Enzymatic and non-enzymatic antioxidant potentials of *Chlorella vulgaris* grown in effluent of a confectionery industry

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Abstract Enzymatic and non-enzymatic antioxidant potentials of *Chlorella vulgaris* have gained considerable importance in recent decades. *C. vulgaris* strain highly tolerant to extreme pH variations was isolated and mass-cultivated in the wastewater from a confectionery industry. *C. vulgaris* showed better growth in wastewater than in improvised CFTRI medium. The microalgal biomass was then screened for the following antioxidants: peroxidase, superoxide dismutase, polyphenol oxidase, glutathione peroxidase, chlorophyll *a*, ascorbic acid, α -tocopherol and reduced glutathione. The total polyphenol content of the strain was also studied. The strain showed a high degree of enzymatic antioxidant activity ($0.195 \times 10^{-5} \pm 0.0072$ units/cell peroxidase, $0.04125 \times 10^{-5} \pm 0.001$ units/cell superoxide dismutase, $0.2625 \times 10^{-5} \pm 0.003$ units/cell polyphenol oxidase and $0.025 \times 10^{-5} \pm 0.003$ glutathione peroxidase). The microalgal biomass also showed, per milligram weight, 0.2182 ± 0.005 μg of ascorbic acid, 0.00264 ± 0.001 μg of α -tocopherol and 0.07916 ± 0.004 μg of reduced glutathione. These results represent the possibility of using *C. vulgaris* grown in confectionery industry wastewater as a source of nutritious supplement, which is highly promising in terms of both economic and nutritional point of view.

Keywords *Chlorella vulgaris* · Confectionery industry effluent · Enzymatic antioxidants · Non-enzymatic antioxidants

Introduction

Use of indigenous microalgal strains as food has been in practice for centuries (Spolaore et al. 2006). Indeed, edible blue-green algae including *Nostoc*, *Arthrospira* (*Spirulina*) and *Aphanizomenon* species have been used as food for thousands of years (Gitta et al. 2001). Microalgae such as *Anabaena cylindrica*, *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Dunaliella salina*, *Porphyridium cruentum*, *Scenedesmus obliquus*, *Spirulina maxima* and *Synechococcus sp.* contain high-value beneficiary nutritional contents. However, the cultivation of microalgae came into practice only a few decades ago (Borowitzka 1999a). In general, plants and microalgae are good sources of natural antioxidants (Rodriguez-Garcia and Guil-Guerrero 2008; Nasirullah and Rakshitha 2009); the antioxidants are produced as a protective mechanism against toxic reactive oxygen species (ROS). These pathways include the photo-respiratory pathway, enzymatic and non-enzymatic pathways, corresponding responsive gene regulation and anatomical ways (Bo-Shao et al. 2008).

During photosynthesis, microalgae absorb freely available solar light, which is converted into chemical energy, later used in the conversion of CO_2 into carbohydrates, and at the same time, generating molecular oxygen. Oxygen is then easily activated by general stress that includes drought, salinity, low temperature, UV-B and others into toxic reactive oxygen species (ROS). Plants and microalgae have developed a protective mechanism, which consists of the production of antioxidant compounds that are able to minimize the concentration of the ROS (Rodriguez-Garcia and Guil-Guerrero 2008). Natural bioactive compounds have the potential to alleviate the biochemical imbalances induced by various toxins associated with free

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radicals (Sachindra et al. 2010). They provide protection without causing any side-effects and therefore, natural antioxidants have been viewed as promising therapeutic agents (Anbuselvam et al. 2007). Microalgal biomass has appeared as a renewable energy alternative and a new dietary source. The biomass digestibility for most microalgae is high and so there is no limitation in using dried whole microalgae as food or feed. Microalgae also represent a valuable source of nearly all essential vitamins, some of which are antioxidants (Becker 1994); vitamins are per se essential, as are antioxidants, when used as food. The high protein content of various microalgal species is one of the main reasons to consider them as an unconventional source of protein (Cornet 1998). In addition, the amino acid pattern of microalgae is suitable for using them as food or feed. As the cells are capable of synthesizing almost all amino acids, they provide essential amino acids to humans and animals (Guil-Guerrero et al. 2004). Moreover, antioxidants are being explored more because of their tremendous therapeutic and pharmaceutical potentials (Borowitzka 1995).

