Sweet-and-salty biocatalysis: fructooligosaccharides production using *Cladosporium cladosporioides* in seawater

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Abstract Production of fructooligosaccharides (FOS) from sucrose was obtained using a bioprocess entirely performed in seawater. The halophilic fungus *Cladosporium cladosporioides* MUT 5506 was grown in a seawater-based medium and mycelium displayed an optimal activity in seawater at 50-60 ºC, being stable up to 60 ºC. Under optimized conditions in seawater (50 °C, sucrose 600 g/L, lyophilized mycelium 40 g/L), *C. cladosporioides* gave a maximum FOS yield of 344 g/L after 72 h with a preponderance of ^{1}F-FOS derivatives (1-kestose 184 g/L, 1-nystose 98 g/L and 1-fructofuranosylnystose 22 g/L), and the noteworthy presence of the non-conventional disaccharide blastose (30 g/L after 144 h). Lyophilized mycelium exhibited good stability in seawater (76% of the initial activity was retained after 15 cycles of reutilization). This proof-of-concept application reports for the first time the production of FOS in a non-conventional medium such as seawater.

Keywords Fructooligosaccharides • fructofuranosidase • *Cladosporium cladosporioides* • seawater • biocatalysis

Introduction

Industrial bioprocesses are characterized by large consumption of fresh water. The development of new (bio) processes should focus not only on efficiency, but also on their sustainability. On Earth, there is a vast quantity of seawater (96.5%) compared to the small amount of fresh water (2.5%) and brackish water (1%) [1]. Therefore, the possible use of non-potable water (i.e., wastewater or seawater) as a medium for large-scale bioprocesses seems to be a remarkable field of research [2,3]. Halotolerant microorganisms producing enzyme of potential industrial interest are of great interest for developing biotransformations in seawater [4,5].

The facultative halophilic fungus *Cladosporium cladosporioides* was recently discovered as an efficient producer of fructooligosaccharides (FOS) from sucrose [6]; lyophilized mycelium of *Cladosporium cladosporioides* gave mainly 1-kestose, 1-nystose, ^{1}F-fructosylnystose, 6-kestose, neokestose, but also blastose, an unconventional disaccharide ([Fru-β(2→6)-Glc]). FOS are prebiotics used as food ingredients due to their beneficial properties [7,8], such as low caloric intake (2 kcal/g), low glycemic impact, prebiotic action, improved gut absorption of Ca^{2+} and Mg^{2+}, lowering blood lipid levels, prevention of urogenital infections, reduced risk of colon cancer and reduction total cholesterol and triglycerides [9,10]. Short-chain ^{1}F-FOS (mostly 1-kestose, 1-nystose, ^{1}F-fructosylnystose,) are produced from concentrated sucrose solutions using fungal transfructosylating enzymes, such as *Aspergillus aculatus* [11]*, Aspergillus japonicas* [12], *Aspergillus oryzae* [13], *Aspergillus niger* [14], *Aureobasidium pullulans* [15] and *Penicillium citrinum* [16].

In this work, we evaluated the possibility to perform FOS production in seawater using lyophilized mycelium of *Cladosporium cladosporioides* grown in seawater; lyophilized mycelia of fungi are easy-to-handle biocatalysts suitable for exploiting mycelium-bound enzymatic activities, often showing remarkable long-term stability [17,18].

Materials and methods

Materials

Sucrose, glucose, fructose, 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma Aldrich. 1-Kestose and 1-nystose were purchased from Fluka. 1-Fructofuranosylnystose was purchased from Megazyme. Yeast extract was purchased from Difco and barley malt flour from Diagermal. Malt extract was from Merck. All reagents and solvents were of the highest available purity and used as purchased. Seawater (pH 7.5) was collected from the Camogli beach (Genova, Italy) and maintained at 4 °C after microfiltration; water salinity of 35 PSU (Practical Salinity Units) was reported by ARPA (Agenzia Regionale Prevenzione e Ambiente) website for this area.

Cultures production

C. cladosporium was previously isolated [1] and deposited at Mycotheca Universitatis Taurinenesis (MUT) as strain MUT 5506. *C. cladosporium* was routinely maintained on MYA slants (Barley malt flour 100 g L^{-1} yeast extract 5 g L^{-1}, agar 15 g L^{-1}, pH 5.6) at 4 °C. *C. cladosporium* was cultivated in 1 L-Erlenmeyer flasks containing 100 mL of liquid medium (Barley malt flour 100 g L^{-1}, yeast extract 5 g L^{-1}, seawater) for 96 h at 28 °C on a reciprocal shaker (150 spm). Mycelium was harvested by filtration at 4 °C and suspended in deionized water, frozen at -20 °C, and finally lyophilized (Alfa Criosec, Milan, Italy) at plate temperature of 25 °C.

