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Potential application of commercial enzyme preparations for industrial production of short-chain fructooligosaccharides

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ABSTRACT

Twenty-five commercial enzyme preparations for use in the food industry were assayed for transfructosylation activity. Three preparations showed high transfructosylation activity from sucrose as well as the ratio of transferase and hydrolase activities. Short-chain fructooligosaccharides (sc-FOS) were not hydrolyzed by the three enzyme preparations after a 12 h reaction time. At a 6 h reaction time, yield and volumetric productivity were in the range from 58.8 to 62.6% (g sc-FOS/100 g initial sucrose) and 52.5 to 55.9 g sc-FOS/L h, respectively. One enzyme preparation was then evaluated for sc-FOS synthesis. Thus, environmental factors influencing the reaction were studied on products. Total sc-FOS concentration was not affected by temperature, pH and enzyme concentration at the studied levels, but high concentrations of sucrose affected the sc-FOS formation. The results suggest that these enzyme preparations can be exploited as a source of food-grade fructosyltransferase, in addition to Pectinex Ultra SP-L.

1. Introduction

Short-chain fructooligosaccharides (sc-FOS) are a mixture of 1-kestose (GF_2), nystose (GF_3) and 1^F-fructofuranosylnystose (GF_4), which have been regarded as prebiotics since the mid-1990s. They have important physiological functions due to their indigestibility in the upper gastrointestinal tract, which stimulate the selective growth of bifidobacteria in the large intestine [1,2]. Sc-FOS have received GRAS status (generally recognized as safe), which has promoted their use as ingredients for both food and feed in East Asia, North America and Europe [3].

Sc-FOS are produced either from sucrose by transfructosylation with fructosyltransferases (β-fructofuranosidase, EC 3.2.1.26 or β-D-fructosyltransferase, EC 2.4.1.9) or from inulin by controlled enzymatic hydrolysis. The transfructosylation process has a greater potential because it is possible to synthesize sc-FOS oligomers of either defined chain length [4] or desired composition mixtures by modulating the reaction time. GF_2 has more sweetening power than other sc-FOS and can be used as a sweetener for diabetics [5], so the reaction should be stopped at the maximum production of sc-FOS.

A fructosyltransferase is considered efficient if it possesses the ability to bind the acceptor, fructosyl moiety, and to exclude H_2O via a double-displacement mechanism, as shown in Fig. 1 [6–9]. This efficiency is confirmed by the synthesis yield because it depends on the relative rates of transfructosylation and hydrolysis [10].

The main disadvantage of the sc-FOS synthesis process is the lack of a reasonably priced and efficient catalyst. Some studies have described screening microorganisms for transfructosylation activity [7,11,12], but this approach is complicated and tedious because a large number of positive hits may still fail. Only a few of these enzymes have the level of transfructosylation activity necessary for industrial applications [10]. Moreover, these isolated enzymes are not yet commercially available. Another approach is the development of heterologous recombinant enzymes that efficiently and selectively synthesize GF_2 [13,14]. These enzymes are not currently used in industrial production of oligosaccharides in Japan, according to a report by Taniguchi [15], because manufacturers are nervous about consumers' response to their use.

Currently, one of the most common alternatives discussed in the literature is the immobilization of fructosyltransferases [16–18], but this process is only justified if the enzyme is expensive or inactivated under reaction conditions. An alternative is to use low-cost commercial enzyme preparations designed for use in the food industry. Many are obtained from filamentous fungi that are considered good producers of fructosyltransferases [19]. In addition to the main enzymatic activity, some enzyme preparations have secondary activities, including transfructosylation, which has been found in Pectinex Ultra SP-L [20].

On the other hand, compared with previous approaches, commercial enzyme preparations have economic and technical advantages, such as low price, versatility and stability of enzymatic

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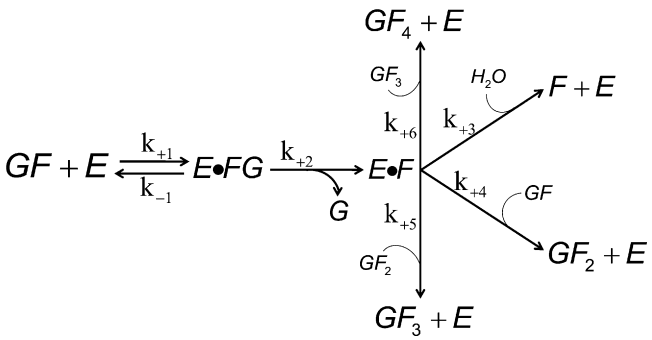

Fig. 1. Schematic representation of the double-displacement mechanism for fructosyltransferases at an early stage of the reaction in which inhibition by glucose may be negligible. GF: sucrose, E: free enzyme, E·FG: covalent sucrose–enzyme intermediate, E·F: covalent fructosyl–enzyme intermediate, G: glucose, F: fructose, GF_2: 1-kestose, GF_3: nystose and GF_4: 1^F-fructofuranosylnystose.

activity under reaction conditions. Because of advances in biotechnology, it is now possible to have more varieties of food-grade enzymes, which increases their potential application in sc-FOS production. More studies are needed to find other preparations with transfructosylation activity and thermal stability as Pectinex Ultra SP-L. In the current study, three commercial enzyme preparations were selected from twenty-five for the synthesis of sc-FOS from sucrose because they showed high transfructosylation activity as well as the ratio of transferase and hydrolase activities. In addition, the effect of reaction conditions on the synthesis was studied by the action of an enzyme preparation previously selected.

2. Materials and methods

2.1. Materials

1-Kestose, nystose and 1^F-fructofuranosylnystose standards were obtained from Wako Chemicals (Richmond, VA, USA). Glucose–oxidase–peroxidase enzymatic kit was purchased from Spinreact (San Esteve de Bas, Spain). Other reagents were purchased from Sigma Chemical (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Enzymes

Twenty-five enzyme preparations from fungal strains that are designed for use in the food industry were kindly donated by Biocatalysts Ltd. (Parc Nantgarw, Wales, UK); Amano Enzyme Co., Ltd. (Elgin, IL, USA); DSM Food Specialties, Unltd (Santiago, Chile); Dimerco Comercial Ltda, Santiago, Chile (AB Enzymes GmbH) and Blumos SA, Santiago, Chile (Novozymes A/S).

