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Production of 1-kestose with intact mycelium of *Aspergillus phoenicis* containing sucrose-1^F-fructosyltransferase

J. A. M. van Balken, Th. J. G. M. van Dooren, W. J. J. van den Tweel, J. Kamphuis, and E. M. Meijer

DSM Research, Bio-organic Chemistry Section, P.O. Box 18, NL-6160 MD Geleen, The Netherlands

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Summary. Favourable reaction conditions for the enzymatic production of 1-kestose by sucrose-1^F-fructosyltransferase, SFT (EC 2.4.1.99) from *Aspergillus phoenicis* CBS 294.80 mycelium were established. The intracellular enzyme SFT works best at 60°C, exhibits a relatively high thermostability and possesses an alkaline pH optimum. An invertase also present in the mycelium of *A. phoenicis* possesses an acidic pH optimum. Consequently, around pH 8.0 sucrose is converted mainly to 1-kestose and nystose while fructose is only formed in relatively small amounts. Under optimal conditions (55°C, pH 8.0 and an initial sucrose concentration of 750 g l⁻¹) a yield of about 300 g 1-kestose per 1.0 l reaction mixture could be achieved after 8 h.

Introduction

Fructans are oligosaccharides and polysaccharides of fructose containing a single glucose moiety. Many enzymes for the production of fructans are known in nature. Inulosucrase (EC 2.4.1.9) converts sucrose into the β (2→1) linked fructan inulin and D-glucose (Bhatia et al. 1955; Dedonder 1952; Edelman and Bacon 1951; Edelman and Dickerson 1966), for example in artichoke (*Helianthus tuberosus*). Levansucrase (EC 2.4.1.10), on the other hand, converts sucrose into β (2→6) linked fructans (Hehre 1951; Hestrin et al. 1956; Reese and Avigad 1966).

It is often suggested (Edelman and Jefford 1968) that the first step in fructan synthesis is catalysed by a different enzyme. This enzyme sucrose fructosyltransferase (Henry and Darbyshire 1980; Pazur 1952; Straathof et al. 1986; Bacon 1954; Jacques 1985) catalyses the initial reaction by transferring a fructosyl group from a sucrose donor to a sucrose acceptor to produce a trisaccharide and glucose.

There are three different ways that coupling can occur, the products of the reactions being 1^F- β -fructosylsucrose (1-kestose, *O*- α -D-glucopyranosyl-(1→2)-*O*- β -D-fructofuranosyl-(1→2)- β -D-fructofuranoside), 6^F-fructosylsucrose (6-kestose, *O*- α -D-glucopyranosyl-(1→2)-*O*- β -D-fructofuranosyl-(6→2)- β -D-fructofuranoside) and 6^G- β -fructosylsucrose (neokestose, *O*- β -D-fructofuranosyl-(2→6)- α -D-glucopyranosyl-(1→2)- β -D-fructofuranoside), of which 1-kestose is the main commercial interest (Meiji Seika 1981, 1982; Hayashi et al. 1990; Muramatsu et al. 1988; Yun et al. 1990). 1-Kestose is not susceptible to decomposition by human and animal digestive enzymes, as a result of which it is a non-caloric and non-cariogenic fructo-oligosaccharide. Consequently, it might be an interesting bodying agent to be used in combination with intense sweeteners. In addition 1-kestose exhibits a stimulating effect on the growth of the beneficial *Bifidobacteria* in the animal intestine, as a result of which animal growth is promoted. Consequently fructo-oligosaccharides are currently used as feed supplements.

It was found that *Aspergillus phoenicis* CBS 294.80, which produces a thermostable inulinase for the industrial production of fructose from inulin (DSM 1981), also produces a sucrose-1^F-fructosyltransferase, SFT (EC 2.4.1.99) and out of the three possible oligosaccharides only 1-kestose was formed (DSM 1989). In the present study we discuss the optimization of the reaction conditions for 1-kestose production.