Biotransformations

The biotransformation under optimized conditions was performed using lyophilized mycelium (400 mg) suspended in a solution of seawater (10 mL) containing in 6 of sucrose and incubated at 50 °C in an orbital shaker at 90 rpm. The biotransformation was followed by HPLC; at different times, aliquots (100 μL) were withdrawn, diluted with 400 μL of water, incubated for 10 min at 90ºC to inactivate the enzymes, and analysed by HPLC.

Lyophilized mycelium was reused in successive cycles. At the end of 24 h, the biotransformation mixture was centrifuged, the mycelium washed twice with seawater and re-suspended in seawater containing sucrose (600 g/L).

Fructofuranosidase activity assays

The enzymatic activity of the lyophilized mycelium was assayed using the dinitrosalicylic acid (DNS) method adapted to a 96-well microplate scale.[2] A calibration curve was performed with a 3 g/L fructose solution. Lyophilized mycelia (10 g/L) were incubated with 1 mL of a sucrose solution (100 g/L) in acetate buffer (20 mM pH 6.0) for 20 min at 50 ºC and 90 rpm. 50 µL of the solution, conveniently diluted to fit into the calibration curve, were added to each well. Then, 50 µL of 10 g/L DNS were added. The plate was incubated for 20 min at 80 ºC to develop colour with a seal plate tape (GeneMate). After cooling, 150 µL of water was added to each well, and the absorbance measured at 540 nm using a microplate reader (model Versamax, Molecular Devices). One unit (U) of activity was defined as that catalysing the formation of 1 µmol reducing sugar per minute. Lyophilized mycelia were incubated in the range of pH 3-9 and temperature 25-80 ºC to evaluate the maximum of activity. Residual activity of lyophilized mycelia was calculated after different periods of time (2 and 24 h) before substrate addition at pH and temperatures in the range of 3-9 and 40-80 ºC respectively. The residual activity was measured at pH 6.0 acetate buffer 20 mM, 50 ºC with the DNS method described above.

HPLC analysis

Analysis of FOS composition was carried out by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS3000 system consisting of an SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, and an autosampler (model AS-HV). All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 × 250 mm Carbo-

Pack PA-1 column (Dionex) connected to a 4 × 50 mm CarboPac PA-1 guard column was used at 30°C. Eluent preparation was performed with Milli-Q water and NaOH. The initial mobile phase (at 0.5 mL/min) was 100 mM NaOH. A gradient from 0 to 200 mM sodium acetate was performed in 50 min at 0.5 mL/min, and 200 mM sodium acetate was maintained for 25 min. The chromatograms were analyzed using Chromeleon software. The identification of the different carbohydrates was done on the basis of standards commercially available or purified in our laboratory [6].

Transferase/hydrolase ratio

The amount of free fructose (measured by HPLC) is a direct measurement of hydrolytic activity whereas the amount of glucose (measured by HPLC) minus the amount of free fructose corresponds to the transferase activity [11]. Accordingly, a transferase/hydrolase index (T/H index) was calculated using the following equation: T/H = ([glucose] − [fructose])/[fructose].

Results

The use of lyophilized mycelium of *Cladosporium cladosporioides* MUT 5506 in freshwater allowed the production of FOSs with a maximum yield of 57% starting from a 600 g/L of sucrose solution. Interestingly, *C. cladosporioides* synthesized a mixture of ^{1}F-FOS, ^{6}F-FOS and ^{6}G-FOS, including the presence of a non-conventional disaccharide (blastose). *C. cladosporioides* was grown in a seawater-based medium and the biotransformation with mycelium-bound β-fructofuranosidase was evaluated using different amounts of lyophilized mycelium in seawater, using sucrose (600 g/L) as substrate at 30 °C (Table 1).

TABLE 1

Maximum FOS production was always registered after 96 h. The highest yields were obtained using 40 g/L of dry mycelium, and this biocatalyst concentration was used in the following

experiments aimed at evaluating the effects of temperature. The reaction rates increased up to 60 °C and then decreased rapidly due to thermal inactivation (Figure 1). Thermal stability in seawater was determined by measuring the residual activity after 24 h of incubation over a temperature range from 25 to 80 °C (Figure 1). Mycelium-bound activity was fully maintained up to 50 ºC, above which a gradual decrease of stability was observed, with significant inactivation (40% of lost activity) at temperatures above 80 ºC. Thus, the best compromise between activity and stability was found at 50 ºC.

