Utilization of Canola Oil and Lactose to Produce Biosurfactant with *Candida bombicola*

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ABSTRACT: The prerequisites for a commercial fermentation process of biosurfactants include the use of low- or negative-cost substrates and maximum conversion yields. Under competitive market conditions, the price of canola oil is expected to decrease in response to its increased supply. Lactose, obtained from cheese whey, is a by-product of the dairy industry. In this work, canola oil with glucose or lactose as carbon sources was used as substrates to produce sophorose lipids (SLs) by means of the yeast *Candida bombicola*. Fermentations were conducted in either shaker flasks or 1-L Bellco (Vineland, NJ) stirred reactors for 5–7 d at 450 rpm and 30°C. The production of SLs reached 150–160 g/L in a medium consisting of 10% glucose, 10.5% canola oil, 0.1% urea and 0.4% yeast extract. When lactose was substituted for glucose, 90–110 g/L SL was obtained. The apolar SL 17-L-([2′-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl]oxy)-octadecanoic acid 1′-4″-lactone 6′,6″-diacetate (SL-1) was the major one (73%) when canola oil was used instead of safflower oil (SL-1, 50%). Use of canola oil generally resulted in increased yields of SLs comparable to the yields obtained when safflower oil was used in the medium. Other literature reports present yields of 70 g/L and 120 g/L SLs, respectively, with these substrates.
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KEY WORDS: Biosurfactants, *Candida bombicola*, canola oil, cheese whey, lactose, sophorose lipids.

The industrial need for surfactants is constantly growing. Surfactants possess both hydrophilic and hydrophobic structural moieties, which in turn impart many unusual properties, including an ability to lower the surface tension of water. The commercial importance of surfactants is evidenced from the increasing trends in their production and the number of industrial applications. The surfactant industry has grown nearly 300% within the U.S. chemical industry during the last decade. The value of U.S. surfactant shipments in 1989 was approximately $3.65 billion, 14% higher than the previous year. U.S. production was estimated to be 15.5 billion pounds (1).

Biosurfactants are attracting recent attention as natural and promising surfactants because they offer several advantages over chemical surfactants, such as lower toxicity, biodegradable nature and ecological acceptability.

In the personal-care sector, penetration by biosurfactants is expected to be rapid. It is predicted that by the year 2000 most cosmetic products will be "bio-cosmetics," including color bases (e.g., bio-lipsticks with fermentation-derived "shikonin"). Sophorose lipids (SLs) from *Torulopsis bombicola* KSM 35 are already used in Japan as a high-value skin moisturizer (2). In the petroleum industry, there are several applications of biosurfactants (some already commercialized). The more important industrial applications of biosurfactants include: (i) microbially enhanced oil recovery; (ii) cleaning of oil tankers; (iii) extraction of bitumen from tar-sands; (iv) pumping of crude oils by use of bio-emulsifiers; (v) demulsification of crude oils; and (vi) viscosity reduction of heavy crude oils (3). In the food area, lecithin, a biosurface-active material, is a widely used food emulsifier. Antibiotic effects of biosurfactants and their inhibitory effect on the growth of the AIDS virus have been reported recently (1). The pulmonary surfactant, essential for normal respiration, is a phospholipid protein complex; many premature infants suffer respiration failure because of the deficiency of this surfactant. The human gene for production of the protein molecule of this surfactant has been isolated and cloned in bacteria; this has opened up the possibility of larger-scale production of this surfactant for medical applications (4).

Interest in biosurfactants is increasing, as they have promise in several commercial applications. At present, the economics of biosurfactant production have not received attention, but economic strategies must be devised if they are to compete with chemical surfactants. The choice of inexpensive raw materials is important to the overall economics of the process because they account for fifty percent of the final product cost. In our previous studies, high-value SLs were biosynthesized with readily available and low-cost substrates, but yields were not high (5). Optimization of SLs production was achieved in a batch fermentation with glucose and safflower oil as substrates (6). Key factors governing the success of biosurfactant production are the development of an economical process that uses low-cost raw materials and gives high product yield and selected biosurfactants for specific applications. Of prime consideration is the cost of production and end use for the biosurfactant (SLs).

