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System Development for Linked-Fermentation Production of Solvents from Algal Biomass

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Five species of the genus *Dunaliella* (*D. tertiolecta, D. primolecta, D. parva, D. bardawil*, and *D. salina*) were examined for glycerol accumulation, growth rate, cell density, and protein and chlorophyll content. The suitability of each algal species for use as a fermentation substrate was judged according to glycerol accumulation and quantities of neutral solvents produced after sequential bacterial fermentations. When grown in 2 M NaCl, with 24 mM $NaHCO_3$ or 3% CO_2 at 28°C and with 10,000 to 15,000 lx of incident light on two sides of a glass aquarium, four of the five species tested produced ca. 10 to 20 mg of glycerol per liter of culture. *Clostridium pasteurianum* was found to convert an algal biomass mixture supplemented with 4% glycerol to ca. 16 g of mixed solvents (*n*-butanol, 1,3-propanediol, and ethanol) per liter. Acetone was not detected. Additionally, it has been demonstrated that *Dunaliella* concentrates of up to 300-fold can be directly fermented to an identical pattern of mixed solvents. Overall solvent yields were reduced by >50% when fermentations were performed in the presence of 2% NaCl. These results are discussed in terms of practical application in tropical coastal zones.

The bioconversion of renewable resources to neutral fuels and solvents represents a potentially important supplement to future energy needs. Although cellulose represents our most abundant natural biopolymer and is therefore an obvious choice as a fermentation substrate, many problems need to be addressed pertaining to its delignification pretreatment (18) and toward improvements in cellulase efficiency (1, 12, 13, 17, 21). The use of marine algal biomass as a fermentation substrate presents some advantages for the utilization and bioconversion of a potentially large renewable resource: (i) algae do not compete with agricultural or forest crops for available land area; (ii) the use of controlled culture systems allows for optimization of growth conditions and harvesting techniques; and (iii) utilization of cell cultures can supply, besides carbohydrates, a nutritionally balanced fermentation mixture rich in nitrogen. It is conceivable that coastal areas and saline lakes, especially in tropical regions, may hold promise for eventually supplying such rapidly growing energy crops (18).

The genus *Dunaliella* represents a group of halophilic microalgae possessing unique characteristics which can be exploited in linked fermentations (9, 11, 22). Members of this genus lack a cell wall and can be lysed in dilute medium (25), thereby eliminating elaborate process development for cell wall breakage. Furthermore, these microorganisms accumulate high intracellular concentrations of glycerol, which presumably serves as an osmotic balance in highly saline environments (3, 4, 8). Our approach to the useful bioconversion of algal biomass has focused on this genus and its sequentially linked microbial transformation to liquid fuels or to commercially important solvents. The demonstration of such linked processes has been reported (16) in preliminary form.

MATERIALS AND METHODS

Microorganisms. A large number of samples were obtained from naturally anaerobic locations. Isolates were selected for their ability to utilize glycerol as a sole carbon source. Three cultures were eventually selected based on the analytical detection of neutral solvents in their growth media. These have been provisionally identified as a *Bacillus* sp., a *Klebsiella* strain, and a *Clostridium pasteurianum* isolate (obtained from D. A. Klein, Colorado State University, Fort Collins). Because of high butanol yields, research focused on *C. pasteurianum*, and all data reported in this paper were obtained with this bacterium. The *Dunaliella* cultures were obtained from the following sources: *D. tertiolecta* from R. R. Guillard of the Woods Hole Oceanographic Institution, Woods Hole, Mass.; *D. salina, D. primolecta*, and *D. parva* from R. C. Starr at the University of Texas, Austin; and *D. bardawil* from the American Type Culture Collection, Rockville, Md. (ATCC 30861).

Growth conditions. Algae were grown in a medium

containing 2 M NaCl, 5 mM KNO_3, 5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.5 mM CaCl, and a micronutrient mixture (8) supplemented with a 10-fold increase in FeEDTA resulting in a final concentration of 15 mM Fe (Dun medium). This increased concentration was found to be necessary for the nonmarine species of *Dunaliella*. Algae were analyzed for cell count microscopically with a standard hemacytometer. Chlorophyll content was determined by the procedure of Arnon (2). Glycerol content and glycerol utilization were measured by the method of Ben-Amotz and Avron (4). Protein was analyzed by the procedure of Lowry et al. (14), as modified by Potty (19).

