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Production of 6-kestose by the filamentous fungus *Gliocladium virens* as affected by sucrose concentration

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ABSTRACT

The filamentous fungus *Gliocladium virens* is able to produce fructooligosaccharides (FOS), fructose-containing sugars, used as functional ingredients to improve nutritional and technological properties of foods. In this work we evaluated FOS production by *G. virens* when grown in a wide range of sucrose concentrations (10–400 g l^{-1}). High sucrose concentrations increased both biomass and FOS production, including 6-kestose, a trisaccharide comprising β (2 → 6) linked fructosyl units, with enhanced stability and prebiotic activity when compared to the typical FOS β (2 → 1) linked. The highest 6-kestose yield (3 g l^{-1}) was achieved in media containing 150 g l^{-1} sucrose after 4–5 days of culture, production being 90% greater than in media containing 10, 30, or 50 g l^{-1} sucrose. After 5 days, FOS production declined markedly, following complete sucrose depletion in the medium. Although most of the β-fructofuranosidases preferentially catalyze sucrose hydrolysis, FOS production in *G. virens* grown in high sucrose concentration, might be attributed to a reverse hydrolysis by these enzymes. In conclusion, high sucrose concentrations increase growth of *G. virens* whilst 6-kestose accumulation in the medium seems to be controlled both by specific properties of β-fructofuranosidases and on the sucrose concentration.

1. Introduction

Quality of life, including attention to the food we consume, is of increasing worldwide interest. In response to the massive demand for healthy and low calorie ingredients, oligosaccharides, especially fructooligosaccharides (FOS), are now widely used to replace sugars in many food products and have received GRAS (generally recognized as safe) status from the Food and Drug Administration (FDA–USA) (Pure Bulk 2010).

FOS are oligomers of fructose mainly represented by 1-kestose (GF_2), nystose (GF_3) and ^{1}F-fructofuranosylnystose (GF_4). These sugars are known as inulin-type FOS (^{1}F-FOS), in which the fructosyl units are attached to sucrose by β (2 → 1) linkages, distinguishing them from other oligomers (Yun 1996; Sangeetha et al. 2005; Ghazi et al. 2007). These oligosaccharides can be obtained by acid or enzymatic hydrolysis of inulin, a linear polymer composed of β (2 → 1) linked fructose residues attached to a terminal sucrose and widely distributed

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in nature as plant storage carbohydrates, mainly in Asteraceae species. The enzymes responsible for inulin hydrolysis are exoinulinases and endoinulinases (EC 3.2.1.7), which promote, respectively, the release of free fructose and FOS, thereby being useful for the production of fructose-enriched syrups and FOS (Carvalho et al. 2007; Singh and Singh 2010).

FOS are usually obtained industrially from sucrose through fructosyltransferases (EC 2.4.1.9) or β-fructofuranosidases (EC 3.2.1.26) with high transfructosylating activity (Sangeetha et al. 2005). These enzymes act on sucrose in a non-proportional reaction, where one molecule of sucrose serves as donor and the other as acceptor of fructose units forming linear or branched chains (Yun 1996).

Frequently the β-fructofuranosidases such as invertases hydrolyze sucrose to glucose and fructose, but depending on its origin may exhibit transfructosylating activity probably due to reverse hydrolysis. Production of FOS by these enzymes occurs under high sucrose concentrations. In contrast, fructosyltransferases have low hydrolytic and high transfructosylating activities even at low sucrose concentrations (Antošová and Polakovi 2001).

The interest in FOS has increased since they were recognized as functional components in foods, acting as prebiotic factors benefiting human nutrition. FOS stimulate the growth of bifidobacteria, reduce plasma levels of cholesterol, phospholipids and triglycerides, relieve constipation and may inhibit the development of tumors and inflammatory bowel diseases (Patel and Goyal 2011). Additionally, FOS are non-cariogenic compounds and excellent calorie-free sweeteners since they are poorly hydrolyzed by digestive enzymes and comprise a safe food for diabetics (Yun 1996; Maiorano et al. 2008; Patel and Goyal 2011). FOS present interesting technological properties since they are easily incorporated into foods, are stable at high and at freezing temperatures, mimic the properties provided by conventional sugars, and may even improve them. In this regard they provide viscosity, humectancy, freezing point depression, thereby increasing the food shelf life (Crittenden and Playne 1996; Yun 1996; Niness 1999; Patel and Goyal 2011).