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MATERIALS AND METHODS

Culture maintenance. The microorganism *Candida bombicola* ATCC 22214 was obtained from the American Type Culture Collection (Rockville, MD). The *C. bombicola* was maintained on yeast malt (YM) agar, consisting of 0.3% Bacto-yeast extract (YE), 0.3% Bacto-malt extract, 0.5% Bacto-peptone, 1% Bacto-dextrose and 2% Bacto-agar, made up to one liter with deionized water. Transfers were made to fresh agar slants in screw-capped tubes to maintain viability each month at 4°C with caps loosely screwed on.

Medium formulation. Glucose was purchased from Sigma Chemical Co. (St. Louis, MO). Lactose and cheese whey were obtained from Adult Food (London, Ontario, Canada). Crude degummed canola oil was obtained from CanAmera Foods (Russell, Manitoba, Canada). YE was purchased from Difco Co. (Detroit, MI). The chemical requirements for growth and lipid production of *C. bombicola* consist of a carbon source, an energy source, water, a nitrogen source, a mineral source and vitamins. The optimized medium, for SLs production, contains 0.1% KH_2PO_4, 0.5% $MgSO_4 \cdot 7H_2O$, 0.01% $FeCl_3$, 0.01% NaCl, 0.4% YE, 0.1% urea, 10.5% canola oil and 10% glucose. The pH was adjusted to 4.5 and the medium was sterilized by autoclaving at 121°C for 20 min. For cultivation in a larger fermenter, sterilization time was increased to 30 min. There was no adjustment of pH during cultivation, and pH decreased toward the end of cultivation to between 3.2 and 3.4.

Inoculum preparation and reactors. For the 500-mL Erlenmeyer flask experiments, inoculation was done with agar slants. A loopful of the cream-colored culture was transferred into a 500-mL Erlenmeyer flask that contained 100 mL of the above medium. The culture was incubated aerobically for one day at 30°C on a rotary shaker (250 rpm). Culture broth (20 mL) was transferred to a Bellco jar glass fermenter (Bellco Glass Inc., Vineland, NJ) with 700 mL medium (3% vol/vol inoculum). The fermenter was magnetically agitated with a Bell-Stir Magnetic stirrer and magnetic plate at a speed of 450 rpm. Aeration was set at 2 vols of air per volume of liquid per minute (vvm). The air supplied to the fermenter was passed though a glass column, packed with cotton to a depth of 10 cm. The temperature was maintained at 30°C by use of an external water bath.

Biomass determination. At regular intervals, samples were withdrawn for analyses. The biomass concentration was determined on the basis of its dry cell weight. Five mL of culture broth was mixed with 10 mL of MeOH/$CHCl_3$ (10:1). The mixture was shaken and centrifuged at 2000 × *g* for 20 min to form a biphasic solution. The upper phase was discarded, and the lower phase (solid) was washed with distilled water, placed in an oven at 105°C for 48 h, to dry the cells, and weighed.

SLs analysis. The SLs (extracellular lipids) were extracted from 5 mL of culture broth with 10 mL ethyl acetate by thoroughly shaking and centrifuging (2000 × *g*) for 20 min. The lipid extract was separated into SL fractions on thin-layer chromatography (TLC) plates with Bakerflex sheets, silica gel IB2-F (J.T. Baker, Phillipsburg, NJ) and chloroform/methanol/water (65:15:2, vol/vol/vol) as the developing solvent. After development, the plate was dried and sprayed with α-naphthol (98%) to detect the monosophorolipids. The SL classes were identified by comparison with standards.

Two methods (A and B) were used for quantitative estimation of SLs. Method A used TLC/flame-ionization detector (FID). In this method, TLC is performed on a thin rod of refractory and chemically stable material with a bonded, sintered partition medium as an outer coating. A 1-μL sample was applied to one end of the rod and then developed with the solvent system chloroform/methanol (90:4). The rods were dried to remove the solvent and were passed through the FID where the separated components were detected. Peaks of sophorolipids on TLC/FID were identified by comparison of TLC and TLC/FID of different fractions after partial separation on a SEP-PAK silica cartridge (Waters, Milford, MA). A 0.5-mL sample of isolated SLs in ethyl acetate was applied on the SEP-PAK cartridge, washed with hexane and eluted by the above TLC solvent mixture. Calibration of the FID detector was done by measuring a set of different SL isolates, correcting for fat content and calculating an average. TLC/FID distinguishes between highly hydrophobic diacetyl-SLs (SL-1) and more hydrophilic groups of monoacetyl-SL and/or SL without acetyl, on which basis the two SL groups were quantitated.