Microalgae can be used to enhance the nutritional value of food and animal feed due to their chemical composition. In addition, they play a crucial role in aquaculture and they can be incorporated into cosmetics too (Spolaore et al. 2006). Among the microalgae, *Chlorella* contains a great variety of chemical components and is a good source of carbohydrates, proteins and vitamins (Matsukawa et al. 2000). Rodriguez-Garcia and Guil-Guerrero (2008) reported that the microalga *C.vulgaris* is a rich source of proteins, eight kinds of essential amino acids, vitamins (B-complex, ascorbic acid), minerals (potassium, sodium, magnesium, iron, and calcium), b-carotene, chlorophyll, “C.G.F.” (*Chlorella* growth factor) and other beneficial substances. The main concerns with respect to the cultivation of microalgae are the relatively low biomass yield and high production costs (Borowitzka 1999b). However, *Chlorella*, *Ankistrodesmus* and *Scenedesmus* species have been used to treat wastewater containing organic pollutants from pulp and paper mills and olive oil mills (Munoz and Guieysee 2006).

Studies have been conducted on microalgae for using them as food (Spolaore et al. 2006) but not much has been reported on antioxidant peptides derived from microalgae and information is scarce on the antioxidative peptides derived from microalgae. In this work, the microalgae *C.vulgaris* is studied as a natural source of antioxidants. *C.vulgaris* is characterized by its ability to grow in wastewater as reported by us recently (Hanumantha Rao et al. 2011). Although treating wastewater using *C.vulgaris* has been broadly studied (de-Bashan et al. 2002 ; Yang et al. 2008), none of the studies has been devoted to the optimization of the extraction of antioxidants from

wastewater-treated microalgae. In this sense, microalgae biomass generated in confectionery industry wastewater consisting of a wide range of organic materials was subjected to a quantitative screening of various enzymatic and non-enzymatic antioxidants. To our knowledge, this is the first study to investigate the antioxidant potentials of *C.vulgaris* cultivated in confectionery wastewater. The confectionery wastewater served as a cheap source of raw material for enhancing the growth of *C.vulgaris*, which might be used as a possible source for antioxidative substances for commercial or pharmaceutical purpose.

Materials and methods

Effluent characteristics and analysis

The raw effluent produced by a confectionery industry (multinational company), situated at Chennai, India is characterized by its organic content, which is composed of easily biodegradable compounds such as sugars, sweeteners, casein, vegetable oils, acacia gums, condensed milk, food colouring and flavouring agents, etc. This confectionery industry uses these ingredients as major raw materials for all types of confectionery products. They use a number of chemicals during the process and they all become a part of the effluent. This liquid effluent is acidic with a pH of 3.8.

Isolation and growth of *C.vulgaris* under *in vitro* conditions

The microalga, *C.vulgaris*, was isolated from confectionery wastewater, and cultured in CFTRI (Central Food Technological Research Institute) medium (Venkataraman and Becker, 1985). Microalgal cultures were examined under a microscope (Olympus HB) and identified according to the monograph of Philipose (Philipose and ICAR Monograph 1967). The taxonomical identification was confirmed by Prof. V. Krishnamurthy, Phycologist, Krishnamurthy Institute of Algology, Chennai, India. The axenic culture of the isolated microalga, *C.vulgaris*, was maintained in Improved CFTRI medium (Venkataraman and Becker 1985) for outdoor cultivation of microalgae. As already reported by us (Hanumantha Rao et al. 2011), *Chlorella* sp. grew well in that medium. Improved CFTRI medium (NaHCO₃, 4.0 g; NPK (15:15:15), 1.0 g; Super phosphate, 0.1 g; MgSO₄·7H₂O, 0.2 g; 1 L distilled water) (Venkataraman and Becker 1985) was used for culturing the microalga and the culture was incubated under laboratory conditions in an environmental chamber illuminated with cool white fluorescent light (3000 lux), in a 12 h light/12 h dark cycle at 24±1°C. Growth (divisions/day) was measured by counting cells using a haemocytometer (Neubauer, Germany).