2.3. Enzyme assays

Transfructosylation and hydrolysis activities were determined by the initial reaction rate of glucose and fructose released from sucrose. Glucose is produced in both cases (transfructosylation and hydrolysis activities), while fructose is exclusively the result of the hydrolytic activity. The reaction mixture consisted of 49 mL of 400 g/L sucrose in 50 mM sodium acetate buffer at pH 5.5, and 1 mL of either the enzyme preparation or diluted enzyme preparation using the same buffer. The mixture was incubated at 50 °C and stirred at 150 rpm. Samples (4 mL) were taken from the reaction mixture at regular intervals for 5 min and the reaction was stopped by heating in boiling water for 10 min, finally, were assayed for glucose (commercial enzymatic kit) and reducing sugars (Somogy–Nelson method). Fructose was calculated as the difference in the concentrations of reducing sugars and glucose. Solid-phase enzymes were dissolved in the same buffer at a certain concentration and tested as described.

The rate of transfructosylation (V_T) was calculated from the following mole balances and ratios of stoichiometric coefficients for chemical species shown in Fig. 1.

- The rate of formation of glucose (G) and covalent fructosyl-enzyme intermediate (EF) is represented by

$$V_G = V_{EF}$$

- The rate of formation of fructose (F), GF_2, GF_3 and GF_4 is represented by (parallel reactions)

$$-V_{EF} = V_F + V_{GF_2} + V_{GF_3} + V_{GF_4}$$

- The mole balance between the two stages of the catalytic mechanism is represented by

$$V_G = V_{EF} = -V_{EF} = V_F + V_{GF_2} + V_{GF_3} + V_{GF_4}$$
$$V_T = V_G - V_F$$

where V_T is the sum of the rates of formation of GF_2, GF_3 and GF_4 and represents the rate of formation of transferred fructose.

One unit of transfructosylation activity (U_T) was defined as the amount of enzyme required to transfer 1 μmol of fructose per min. One unit of hydrolytic activity (U_H) was defined as the amount of enzyme required to release 1 μmol of fructose per min.

2.4. Confirmative experiment for sc-FOS synthesis

9 U_T/g sucrose was diluted in the same buffer as in Section 2.3 to a final volume of 0.5 mL and added to 19.5 mL of 550 g/L sucrose solution. Other experimental conditions were the same as previously stated, except for 0.4 mL aliquot that was withdrawn from this reaction mixture for analysis of carbohydrates. The experimental outputs were yield ($Y_{P/S}$) and volumetric productivity (Q_P). Yield was defined as sc-FOS grams per 100 g of initial sucrose. Volumetric productivity was defined as sc-FOS grams per reactor volume and time (g/L h). Sucrose conversion was defined as the ratio of transformed sucrose in the reaction relative to the initial sucrose.

In order to compare the yields achieved for each fructooligosaccharide during synthesis with the theoretical yield, the relative yield was defined as follows [11]

$$2GF \rightarrow GF_2 + G \quad Y^*_{GF_2} = \frac{2c(GF_2)}{c_0S}$$

$$2GF_2 \rightarrow GF_3 + GF \quad Y^*_{GF_3} = \frac{3c(GF_3)}{c_0S}$$

$$2GF_3 \rightarrow GF_4 + GF_2 \quad Y^*_{GF_4} = \frac{4c(GF_4)}{c_0S}$$

$$Y^*_{sc\text{-}FOS} = Y^*_{GF_2} + Y^*_{GF_3} + Y^*_{GF_4}$$

where $c(GF_2)$, $c(GF_3)$, $c(GF_4)$, c_0S are the molar concentrations of 1-kestose, nystose, 1^F-fructofuranosylnystose and initial sucrose, respectively.

2.5. Effect of reaction conditions on the performance of sc-FOS formation

In order to evaluate the performance of an enzyme preparation previously selected as a biocatalyst for synthesis of sc-FOS,

experiments were conducted by the one-factor-at-a-time method. Thus, reactions were performed in 25 mL flasks and stirred by a magnetic stirrer at 150 rpm. 100 μL of suitably diluted enzyme was added to 19.9 mL of sucrose solution in buffer at the corresponding pH of the reaction. Operating parameters such as temperature, pH and concentrations of sucrose and enzyme were designated as independent variables in the experiments. Experimental yield ($Y_{P/S}$) and volumetric productivity (Q_P) were reported when the sucrose conversion in the reaction medium was 0.8 wherein the GF_2 concentration had a maximum value. It was due to the fact that the total sc-FOS concentration did not have a maximum concentration under the conditions tested (see below).

The effect of temperature on reaction products was studied in the range from 45 to 60 °C and the remaining variables were maintained at pH 5.5, 600 g/L sucrose and 5.4 U_T/mL. The influence of pH was tested in the range from 4.5 to 6.5 (50 mM sodium acetate buffer at pH 4.5, 5.0, 5.5, 6.0 and 50 mM sodium phosphate buffer at pH 6.5) and the other variables were constant as above-mentioned values at 50 °C. To study the effect of sucrose concentration, the experimental conditions were 50 °C, pH 5.5, 5.4 U_T/mL and initial sucrose concentration was varied over the range of 400–800 g/L. Finally, the effect of enzyme concentration on sc-FOS formation was determined in the range from 4.2 to 15 U_T/mL, keeping other variables at 50 °C, pH 5.5 and 600 g/L sucrose.

2.6. Thermal stability of free enzyme

Thermal stability under non-reactive conditions was assayed in 50 mM sodium acetate buffer at pH 5.5. In order to mimic the thermal stability under reactive conditions, sucrose was replaced by 600 g/L methyl-α-D-glucopyranoside in the same buffer [21]. 0.5 mL aliquots were withdrawn from the reaction mixture at different times and residual activity was measured by the initial reaction rate of reducing sugars using sucrose as substrate.

