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Culture and biofuel production efficiency of marine microalgae *Chlorella marina* and *Skeletonema costatum*

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In the present study, microalgae were cultured under indoor and outdoor systems with high density and biomass. Their chemical analysis showed high lipid (28.2 and 21.6% respectively) and fatty acid methylesters (FAME) content. Fatty acids such as palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2) were reported to be high in *C. marina* and *S. costatum*. *S. costatum* is containing more eicosapentaenoic acid (EPA). Marine diatom, *S. costatum* is efficiently converting the algal oil into biodiesel (87%) followed by *C. marina* which is produced the 70% of the biodiesel. Biodiesel yield of presently studied marine microalgae was being comparatively higher than that of existing oil crops. It is clearly implied that, marine microalgae *C. marina* and *S. costatum* were considered as a promising feedstock for biodiesel production in near future.

[Keywords: Biofuel, *C. marina,* FAME, *S. costatum*]

Introduction

Microalgae have been suggested as a potential candidate for fuel production because of a number of advantages including higher photosynthetic efficiency, higher biomass production and higher growth rate besides short life span due to their simple structures compared to other energy crops[1]. Moreover, biodiesel from microalgae oil is similar in properties to the standard biodiesel, and is also more stable according to their flash point values. Microalgae are the largest primary producers of any aquatic ecosystem and it can be considered as a source of high-lipid material for the production of biofuel because the photosynthetic conversion of microalgae is an efficient and no alternative competent and carbon dioxide released is fixed[2]. It is estimated that the biomass productivity of microalgae could be 50 times more than that of switch grass, which is the fastest growing terrestrial plant[3]. So the present study was concentrated on the culture of marine microalgae *C.marina* and *S.costatum* for biodiesel production.

Materials and Methods

Microalgae culture

Marine microalgae, *Chlorella marina* and *Skeletonema costatum* strains were obtained from the Central Institute of Brackishwater Aquaculture, Chennai. Indoor algal stock culture was maintained in special air conditioner room. Stock cultures were kept in 1 and 2 litre culture flasks, 5 and 15 litre plastic containers. Seawater was filtered using filter bag (5 micron) and sterilized using autoclave and after cooling water was transferred to the culture flask plugged with cotton or covered by aluminum foil. Vessels are used for algal culture was sterilized properly and dried in an oven before use. The Conway's medium was used for indoor culture. About 10 ml of inoculum in the growing phase was transferred to the culture flasks and the culture has been provided with 12:12 hrs light and dark cycle with 5000 lux by using two tube lights. After 8-10 days, the maximum exponential phase was obtained. Temperature and salinity was maintained in the range between 23 and 25°C and 28 and 30 ppt. respectively for entire culture period, continuous aeration was provided for culture.

For large scale production of microalgae 100 liters FRP tanks were used. The grown stock culture was used as an inoculum for mass culture. For efficient growth of algae, commercial fertilizers namely, ammonium sulphate, super phosphate and urea were added in the ratio of 100 gm; 10 gm and 10 gm respectively for 100 liters of seawater. For 100 litres

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of seawater, 2 litres of inoculum was added for culturing in tank. Continuous and vigorous aeration was provided to culture which keeps the culture always in suspension. Continuous aeration was helpful for uniform distribution of nutrient in the medium.

Algal biomass was harvested by filtering the culture through Millipore filtering equipment using filter paper (0.45 cm dia.). Obtained biomass was scrapped using sterile blades, washed (twice with distilled water), dried and weighed gravimetrically (g/l). Algal biomass was also harvested by centrifugation at 3000 rpm for 10 min. Cell pellet was washed twice with distilled water. Collected cell pellets were dried in an hot air oven at 80°C for 40 min. and the weight was determined gravimetrically (g/l). Density of algae in culture system was determined by cell counts using Sedgewick counting chamber under light microscope followed by the method of[4]. Biomass of microalgae was estimated by the standard method of[5]. 10 mL of algal culture sample was filtered by using Millipore filtering system fitted with a 4.5 cm diameter GF/C filter paper by applying low suction. Before filtering the sample, a thin bed of magnesium carbonate (2 mL) was made for effective filtration. After filtration, the filtrate was removed and ground with 90% acetone using mortar and pestle. The ground samples were transferred to screw cap test tubes and covered using black cloth and incubated in the refrigerator for 24 hrs. Again the contents were grinded with 90% acetone and centrifuged at 3000 rpm for 10 min. The optical density was measured at different wavelengths of 630, 645 and 665 nm for chlorophyll *a* estimation.

