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Conversion of sucrose to isomaltulose by *Klebsiella planticola* CCRC 19112

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An isomaltulose-producing bacterium was isolated and taxonomically characterized. Its morphological and biochemical properties conform best to those described for *Klebsiella planticola*. When cultured under optimal conditions, the organism simultaneously converted sucrose into both isomaltulose (α-D-glucopyranosyl-1,6-fructose) and trehalulose (α-D-glucopyranosyl-1,1-fructose) with substrate conversion rates of 80% and 15%, respectively. Sucrose and Bacto-tryptone were the most effective carbon and supplemental nitrogen sources, respectively, for producing cells of high isomaltulose-forming ability. None of several inorganic salts tested had any significant effect. The major product formed in the reaction mixture was verified to be isomaltulose by co-chromatography and IR spectroscopy.

Keywords: sucrose; isomaltulose; trehalulose; isomaltulose synthase; *Klebsiella planticola*

Introduction

Isomaltulose (α-d-glucopyranosyl-1,6-fructose, commonly referred to as palatinose), is a reducing sugar and structural isomer of sucrose found naturally in honey and sugarcane extract. It has similar physical properties to sucrose except that its sweetness is about 42% that of sucrose. Recently, isomaltulose and its hydrogenated derivative, palatinitol (isomaltitol), have attracted the attention of researchers, thanks to their industrial applications either as a sucrose substitute in food processing [7,11,16] or as a starting material for producing special chemicals, such as surfactants and polymers [8,9,18]. Both surfactants and polymers are manufactured by the chemical industry in large quantities, mostly from downstream products of the petroleum industry. The increased concern of environmental contamination caused by these hard-to-degrade substances has urged scientists to pursue alternative materials such as the hydrophilic and biodegradable carbohydrates, for their production.

Dental caries is the most prevalent disease among civilized human populations [14,23,25]. The oral flora is an environment with many acid-producing microorganisms including *Streptococcus mutans*. As fewer acids and insoluble glucans are produced from isomaltulose than from sucrose by *Streptococcus mutans* during oxidative degradation, isomaltulose is thus considered an ideal substitute for sucrose in preventing dental caries. It has been reported that the incidence of dental caries in permanent dentition increases with the increasing age of children in Taiwan, and as many as 94% of children at age 17 were reported to be affected [20]. One critical measure generally recognized for effectively reducing dental caries is to minimize sucrose consumption or to substitute sucrose with non-metabolizable sweeteners in food systems [1]. To meet the increasing demand for isomaltulose, the development of an industrial scale production of isomaltulose is desirable.

Isomaltulose was first demonstrated by Stodola *et al* [22] as a by-product during dextran production from sucrose by *Leuconostoc mesenteroides*. Since the isolation of the isomaltulose-producing *Protaminobacter rubrum*, the metabolic pathway of isomaltulose formation has been well studied [3,26]. The trivial name isomaltulose synthase was proposed for the enzyme that catalyzes the bioconversion [3]. To date, enzymes capable of converting sucrose to isomaltulose have been demonstrated in *Serratia plymuthica*, *Erwinia carotovora* var *atroseptica*, *Erwinia rhapontici* and *Klebsiella planticola* [2,3,6,10,12,26]. Immobilized cells of these organisms have been used in the industrial production of isomaltulose [4,5,18]. To be cost-effective, such processes require those microorganisms used to retain isomaltulose-producing activities during prolonged reactions and recycling.

In order to expand the spectrum of the biotechnology industry in this country and to provide the people with a functional sweetener as a sucrose substitute, a process converting sucrose, a major agricultural product in Taiwan, to isomaltulose is worth exploring. The success in establishing such a process would not only increase the economic margin of sucrose but provide a non-cariogenic sucrose substitute critical for the improvement of oral hygiene. In this paper, we describe the isolation and the taxonomic characterization of a newly isolated bacterium capable of producing isomaltulose in a culture medium containing sucrose, and investigate conditions optimal for cell growth and isomaltulose production.