Mass cultivation of *C. vulgaris* in confectionery wastewater using a sloping pilot plant

C. vulgaris was cultivated in the confectionery wastewater in a sloping pilot plant. Light intensity was measured using a lux meter (Lutron LX 101A). The pilot sloping pond was constructed with a dimension of 2 m (length)×2 m (width)×0.75 m (depth) with a sloping angle of 15°, with the sloping surface made of galvanized iron (GI) sheet. The dimension of the sloped area was 1.5 m². The sloping pilot pond was constructed to enhance sunlight exposure and aeration to the algal cells so as to drastically increase the growth of *C. vulgaris*. The principle of the sloping design is to create a turbulent flow while the algal suspension flows through the sloping surfaces or trenches present on the slope. A pump returns the algal medium from the lowest point to the top of the slope. The turbulence is produced by gravity, according to the flow speed of the liquid given by the slope of the surface (Becker 1994).

Biochemical assays

Extraction of crude biomass

For the extraction of crude biomass, 10 ml of *C. vulgaris* culture was retrieved from the pond, centrifuged at 2000× g for 10 min, and the pellet was purified by washing twice with all quartz double-distilled water. This sample was used for extracting different antioxidants, and the amounts of enzymatic and non-enzymatic antioxidants as well as other biochemical parameters were determined.

Extraction and estimation of chlorophyll 'a' and biochemical studies

Microalgal cells were pelleted by centrifugation at 2000× g for 10 min. To the pellet, 10 ml of 90% acetone was added and sonicated for 15 min using an ultrasonic processor (Vibronics P2). After extraction, it was again centrifuged at 2000× g for 15 min and the clear supernatant was used for the estimation of chlorophyll pigments using a double-beam UV-visible spectrophotometer (Shimadzu UV - 1650 PC, Japan) (Jeffrey and Humphrey 1975). The biochemical estimation of algae biomass was performed, total carbohydrates by Anthrone method of Pons et al. (1981), total protein according to Lowry et al. (1951) and total lipid content according to Bligh and Dyer (1959).

Extraction and estimation of enzymatic antioxidant activity

The enzyme peroxidase was estimated by purpurogalli method, i.e. extracted using 0.1 M phosphate buffer (pH 6.5) in a homogenizer and peroxidase activity was measured

(Reddy et al. 1985). Superoxide dismutase activity was measured by incubating the microalgae biomass extract containing 300 µl of each of 50 mM potassium phosphate buffer (pH 6.5), 45 mM methionine, 5.3 mM riboflavin, 84 mM NBT (NitroBlue Tetrazolium) and 20 mM potassium cyanide. To the test, 300 µl of the sample was added and the final volume was adjusted to 3 ml with distilled water. The tubes were placed in an aluminium foil lined box, maintained at 25°C, equipped with 15 W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600 nm after exposure to light for 10 min; the maximum reaction was evolved in the absence of the enzyme (Misra and Fridovich 1972). Phenol oxidases are copper proteins of wide occurrence in nature, which catalyse the aerobic oxidation of certain phenolic substrates to quinines, which are then auto-oxidized to dark brown pigments generally known as melanins. The polyphenol oxidases (PPO) comprise catechol oxidase and laccase.

Polyphenol oxidase activity was estimated by adding 0.1 M phosphate buffer (pH 6.5) and catechol solution (0.01 M) to the enzyme extract (in 0.01 M Tris–HCl buffer, pH 6.5). The change in absorbance was recorded using the spectrophotometer at 495 nm for every 30 s up to 5 min (Esterbauer et al. 1977). One unit of catechol oxidase is defined as the amount of enzyme that catalyses the conversion of 1 µmole of dihydrophenol to 1 µmole of quinone per minute under the assay conditions. Glutathione peroxidase activity was estimated by allowing known amount of enzyme extracted from microalgae to react with H₂O₂ in the presence of GSH at 37° C for 10 min and the remaining GSH was measured (Rotruk et al. 1973).