Biodiesel production

Biodiesel from microalgae was produced by direct Trans-esterification method of[6]. Dried algal biomass (1.5 g) or wet algal biomass (1.5 g dry weight equivalent) was placed in a glass test tube and mixed with 3.4 mL of methanol, 0.6 mL of sulfuric acid and 4.0 mL of solvent (chloroform). The reaction mixture was heated at 90°C for 40 min and the samples were mixed well during heating. After the reaction was completed, the tubes were allowed to cool at room temperature. Two mL of distilled water was added into the tube and mixed well for 45 seconds. Samples were centrifuged at 3000 rpm for 10 min. to accelerate phase separation. Organic layer that contained biodiesel (FAME) was collected and transferred to a pre weighed glass vial. Solvent was evaporated using N_2, and the mass of biodiesel was determined gravimetrically.

Estimation of total lipid and fatty acids

Moisture content of the microalgae was estimated by drying the known quantity of wet samples in glass container and samples are dried under an oven at 60°C until the samples have dried properly. Difference in weight between wet weight and dry weight was calculated and expressed as percentage of moisture content of the sample. Lipid concentration of cultured algal sample was estimated by the method of[7]. 10 mg of dried sample was homogenized in 10 ml of chloroform: methanol mixture (2:1 v/v). Homogenate was centrifuged at 2000 rpm for 10 min. Supernatant was washed with 0.9% saline solution (KCl) to remove the non-lipid contents and allowed to separate. The upper phase was discarded by siphoning. Lower phase was allowed to dry under oven and the weight was calculated.

About 0.45 g of the substance was examined into a 10 mL of volumetric flask and the substance was dissolved in hexane containing 50 mL of butylhydroxytoluene per litter. Sample was diluted to 10 mL with the same solvent. 2.0 mL of solution was transferred into a quartz tube and the solvent was evaporated with a gentle current of nitrogen. One and a half ml of 20 g/l solution of sodium hydroxide was added in methanol and covered with nitrogen cap tightly with a polytetrafluoroethylene lined cap mix and solution was kept in a water bath for 7 min. Solution was cooled and added 2 mL of boron trichloride-methanol solution and covered with nitrogen cap tightly and mixed and heated in a water bath for 30 min. Solution was cooled to 40-50°C and 1 mL of trimethylpentane was added. Cap and the sample was vortex vigorously for 30 s. Saturated sodium chloride solution was added and covered with nitrogen cap and vortex the sample for at least 15 s. Upper layer was allowed to become clear and transferred to a separate tube. Methanol layer was shaken properly once again with 1 mL of trimethylpentane and to combine the trimethylpentane extracts. Wash the combined extracts with 2 quantities, each of 1 mL of water and dry over anhydrous sodium sulphate, two solutions were prepared for each sample. 1 µl of sample was injected twice, quality and quantity of fatty acids were

identified by Gas Chromatography installed with fused silica column in the size of l = 30 m, Ø = 0.25 mm. Helium was used as carrier gas for chromatography R where oxygen scrubber is applied.

Results

Density and biomass of microalgae

In the present study, *Chlorella marina* and *Skeletonema costatum* were cultured successfully in both in-door and out-door systems with maximum biomass. Cell growth and biomass of *C.marina* and *S.costatum* was clearly indicated that, their production efficiency and high growth rate with cost effective and environment friendly nature. Cells were found to multiply and increase day by day for entire culture period. On the 10th day onwards a dark green coloured bloom was obtained with a maximum cell density in *C.marina*. Whereas in case of *S.costatum* a dark brown coloured bloom was reported. Average growth in terms of density of *C.marina* cultured in indoor system was ranged between 38200.3 and 486547.5 cells/ml (Fig. 1). Maximum density was obtained on 10th day of the culture whereas the minimum was obtained at 1st day. Biomass in terms of chlorophyll 'a' concentration was reported in the ranged between 0.2466 and 0.7923 mg/10 mL (Fig. 2). Maximum biomass was obtained during 10th day of culture, whereas the minimum density was observed during the first day. After 10th day the declining phase was started. In out-door system, the cell density was varied from 78103.7 to 1045679 cells/mL. Maximum cell density was obtained during 10th day of culture whereas minimum density was recorded during the initial day (Fig. 3). The maximum biomass of 1.0964 mg/10 mL was recorded during 10th day. However the least biomass of 0.2979 was obtained at first day onwards (Fig. 4).