2.7. Analysis of carbohydrates

The samples were analyzed for their carbohydrate composition by high-performance liquid chromatography (HPLC) using a Perkin-Elmer Series 200 machine with a refractive index detector and autosampler. Chromatography was performed on a Benson Polymeric BP-100 Ag^+ column. Column and detector temperatures were maintained at 50 °C and 45 °C, respectively. Samples (10 μL) were eluted with Milli-Q water at a flow-rate of 0.4 mL/min. The data obtained were analyzed using the TotalChrom software (version 6.3.1).

2.8. Statistical analysis

All experiments were performed in duplicate, except for kinetic experiments of thermal inactivation of free enzyme which were conducted in triplicate. The average values with standard deviation are reported. Results of the enzymatic activities were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Differences were considered significant at p-values < 0.05. Data were analyzed using Microsoft® Excel 2007 (Microsoft Corporation) and Statistica 8.0 (StatSoft, Inc. 2007, USA).

3. Results and discussion

3.1. Screening of enzyme preparations for transfructosylation activity

The twenty-five enzyme preparations have several known activities for their use in the food industry, but the current preparations were assayed using sucrose to measure conversion into transfructosylation products. The transfructosylation and hydrolysis activities of these preparations are shown in Fig. 2a and b.

Enzyme preparations exhibited one or both activities, except for Pectinex Smash XXL, Cellulase 13L and Depol 692L. In order to select enzyme preparations for subsequent experiments were performed three Dunnett's multiple comparison test. Pectinex Ultra SP-L and Maxinvert L 10000 were used as the control preparations because these enzymes are known to have efficient transfructosylation and hydrolysis activities, respectively [21,22]. Pectinex Ultra SP-L is marketed for use in the processing of fruit juice and contains pectinolytic and cellulolytic enzymes from *Aspergillus aculeatus*. Maxinvert L 10000 is marketed in the food industry as an invertase from a strain of *Saccharomyces cerevisiae*.

The selection criteria when screening enzymes for fructosyltransferases include transfructosylation activity and the ratio of transferase and hydrolase activities (U_T/U_H). This ratio expresses the maximum efficiency for transfructosylation under reaction conditions such as sucrose concentration, temperature and pH of the reaction medium because the activities were measured as initial reaction rates.

According to Dunnett's test, preparations with greater transfructosylation activity relative to Pectinex Ultra SP-L were in the following order: Rohapect CM > Maxinvert L 10000 > Protease M > Protease AD-S > Viscozyme L. The p-value was < 0.001 for all of them. Pectinex Smash was considered because it showed greater activity than did Pectinex Ultra SP-L, although this activity was not significant ($p = 0.64$). In addition, Pectinex Smash showed the highest ratio of transferase and hydrolase activities (U_T/U_H). The hydrolysis activity of these enzyme preparations relative to Maxinvert L 10000 were in the following order: Rohapect CM > Protease M = Protease AD-S > Viscozyme L > Pectinex Smash. The p-value was < 0.01 for all of them. In addition, according to Dunnett's test, higher ratios of transferase and hydrolase activities relative to Pectinex Ultra SP-L were in the following order: Pectinex Smash ($p < 0.001$) > Rohapect CM ($p < 0.001$) > Viscozyme L ($p = 0.019$) > Depol 40 L ($p = 0.049$).

These results highlight the need to characterize an enzyme preparation not only by name but also experimentally because some of these enzyme preparations exhibited high levels of transfructosylation activity while others show hydrolytic activity towards sucrose [3]. This phenomenon could be explained by the presence of secondary activity (transfructosylation and hydrolysis) in addition to their primary marketed activity.

Of the twenty-five enzyme preparations, three were selected for further experiments. The three included Vizcozyme L (from *A. aculeatus*), Pectinex Smash (from *A. aculeatus/Aspergillus oryzae*) and Rohapect CM (from *Trichoderma reesei*); two control enzyme preparations were also included in the further experiments.

3.2. Confirmation of sc-FOS synthesis

The kinetic of the transfructosylation products for the five enzyme preparations was assayed using sucrose as substrate. Fig. 3 shows two HPLC chromatograms obtained from aliquots of the reaction mixtures catalyzed by Rohapect CM and Maxinvert L 10000. The fructose peak was very small compared with those of glucose for the four enzyme preparations (Pectinex Ultra SP-L, Rohapect CM, Viscozyme L and Pectinex Smash) and confirmed transfructosylation activity at high sucrose concentrations.

On the other hand, a large amount of sucrose was hydrolyzed by Maxinvert L 10000, as the fructose peak was almost equal to that of glucose, confirming the selectivity for hydrolysis by this enzyme. Additionally, the synthesis of oligofructans was not selective because two unidentified disaccharides (peaks 7 and 8, in Fig. 3) were observed throughout the time course of the reaction.