Laboratory-scale mass culture of all algal species was carried out in a 100-liter aquarium illuminated from two sides with 10,000 to 15,000 lx on each side (measured at the surface). The temperature was maintained between 27 and 30°C, and the culture was mixed by vigorous aeration. Enrichment with a carbon source, essential for rapid growth, was provided by the addition of sodium bicarbonate (ca. 24 mM, with an initial pH of 7.8). The pH of the solution increased to 9.0 within 3 to 5 days and finally reached 9.5, at which point growth was inhibited. Alternately, the medium contained 2 mM $NaHCO_3$ and was sparged with CO_2-enriched air (3% CO_2). In this treatment, the initial pH was ca. 5.0 and reached pH 8.0 at the end of 12 days. The pH rise was generally proportional to the growth rate.

All bacterial cultures were maintained on slants of tryptic soy agar (Difco Laboratories, Detroit, Mich.) except for the *C. pasteurianum* culture, which was kept as a spore inoculum over sterile soil. Before inoculation into unsupplemented algal concentrates, each bacterial strain was transferred several times in an algal extract-glycerol broth containing a 10-fold concentration of algae and 2% glycerol. Inoculations were adjusted to a final volume of 10% of the fermentation mixtures. All fermentations were conducted in 125-ml Erlenmeyer flasks (100-ml volume) for 5 days at 30°C.

Solvent analyses. Algae were harvested by continuous-flow centrifugation at 1,500 × *g* in a Sorvall RC2-B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), and concentrates were prepared by suspension in distilled water. Solvent determinations were performed on culture supernatants by gas chromatography with a stainless steel column (1.82 m by 0.31 cm) packed with Porapak Q (Supelco Inc., Bellefonte, Pa.). Analyses were performed on a model 2400 Varian gas chromatograph (flame ionization detector) with the column temperature at 190°C, the injector at 185°C, the detector at 235°C, and the carrier gas at 30 ml/min. The gas chromatograph was coupled to a Varian CDS-111L chromatography data system, and quantification was achieved by internal standardization with ethyl acetate. The identification of 1,3-propanediol was accomplished using a Finnigan 4000 gas chromatograph-mass spectrometer data system with a Tekmar accessory (Finnigan/MAT Co., Inc., San Jose, Calif.).

RESULTS

Algal growth studies. Since the purpose of this project was to compare five species of the genus *Dunaliella* as potential substrates for subsequent bacterial fermentations, it was important to determine growth conditions, allowing for maximum glycerol synthesis and biomass production. For *D. tertiolecta*, details of the relationship between NaCl concentration in the medium and algal performance are shown in Fig. 1. This alga grew adequately in 0.2 to 1.6 M NaCl, with, however, some reduction of growth at a 2 M concentration. The glycerol content (per cell) increased linearly between 0.2 and 2 M NaCl, whereas the protein content remained unchanged. These relationships shifted dramatically at 3.2 M NaCl. Here, the glycerol content per cell increased, apparently the result of a larger cell size, which in turn was related to a low division rate (Fig. 1). The increase in cell size was observed microscopically. From these preliminary data, 2 M NaCl was selected as the optimum salt concentration for comparison of the five *Dunaliella* species. At this NaCl concentration, growth rates and the glycerol yield (per volume) were optimal. Cell densities of the rapidly growing species, *D. tertiolecta* and *D. primolecta*, were near maximum after 5 days. The more slowly growing species, especially *D. salina*, reached reasonable cell densities only after 12 days. The cultures were therefore tested on days 5 and 12 and harvested after 12 days of cultivation. The rapidly growing species maintained their maximum cell numbers over the 12-day period.