In case of *S.costatum,* the maximum density of was reported as 209564 cells/ml. Whereas the minimum density was 1093.6 cells/l. Minimum density was obtained during 1st day onwards. Similarly the maximum density was observed during 12th day (Fig. 5). Biomass was observed in the ranged between 0.0126 and 0.1054 mg/10 mL. Maximum biomass was procured during 12th day of the culture and the minimum was reported at 1st day (Fig. 6). In out-door culture, the maximum density was recorded as 812345.8 cells/ml and minimum was 213200 cells/mL. Maximum density was observed on 10th day onwards and minimum was during 1st day (Fig. 7). Biomass of *S. costatum* was reported with maximum value of 0.6198 mg/10ml whereas the low biomass was 0.1125. Low and high biomass was observed during 1st and 12th days respectively (Fig. 8). Culture of *S. costatum* was appeared with dark brown coloured bloom at exponential phase (12th day).

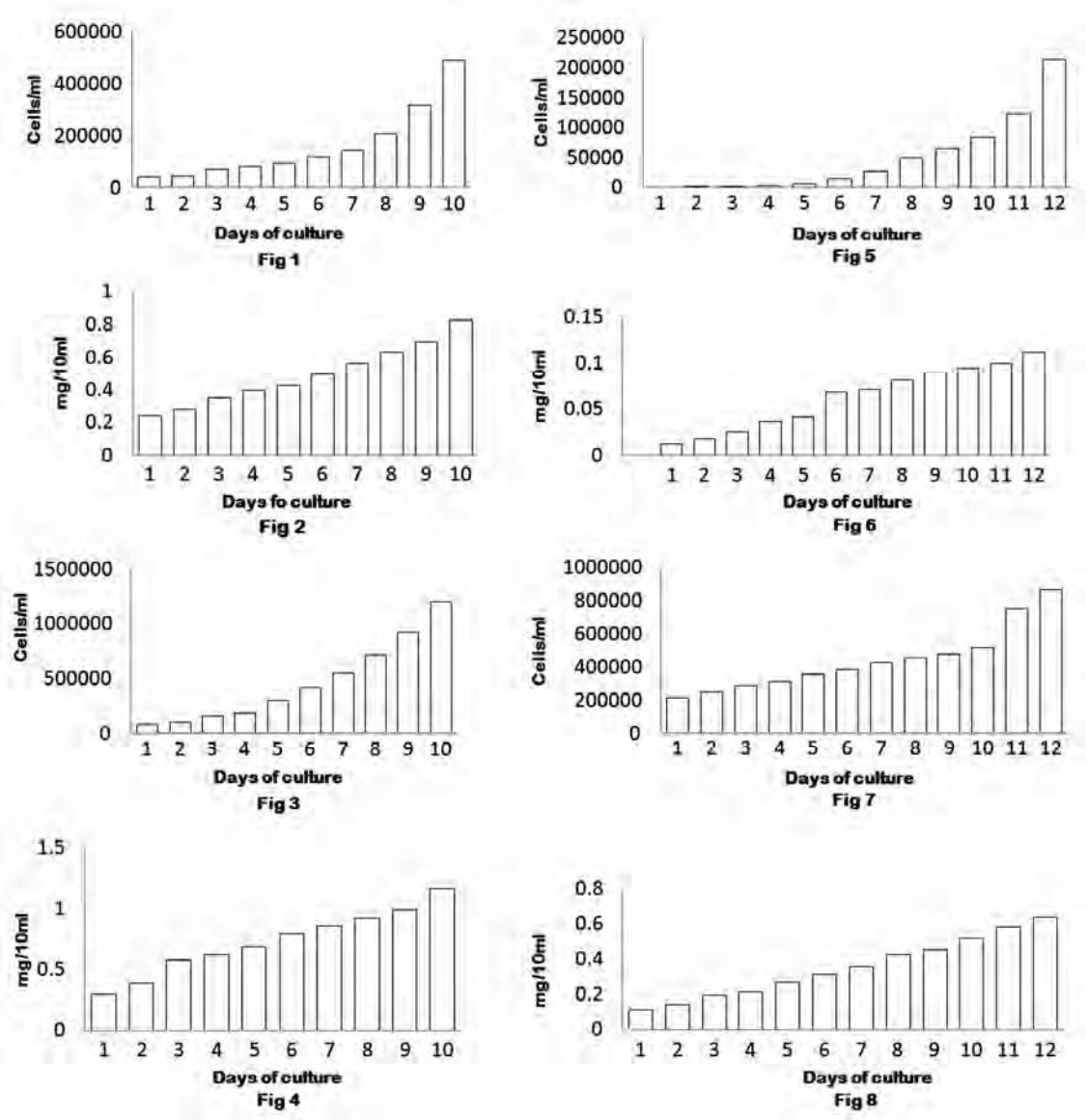
Biodiesel Yield

In the present study**,** the biodiesel was extracted from the algal biomass through direct transesterification. Direct Trans-esterification resulted the maximum biodiesel yield of 87% in diatom *S. costatum* whereas the green alga *C. marina* showed the oil yield of 70% (Table 1). Oil yield of oil seed crops such as Jatropha, coconut, groundnut, white soy bean, castor and red soy bean were found to reported as 62.2, 57.4, 51.2, 37.8, 33.4 and 9.4% for respectively. Biodiesel yield in terms of weight (g/1.5 g DW of biomass) relative to the algal oil present in the biomass was 1.305; 1.049; 0.933; 0.862; 0.768; 0.568; 0.501 and 0.141 for *S. costatum*, *C. marina*, Jatropha, coconut, groundnut, white soy bean, castor and red soy bean respectively (Table 1).

Characterization of oil