Extraction and estimation of non-enzymatic antioxidants

For estimation of ascorbic acid, 1 g of the microalgae sample was ground and homogenized in 5 ml of 4% trichloroacetic acid (TCA); the volume was made up to 10 ml with double-distilled water and centrifuged at 2000× g for 10 min. The supernatant was treated with activated charcoal or bromine. Dehydroascorbic acid reacts with 2, 4 - dinitro phenyl hydrazine to form osazones, which dissolve in sulphuric acid to give an orange coloured solution whose absorbance was measured spectrophotometrically at 540 nm (Roe and Kuether 1943). Tocopherol was estimated using Emmerine–Engel reaction, which is based on the reduction of ferric to ferrous ions by tocopherol, which then forms a red colour with 2,2 dipyridyl. Tocopherols were first extracted with xylene and was read at 460 nm (Rosenberg 1992). For the estimation of reduced glutathione, 1 g of sample was homogenized to a 20% homogenate using 5% TCA. The precipitated protein was centrifuged at 1000×g (4°C) for 10 min. The homogenate was cooled on ice and 0.1 ml of supernatant was used for the estimation of reduced

glutathione. Reduced glutathione was measured by reacting it with 5, 5'-dithio-bis 2-nitro benzoic acid (DTNB) and the compound formed was read spectrophotometrically at 412 nm (Moron et al. 1979). Polyphenols were extracted from the microalgal culture using 80% ethanol. Total phenol estimation was carried out using the Folin–Ciocalteu reagent. Phenols react with phosphomolybdic acid in Folin–Ciocalteu reagent in alkaline medium and produce a blue coloured complex (Molybdenum blue) and the colour intensity was measured at 650 nm (Malik 1980).

Statistical analysis

All analyses were carried out in triplicate and the results were reported as mean $\pm$ standard deviation (SD).

Results and discussion

Growth study of *C. vulgaris*

Growth rate of microalgal cells in both improvised CFTRI medium and confectionery wastewater was determined. Growth of *C. vulgaris* was better in confectionery effluent than in improvised CFTRI medium (Fig. 1). This showed the efficacy of confectionery wastewater as a better microalgal growth medium when compared to CFTRI medium, which is commonly used for microalgal cultivation (Hanumantha Rao et al. 2011). The increased growth of *C. vulgaris* in the confectionery effluent can be attributed to its ability to grow under heterotrophic and mixotrophic conditions, as seen in some microalgal strains (Garcia et al. 2010). Tsavalos and Day (1994) reported that, the basic culture medium composition for heterotrophic cultures is similar to the autotrophic culture with the sole exception of adding an organic carbon source. Mixotrophic growth regime is a variant of the heterotrophic growth regime,

Fig. 1 Growth rate of *C. vulgaris* in Improvised CFTRI medium and Confectionery effluent. The values plotted in this graph are mean of three replicates ($n=3$)

where CO_2 and organic carbon are simultaneously assimilated and both respiratory and photosynthetic metabolic pathways operate concurrently. As confectionery effluent contains 11.412 g/L of glucose, the physiological function of the nutrient requirement and the mode of carbon nutrition in *C. vulgaris* can shift rapidly from autotrophy to heterotrophy or mixotrophy. This is based on the ability of the photoautotrophic algae to utilize both inorganic and organic carbon substrates. *C. vulgaris* has been reported to utilize hexoses such as glucose or 6-deoxyglucose together with protons in 1:1 stoichiometry (Komor and Tanner 1976) from the surrounding media for their growth.

The phycoremediation carried out in the pilot plant stabilized the pH of confectionery effluent at approximately 8.3 and also enabled the removal of organic and inorganic nutrients (Effluent treatment data will be published separately). At the end of the treatment process, the microalgal biomass was harvested and the pellet was washed three times with double-distilled water for chemical analysis. The total protein content in the pellet was $0.6001 \mu\text{g} / 10^6$ cells, the total carbohydrate content $0.4323 \mu\text{g} / 10^6$ cells, the total lipid content $0.6923 \mu\text{g} / 10^6$ cells and the chlorophyll content $0.0068 \mu\text{g} / 10^6$ cells. The laboratory-maintained *C. vulgaris* culture was also estimated for total protein, total carbohydrate, total lipid and total chlorophyll 'a'. The total protein, carbohydrate, lipid and chlorophyll contents were $0.3501 \mu\text{g} / 10^6$ cells, $0.3789 \mu\text{g} / 10^6$ cells, $0.0514 \mu\text{g} / 10^6$ cells and $0.0077 \mu\text{g} / 10^6$ cells respectively (Fig. 2). When compared, total protein, total carbohydrate and total lipid were higher in effluent-grown microalgal cells than in laboratory-maintained culture. The reason for this higher growth and higher nutrient content could be that this strain of *C. vulgaris* was isolated from the same confectionery effluent. Chlorophyll 'a' was lower in effluent-grown microalgal cells compared to that of the laboratory-maintained culture because sugars present in the effluent serve as a