Materials and methods

Organism. *A. phoenicis* CBS 294.80, formerly employed for fructose production from inulin (DSM 1981), was used to produce 1-kestose from sucrose. Permanent stock cultures were maintained on malt agar at 10°C.

Precultivation and fermentation. For the production of spores, *A. phoenicis* CBS 294.80 was precultivated in a medium (pH 6.8) of the following composition: FeSO₄·7H₂O, 0.1 g l⁻¹; MnSO₄·4H₂O, 0.1 g l⁻¹; K₂HPO₄, 5.0 g l⁻¹; NaCl, 1.0 g l⁻¹; yeast extract (Difco, Detroit, MI, USA), 3 g l⁻¹; MgSO₄·7H₂O, 0.2 g l⁻¹; sucrose, 100 g l⁻¹ and agar, 20 g l⁻¹. Cultivation took place at 28°C for 8 days.

Fermentation of mycelia was carried out in 300-ml erlenmeyer flasks containing 100 ml medium. The fermentation medium (same as precultivation medium minus agar) was inoculated with 10^8 spores l^{-1} and incubated for about 2 days on a rotary shaker (200 rpm) at $30^\circ C$.

Mycelium was separated from the fermentation liquid by filtration. As SFT of *A. phoenicis* is closely associated with mycelium, whole mycelium cells were used after a single wash with 0.15 M NaCl.

Enzyme assays. The SFT and invertase activity were measured by adding a specified amount of dry mycelium to 100 ml reaction mixture containing sucrose and 0.1 M buffer. During incubation (200 rpm) at a specified temperature and pH, samples were taken and analysed for glucose, fructose, sucrose, 1-kestose, nystose and other oligosaccharides.

The SFT activity was expressed in units of 1-kestose + nystose produced per minute per gram dry weight. The invertase activity was calculated from the amount of fructose liberated per minute per gram dry weight. The ratio of the SFT activity on sucrose versus the invertase activity on sucrose characterizes the process, i.e. the higher the ratio the more kestose and the less fructose is produced.

Analytical methods. Glucose, fructose, sucrose and the oligosaccharides 1-kestose and nystose were qualitatively determined by TLC on plates of silica gel F254 (5635, Merck, Darmstadt, FRG) and developed in a solvent system of chloroform-ammonium hydroxide-methanol (60:20:54). Spots were visualized by spraying with 1-naphthol or phosphomolybdate.

For quantitative analyses an HPLC equipped with an Aminex HPX-87C column (300×7.0 mm; BioRad, Richmond, Calif., USA) and connected to a Perkin Elmer (Überlingen, FRG) R401 refractive index detector was used. Water was used as the eluent and the flow rate was 0.6 ml/min. The operating pressure was 22 bar and the temperature $85^\circ C$.

The samples were appropriately diluted before injection. Reference samples of 1-kestose, nystose and fructonystose were obtained from Meiji Seika Kaisha (Tokyo, Japan).

The ^{13}C NMR spectrum was recorded with a Varian XL200 (200 MHz) spectrometer.

Results

Selection of *A. phoenicis*

Recently *A. phoenicis* was isolated, which produces an extracellular inulinase. This thermostable enzyme has proved very suitable for producing high percentage fructose syrups from inulin (DSM 1981). In searching for broader applications of this commercially attractive mould the enzymatic production of non-digestible oligosaccharides from sucrose was investigated. As shown later, *A. phoenicis* indeed contained an SFT that catalyses the reactions depicted in Fig. 1.