The *C. marina* and *S. costatum* have proved the capability of producing biodiesel due to availability of rich total lipid (Table 2). Total lipid content of algae *C. marina* and *S. costatum* was 28.2 and 21.6% respectively. Whereas the lipid content of edible crops such as coconut, groundnut, white soy bean and red soy bean were: 47.8, 42.33, 3.99 and 3.33% respectively. Likewise the lipid content of non edible crops such as castor and Jatropha was found to report as 43.5 and 50.83% respectively.

The fatty acid composition of microalgae can support for their high biofuel efficiency. In general, *C. marina* contain high level of poly unsaturated fatty acids such as 18:2 with 20% and 18:3 with 19.4% followed by mono unsaturated fatty acids such as 16:2 with 9.5%, 16:3 with 9% and saturated fatty acid (14:0) with 9% other fatty acids are observed in the minimum percentage. In case of *S. costatum*, the maximum level of poly unsaturated fatty acids such as 20:5, 18:3, and 18:2 were observed in the percentages of 20.07, 20.0 and 18.2 respectively. The saturated fatty acid namely 16:0 was found in the percentage of 15.96. Fatty acids such as 16:3, 16:2, 18:1 and 18:0 were reported as 10.65, 8.56, 4.0 and 1.0% respectively (Table 3). In the present study, both the microalgae species showed considerable level of fatty acids (Table 3).



Figs 1-8—(1) Daily growth of *C. marina* in indoor culture; (2) Daily biomass of *C. marina* in indoor culture; (3) Daily growth of *C. marina* in out-door culture; (4) Daily biomass of *C. marina* in out-door culture; (5) Daily growth of *S. costatum* in indoor culture; (6) Daily biomass of *S. costatum* in indoor culture; (7) Daily growth of *S. costatum* in out-door culture and (8) Daily biomass of *S. costatum* in out-door culture.