Tables 1 and 2 provide a general overview of some characteristics of the five species exam-

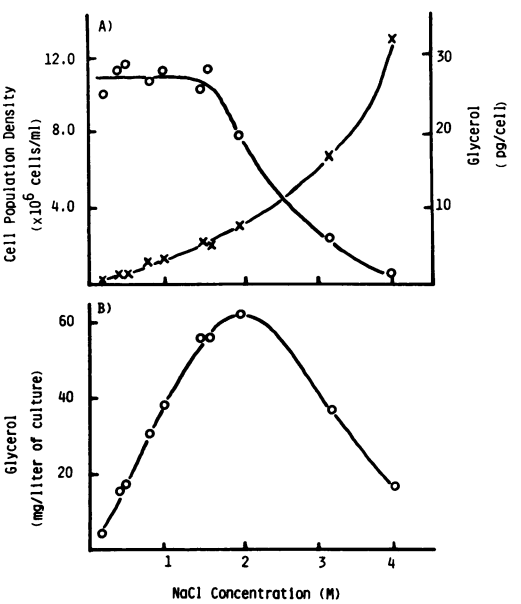

FIG. 1. Effect of NaCl concentration on (A) cell population density (○) and glycerol content per cell (×) and (B) total glycerol production per liter by *D. tertiolecta* after 5 days of growth.

TABLE 1. Comparison of five *Dunaliella* species grown in Dun medium with 24 mM $NaHCO_3$ or 3% CO_2 as a carbon source

Species	Cell no.[a] (10^6/ml)		Glycerol (pg/cell)		Chlorophyll (pg/cell)		Glycerol (mg/liter of culture medium)	
	$NaHCO_3$	CO_2 air	$NaHCO_3$	CO_2 air	$NaHCO_3$	CO_2 air	$NaHCO_3$	CO_2 air
D. bardawil	0.98	1.79	19.9	12.24	4.5	3.52	19.7	21.8
D. tertiolecta	2.43	3.86	8.94	7.77	2.1	1.23	21.3	22.2
D. parva	2.30	1.13	9.53	6.45	2.2	3.08	21.8	9.9
D. salina	0.38	1.60	27.6	7.94	6.2	2.99	10.5	12.6
D. primolecta	2.17	3.36	8.9	4.58	2.5	1.36	18.6	15.5

[a] Cell densities were obtained after 5 days of growth (8 days for *D. salina*) in 100-liter aquaria.

ined in 24 mM bicarbonate or in 3% CO_2, respectively. Generally, growth in media supplied with 3% CO_2 was more rapid, and final cell densities were slightly higher. However, overall yields of glycerol per milliliter of culture medium were almost identical.

The highest cell density was exhibited by *D. tertiolecta* in both 3% CO_2 medium and the bicarbonate medium (Table 1). Although *D. bardawil* and *D. tertiolecta* produced equivalent amounts of glycerol per milliliter of either culture medium, a greater yield of potentially fermentable carbon was obtained from *D. tertiolecta* due to an elevated cell density. For this reason, *D. tertiolecta* was used in most later fermentation linkage feasibility and optimization studies. In bicarbonate medium, *D. salina* cell counts were the lowest of all five *Dunaliella* species tested, and the resultant total glycerol yield was only half the level of the other algae. When 3% CO_2 was bubbled through the growth medium, the cell density of *D. salina* increased by almost fourfold, but the total glycerol yield increased by only 20%. Also, *D. parva* grew poorly in the CO_2-sparged medium and produced the lowest glycerol yield of all cultures in both media.

The data presented in Table 2 lists the glycerol, protein, and chlorophyll contents of algal concentrates grown with bicarbonate versus 3% CO_2. Except for *D. salina*, the algae grown with bicarbonate produced comparable amounts of all three components. When grown with 3% CO_2, however, *D. bardawil* produced approximately twice the protein when compared with other *Dunaliella* species.

Fermentations. In the first series of experiments, the fermentation of *Dunaliella* biomass by *C. pasteurianum* was studied as a function of added glycerol (Table 3). The solvent yield varied both with algal species and with glycerol concentration. The highest solvent yield of 1.6% (>16 g/liter) was obtained using 10-fold concentrates of *D. bardawil* supplemented with 4.0% glycerol, with butanol comprising approximately half of the fermentation products. However, using *D. parva* as the fermentable substrate yielded 1% total solvents in the fermentation broth, with butanol comprising 93% of the products. *D. tertiolecta* and *D. salina* both yielded in excess of 1.4% total solvents after fermentation. The fermentation of *D. primolecta* and *D. parva* biomass resulted in less total solvent production (1.0%) but produced the highest distribution into butanol, 0.75 and 0.94%, respectively.