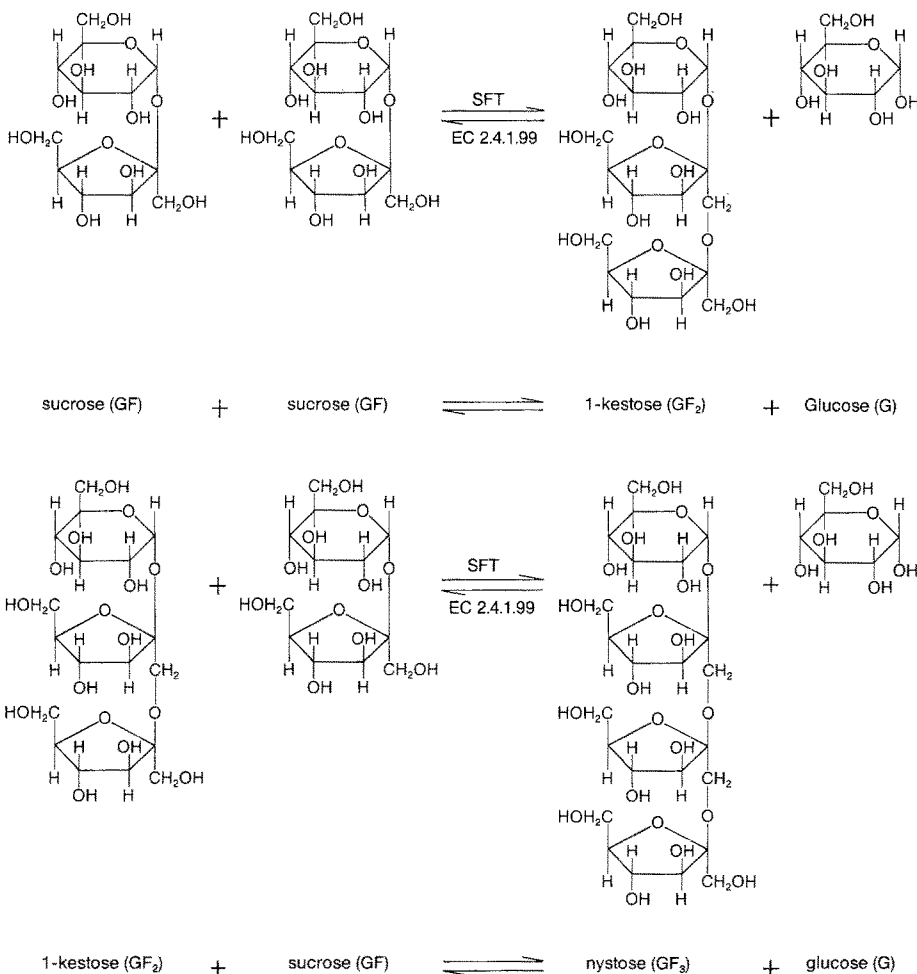


Fig. 1. Sucrose-1^F-fructosyltransferase (SFT)-catalysed reactions

Optimal cultivation conditions for mycelium containing high SFT activity

Using the media described in Materials and methods ca. 30 g wet fungal mycelium/l was obtained. Sucrose is necessary for SFT activity but the sucrose concentration in the fermentation medium is not very critical. The addition of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g l^{-1}) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g l^{-1}) to the fermentation liquid stimulated SFT activity.

Isolation and identification of the products

To isolate the main product, the reaction mixture (1.9 g wet mycelium in 100 ml for 3 h at 50°C , pH 8.0 in 500 g l^{-1} sucrose) was separated from the mycelium by filtration and subsequently freeze dried. The freeze-dried sample was dissolved in a small amount of distilled water and applied to a silica gel column ($6.5 \times 15 \text{ cm}$). The column was fully washed with methanol and eluted with a solvent system of chloroform-ammonium hydroxide-methanol (10:10:30) to remove mono- and disaccharides. Trisaccharides were eluted with a solvent system of chloroform-ammonium hydroxide-methanol (10:10:20). Trisaccharide-containing fractions (TLC analyses) were combined and freeze dried. As shown by HPLC, the remaining material contained only 1-kestose. Moreover, hydrolysis of the product with 4 N HCl, followed by neutralization (NaHCO_3), showed that the trisaccharide contains glucose and fructose as monomeric units in a ratio of 1:2 (HPLC analyses). It was confirmed by means of ^{13}C -NMR that the synthesized product was indeed 1-kestose.

In addition to 1-kestose also a small amount of nystose was produced (HPLC analysis). However, the reaction product contained no fructonytose.