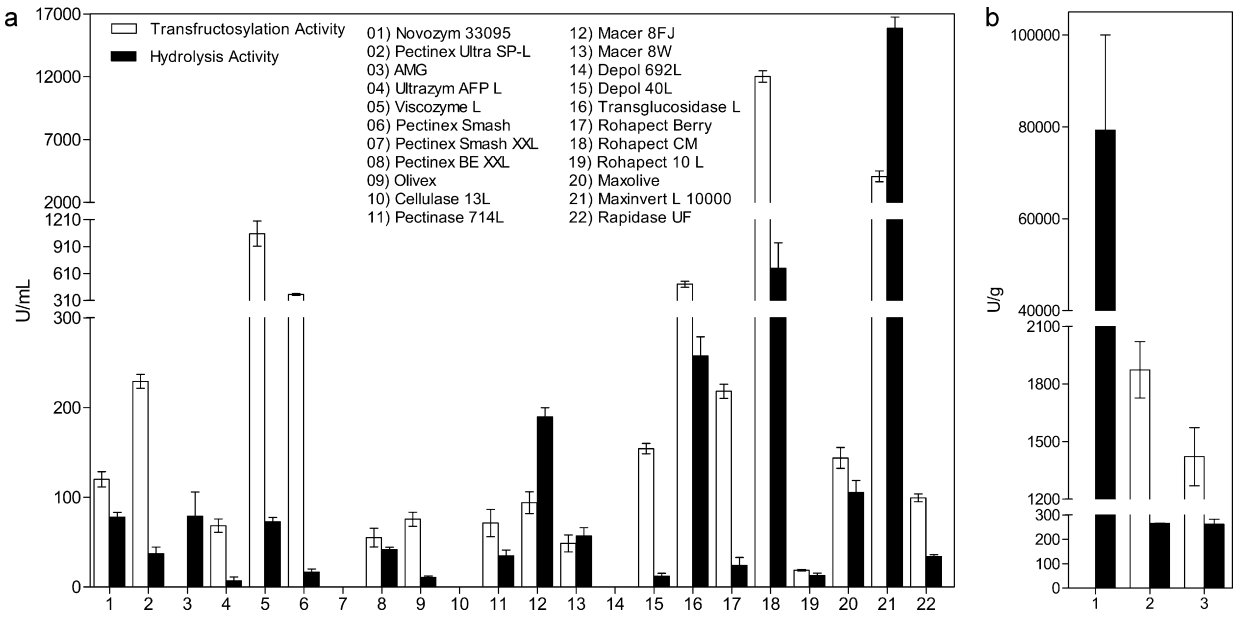

Fig. 2. (a) Transfructosylation and hydrolysis activities of commercial food-grade enzyme preparations in liquid phase. Enzyme preparations were from Novozymes (1)–(9), Biocatalysts (10)–(15), Amano Enzyme (16), AB Enzymes (17)–(19) and DSM Food Specialties (20)–(22). (b) Transfructosylation and hydrolysis activities of commercial food-grade enzyme preparations in solid phase. Sucrase/Invertase (1), Protease M (2), Protease AD-S (3). Enzyme preparations were from Biocatalysts (1), Amano Enzyme (2–3).

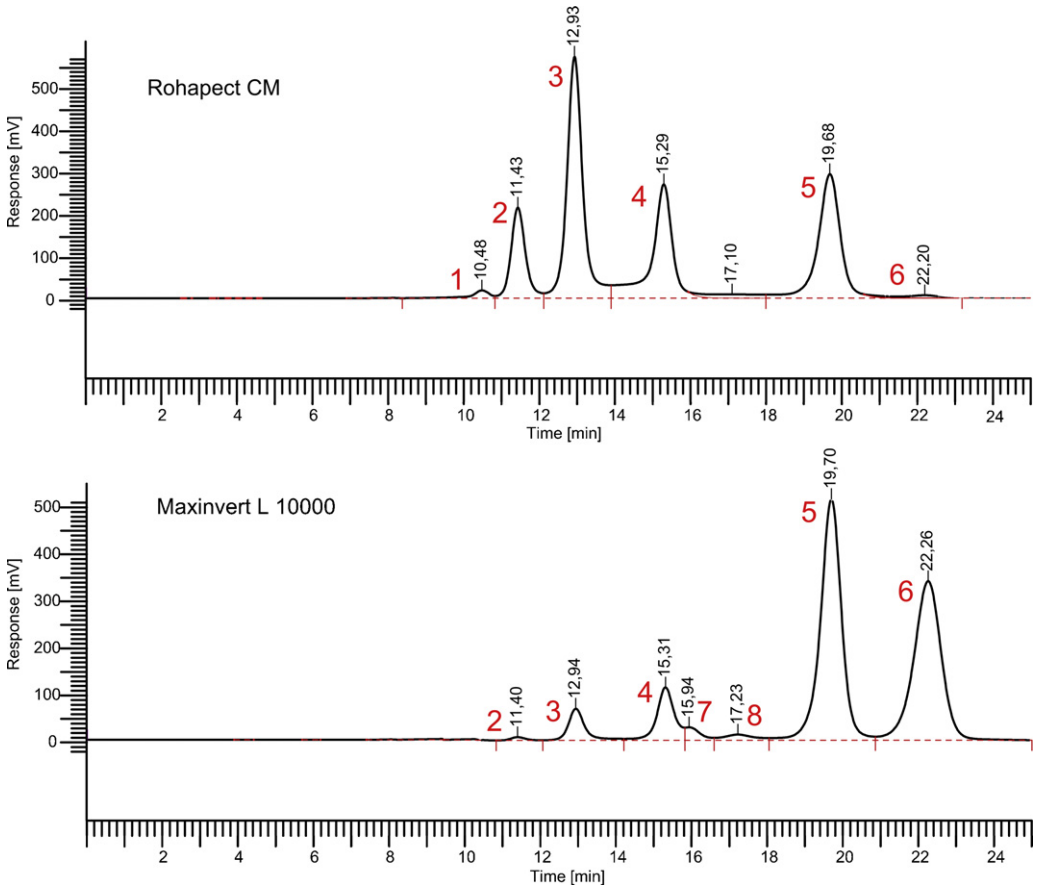

Fig. 3. Carbohydrate profiles obtained by HPLC from reactions catalyzed by Rohapect CM and Maxinvert L 10000 at 4 and 3 h reaction times, respectively. Experimental conditions were 536.2 g/L sucrose in 50 mM sodium acetate buffer (pH 5.5), 9 U_T/g sucrose at 50 °C. The components identified were (1) GF_4, (2) GF_3, (3) GF_2, (4) GF, (5) G, (6) F, (7) and (8) disaccharides.

