapidly with higher X_m (700.0±1.92 mg L^{-1}) and P_x (92.8 ± 2.40 mg $L^{-1}day^{-1}$) values than those in other cultures (Figure 1C). In photoautotrophic and mixotrophic cultures with 10 g L^{-1} of lactose, the X_m was reached at day 5 (318.0±19.2 and 311.6±21.8 mg L^{-1} respectively), while X_m was observed at day 7 for mixotrophic cultures at 2.5 g L^{-1} of lactose (700.0±1.92 mg L^{-1}) and 5.0 g L^{-1} of lactose (549.0±9.64 mg L^{-1}). The higher initial lactose concentration (10 g L^{-1}) in CW prompted a significantly shorter log phase, possibly due to repression of the chlorophyll in the presence of glucose, as reported in the red alga *Galdieria partita* (Stadnichuk et al., 1998). However, lactose concentration above 2.5 g L^{-1} did not support cell growth and presumably could not be used to enhance the cell concentration of *D. tertiolecta* (Figure 1C). Similar results were reported by Velu, Peter, and Sanniyasi (2015) using *D. tertiolecta* and lactose (10 g L^{-1}) as a carbon source, and they observed no difference in the maximum growth rate in mixotrophic and photoautotrophic cultivation.

The mixotrophic growth of some microalgae significantly improves cell concentration, growth rate, and cell productivity, thus decreasing production costs and providing opportunities to recycle nutrients present in food wastewater effluents (Melo et al., 2018; Patel et al., 2019). CW has already been reported as an excellent carbon source for microalgae mixotrophic cultivation, mainly *Chlorella* sp., *Scenedesmus* sp., and *Dunaliella* sp., the microalgae most studied for growth in pretreated dairy effluents (Girard, 2014, Patel et al., 2019; Zanette et al., 2019). Thus, Whangchai et al. (2021) claimed that mixotrophic cultures are less susceptible to photoinhibition because of their capability to use greater light energy and increased saturation limit for photosynthesis mixotrophic cultures.

CW is mainly composed of lactose, which can easily support and/or stimulate the growth of some microalgae after hydrolysis. Lactose can be used as an organic carbon source for *C. vulgaris* and *T. obliquus* growth, but concentration above 2.5 g L^{-1} cannot be effectively used for *D. tertiolecta* growth. In addition, the cell concentration of *D. tertiolecta* was considerably lower than that of other microalgae using the same lactose concentrations as organic carbon sources. Previous studies have shown that *C. minutissima*, *N. oculata*, *Scenedesmus* sp., and *D. tertiolecta* exhibit β-galactosidase activity (Bentahar et al., 2018; Zanette et al., 2019) that yield glucose and galactose. Specific transmembrane transporters uptake these monosaccharides (Stadler, Wolf, Hilgarth, Tanner, & Sauer, 1995; Mandal & Mallick, 2009) that are useful in cell growth by oxidative carbon metabolism (Davies, Apte, Peterson, & Stauber, 1994; Zanette et al., 2019). Therefore, this result explains why cell growth was significantly higher in mixotrophic cultures than in photoautotrophic cultures, since the Calvin cycle (photosynthesis) and oxidative carbon metabolism occur simultaneously and independently in mixotrophic cultures (Marquez, Sasaki, Kakizono, Nishio, & Nagai, 1993; Choi, Patel, Hong, Chang, & Sim, 2019).

Conclusion

The present study investigated the possibility of microalgal biomass production in CW, a dairy industrial waste. *C. vulgaris* exhibited promising growth, while *T. obliquus* and *D. tertiolecta* growth were inhibited at higher lactose concentrations in CW. The addition of CW with 10 g L^{-1} lactose resulted in higher cell concentration and cell productivity under mixotrophic conditions than under photoautotrophic conditions in *C. vulgaris* cultures. The results show that CW utilization is a promising method for improving the microalgal biomass yield with wide biotechnological applications, including pharmaceutical, nutraceutical, and regenerative medicine, owing to bioactive molecules that may lead to the discovery of new drugs.

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