Discussion

The present result shows that, the density and biomass of *C. marina* was slow during first and second day. After that the growth was rapid and it attains maximum density on 10th day onwards it might be due to the provision of 12:12 hrs light:dark condition[8]. Optimal growth of *C. marina* was reported on 12th day of culture when the algae provided with 24 hrs of light and the lag phase of *C. marina* was extended when they illustrated at 16:8 hrs light:dark[9]. However, the low cell numbers was indicated that in the absence of CO_2 the algae were not able to grow. Presently maintained salinity of 28-30 ppt was appeared to more suitable for the growth of the algae[10]. Present study partly supported by[11] who reported that lower growth of *T. suecica* related to an increase in salinity from 31 to 36 ppt.

Nutrients were also one of the important parameter to maintain the algal density and biomass in great success. In our experiment, the algae were cultured

Table 1—Comparative oil yield of marine microalgae and oil crops

Crops	Oil Yield (g/1.5 g DW)	Oil Yield in Percentage
Castor	0.501	33.4
Jatropha	0.933	62.2
White soy bean	0.568	37.8
Red Soy bean	0.141	9.4
Coconut	0.862	57.4
Groundnut	0.768	51.2
Chlorella marina	1.049	70.0
Skeletonema costatum	1.305	87.0

Table 2—Total lipid content of microalgae and oil crops

Crops	Lipid Content (%)
Castor	43.5
Jatropha	50.83
White soy bean	4.0
Red Soy bean	3.33
Coconut	47.8
Groundnut	42.33
Chlorella marina	28.2
Skeletonema costatum	21.6

Table 3—Fatty acid composition of *C. marina* and *S. costatum*

Fatty acids	*C. marina* (%)	*S. costatum* (%)
14:0	9±0.5	-
16:0	25.4±1.2	15.96±0.8
16:1	2±0.3	1.56±0.8
16:2	9.5±0.5	8.56±0.4
16:3	9±0.1	10.65±1.3
18:0	1.2±0.2	1.0±0.5
18:1	4.5±0.4	4.0±0.5
18:2	20±0.6	18.2±0.9
18:3	19.4±0.2	20±0.5
20:5	-	20.07±1.2
14:0	9±0.5	-
16:0	25.4±1.2	15.96±0.8

using Conway's medium. In addition, silicate was added in the medium for efficient growth of *S. costatum*. Presently used medium was found to more suitable for culture of *C. marina* and *S. costatum* so that the maximum cell numbers and biomass was achieved presently[12-14] who described that, the importance of nitrate, phosphate and silicate in the culture medium for the higher growth of *Thalassiosira fluviatilis* and *Skeletonema costatum*[15] who justified that, the nutrients and temperature can limits the growth in *Skeletonema costatum*, *Chaetoceros* sp. *Cylindrotheca closterium* and *Thalassiosira* sp. In the present culture, temperature was maintained in the range between 29 and 30°C by providing partial shade which results the good density and biomass of cultured algae[14].

The net growth rate was highest for *C. marina* which grew at 48×10^7 cells/L. after which the culture entered into the stationary phase and decayed. In the present study a huge density was obtained than previous report[16] who reported the maximum cell density of 85×10^6 in dinoflagellate, *Karlodinium veneficum* for biodiesel production. Maximum biomass obtained presently is several folds higher than that of previous report[16] might be due to the increase in wet weight during the exponential phase. Among the two species studied presently, the *C. marina* showed the faster growth rate and maximum density than *S. costatum* might be due to their shorter life span and physiological condition and smaller size. Diatom *S. costatum* is chain forming one and it may occupy more space in the culture tank. But the biomass (wet weight) of *S. costatum* was comparatively higher than that of *C. marina*. Similarly, *S. costatum* showed best performance in terms of growth in out-door culture, it was high enough to yield a large biomass in culture within a reasonable period of time. Since the *S. costatum* has chain forming nature which could increase the total biomass, so that we can get more raw material for biofuel production. Present results suggested that, in terms of growth phase and biomass both algae resulted the highest yields[17]. Economic biodiesel production from microalgae requires enormous bulk of algal biomass. Outdoor mass culture of *C. marina* and *S. costatum* was maintained in 100 litres of FRP tanks it is relies on natural light for illumination. Thus, setting up, operating and maintenance cost is less as compared to bioreactors[18]. Maximum biomass productivity was obtained for *C. marina* and *S. costatum* in outdoor mass culture provided with partial shade by using waste cardboard and this could reduce the contamination and also excess evaporation.