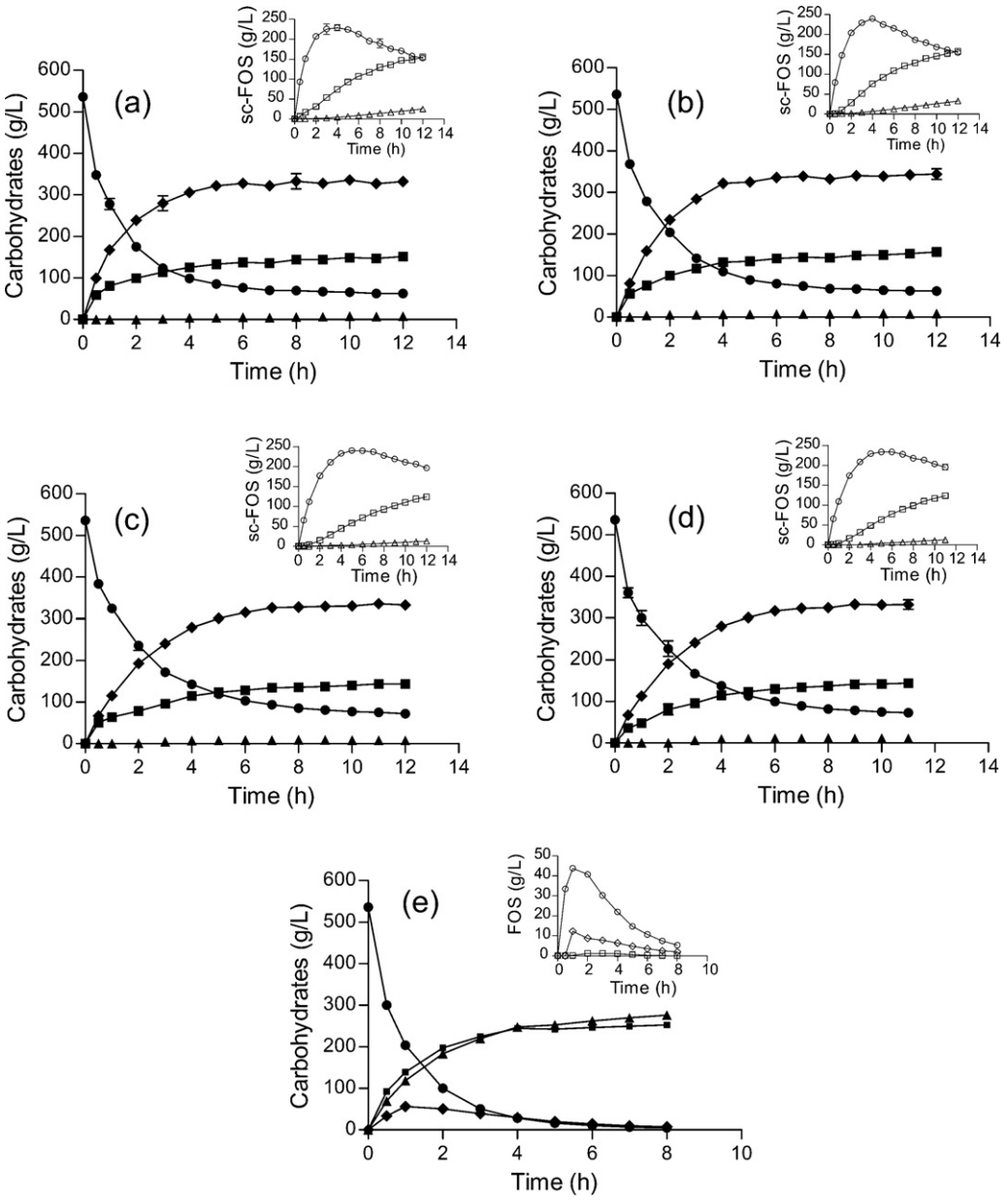

Fig. 4. Time course of the sc-FOS synthesis from sucrose catalyzed by Pectinex Ultra SP-L (a), Rohapect CM (b), Viscozyme L (c), Pectinex Smash (d) and Maxinvert L 10000 (e). Experimental conditions were 536.2 g/L sucrose in 50 mM sodium acetate buffer (pH 5.5), 9 U_T/g sucrose at 50 °C. Sucrose (●), glucose (■), fructose (▲), total sc-FOS (♦), GF_2 (○), GF_3 (□), GF_4 (△) and disaccharide (◊). Remaining sucrose concentration of the reaction catalyzed by Maxinvert L 10000 was equivalent to the sum of remaining sucrose and the other disaccharide which is not shown in Table 1.

This observation agrees with the two disaccharides identified by Straathof et al. [23] and Farine et al. [24] using an invertase from *S. cerevisiae*.

Retention times of the standards and oligofructans synthesized by Pectinex Ultra SP-L were taken as references. The results suggested that oligofructans synthesized by the three enzyme preparations were sc-FOS. Fig. 4 shows typical batch kinetics for sucrose conversion into sc-FOS by the five enzyme preparations. These profiles, except for the reaction catalyzed by Maxinvert L 10000, were quite similar and are in agreement with previously reported results for the synthesis of sc-FOS [7,11,25]. The four enzyme preparations exhibited a high ability to transfer fructosyl moieties to an acceptor other than H_2O, as a plateau of the total sc-FOS concentration was observed from 6 to 12 h of reaction time. Total sc-FOS concentration for Pectinex Ultra SP-L and Rohapect CM was time-dependent up to 6 h of reaction time when the average sucrose conversion was 0.85. For the four enzyme preparations, GF_2 concentration was decreasing after a 6 h reaction time with a corresponding linear increase in GF_3 and GF_4 concentrations. This reaction is independent of the overall kinetic control mechanism because sc-FOS were not transformed into fructose and glucose as the reaction time progressed. The reaction time is an important control parameter with which it is possible to obtain a specific mixture of sc-FOS.

The estimated yield, volumetric productivity and ratio R (grams sc-FOS/mL enzyme) for each reaction was determined after a 6 h reaction time as shown in Table 1. The outputs of the reaction catalyzed by Maxinvert L 10000 were estimated at the peak of fructans (1 h reaction time). These results, except for the ratio *R*, are comparable to that obtained by the action of Pectinex Ultra SP-L, which has been reported for the synthesis of sc-FOS in free and immobilized forms with conversion efficiency above 70% based on the amount

Table 1
Kinetic parameters after a 6 h reaction time for the synthesis of sc-FOS from sucrose catalyzed by commercial enzyme preparations.