Materials and methods

Chemicals

Sucrose (superior white fine granulated sugar, purity >99.6%, Taiwan Sugar Co) was purchased from a local market. Isomaltulose was obtained from Wako Pure Chemical Industries (Osaka, Japan) and used as the control for

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analysis. Tryptic soy broth, yeast extract, Bacto-tryptone and Bacto-agar were obtained from Difco Laboratories (Detroit, MI, USA). HPLC grade acetonitrile was procured from Alps Chem Co (Hsinchu, Taiwan). All other chemicals were of reagent grade and purchased from commercial suppliers.

Isolation of isomaltulose-producing microorganisms

Microorganisms were grown from inoculum samples, taken from molasses, honey, sugarcane stalks, soil of sugarcane field, and the soils, sewage and leaves around the campus, in an enriched culture medium containing a high concentration of sucrose (15%) as the major carbon source. Seven days later, growth was transferred to a fresh medium for further selection. Following serial dilutions with sterile water, the microorganisms were isolated on tryptic soy agar plates using a direct isolation technique. Four isolates which significantly transformed sucrose into isomaltulose were selected. After purification by the standard streaking technique, the selected isolates were subjected to a second screening test for isomaltulose formation. All four strains were bacteria. The strain (No. 356) which exhibited the highest isomaltulose-forming activity obtained from a sample of plant leaves collected on this campus, was studied further. The bacterial strain was identified, according to Bergey's Manual [17], as *Klebsiella planticola*. It has been deposited in the Culture Collection and Research Center of the Food Industry Research and Development Institute, Hsinchu, Taiwan with an accession number CCRC 19112. The organism was frozen in 3% tryptic soy broth containing 50% (v/v) glycerol at –30°C for long-term storage or maintained on 3% tryptic soy agar medium at 4°C with monthly subculture. An electron micrograph of the negatively stained cell from a 24-h culture is shown in Figure 1.

Media and culture conditions

The medium used for the isolation of isomaltulose-producers from natural resources contained a high concentration of sucrose as the sole carbon source. It was composed of 150 g sucrose, 30 g corn steep liquor, 2 g yeast extract, 2 g Na_2HPO_4 and 3 g NaCl per liter, which was adjusted to pH 7.0 prior to sterilization by autoclaving. For comparing the isomaltulose-forming abilities, the microorganisms were cultured in 3% tryptic soy broth agar slant at 30°C for 24 h. Five milliliters of a 50% sucrose solution were then added. After static incubation at 30°C for 2 days, isomaltulose and other sugars in the supernatant were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

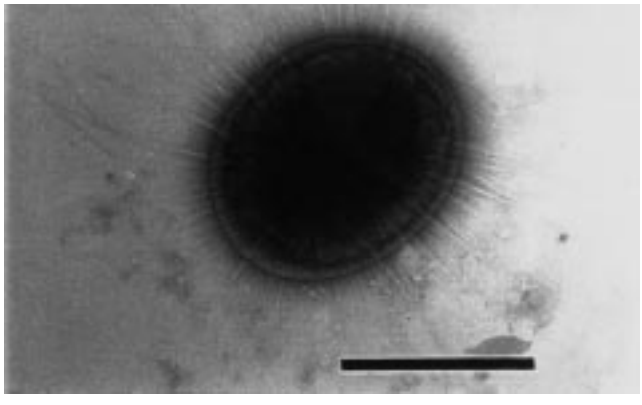

Figure 1 An electron micrograph of a cell of *K. planticola* 356. The cell was negatively stained with 2% phosphotungstic acid. Bar = 1 μm.

Preparation of cell suspension

A loopful of cells, grown on a 3% tryptic soy agar slant at 30°C for 24 h, was inoculated into 50 ml of 3% tryptic soy broth in a 250-ml Erlenmeyer flask and cultivated at 30°C for 12 h in a reciprocating shaker water bath set at 125 opm and a 3.85-cm stroke. Aliquots (2 ml) of the culture broth were transferred to 500-ml Hinton flasks containing 100 ml medium. Unless otherwise specified, the medium contained 70 g sucrose, 30 g tryptic soy broth, 20 g Bacto-tryptone and 5 g NaCl in 1 L distilled water adjusted to pH 7.0. The flasks were incubated at 30°C in a rotary shaker set at 125 rpm and a 7-cm stroke. After 18-h cultivation, cells at their early stationary phase were harvested by centrifugation at $5000 \times g$ for 10 min at room temperature and washed three times with sterile 0.85% saline solution. The cell pellets were resuspended in the same solution at the desired concentrations.