Solvent yields were substantial at a 1% glycerol supplementation, and this substrate level was found to occur in Ca. 300-fold concentrates of the algae. Such concentrates are viscous as the result of high polymer content but were found to be acceptable for flask fermentation.

The use of algal biomass and accumulated glycerol as fermentation substrate necessitates concentration of the cellular photosynthate to achieve subsequent solvent yields which might be comparable to those obtained with carbohy-

TABLE 2. Composition of 300-fold concentrates of five *Dunaliella* species grown in Dun medium with 24 mM $NaHCO_3$ or 3% CO_2 as a carbon source

Species	Composition per ml of concentrate[a]					
	Glycerol (mg)		Protein (mg)		Chlorophyll (mg)	
	$NaHCO_3$	CO_2 air	$NaHCO_3$	CO_2 air	$NaHCO_3$	CO_2 air
D. tertiolecta	5.06	4.90	4.77	4.0	0.77	0.55
D. primolecta	5.00	4.61	5.28	6.6	0.80	0.63
D. bardawil	5.53	6.43	3.25	13.8	0.70	1.10
D. parva	4.83	5.18	3.15	4.1	0.57	1.30
D. salina	1.74	5.26	1.96	5.6	0.30	1.58

[a] Normalized to a 300-fold concentrate of the original culture.

TABLE 3. Comparison of solvent production: *C. pasteurianum* with each species of *Dunaliella* (10-fold concentrate) with added glycerol

Algal source	Protein content (mg/liter)	% Added glycerol	Product distribution (g/liter)				Total solvent production (g/liter)
			n-Butanol	1,3-Propanediol	Ethanol	Acetate	
D. tertiolecta	158.0	4.0	6.37[a]	4.87	1.80	1.21	14.25
		1.0	1.71	1.78	0.33	0.42	4.24
		0.5	0.78	0.81	0.15	0.35	2.09
		0.0	0.25	0.01	0.09	0.80	1.15
D. primolecta	108.0	4.0	7.52	2.09	0.71	0.04	10.36
		1.0	1.12	0.02	0.01	0.01	1.16
		0.5	0.14	0.01	0.01	0.01	0.17
		0.0	0.01[b]	0.01	0.01	0.01	0.04
D. parva	104.0	4.0	9.42	0.53	0.15	0.02	10.12
		1.0	0.36	0.04	0.01	0.01	0.42
		0.5	0.08	0.01	0.01	0.01	0.11
		0.0	0.02	0.01	0.01	0.01	0.03
D. salina	65.0	4.0	6.28	6.94	0.81	0.18	14.21
		1.0	1.62	2.26	0.14	0.36	4.38
		0.5	0.70	1.62	0.06	0.33	2.71
		0.0	0.21	0.68	0.02	0.28	1.19
D. bardawil	176.0	4.0	7.76	6.15	1.36	0.79	16.06
		1.0	2.47	0.90	0.37	0.45	4.19
		0.5	1.18	0.45	0.20	0.47	2.30
		0.0	0.28	0.06	0.08	0.22	0.64

[a] Values represent the mean of three replicates. Standard errors were always less than ±5%.
[b] Limit of detection.

drates derived from more traditional biomass resources. The actual linkage of photolithotrophically grown biomass to neutral solvent end product formation by *C. pasteurianum* is shown in Table 4. Unsupplemented algal biomass was harvested and concentrated 10-, 150-, and 300-fold and served as the fermentable substrate. Total solvents produced (at a 300-fold concentration) ranged from 2 to 6.5 g/liter, with the major products distributed between butanol and 1,3-propanediol. In each case, a concentration of at least 150-fold algae was required to stimulate fermentation. *D. bardawil* served as the best unamended substrate, providing 6.5 g of mixed solvent per liter at a 300-fold concentration. These data coincided with elevated protein and glycerol values for *D. bardawil* biomass.

The effect of salt on solvent formation by *C. pasteurianum* is shown in Table 5. These data demonstrate a severe inhibition (>50%) of solvent formation at 2% NaCl and almost total inhibition at 3% NaCl. Solvent concentrations actually increased to peak levels (12 g of total solvents per liter) at 1% NaCl and declined sharply at salt concentrations approaching 2%. However, it should be emphasized that the harvesting procedures used in this research provided a fermentable biomass substrate with salt concentrations of below 1%.