Kinetic Parameters	Pectinex Ultra SP-L	Rohapect CM	Viscozyme L	Pectinex Smash	Maxinvert L 10000[a]
$Y_{P/S}$ sc-FOS	61.1 ± 0.3	62.6 ± 0.5	58.8 ± 1.2	59.1 ± 0.3	10.5 ± 0.1
$Y_{P/S}$ sc-FOS[b]	71.3 ± 0.5	73.6 ± 0.7	72.8 ± 1.9	72.5 ± 0.6	16.9 ± 0.3
$Y_{P/S}$ GF_2	39.6 ± 0.3	40.1 ± 0.4	44.6 ± 0.9	43.6 ± 0.3	8.2 ± 0.2
$Y_{P/S}$ GF_3	19.9 ± 0.3	20.2 ± 0.1	13.3 ± 0.3	14.5 ± 0.0	0.04 ± 0.0
$Y_{P/S}$ GF_4	1.6 ± 0.2	2.3 ± 0.0	0.9 ± 0.1	1.0 ± 0.0	–
$Y_{P/S}$ disaccharide[c]	–	–	–	–	2.3 ± 0.1
Q_P sc-FOS	54.6 ± 0.3	55.9 ± 0.4	52.5 ± 1.1	52.8 ± 0.3	56.3 ± 0.4
R	15.9 ± 0.1	839.0 ± 6.6	65.7 ± 1.3	23.5 ± 0.1	43.3 ± 0.3

$Y_{P/S}$ (g/100 g initial sucrose), Q_P (g/L h) and R (g sc-FOS/mL enzyme preparation).
[a] Kinetic parameters were estimated at the peak of oligofructans (1 h reaction time).
[b] $Y_{P/S}$ (g/100 g transformed sucrose).
[c] Concentration was calculated using the calibration curve of sucrose.

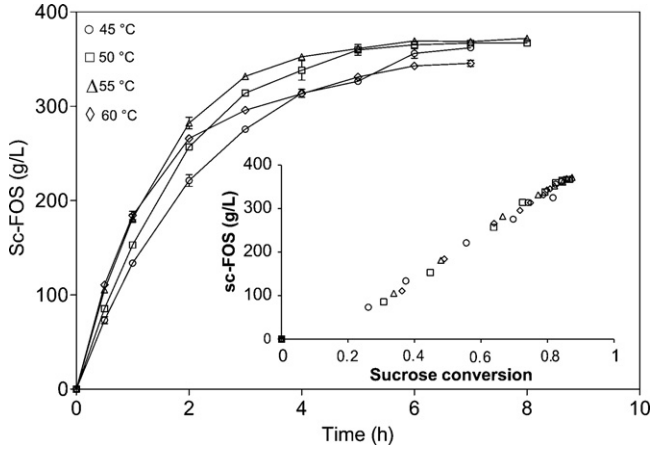

Fig. 5. Effect of temperature in the synthesis of sc-FOS from sucrose catalyzed by Rohapect CM. Experimental conditions were 600 g/L sucrose in 50 mM sodium acetate buffer (pH 5.5) and 5.4 U_T/mL.

of sucrose consumed or 62% (w/w) of total carbohydrates in the syrup [26–28]. Fructosyltransferases from a variety of microorganisms have been used for sc-FOS synthesis from a sucrose solution between 50 and 70% (w/v) in which the yield is in the range from 55 to 60% of the total carbohydrates in the mixture [19,25,29]. Our results are in agreement with literature results, although this process by the application of enzyme preparations is neither optimized nor scaled. The results and the low cost of these enzyme preparations support their use to scale up sc-FOS production with promising results.

Table 2 shows that the reaction catalyzed by Pectinex Ultra SP-L and Rohapect CM reached approximately 90% of theoretical yield, while the reaction catalyzed by Viscozyme L and Pectinex Smash were above 80% theoretical yield after a 6 h reaction time. The results for the last two enzyme preparations are consistent with that reported for fructosyltransferase of *Aureobasidium pullulans* [11]. In contrast, the yield achieved in the reaction catalyzed by the mycelium of *Aspergillus sp.* N74 was 70% of theoretical yield, which was then reduced to 57% at the end of the reaction time [30].

Table 2
Relative yields after a 6 h reaction time in the synthesis of sc-FOS from sucrose catalyzed by enzyme preparations.

Relative yield	Pectinex Ultra SP-L	Rohapect CM	Viscozyme L	Pectinex Smash
$Y^*_{sc\text{-}FOS}$	0.88 ± 0.00	0.89 ± 0.01	0.82 ± 0.02	0.83 ± 0.00
Y^*_{GF2}	0.54 ± 0.00	0.54 ± 0.00	0.60 ± 0.01	0.59 ± 0.00
Y^*_{GF3}	0.31 ± 0.00	0.31 ± 0.00	0.20 ± 0.00	0.22 ± 0.00
Y^*_{GF4}	0.03 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.00

Volumetric productivity is directly dependent on the amount of enzyme added to the reaction mixture. Using 9 U_T/g sucrose, volumetric productivity after a 6 h reaction time reached higher values than the literature results by batch operation (3.25, 6.61 g/L h) [31,32], solid-state fermentation (10.76 g/L h) [33] and semi-continuous operation (45 g/L h) [16].

The quality of an enzyme preparation can be quantified in terms of enzyme concentration which is reflected in levels of transfructosylation activity by the ratio *R*. The quality was not referring to the enzyme efficiency, since the four preparations exhibited high selectivity for sc-FOS. The smaller volume of enzyme preparation added to the reaction mixture was for Rohapect CM. Further experiments were performed with this enzyme preparation that had 12,000 U_T/mL.

3.3. Effect of temperature

Fig. 5 shows that the increase of temperature in the reaction system had no significant effect on total product concentration throughout the sucrose conversion for the synthesis of sc-FOS. Yield was not significantly affected by temperature as presented in Table 3, but a small increase was observed for GF_3 and GF_4 contents at 55 °C (data not shown). The temperature caused an increase in

Table 3
Effect of environmental factors in the reaction system on yield ($Y_{P/S}$) and volumetric productivity (Q_P) for the synthesis of sc-FOS from sucrose catalyzed by Rohapect CM.