Conversion of sucrose into isomaltulose by cell suspension

Reactions were conducted in test tubes (16×150 mm) containing 5 ml of a 50% or 60% sucrose solution and the specified number of cells at 40°C in a reciprocating shaker water bath set at 30 opm and a 3.85-cm stroke. The temperature used has been determined to be optimal for this reaction. The reactions were terminated by heating the tubes for 10 min in a boiling water bath.

Determination of isomaltulose-forming activity

The activity of isomaltulose synthase (EC 5.4.99.10) [19] was assayed by measuring the formation of isomaltulose from the sucrose substrate. The reaction mixture contained 5 ml of a 60% sucrose (Sigma, St Louis, MO, USA) in 0.1 M acetate buffer (pH 5.0), and 1 ml of an appropriately diluted whole culture sample. The reaction was allowed to proceed at 40°C, with constant shaking, for 4 h and terminated by boiling in a water bath for 10 min. One unit (U) of isomaltulose synthase activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of isomaltulose per min under the assay conditions.

Analytical methods

Cell growth was monitored by measuring the optical density of the culture broth at 660 nm (OD_{660}). Qualitative and quantitative determinations of isomaltulose, trehalulose, sucrose and other major sugars were conducted by TLC and HPLC. TLC analysis was performed on silica gel plates and developed $3\times$ with acetonitrile:water (90:10, v/v) at room temperature. The plates were then sprayed with aniline-diphenylamine-85% phosphoric acid-acetone (4 ml:4 g:20 ml:200 ml) and heated at 100°C for 5–8 min. HPLC analysis was performed on a LiChrospher 100 NH_2 (5 μm, 4×250 mm) column. Samples were eluted isocratically with acetonitrile:water (90:10, v/v) at a flow rate of 2.0 ml min^{-1} and detected upon passage through a

Bischoff 8110 refractive index detector (Leonberg, Germany). The detector was coupled to a Chromatocorder for quantification. The retention times for isomaltulose, trehalulose and sucrose were 9.2, 10.7 and 8.2 min, respectively, and the correlation coefficients of sugar concentration and peak area for isomaltulose and sucrose were 0.9981 and 0.9991, respectively. For the quantitative determination of trehalulose, it was assumed that, at the same concentration, the refractive indices of sucrose, isomaltulose, and trehalulose solutions are identical. The infrared absorption spectra were determined in a Perkin-Elmer 983G spectrophotometer. TLC, HPLC, and infrared spectroscopy were used to confirm the authenticity of isomaltulose produced in the reaction mixture.

Results

Taxonomic studies of strain 356

The morphological, cultural, and physiological characteristics of strain 356 were studied by conventional methods. Microscopic examinations were used to establish the morphology of the isolate. The strain possesses fimbriae. After 24-h shaker culture in a tryptic soy broth at 30°C, cells exhibited a morphology of rod with round ends, with a dimension of $0.9–1.5 \times 0.75–1.0\ \mu$m. Figure 1 shows an electron micrograph of a negatively stained cell. Isolate 356 is Gram-negative, catalase-positive, oxidase-negative, facultatively anaerobic, non-motile, and capsulated. Table 1 summarizes some additional characteristics of the isolate. Based on these characteristics, strain 356 conforms best to the description of *Klebsiella planticola* in Bergey's Manual [17]. To date, only one other report described the production of isomaltulose by this species [26].

Optimization of culture medium for the expression of isomaltulose-forming activity

Using a basal medium containing 1% sucrose and 3% tryptic soy broth, the growth profile of *K. planticola* 356 (CCRC 19112) was studied. Following a 16–18 h cultivation as the cells entered the late exponential or early stationary phase, the level of intracellular isomaltulose-forming activity reached its maximum. No isomaltulose-forming activity could be detected in the medium. Prolonging the time of culturing did not significantly increase the level of enzyme activity. To obtain cells with a higher conversion activity, the effect of medium composition on isomaltulose-forming activity of *K. planticola* 356 (CCRC 19112) was examined.