Optimal pH for SFT activity

The effect of pH on SFT activity by resting cells was studied using 2191 mM sucrose at 55°C . The pH of the reaction mixture was adjusted to 5.0 and 5.5 with 0.1 M sodium-acetate buffer and to 6.0–9.5 with 0.1 M Sørensen buffer. The optimum pH for SFT activity (FT) was found to be between 7.5 and 9.0 (Fig. 2). The competing invertase activity (I) on the other hand was very low at pH values above 7.0. Consequently an optimum FT/I ratio was found at around pH 8.0.

Optimum temperature for SFT activity and stability

Using an initial sucrose concentration of 2191 mM at the optimal pH for SFT activity (pH 8.0), the effect of temperature on SFT activity was investigated (Fig. 3). The rate of oligosaccharide production increased with increase in temperature. However, at 65°C there was a

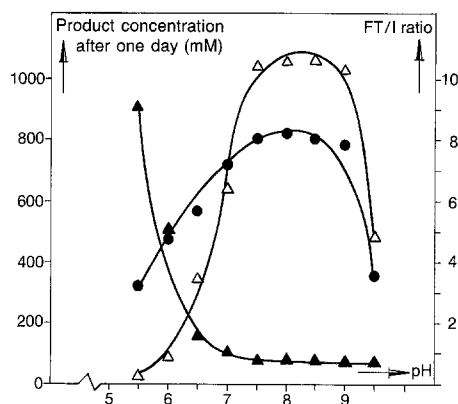


Fig. 2. Effect of pH on product formation by SFT and invertase. The incubation mixtures (100 ml) contained 0.3 g dry weight mycelium: ●, SFT activity (FT), 1-kestose + nystose; ▲, invertase activity (I), fructose; △, FT/I ratio

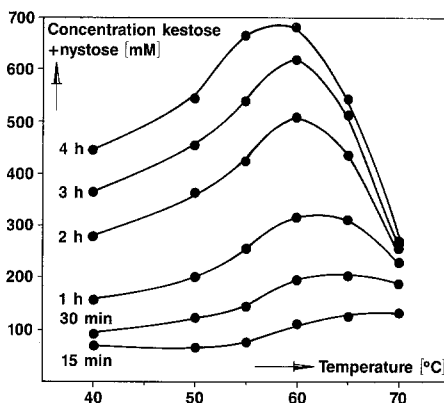


Fig. 3. Time-dependence of product formation by SFT at various temperatures. The incubation mixtures (100 ml) contained 0.31 g dry weight mycelium

decrease in the total amount of oligosaccharides formed. The highest production was observed at 60°C . The composition of the product mixture at the six temperatures tested is given in Table 1.

To study the effect of temperature on SFT stability, recycling experiments at four different temperatures (50 , 55 , 59 and 62°C) were performed (Fig. 4). The incubations were performed at pH 8.0 using 2191 mM sucrose. Although each run lasted for 20 h, SFT activity was determined during the first hour of incubation. After each run, fungal mycelium was separated by filtration and fresh sucrose solution (2191 mM) was added. At a temperature of 50°C only minor inactivation took place; after six runs (120 h) 75% of the initial activity still remained. Even at 55°C almost no inactivation occurred. At 59°C , however, 50% of the initial activity was lost after one run, while at 62°C only 16% remained after a single run (Fig. 4). Consequently, in view of the relatively low stability at about 60°C the preferred temperature for fructo-oligosaccharide production is 55°C .