Direct trans-esterification using chloroform as solvent, marine microalgae have resulted the high biodiesel yield than indirect trans-esterification[6]. Due to the inherent nature of single stage reaction, direct trans-esterification was much less time consuming than the oil extraction and transesterification. It also avoided the potential lipid loss during the extraction stage, as a result the direct methylation led to a higher crude biodiesel yield. Similarly, the oil yield is also

solvent dependent. Biodiesel yield was found to report very low when no solvent was used in the direct transesterification it indicating the essential of solvent for reaction. Direct transesterification with chloroform solvent was resulted much better on FAME yield also the crude biodiesel yield[6]. Presently the acid was used as a catalyst for the transesterification because of the high free fatty acid value and thus soap formation in the alkali-transesterification. Therefore in the present study the acid catalyst used for making biodiesel from the algal biomass in order to avoid soap formation. Algal biodiesel production by direct methylation producing FAME from biomass has been reported in some algal species by earlier workers[19,20].

A presently investigated microalgae viz. *C. marina* and *S. costatum* seems to be a good option for biodiesel production due to its considerable composition of fatty acids, as they can have a significant effect on the characteristics of biodiesel produced. Result on the fatty acids analyses of *C. marina* and *S. costatum* showed that both of them are synthesized C14:0, C16:0, C16:1, C16:2, C18:0, C18:1, C18:2 and C18:3 fatty acids. Observed fatty acids are responsible for rich biodiesel yield in marine microalgae than other crops[21]. Fatty acid composition of microalgae may ascribed by different nutritional and environmental factors, cultivation conditions and growth phases[22-23]. Although the microalgae oil yield is strain-dependent it is generally much greater than other vegetable oil crops, as shown in Table 1 that compares the biodiesel production efficiencies and other crops, including the amount of oil content in a dry weight basis and the oil yield in percentage[6]. Present study (Table 3) found that the lipid content of crop plants was comparatively higher than microalgae. Even though, the microalgae produced more biodiesel it might be due to the high conversion efficiency of algal oil into biodiesel and biodiesel productivity with a clear advantage for microalgae[1,17,24-29].

Marine microalgae *C. marina* and *S. costatum* possess a favorable fatty acids profile that can be utilized for biodiesel production with high oxidation stability. Lipids are produced in algal cells as they utilize both inorganic (CO_2 from atmosphere) and organic form of carbon (energy substrate from growing ambience). Various classes of lipids (both polar and neutral) such as triglycerides, phospholipids, cholesterol, etc, are produced among which triglycerides are the major raw lipid precursor for biodiesel production. Triglycerides are mostly found in marine microalgae such as *C. marina* and *S. costatum* so that they can releases high oil yield than other crop seeds[30]. Presently examined *C. marina* showed the equal oil yield of *Botryococcus braunii* whereas the *S. costatum* showed more than that of *C. marina* and *Botryococcus braunii*. Reason may be the presence of more poly unsaturated fatty acid especially 20:5n-3 in *S. costatum*. Thus it was feasible to use algae oil production to completely replace fossil diesel. Aside from higher oil yields, marine microalgae are able to grow extremely rapidly, generally doubling their biomass within a day. Moreover, they can grow on non cultivable land or in coastal land which is not suitable for agriculture, avoiding competition for land with crops and tapping into freshwater resources for irrigation. Another reason why microalgae are attractive is that they can assimilate carbon dioxide as the carbon source for growth[30]. This contributes to both atmospheric CO_2 emission mitigation and high microalgae biomass production. In addition, microaglal oils are similar to those produced by crops such as soybean, and can be used directly to run an existing diesel engines or as a mixture with crude oil diesel[31]. Commercial production of biodiesel from microalgae practically successful in developed countries. But in India, using marine microalgae as feedstock for biofuel production is still absent[32]. It is clearly implicit that the marine microalgae *C. marina* and *S. costatum* were considered to be the promising candidate species for biodiesel production.

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