Table 1 Taxonomic characteristics of strain 356

Characteristic	Result
Indole production	–
Methyl red test	–
Voges–Proskauer test	+
Gas production from lactose at 44.5°C	–
Growth at 10°C	+
Lysine decarboxylase	+
Urease	+
Ornithine decarboxylase	–
H_2S production	–
Utilization of:	
d-Glucose	+
Citrate	+

(1) Effects of carbon sources: Many carbon sources supported the formation of isomaltulose synthase activity of *K. planticola* 356 (CCRC 19112). Among them, sucrose and fructose were most effective, as shown in Table 2. Sucrose was chosen for subsequent studies because of its availability and lower cost. Figure 2 shows the effect of sucrose concentration on the isomaltulose-converting activity in the cells. The isomaltulose-forming activity reached its maximum at a sucrose concentration of 7% and remained constant up to 10%.

(2) Effects of inorganic salt: Various inorganic salts tested did not show a significant effect on isomaltulose-forming activity, as shown in Table 3. Nevertheless, a slight but reproducible effect of sodium chloride was seen and therefore 0.5% sodium chloride was used in subsequent studies.

(3) Effects of supplemental nitrogen sources: No remarkable differences in isomaltulose-forming activity were observed for all the nitrogen sources tested (Table 4). However, organic nitrogen sources appeared to be more effective than inorganic ones. When 1% of Bacto-tryptone, Bacto-peptone, soybean flour or casein was supplemented to the basal medium, the conversion activity of the cells was generally higher than that obtained from cells grown on ammonium compounds. As 1% Bacto-tryptone resulted in the highest isomaltulose-forming activity, it was used in subsequent experiments.

(4) Time course of isomaltulose production: To follow the time course of isomaltulose formation, a 1-ml cell suspension was incubated with 5 ml of a sucrose solution (50%) in a test tube at 40°C for 24 h with agitation. Aliquots of the reaction mixture were sampled and analysed for the amounts of isomaltulose formed. Following inactivation of the reaction by boiling for 10 min in a water bath, the solution was cooled and centrifuged. Isomaltulose in the supernatant was determinated by HPLC. Figure 3 shows a typical profile of the conversion of sucrose into isomaltulose using washed cells of *K. planticola* 356 (CCRC 19112) grown in the optimized medium. The conversion of sucrose was very rapid during the first 1–2 h and nearly reached completion in 8 h. As the maximum level of conversion was obtained after 4 h of incubation, later experiments were carried out for 4 h. The yield, represented by the ratio of the concentration of isomaltulose formed over that of sucrose used (50%), was more than 76%.

Isolation and identification of the transformation products

For identification of the products formed, TLC was carried out to separate the products in the filtrate of the culture broth or the reaction mixture. A major spot was detected after spraying the plate with aniline-diphenylamine-85% phosphoric acid-acetone [21]. The mobility of the spot was similar to that of authentic isomaltulose. For large-scale

Table 2 Effects of carbon source on isomaltulose synthase production by *K. planticola* 356 (CCRC 19112)

Carbon source (2%, w/v)	Growth (OD_{660})	Isomaltulose synthase activity (U ml^{-1})	Specific productivity
Control	0.34	0.29	0.87
Glucose	0.45	2.21	4.97
Fructose	0.58	5.20	9.00
Sucrose	0.59	4.82	8.24
Isomaltulose	0.56	3.42	6.16
Maltose	0.60	2.16	0.62
Lactose	0.73	1.94	0.66

Basal medium contained 30 g tryptic soy broth in one liter distilled water. Cells were cultured at 30°C for 18 h at 125 rpm in 500-ml shaker flasks, each containing 100 ml of basal medium. The values presented are the means of at least two experiments. Specific productivity was calculated by dividing the isomaltulose synthase activity (U ml^{-1}) by the cell growth (OD_{660}).