Method B involves a solvent extraction of sophorolipids with ethyl acetate, removing the solvent, and drying. A 50-mL sample of fermentation broth was centrifuged at 5000 rpm for 20 min. The supernatant was removed and placed into a 250-mL separatory funnel, and 25 mL distilled water and 100 mL ethyl acetate were added to the cell pellet, which was resuspended and centrifuged. The supernatant was placed into the same separatory funnel as above. The mixture of broth and ethyl acetate formed two phases. The upper phase was transferred to a round bottom flask. The lower phase was extracted with an equal volume of ethyl acetate, three times, for complete recovery of SLs. The round-bottom flask was placed on a roto-evaporator (Buchi RotoVapor-R; Flawil, Switzerland) and the ethyl acetate was evaporated under vacuum at 80°C. The residue was washed with hexane, three times, to remove fatty acids, and then dried in the oven to constant weight.

TLC/FID offers an advantage of small sample size for estimation of SL content and complete separation of SLs from oils.

Sugar determination. The method for the determination of sugar concentration was done in the water phase after extraction of SLs by the dinitrosancyclic (DNS) method (7).

Surface and interfacial tension measurements. The measurements of the surface and interfacial tensions were carried out with a Fisher (Cincinnati, OH) Autotensiomat, which is a modified duNoüy surface tensiometer, equipped with a motorized sample stage and a sensitive strain gauge connected to a platinum ring.

RESULTS AND DISCUSSION

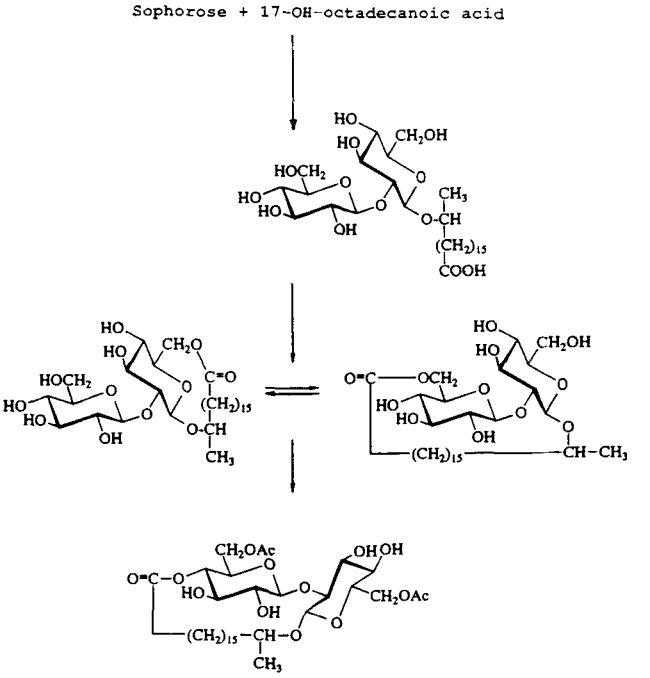
The prospect for producing biosurfactants by the yeast *C. bombicola* largely depends on identifying an inexpensive and abundant feedstock. The objective in the commercial production of SLs can be described as "optimum quality and quantity of SLs at minimum cost." A variety of carbon sources can be used for SL production, including hydrocarbon, carbohydrate and vegetable oil sources. The optimal yields are obtained when both carbohydrate and vegetable oil are used as substrates together.

Product yields, based on substrate, must be considered when choosing a feedstock. For example, although biosurfactants from *Corynebacterium fascians* can be produced on sucrose or hexadecane, yields were over twenty times higher on the latter, thus offsetting the higher cost of the hydrocarbon.

A potential source of substrates and the least expensive is waste streams. In addition, waste treatment costs can be offset by the production of valuable co-products. Such carbon sources can often be obtained at little or no cost. For example, considerable amounts of whey (4–5% lactose content) are produced during cheese processing. To utilize the lactose effectively, a given organism must be able to consume both the lactose and its hydrolysis products, glucose and galactose. *Candida bombicola* is such an organism.