A soil isolate, tentatively classified as a member of the genus *Bacillus*, converts glycerol-amended algal mixtures into ethanol to a final concentration of 7.0 to 9.6 g/liter. Additionally, a *Klebsiella* sp. isolated from sewage sludge converts the algal biomass-glycerol supplement solely to 1,3-propanediol (trimethylene glycol) at a final concentration of 4.2 to 5.3 g/liter (data not shown). These alternate transformations of the algal photosynthate are under investigation.

DISCUSSION

All five species of the genus *Dunaliella* grew well and produced near-maximum levels of glycerol at 2 M NaCl. Considerable variation in cell yield was obtained with *D. salina* despite an attempt to optimize media and culture conditions. Although *D. bardawil* never reached high cell densities under any of the stated conditions, the yield of glycerol per volume of culture medium was comparable to that of other *Dunaliella* species. This can most likely be attributed to a larger cell size (11 ± 1.9 μm) which reflected increased glycerol concentrations per cell vol-

TABLE 4. Solvent production by *C. pasteurianum*, utilizing algal concentrates

Alga	Fold concn	Initial glycerol (μmol/ml)	% Glycerol utilization	Product distribution (g/liter)				Total solvent production (g/liter)
				n-Butanol	1,3-Propanediol	Ethanol	Acetate	
D. bardawil	300	111.80	93.1	2.73	2.81	0.13[a]	0.90	6.57
	150	54.30	96.5	1.87	0.97	0.07	0.26	3.17
	10	3.73	97.3	0.08	0.10	0.01[b]	0.15	0.34
D. primolecta	300	49.20	94.9	1.24	0.77	0.02	0.20	2.23
	150	23.90	96.8	0.62	0.30	0.01	0.01	0.94
	10	1.54	96.4	0.07	0.01	0.01	0.01	0.10
D. tertiolecta	300	50.00	94.6	1.61	0.29	0.16	0.20	2.26
	150	25.35	95.7	0.89	0.20	0.08	0.06	1.23
	10	1.66	93.1	0.15	0.01	0.02	0.01	0.19
D. parva	300	61.40	97.5	1.34	1.50	0.10	0.22	3.16
	150	31.40	98.0	0.66	0.43	0.05	0.01	1.15
	10	2.35	97.2	0.02	0.12	0.01	0.01	0.16
D. salina	300	47.50	95.9	0.67	1.17	0.05	0.28	2.17
	150	21.25	95.3	0.54	0.46	0.05	0.06	1.11
	10	1.74	95.1	0.11	0.01	0.02	0.06	0.20

[a] Values represent the mean of three replicates. Standard errors were always less than ±5%.
[b] Limit of detection.

ume. The two marine species, *D. primolecta* and *D. tertiolecta*, appeared to be well-suited for biomass production after acclimation to high-salt conditions. These two species proved to be relatively easy to cultivate under both bicarbonate and forced-air conditions and provided comparable amounts of glycerol per cell. They would, therefore, be possible species of choice for biomass production. However, *D. bardawil*, despite lower final cell densities, did produce a similar amount of glycerol in comparison with *D. tertiolecta*. In these studies, *D. bardawil* biomass provided the best fermentation substrate.

The selection of an alga for mass cultivation will be to a large extent determined by the type of cultivation system used and the quality of water available. In an intensive system where growth rate is important, a rapidly dividing species such as *D. tertiolecta* would be advantageous, but if the time required for culture development is not critical, slower-growing *D. bardawil* might be more suitable. The possibility of commercial cultivation of *Dunaliella* species as energy crops in marginal, brackish environments was given impetus by the data in Table 1 which demonstrated comparable growth rates, biomass, and glycerol accumulations, using bicarbonate versus CO_2 as the carbon source.

The fermentation of algal biomass was rapid and efficient based upon glycerol as the major fermentation component. When calculated on a molar basis, ca. 80 to 100% of the accumulated glycerol was converted to solvents by the fermenting bacteria. These efficiencies may be explained by the high protein and general nutrient content of the available algal biomass. With 300-fold algal concentrates, solvent production was roughly equivalent to the yield from a 1% glycerol-supplemented medium (Table 2).