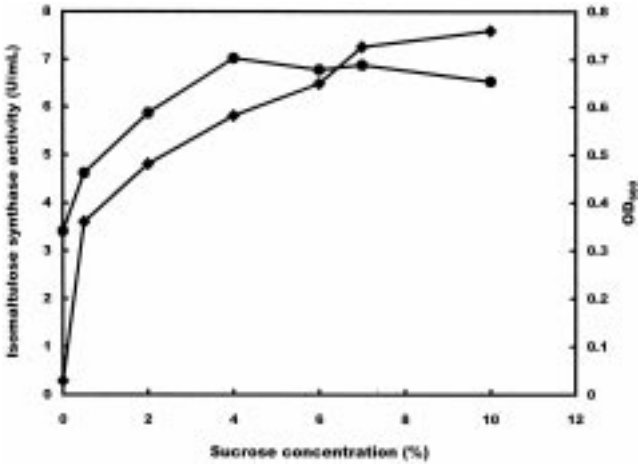

Figure 2 Effect of sucrose concentration in the growth medium on the induction of isomaltulose synthase activity (—◆—) of *K. planticola* 356 (CCRC 19112). Cells were cultured for 18 h at 30°C in 100 ml medium containing the indicated concentrations (%, w/v) of sucrose in 500-ml shaker flasks under constant agitation at 125 rpm in a rotary shaker. The basal medium contained 30 g tryptic soy broth per liter distilled water. (—●—) OD_{660}.

Table 3 Effects of inorganic salts in culture medium on isomaltulose synthase production by *K. planticola* 356 (CCRC 19112)

Inorganic salt (0.5%, w/v)	Isomaltulose synthase activity (U ml^{-1})
NaCl	9.32
KCl	8.31
Na_2SO_4	8.64
Na_2CO_3	8.04
K_2HPO_4	8.78
KH_2PO_4	8.91
NaH_2PO_4	9.05
Control	8.37

Basal medium contained (per liter of distilled water): 30 g tryptic soy broth and 70 g sucrose. Cultivation conditions were the same as described in Table 2. The data presented are the means of at least two experiments. The deviations from the means are less than 10%.

recovery of the product, acetone was added slowly to 50 ml of the filtrate (about 42% isomaltulose) while stirring. A thick paste-like substance appeared. The paste was removed by decantation and redissolved in 100 ml of 95% ethanol

Table 4 Effects of additional nitrogen sources on isomaltulose synthase production by *K. planticola* 356 (CCRC 19112)

Nitrogen source (1%, w/v)	Isomaltulose synthase activity (U ml^{-1})
Bacto-peptone	10.18
Bacto-tryptone	11.08
Casein	9.08
Soybean flour	10.80
Urea	10.53
Ammonium sulfate	9.91
Ammonium chloride	9.32
Sodium nitrate	7.02
Control	9.32

Basal medium contained (per liter of distilled water): 30 g tryptic soy broth, 70 g sucrose and 5 g NaCl. Cultivation conditions were as described in Table 2. The data presented are the means of at least two experiments. The deviations from the means are less than 10%.

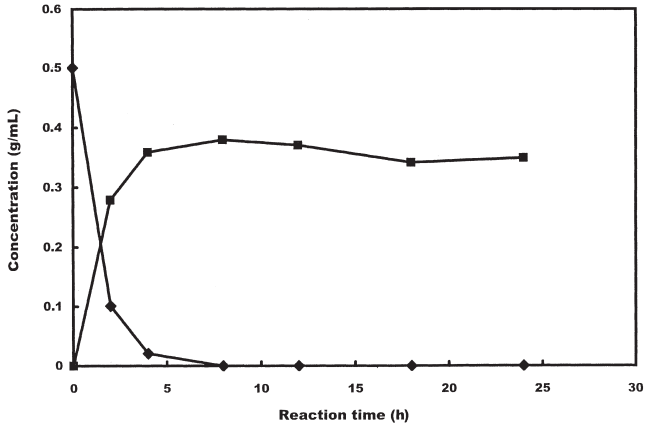

Figure 3 Time course of isomaltulose formation by resting cells of *K. planticola* 356 (CCRC 19112) during a 24-h incubation. Reactions were conducted at 40°C in test tubes (16 × 150 mm) containing 5 ml of a 50% sucrose solution and 1 ml of the cell suspension in a reciprocating shaker water bath adjusted to 30 opm. Cell suspensions were prepared as described in Materials and Methods. The optical density (OD_{660}) of the cell suspension, after 200 × dilution, was 0.446. The broth was sampled at different time intervals and the concentrations of isomaltulose (—■—) and sucrose (—◆—) were determined.

in a water bath at 80°C. After cooling, the white precipitate formed was collected and washed twice with 95% ethanol. The residue, which was dried to a constant weight of 14.7 g, appeared as crystalline powder. The calculated yield of the product from sucrose was 49%. The product thus obtained co-migrated with an authentic isomaltulose during TLC analysis and had a retention time and IR spectrum (data not shown) identical to those of the authentic isomaltulose.