Regardless of carbohydrate or fatty acid used, the same SL is obtained, namely, sophorose, acetylated at position 6′ and 6″ and linked to 17-hydroxy-octadecanoic acid. This suggests that a highly specific enzymatic sequence is involved in the *de novo* synthesis of sophorose and its subsequent linkage to the hydroxy fatty acid. *Candida bombicola* can synthesize both the lipid and the sugar moiety *de novo* when grown on carbohydrates solely. In the presence of a lipophilic carbon source, only the sophorose sugar is formed *de novo*. Scheme 1 shows a postulated last step in the reaction of sophorose with 17-hydroxy-octadecanoic acid to form SLs that include both the acetylated and lactonized derivatives.

In our previous report, nitrogen exhaustion was thought to switch on biosurfactant production, and the results demonstrated that SLs were secondary metabolites (5,6). Figure 1 shows the effect of yeast extract content on the production of SLs in the cultivation of *C. bombicola* in a 1-L fermenter in a medium containing 10.5% glucose and 10.5% canola oil. As shown in Figure 1, best yields of the SLs were obtained at 0.4% YE. Below 0.2% YE and above 0.6% YE, SL biosynthesis diminished dramatically. These results indicate that nitrogen concentration strongly controls SL biosynthesis, in agreement with our earlier report. Limitation of nitrogen leads to increased activity of the enzymes involved in SL production, which are not fully active until cell growth has ceased. A maximum of about 160 g/L SLs was obtained at 0.4% YE. The major SL found was 17-L-([2′-O-β-D-glucopyranosyl-β-D-glucopyranosyl]-oxy)-octadecanoic acid 1′-4″-lactone 6′,6″-diacetate (SL-1), which increased to 73 from 50% when canola oil was used instead of safflower oil as the lipophilic carbon source. Use of canola oil resulted in yields of SLs comparable to those obtained when safflower oil was used in the medium (6). Other data from literature report yields of 70 g/L, and 120 g/L SLs with these substrates.



(SL-1)

SCHEME 1

Canola oil and glucose utilization, cell growth and SL production by *C. bombicola* in shaker flasks are shown in Figure 2. After 3 d of fermentation, the biomass content was

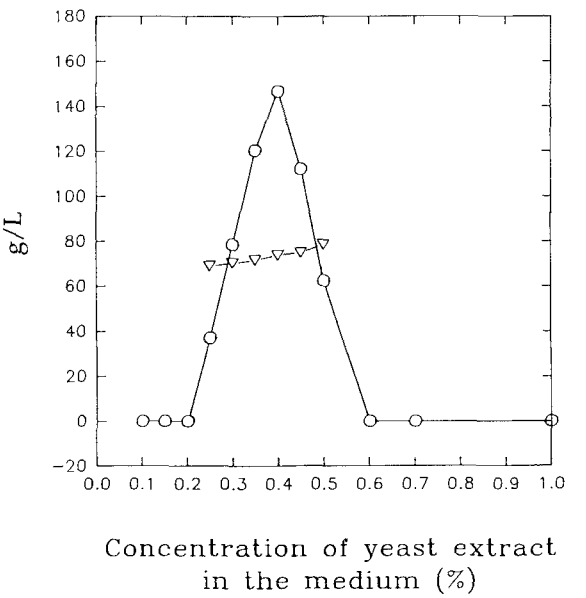


FIG. 1. Effect of yeast extract on the production of sophorose lipids (SLs) in the cultivation of *Candida bombicola* on 10% glucose and 10.5% canola oil. ○, Highest concentration of sophorose lipids in the cultivation. ▽, Concentration of SL-1 in the mixture of SLs.