FIGURE 1

The time-course of FOS production carried out under optimized conditions (40 g/L of lyophilized mycelium, 50 °C, 600 g/L of sucrose) in seawater is reported in Figure 2, while total FOS production and transferase/hydrolase ratio (expressed as T/H index) are reported in Table 2.

TABLE 2

The highest amount of FOS (344 g/L) was reached after 72, with a preponderance of ^{1}F-FOS derivatives (1-kestose 184 g/L, 1-nystose 98 g/L and 1-fructofuranosylnystose 22 g/L at 72 h) and significantly lower concentrations of 6-kestose (12 g/L), neo-kestose (11 g/L), and neo-nystose (6 g/L). The highest amount of the non-conventional disaccharide blastose was observed at 144 h (30 g/L). The ratio between transfructosylating and hydrolytic activity (T/H index) was very high until 72 h and decreased at prolonged times, as expected for fructofuranosidase-catalyzed biotransformations [19].

The reuse of lyophilized mycelium was studied in a batch reactor measuring the activity in a sequence of reaction cycles in seawater. Figure 3 illustrates the operational stability of the lyophilized mycelium in 15 successive reaction cycles of 24 h each. Lyophilized mycelium of *C. cladosporioides* retained 76% of the initial activity after 15 cycles of reutilization.

FIGURE 3

Discussion

177 Fructofuranosidases can catalyze the synthesis (from sucrose) or hydrolysis of
178 fructooligosaccharides (FOS) [19]. During the synthesis of FOS, one molecule of sucrose acts as
179 fructose donor (releasing one molecule of glucose) and another is the fructose acceptor for GF2
180 synthesis; the extension of the FOS chain involves GF2 as fructose acceptor and another
181 molecule of sucrose as fructose donor, thus generating GF3 [20]. Alternatively,
182 fructofuranosidases may also catalyse the hydrolysis of FOS, thus releasing fructose. Therefore,
183 the maximal fructooligosaccharides production depends on the ratio between transfructosylation
184 and hydrolysis, deductible from the relative amounts of glucose and fructose formed during the
185 biotransformation [19].
186 The more efficient bioprocesses for producing FOS described in literature furnish yields in the
187 range of 54-61% of total FOS starting from 400-700 g/L of sucrose in freshwater [21]. In all
188 these bioprocesses, the transfructosylation/hydrolysis ratio is a key-factor for determining high
189 yields of FOSs. In a recent work, we reported that a strain of the filamentous fungus
190 *Cladosporium cladosporioides* (now deposited at Mycotheca Universitatis Taurinenesis as strain
191 MUT 5506) showed high mycelium-bound transfructosylating activity, furnishing a maximum
192 FOS yield of 56%, when used in a conventional aqueous buffer [6]. The FOS mixture contained
193 ^{1}F-FOS, ^{6}F-FOS and ^{6}G-FOS, including the presence of a non-conventional disaccharide,
194 characterised and identified as blastose. The FOS yields observed using *C. cladosporioides* MUT
195 5506 are comparable with the ones described for the industrial biotransformation carried out
196 with enzymes from *Aspergillus niger* or *Aureobasidium pullulans*, but, in the case of *C.*
197 *cladosporioides*, a wider array of FOS with different glycosidic bonds is produced.
198 High yields of FOS depends on enzyme properties, but also on biotransformation conditions
199 suitable for favouring transfructosylation over hydrolysis (i.e., temperature, high sucrose
200 concentration and high salt concentration with consequent low water activity). During the
201 physiological characterization of *C. cladosporioides* MUT 5506, we noticed that this strain is a
202 facultative halophilic microorganism, being able to grow at different concentration of NaCl and

also in microfiltered seawater added with the necessary nutrients. This observation led us to investigate the transfructosylating activity of the lyophilized mycelium of *C. cladosporioides* MUT 5506 grown in seawater. The prospect to perform bioprocesses avoiding the consumption of drinkable water seems a remarkable way for optimizing the use of natural resources in sustainable bioprocesses [2].