There is an increasing interest in novel molecules with prebiotic and physiological effects. Some fungi are able to synthesize levan-type FOS containing two fructose units linked by β (2 → 6) linkages (^{6}F-FOS: 6-kestose), or neolevan-type FOS containing a fructose unit also linked by this type of linkage to a glucose (^{6}G-FOS: neokestose, neonystose, or neofructofuranosylnystose). Such FOS exhibit increased prebiotic activity when compared to the usual inulin-type FOS (Marx et al. 2000; Kilian et al. 2002). Okada et al. (2010) reported four novel trisaccharides in beverages produced by fermentation of an extract of fruits and vegetables by yeasts (*Zygosaccharomyces* spp. and *Pichia* spp.) and lactic acid bacteria (*Leuconostoc* spp.). The O-β-D-fructopyranosyl-(2 → 6)-D-glucopyranose produced during the fermentation was not cariogenic and was selectively used by the beneficial intestinal bacteria, *Bifidobacterium adolescentis* and *B. longum*, but was not used by unfavorable bacteria.

Gliocladium virens, a saprophytic filamentous fungus (Ascomycota, Hypocreales) isolated from the rhizosphere of *Vernonia herbacea*, a native inulin-accumulating Asteraceae from the Brazilian savanna, has the ability to synthesize FOS in sucrose-based medium. Additionally, invertase activity was detected early during fungal development, indicating that *G. virens* is a promising fungus for the production of FOS and enzymes with activities on fructose-based carbohydrates (Pessoni et al. 2009). In this work we analyzed the influence of sucrose concentration on FOS production by *G. virens* aiming to increase the knowledge on the utilization of filamentous fungi in the production of such sugars.

2. Materials and methods

2.1. Biological material and cultivation

Gliocladium virens J.H. Mill., Giddens & A.A. Foster, originally isolated from the rhizosphere of *V. herbacea* (Cordeiro-Neto et al. 1997), was obtained from the URM culture collection at the Federal University of Pernambuco, Brazil (URM number 3333). To obtain the inoculum, the fungus was cultured in potato dextrose agar (PDA) for 7 d at 28 °C. Plugs (6 mm diameter) of agar-containing the mycelium were transferred to 250 ml Erlenmeyer flasks containing 100 ml Czapek medium constituted by the following components in g l^{-1}: $NaNO_3$ (3), KH_2PO_4 (1), KCl (0.5), $MgSO_4 \cdot 7H_2O$ (0.5), $FeSO_4 \cdot 7H_2O$ (0.01). Sucrose was employed as carbon source at 10, 30, 50, 100, 150, 300, and 400 g l^{-1}. The flasks were kept at 28 °C with constant shaking (140 rpm). After the cultivation period, the mycelial mass was separated by filtration, dried, and the dry matter determined gravimetrically. The culture filtrates were used for sugar, protein, and enzymatic analyses. All experiments were performed in triplicate.

2.2. Quantification of total sugars and extracellular proteins

The concentration of total sugars in the culture filtrates was determined by the phenol–sulfuric method (Dubois et al. 1956). An equimolar mixture of glucose and fructose was used as standard. The protein content was determined according to Bradford (1976), using bovine serum albumin as standard.

2.3. Fructooligosaccharide (FOS) analysis

Samples of culture filtrates, containing 5 mg of total sugars, were deionized by ion exchange chromatography in columns (10 × 1 cm) containing cationic (Dowex® 50Wx8) and anionic (Dowex® 1x8) resins from Sigma–Aldrich. The deionized samples, containing 2 mg ml^{-1} of total sugars, were filtered through nylon membranes (0.45 μm) and analyzed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC/PAD) in a Dionex ICS-3000 system with CarboPac PA-1 column (4 × 250 mm), using a gradient of 500 mM sodium acetate in 150 mM sodium hydroxide (Shiomi et al. 1991; Vieira et al. 1995). The identification of the peaks was based on retention times compared to those of standards while their quantification was based on the peak area compared to those of the standards. Additionally, confirmation of the peak identities was carried out by spiking the sample with authentic standards.