Fig. 2 Biochemical composition of *C. vulgaris* grown in Improvised CFTRI medium and Confectionery effluent. The values plotted in this graph are mean of three replicates ($n=3$)

carbon source for the algae and this may be the reason for low chlorophyll content. Lipids were high in pilot plant cultures, the reason being sugars help in increasing the lipid content of *Chlorella vulgaris* (Han et al. 2006). This could be attributed to the mixotrophic nature of the microalga in the presence of high organic nutrient levels.

Enzymatic antioxidants of *C. vulgaris*

Microalgae represent an almost untapped resource of natural antioxidants, due to their enormous biodiversity, much more diverse than higher plants. However, not all groups of microalgae can be used as natural sources of antioxidants, due to their widely varied contents of target products, growth rate or yields, ease of cultivation, and / or other factors (Li et al. 2007). Takekoshi et al. (2005) have reported that the unicellular microalga *C. vulgaris* expresses various pharmacological effects both in animals and humans. Antioxidant enzymes such as superoxide dismutase (SOD) play a key role in the removal of reactive oxygen species produced in microalgae during various physical-chemical stress responses (Santos et al. 1999).

In this study, 0.04125×10^{-5} U/cell of superoxide dismutase and 0.195×10^{-5} U/cell peroxidase activity were recorded. It is well known that reactive oxygen species such as superoxide (O_2^-), hydroxyl radicals ($OH^\cdot$) and hydrogen peroxide (H_2O_2) are produced in cells when exposed to environmental stress, e.g. exposure to high light intensities, UV radiation, metals, etc. (Li et al. 2006). Glutathione peroxidases are antioxidant enzymes protecting various organisms from oxidative stress by catalyzing the reduction of hydroperoxides at the expense of glutathione (Roy et al. 2005). In the present study, the levels of glutathione peroxidase and polyphenol oxidase in *C. vulgaris* were 0.025×10^{-5} µg/cell and 0.2625×10^{-5} µg/cell respectively (Table 1). There are many reports regarding reactive oxygen species (ROS) in *Chlorella* sp. In *Chlorella*

vulgaris, many antioxidants are present to trap the singlet oxygen. In this study, the antioxidants were estimated and these antioxidants participate in scavenging the singlet oxygen (Anbuselvam et al. 2007). Biotic and abiotic environmental stresses are known to affect plants by causing excess accumulation of ROS, which can react with certain biomolecules, and alter or inactivate the biochemical activities (Choudhary et al. 2011). To survive under stress conditions, each microalgal cell possesses a complex array of enzymatic and non-enzymatic antioxidant defence systems.

Superoxide dismutase is the first enzyme of the enzymatic antioxidative pathway to convert superoxide anion into peroxides, which are scavenged by catalase. Photosynthesizing plant cells including microalgae are exposed to a intense light and high oxygen levels. Photo-oxidative damage occurs upon *in vivo* oxygenation of a susceptible molecule by active oxygen, or other free radicals generated under such conditions. The absence of such damage in microalgae suggests that these cells possess protective, antioxidative mechanisms and compounds. They maintain an acceptably low concentration of oxygen, and other harmful agents, thereby preventing the deleterious oxygenation of target molecules. Thus the ability of microalgae to thrive under such extremely oxidizing conditions leads to the production of various antioxidants (Matsukawa et al. 2000). There are a number of reports on the evaluation of antioxidant activity of some species belonging to the genera of *Botryococcus* (Rao et al. 2006), *Chlorella* (Wu et al. 2005), *Dunaliella* (Herrero et al. 2006), *Nostoc* (Li et al. 2007), *Spirulina* (Jaime et al. 2005), etc.