Lyophilized mycelium of *C. cladosporioides* MUT 5506 grown in a seawater-based medium produced 344 g/L (57% w/w) of total FOSs from 600 g/L of sucrose in seawater under optimized conditions after 72 h. It should be pointed out that in all the experiments, seawater was only microfiltered, with no further optimization/modification of the medium (e.g. pH, salt composition). Interestingly, the overall performances were very similar (or even slightly better) compared with what observed in a conventional buffer [6]. Hence, the use of seawater (both as growth and biotransformation medium) resulted suitable for the overall process of FOS production. The transfructosylating/hydrolysis ratio (expressed in this work as T/H index, see Materials and methods) decreased during the reaction, indicating that fructooligosaccharides were progressively hydrolysed; this phenomenon is commonly observed with fructofuranosidase-catalyzed reactions [22,23]. Preliminary tests about the stability of the mycelium-bound enzyme(s) in seawater indicated that 76% of the original activity was maintained after 15 cycles of biotransformation, opening the possibility of studies for setting up continuous processes.

In conclusion, the production of fructooligosaccharides using lyophilized mycelium of the halophilic fungus *Cladosporium cladosporioides* MUT 5506 was entirely accomplished in seawater. This proof-of-concept application reports for the first time the production of alternative sweeteners by using a bioprocess where the biocatalyst was produced and used in a non-conventional medium, such as seawater. Purification of FOS from salts and other impurities (including the main by-product glucose) can be efficiently performed by nanofiltration [24] or simulated moving bed (SMB) chromatography [25]; more recently, a simple and effective

process using activated charcoal for directly purifying FOS from a fermentative broth was proposed [26].

Acknowledegements

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338

339 **Figure legends**

340

341 **Fig. 1.** Effect of temperature on activity and stability of lyophilized mycelium of *C.*
342 *cladosporioides* in seawater (pH 7.5). Stability is defined as residual activity determined at 50 °C
343 after pre-incubation of the lyophilised mycelia for 24 h in seawater at different temperatures.
344 Standard deviations were lower than 5%.

345 **Fig. 2.** Time-course of FOS production with lyophilized mycelium of *C. cladosporioides*.
346 Reaction conditions: 600 g/L sucrose, 40 g/L lyophilized mycelium, seawater (pH 7.5), 50 °C.
347 Results are the average of three triplicates, with standard deviations lower than 5%. Standard
348 deviations were lower than 5%.

349

350 **Fig. 3** Reuse assay of lyophilized mycelium of *C. cladosporioides* in repeated-batch reactions.
351 After each reaction cycle (24 h), the lyophilized mycelium was separated from the reaction
352 medium and washed.

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Table 1 Maximum FOS production and fructofuranosidase activity. FOS production after 96 h of biotransformation with different amounts of *Cladosporium cladosporioides* lyophilized mycelium in seawater (pH 7.5), with an initial sucrose concentration of 600 g/L.

Amount of mycelium (g/L)	Activity (U/g)	Maximum FOS production (g/L)
10	6.9	108
20	7.9	164
30	8.9	188
40	8.8	208
50	7.4	194

Table 2 Time-course of total FOS production and T/H index. T/H = ([glucose] − [fructose])/[fructose]

Time (h)	Total FOS production (g/L)	T/H index
24	176	5.0
48	291	4.8
72	344	4.7
96	340	4.1
120	309	3.4
144	292	2.7

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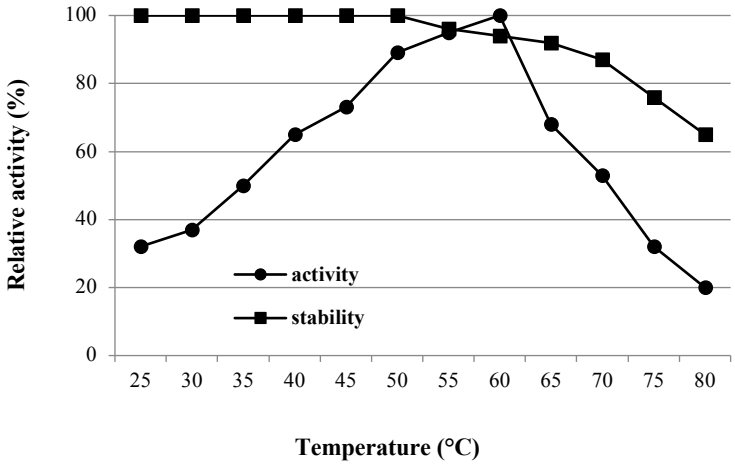