Factor	Treatment (level)	$Y_{P/S}$[a] (g/g initial sucrose)	Q_P[a] (g/L h)
Temperature (°C)	45	54.2 ± 0.3	65.0 ± 0.4
	50	56.3 ± 1.7	84.5 ± 2.6
	55	57.6 ± 0.3	95.6 ± 3.1
	60	57.1 ± 0.1	57.1 ± 0.1
pH	4.5	53.8 ± 0.4	66.0 ± 2.5
	5.0	56.9 ± 0.4	83.5 ± 2.0
	5.5	56.8 ± 0.5	85.2 ± 0.7
	6.0	56.6 ± 0.3	84.9 ± 0.5
	6.5	54.7 ± 0.2	82.0 ± 0.3
Sucrose (g/L)	400	57.0 ± 0.8	77.0 ± 1.0
	500	56.9 ± 1.5	84.3 ± 3.4
	600	56.0 ± 2.2	84.6 ± 3.3
	700	56.6 ± 2.1	73.6 ± 6.2
	800	54.1 ± 1.1	69.3 ± 4.1
Enzyme (U_T/mL)	4.2	60.0 ± 0.2	62.0 ± 0.2
	5.4	56.0 ± 2.2	84.6 ± 3.3
	6.6	58.6 ± 0.3	100.0 ± 0.5
	7.8	57.9 ± 0.6	130.4 ± 6.7
	10.8	56.8 ± 0.1	166.5 ± 0.2
	15	56.1 ± 1.6	206.7 ± 18.6

[a] $Y_{P/S}$ and Q_P were calculated as sucrose conversion in the reaction mixture was 0.8.

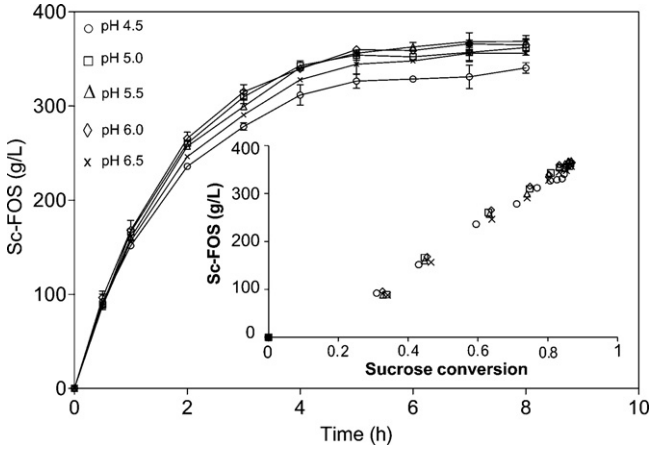

Fig. 6. Effect of pH in the synthesis of sc-FOS from sucrose catalyzed by Rohapect CM. Experimental conditions were 50 °C, 600 g/L sucrose in 50 mM sodium acetate buffer (pH 4.5–6.0) or 50 mM sodium phosphate buffer (pH 6.5) and 5.4 U_T/mL.

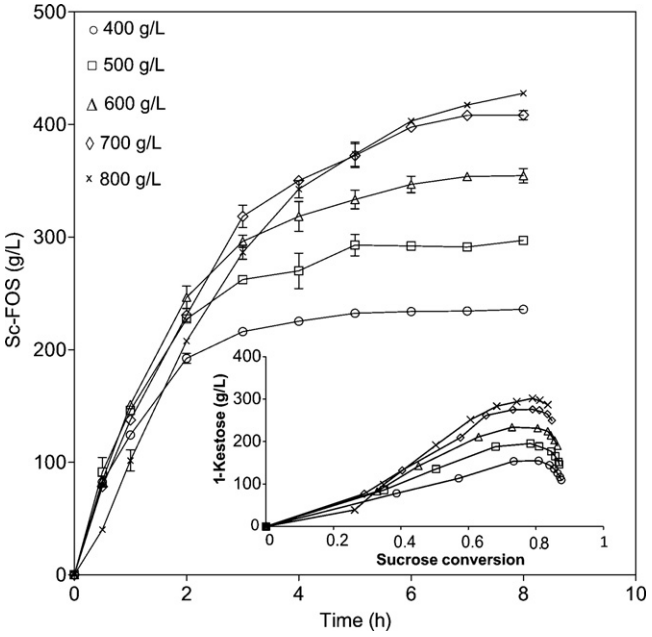

Fig. 7. Effect of initial sucrose concentration in the synthesis of sc-FOS catalyzed by Rohapect CM. Experimental conditions were 50 °C, sucrose solution in 50 mM sodium acetate buffer (pH 5.5) and 5.4 U_T/mL.

the reaction rate in the monitored range from 45 to 55 °C which was reflected in the volumetric productivity (Table 3). Similar behavior has been reported for other fructosyltransferases [11,34]. Volumetric productivity decreased at the temperature of 60 °C because the catalytic activity was slightly lost after a 2 h reaction time due to thermal inactivation. In contrast, fructosyltransferase of Pectinex Ultra SP-L is most active at 65 °C [21,26].

3.4. Effect of pH

Fig. 6 illustrates the effect of pH on total product concentration during the synthesis of sc-FOS. In the pH range from 5.0 to 6.5, no significant differences were observed in the profiles of total sc-FOS concentration and their composition according to the polymerization degree (data not shown) at a same sucrose conversion. In addition, the yield was slightly affected by pH in this range as shown in Table 3. Our results are in agreement with results previously reported for a fructosyltransferase of *A. pullulans* [11]. Fructosyltransferase of Rohapect CM was most active at pH 5.0–6.5 which was shown in the corresponding volumetric productivity of sc-FOS (Table 3). In contrast, invertase from *S. cerevisiae* exhibits relatively high activity on a more acidic pH range (3.5–5.5) [22].

3.5. Effect of initial sucrose concentration

Initial sucrose concentration is a key factor influencing sc-FOS formation, which increases the availability of fructosyl acceptors and decreases the availability of water. Fig. 7 shows that the concentrations of total sc-FOS and GF_2 increased to the same extent by increasing the initial sucrose concentration in the range from 400 to 700 g/L, but the increase was relatively small for all of sc-FOS at the level of 800 g/L sucrose. No significant differences were observed for yield of sc-FOS in the range from 400 to 700 g/L as shown in Table 3. Our results are in agreement with the ones reported by other authors [25,34]. The decrease in volumetric productivity at the levels of 700 and 800 g/L sucrose (Table 3) can be explained according to the theory that attributes the decline in the rate of enzymatic reactions to the effect of thermodynamic non-ideality [35], inhibition by sucrose [36], mass transfer limitations [37] or low water activity at high concentration of saccharides [38].