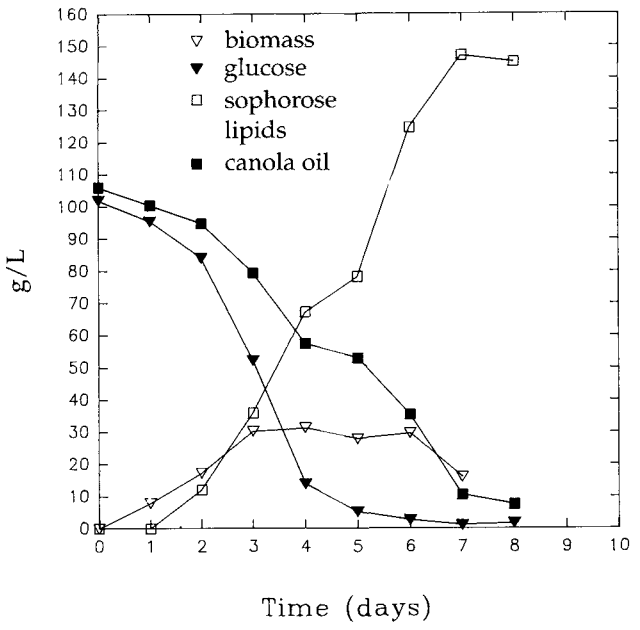


FIG. 2. The time course of cultivation of *Candida bombicola* in shake flasks in medium containing 10% glucose, 10.5% canola oil and 0.4% yeast extract.

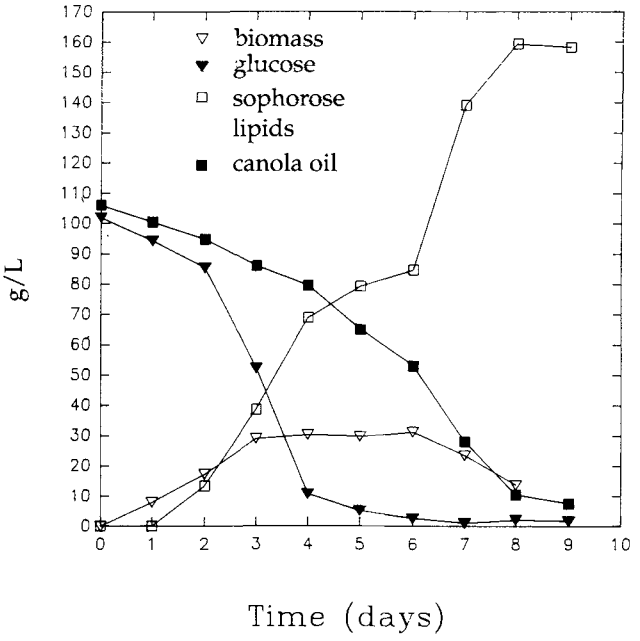


FIG. 3. The time course of cultivation of *Candida bombicola* in 1-L fermenters in medium containing 10% glucose, 10.5% canola oil and 0.4% yeast extract.

31 g/L, which remained constant till day 6, then decreased. Lipid accumulation started in the middle of the exponential growth phase and increased rapidly until growth ceased, at which time nitrogen was nearly exhausted. Glucose was more quickly converted to biomass and SLs than canola oil. Glucose was nearly completely consumed after 4 d, whereas canola oil was nearly consumed after 7 d. At the beginning of the fermentation, mainly glucose with a small amount of canola oil was converted to biomass, then to SLs. The highest yield of SLs (150 g/L) occurred at 7 d of fermentation. The results suggest that enzymes for biosynthesis of SLs were produced during the exponential growth phase. When nitrogen was nearly exhausted, biosurfactant synthesis was switched on.

Figure 3 shows the biomass production, SLs production, canola oil and glucose utilization from the growth medium in a 1-L fermenter, which were similar to those observed in the flask cultures. A yield of about 160 g/L of SLs was obtained.

Because lactose can be easily obtained from cheese whey, lactose was used to replace glucose to repeat above experiments in both shaker flasks and the 1-L fermenter. The results obtained in both instances were similar. Figure 4 shows the time course of cultivation of *C. bombicola* in the 1-L fermenter in medium containing 10% lactose, 10.5% canola oil and 0.4% YE. Conversion of canola oil and lactose of approximately 45% to SLs was achieved in the 1-L fermenter, compared to 80% from glucose and canola oil. SLs were detected after 3 d of fermentation, again when depletion of nitrogen in the growth medium had occurred. The maximum production of 90–110 g/L SLs was obtained after 8 d of fermentation. The maximum production of SLs from lactose and canola oil decreased, compared to that from glucose and canola oil. When 20% of solid cheese whey (70% lactose) replaces lactose in the above medium, about 12 g/L SLs were obtained. These data demonstrate the potential for using canola oil, lactose and cheese whey for commercial fermentation production of SLs. A comparison of SL production from various substrates is shown in Figure 5. The data are from our previous reports (5,6) and the present report. The highest production of SLs is that from canola oil and glucose. The next was from safflower oil and glucose. It also showed that the highest production of SLs was obtained when both vegetable oil and sugar were used as substrates.