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402
403 **Figure 1.**

404

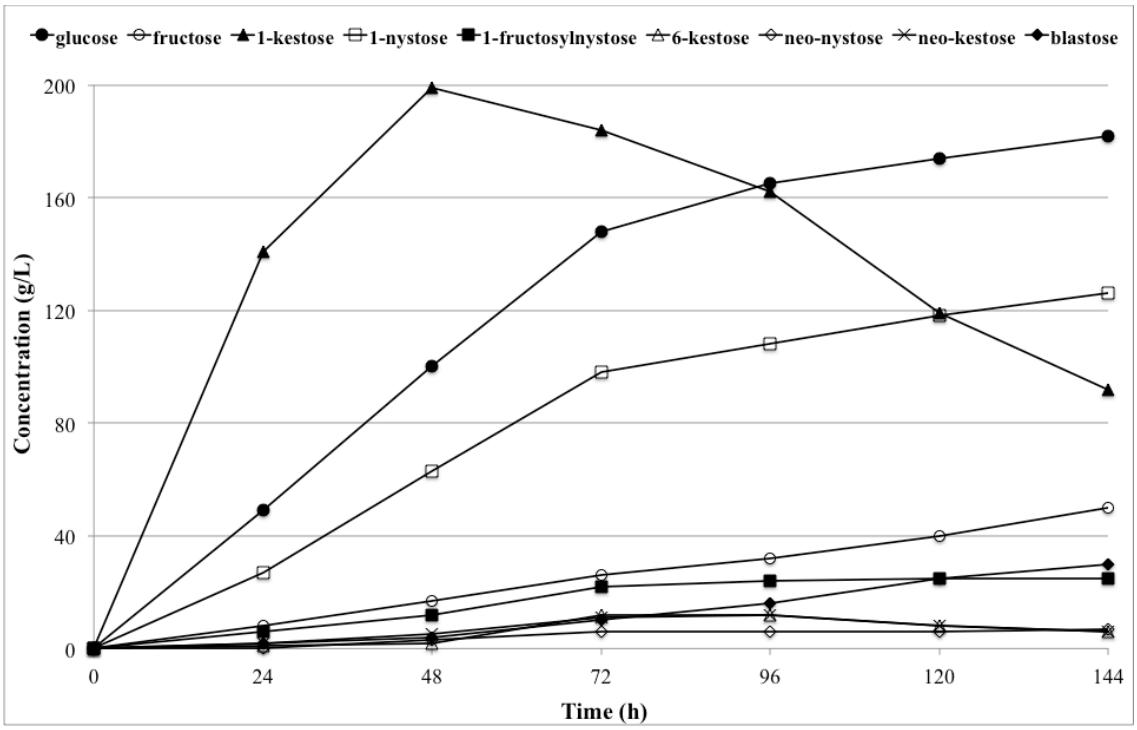
glucose
fructose
1-kestose
1-nystose
1-fructosylnystose
6-kestose
neo-nystose
neo-kestose
blastose
Concentration (g/L)
200
160
120
80
40
0
0
24
48
72
96
120
144
Time (h)

Figure 2.

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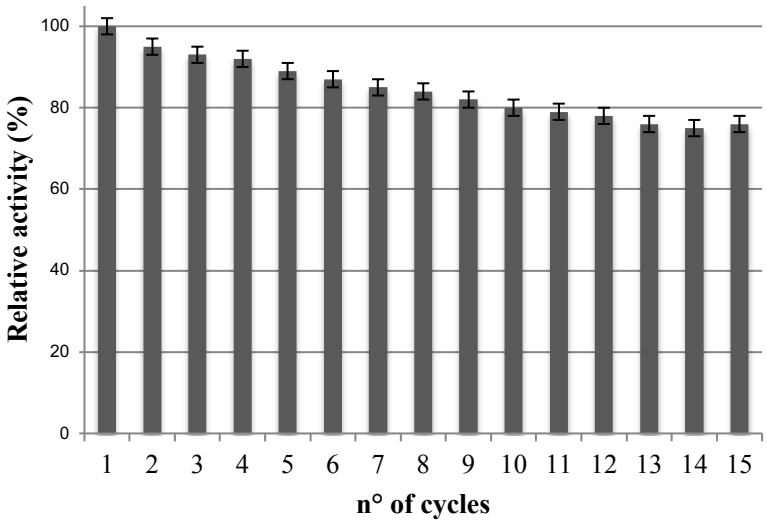

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412 **Figure 3**