3.6. Effect of enzyme concentration

As shown in Fig. 8, the total sc-FOS concentration was not affected by enzyme concentration throughout the sucrose conversion during the synthesis of sc-FOS as also reported Ballesteros et al. [10]. Therefore, the yield was not significantly affected by enzyme concentration (Table 3). As expected, the increase in enzyme concentration only increased the reaction rate which was reflected in the linear volumetric productivity (Table 3) and a slight decrease in the GF_2 composition when sucrose conversion in the reaction medium was 0.8 (data not shown).

It is noteworthy that the fructosyltransferase enzyme of Rohapect CM exhibited high selectivity for the production of sc-FOS under all conditions tested. Sc-FOS production was proportional to the sucrose conversion during the synthesis up to about 0.89. Sucrose remaining in the reaction mixture was due to the fact that GF_2 acts as a donor and acceptor to form GF_3 and also due to inhibition by glucose [36].

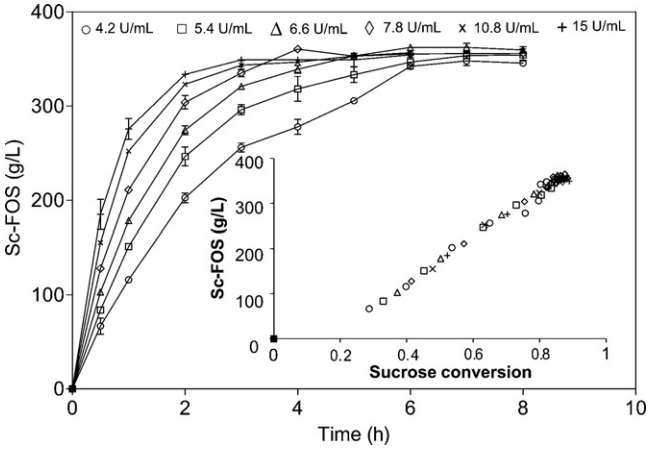

Fig. 8. Effect of enzyme concentration in the synthesis of sc-FOS from sucrose catalyzed by Rohapect CM. Experimental conditions were 50 °C, 600 g/L sucrose in 50 mM acetate buffer (pH 5.5).

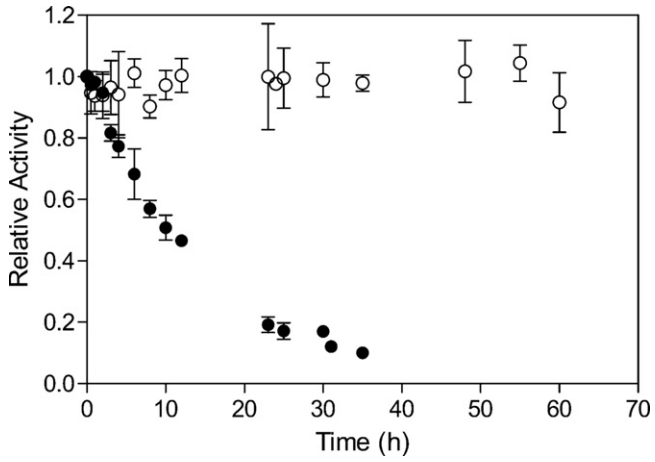

Fig. 9. Thermal inactivation kinetics of fructosyltransferase of Rohapect CM under non-reactive conditions (●, 50 mM sodium acetate buffer at pH 5.5) and reactive conditions (○, 600 g/L methyl-α-D-glucopyranoside in the same buffer) at 50 °C and 192 U_T/mL of incubation medium.

3.7. Thermal stability of free enzyme

For any enzymatic process, long-term stability of the catalyst is a prerequisite for the success of large-scale operation [39]. Thermal inactivation under non-reactive and reactive conditions of free enzyme (Rohapect CM) at 50 °C is presented in Fig. 9. Reactive conditions were mimicked by replacing sucrose with methyl-α-D-glucopyranoside. Results clearly show a stabilizing effect at high concentrations of methyl-α-D-glucopyranoside on enzyme activity after a 60 h incubation time. In contrast, inactivation rate was faster in buffer solution in which the half-life was approximately 10.2 h. Similar behavior has been reported for Pectinex Ultra SP-L at the same concentration of this glycoside during incubation [21]. With these results, it is not surprising that high concentrations of sucrose and sc-FOS may cause a protective effect in the same extent as that exerted by methyl-α-D-glucopyranoside on the transfructosylation activity due to the fact that sucrose has been recognized as one of the key stabilizers of proteins [39–41].

4. Conclusions

Three low-cost enzyme preparations were selected from a screening for transfructosylation activity. These preparations and Pectinex Ultra SP-L exhibited a high ratio (U_T/U_H), selectivity for the synthesis of sc-FOS and did not hydrolyze the produced sc-FOS after a 12 h reaction time. Pectinex Ultra SP-L, Rohapect CM, Viscozyme L and Pectinex Smash could serve as a source of food-grade fructosyltransferase for the inexpensive and efficient production of sc-FOS.

Sc-FOS synthesis catalyzed by Rohapect CM was not influenced by operating variables such as temperature, pH, concentrations of initial sucrose and enzyme. Yield was not affected by these variables within the ranges studied, except at the levels of pH 4.5 and 800 g/L sucrose. Operational variables affected the reaction rate being reflected in the volumetric productivity, but did not result in any change in the sc-FOS formation. In addition, Rohapect CM was thermally stable at 50 °C when sucrose was replaced by methyl-α-D-glucopyranoside. Volumetric productivity of sc-FOS at initial sucrose concentrations above 700 g/L can be further improved by response surface methodology. This strategy has been successfully applied to Rohapect CM and the results will be reported in another paper.

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