Discussion

Isomaltulose synthase (EC 5.4.99.10) catalyzes the conversion of sucrose into both isomaltulose and trehalulose [3,13,15]. In this study, the isolated bacterium, *Klebsiella planticola* 356 (CCRC 19112), converted sucrose into isomaltulose (76–84%), trehalulose (14–16%) and trace amounts of glucose (2–6%) and fructose (2–3%) when grown in a sucrose medium. As compared to the other isomaltulose-producing strain of *K. planticola* reported by Tsuyuki *et al* [26], the strain produces more isomaltulose (76–84 *vs* 57–68%) but less trehalulose (14–16 *vs* 25–30%) from the sucrose medium. Optimization of medium composition and cultural conditions for the previously reported isolate [26] were however not described. The extent of conversion of sucrose into isomaltulose could be optimized by increasing the density of cells in the reaction system. When cultured in basal medium, the strain could accumulate a high isomaltulose-forming activity ranging from 9.0 to 12.0 U ml^{-1} at the early stationary phase. The enzyme appeared to be localized in the periplasmic space or bound to the plasma membrane of the cells when cultured under a variety of media containing various carbon sources. The bacterium preferentially utilizes mono- and di-saccharides over polysaccharides. The highest isomaltulose synthase activity was obtained with fructose in the medium. A comparable, but slightly lower, enzyme activity was also seen using sucrose in the medium. Other carbon sources tested, including glucose, maltose, lactose, and isomaltulose, supported less enzyme production under the conditions used (Table 2). It has been reported that the enzyme could be induced by sucrose or isomaltulose [3,5,13]. In our studies, fructose and sucrose appeared to be the two that had significant effects on enzyme induction. The purified enzyme exhibited a stringent substrate specificity, utilizing sucrose efficiently, but not all other substrates tested, including isomaltulose, trehalose, lactose, maltose, raffinose, and glucose + fructose (data not shown).

For industrial applications, it is desirable to produce cells with high isomaltulose-forming activity. However, it has been reported that viable cells were not absolutely required for isomaltulose formation [2]. The large-scale production of catalytically active cells of *K. planticola* 356 has been problematic both for laboratory studies as well as industrial applications. The composition of culture medium appeared to be an important factor affecting isomaltulose synthase expression. A couple of practical measures were therefore taken to improve the substrate conversion. First, the conversion of sucrose to isomaltulose was carried out with the harvested cells resuspended in the buffer or in saline solution. Second, a medium with concentrated sucrose (50–60%) lacking other nutrients was used so that sucrose could not be effectively utilized to support the growth of cells and the induction of other enzymes, which tend to produce side-products. Figure 3 was a typical example of experiments based on such strategies. The conversion of sucrose into isomaltulose was probably preceded by the intramolecular transglucosylation. The ability of the isolate to convert sucrose into isomaltulose in a reaction mixture containing a high concentration of sucrose is advantageous, because the equilibrium between isomaltulose and sucrose in the reaction favors the formation of isomaltulose. In addition, a medium with concentrated sucrose may also prevent the growth of contaminating microorganisms, thereby greatly reducing not only the size of equipment and the volume of syrup required for isomaltulose production but also the amount of water that needs to be removed prior to product crystallization.

In conclusion, we have succeeded in obtaining an isomaltulose-producing bacterium which could efficiently convert sucrose into isomaltulose. The biocatalytic procedure established herein for isomaltulose production is highly efficient, requiring a relatively simple operation. A protocol for whole-cell immobilization of this isolate was previously developed in this laboratory [24], which has the potential for large-scale applications. Further investigations on the kinetics of the enzyme reactions and the molecular mechanisms involved are apparently needed to better utilize the enzymes produced by *K. planticola* 356 (CCRC 19112). The development of a pilot-scale procedure for isomaltulose production is currently underway in this laboratory.

Acknowledgements

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