The novelty of this research lies in the demonstration of a direct linkage between a halophilic and photolithotrophic biomass resource and the classic butanol fermentation (15, 16, 23). In experiments designed to test direct bioconversion of algal biomass (Table 4), *D. bardawil* again demonstrated superior potential as a fermentation substrate. With 300-fold algal concentrates, the fermentation of *D. bardawil* biomass produced approximately twice as much *n*-butanol as did substratum from other *Dunaliella* species.

The fermentation of *Dunaliella* biomass and accumulated glycerol (Tables 3 and 4) with a *Clostridium* sp. produces a solvent mixture rich in butanol and 1,3-propanediol, thus differing markedly from the classic butanol-acetone-ethanol fermentation that uses a number of well-described clostridia and easily fermentable substrates such as molasses or corn steep liquor (20). Acetone was not detected, and ethanol was usually present at less than 10% of the recoverable solvents.

To develop an operational fermentation system based on *Dunaliella* species, salt tolerance by the bacterial fermenters is of crucial importance. Since washing of the osmotically sensitive algae after collection appears to be impracti-

TABLE 5. Effects of NaCl on solvent formation, using *C. pasteurianum* and 10-fold concentrates of *D. tertiolecta*

NaCl (%)	Product distribution (g/liter)				Total solvent production (g/liter)
	Butanol	1,3-Propanediol	Ethanol	Acetate	
0.0	4.83	1.59	1.88[a]	0.92	9.22
0.1	5.85	2.42	1.94	0.74	10.95
0.5	7.23	2.39	2.04	0.56	12.22
1.0	7.37	2.43	1.81	0.45	12.06
2.0	3.14	3.93	0.42	0.49	7.98
3.0	0.08	0.01	0.07	0.53	0.69

[a] Data represent the mean of duplicate analyses.

cal, the final algal biomass will always contain relatively high NaCl concentrations. Our algal biomass generally contained between 0.1 and 0.5 M NaCl after concentration. This wide range of values reflects differences in pelleted cell density during harvesting of the algae. The highest concentrations in the actual fermentation medium reached 0.2 M, which is well within the tolerance of the fermenting bacteria used. The data presented in Table 5 indicate an increase in solvent production to 1.0% salt, with partial inhibition at 2.0% and total inhibition at 3.0%. Although no salt inhibition of solvent production was observed in our studies with algal concentrates, the success of large-scale algal cultivation linked to bacterial fermentations may depend on salt removal or the isolation of solvent-producing, salt-tolerant clostridia.

The feasibility of the described fermentation process is best evaluated by comparison with the now classical butanol fermentation with *Clostridium acetobutylicum* (20). The use of molasses or pure glucose as a fermentation substrate has routinely produced 10 to 20 g of mixed solvents per liter. In our investigation, the use of 10-fold algal concentrates supplemented with 4% glycerol resulted in 14 to 16 g of mixed solvents per liter.

Solvent yields might be further enhanced by isolating related bacteria with elevated proteolytic activity. The algal concentrate contains 16 to 21 mg of protein per ml of 350-fold concentrate, which can be utilized for cell growth as well as for solvent formation. Also, the bioconversion of highly proteinaceous biomass to fatty acids is worthy of further study. If the level of fermentable substrates (algal biomass and glycerol) can be increased to 3 to 4%, the solvent yields would then be comparable to results obtained from more traditional biomass resources (5, 7, 10, 24). Although similar levels of total solvents can be obtained with the addition of 3 to 4% glycerol, this linked algal system needs further research to facilitate the processing procedures for optimal biomass yield. In this regard, the recovery of glycerol from the extracellular medium by the application of suitable membrane or other separation techniques (18) could increase the fermentable substrate concentration to 3 to 4% at low energy input. At this point, it would be conceivable to begin to examine cost comparisons, especially in tropical coastal areas, to evaluate the economic feasibility of this linked-fermentation process.

The use of algal biomass as a substrate for solvent-producing bacteria adds a new dimension to the concept of using renewable resources as petroleum substitutes and chemical feedstocks (6, 21). Also, in less-developed countries, the use of food crops as fermentation substrates has questionable merit, and the food-versus-fuel argument need not be addressed in this type of algal system.

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