From the measurements of surface and interfacial tension, it was found that SL obtained from glucose or lactose plus canola oil could lower the surface tension of water (air–water interface) from 72 to 33 mN/m, and the interfacial tension (oil–water interface) for water against *n*-hexadecane from 40 to 1 mN/m, which shows that it is a good surfactant. There were no significant differences in the surface and interfacial tension of the SLs made from various vegetable oils and sugars.

The commercial interest in SLs production has triggered our research toward the synthesis of this high-value biosurfactant. The key to the successful commercial production of SLs lies in using inexpensive and plentiful substrates. In this work, several low-cost substrates were used to produce SLs to reduce final product cost. The data show that a wide range of carbon sources, including agricultural renewable resources like sugars and oils are suitable carbon sources for the high-yield production of SLs with good surfactant properties. There is still research to be done in this area. One question is: Is it more economical to use bulk, readily available by-product materials to produce biosurfactants? Also, further improvements in reactor design, such as the two-stage continu-

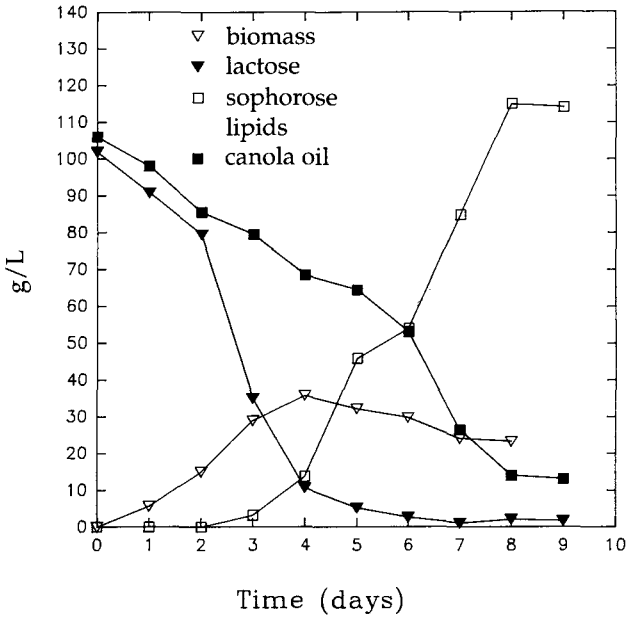


FIG. 4. The time course of cultivation of *Candida bombicola* in 1-L fermenters in medium containing 10% lactose, 10.5% canola oil and 0.4% yeast extract.

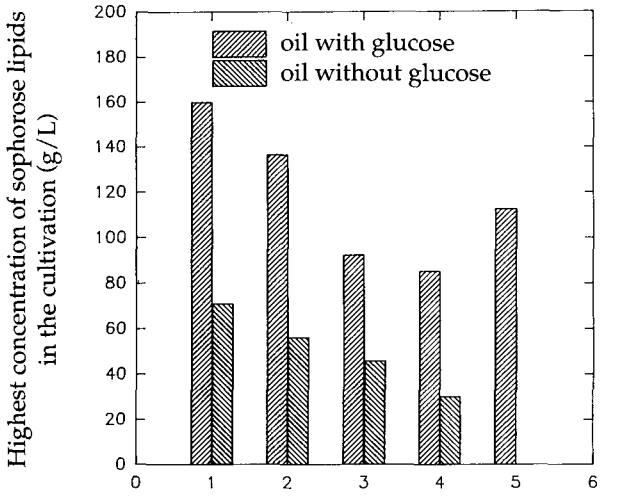


FIG. 5. Comparison of production of sophorose lipids by various substrates; 1. canola oil, 2. safflower oil, 3. sunflower oil, 4. olive oil, 5. canola oil + lactose.

ous system, can greatly enhance the productivity of SL, and this could the focus of future research.

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