Non-enzymatic antioxidants of *C. vulgaris*

The non-enzymatic antioxidants from confectionery effluent-grown *C. vulgaris* are summarized in Table 1 and the amounts obtained are also presented. In general, *Chlorella* sp. contains more chlorophyll per g than any other land or sea plants. In this study, we obtained 0.0335 µg chlorophyll/mg wet weight of *C. vulgaris*. Phenolic compounds serve as important antioxidants because of their ability to donate a hydrogen atom or an electron to form stable radical intermediates (Singh et al. 2009). The total phenolic contents of the cell mass or extracellular substances of the isolated microalgae were determined and expressed as gallic acid equivalent (GAE). Total phenols play a significant role in the regulation of plant metabolic processes and overall plant growth as well as lignin synthesis (Lewis and Yamamoto 1990). On the other hand, phenols act as substrates for many antioxidant enzymes. In the present study, the total polyphenols of *C. vulgaris* was estimated to be 4.6875×10^{-5} µg/mg wet

Table 1 Enzymatic and non-enzymatic antioxidant levels in *Chlorella vulgaris*

Enzymatic antioxidants	Amount (Mean±SD) (n=3)
Peroxidase (units/cell)	$0.195 \times 10^{-5} \pm 0.0072$
Superoxide dismutase (units/cell)	$0.04125 \times 10^{-5} \pm 0.001$
Polyphenol oxidase (units/cell)	$0.2625 \times 10^{-5} \pm 0.003$
Glutathione peroxidase (µg/cell)	$0.025 \times 10^{-5} \pm 0.003$
Non-enzymatic antioxidants	Amount (Mean±SD) (n=3)
Chlorophyll a (µg/mg wet weight)	0.0335 ± 0.002
Ascorbic acid (µg/mg wet weight)	0.2182 ± 0.005
α-tocopherol (µg/mg wet weight)	0.00264 ± 0.001
Reduced glutathione (µg/mg wet weight)	0.07916 ± 0.004
Polyphenols (µg/mg wet weight)	$4.6875 \times 10^{-5} \pm 0.067$

weight. Phenols protect the cells from potential oxidative damage and increase the stability of cell membrane (Burguières et al. 2007). Ascorbic acid or vitamin C is a water-soluble antioxidant that can reduce radicals from a variety of sources. It also appears to participate in recycling vitamin E radicals. Interestingly, vitamin C also functions as a pro-oxidant under certain circumstances. Ascorbic acid present in the effluent-grown microalgae was 0.2182 µg/mg wet weight.

One of the important functions of these non-enzymatic antioxidants is to scavenge OH radicals. The level of glutathione in the effluent-grown *C. vulgaris* was 0.07916 µg/mg wet weight (Table 1). GSH appears to be one of the most important antioxidants that occurs in biological systems where it plays an important function in the detoxification of a variety of xenobiotics (Lei et al. 2006), and changes in its concentration are important in protecting the plant from a wide range of stresses including oxidative stress (Lamoureux and Rusness 1989). Elevated levels of GSH protect cellular proteins against oxidation through the glutathione redox cycle and also directly detoxify reactive oxygen species. α-Tocopherol obtained from confectionery industry effluent-grown *C. vulgaris* was 0.00264±0.001 respectively. Tocopherol compounds play an important role as antioxidant in microalgal cells and act as stabilizer of membrane lipids (Nocto and Foyer 1988). Tocopherol acts as a chain-breaking antioxidant and as chemical scavengers of O₂ radicals, i.e. it is capable of repairing oxidizing radical directly, thereby preventing the chain propagation step during lipid peroxidation (Karpinski et al. 1999). Regeneration of the reduced form of TOH from its oxidized form can be achieved by Ascorbic acid (AA) or Glutathione GSH.

Conclusion

In this study, we examined the antioxidant potentials of *C. vulgaris* grown in confectionery industry wastewater. Using confectionery industry wastewater as a medium reduced the cost for culturing algal biomass. Moreover, *C. vulgaris* cultivated in wastewater responds to stress by increased production of antioxidants or elevated activity of protective enzymes. The adaptability of the microalgal species to grow in extreme environments made *C. vulgaris* mass cultivation in confectionery wastewater possible, and the antioxidant potential of the same studied here promises commercial significance. Thus wealth is created from waste, which may enhance the possibility of its use in pharmaceutical and cosmetic applications; more importantly, non-enzymatic antioxidants have appreciable potential value as medicinal products and as an additive in pharmaceutical, food or cosmetic applications.

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