Table 1. Analysis of the incubation mixture after enzymic conversion of sucrose at various temperatures after 4 h incubation by mycelia possessing sucrose-1^F-fructosyltransferase activity

T (°C)	Fructose (mM)	Glucose (mM)	Sucrose (mM)	1-Kestose (mM)	Nystose (mM)	Total oligosaccharides
40	<7	402	1278	424	24	448
50	<7	520	1022	520	26	546
55	42	652	876	595	71	666
60	42	687	803	620	77	697
65	42	529	1058	494	53	547
70	83	320	1533	248	9	257

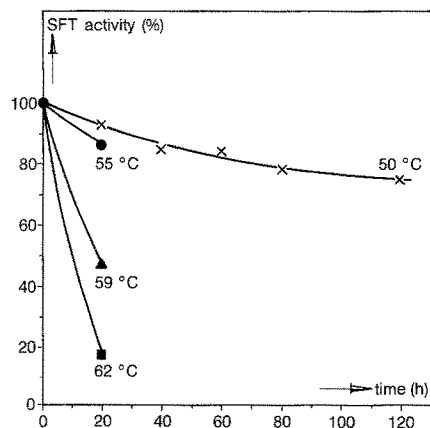


Fig. 4. Temperature stability of SFT activity: 100% corresponds with activities of 825, 1050, 1275 and 1250 μmol of product formed per minute per gram dry weight for 50°C, 55°C, 59°C and 62°C, respectively

Effect of different concentrations of substrate

In the reaction mixture the concentration of sucrose was varied. The reactions were carried out at 60°C, pH 8.0, for 8 h. The results are presented in Fig. 5.

The initial SFT activities were almost equal for all sucrose concentrations tested. However, at an initial sucrose concentration of 2410 mM (825 g l^{-1}) a significantly lower fructo-oligosaccharide yield (w/w) was observed. The FT/I ratios for these incubations all varied between 12 and 15. Based on these results a sucrose concentrations of 2191 mM (750 g l^{-1}) is recommended.

Product inhibition

Since the formed fructo-oligosaccharides, 1-kestose and nystose, may have an effect on SFT activity this possibility was investigated. Because insufficient amounts of pure 1-kestose and nystose were available, appropriate amounts of end-product were mixed with a 2191 mM sucrose solution. The composition of the pertinent end-product (obtained after 8 h incubation) was: fructose, 60 mM; glucose, 740 mM; sucrose, 627 mM; 1-kestose, 656 mM; nystose, 100 mM.

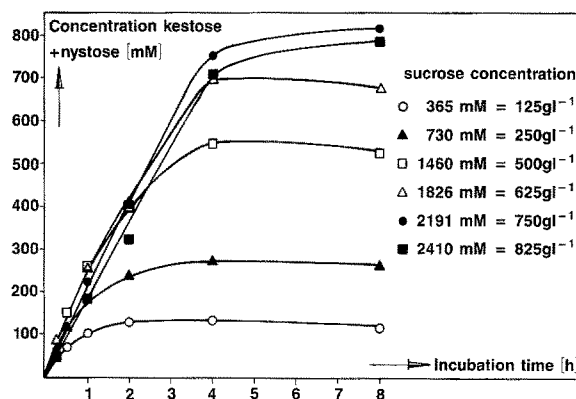


Fig. 5. SFT activity at various initial sucrose concentrations. All reaction mixtures (100 ml) contained 0.25 g dry weight mycelium

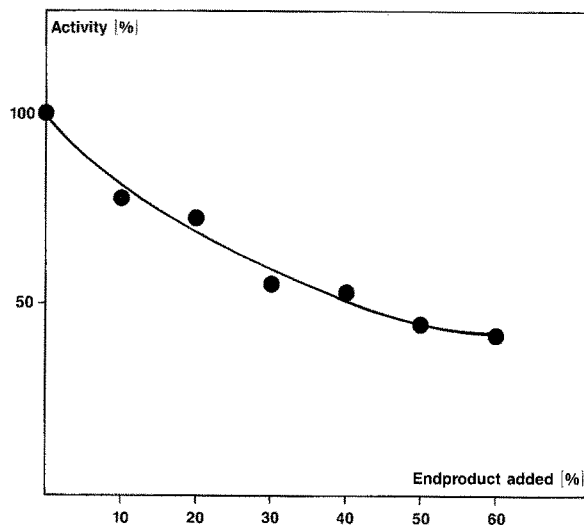


Fig. 6. Inhibition of SFT by the end-product: 100% corresponds with an SFT activity of 1300 μmol kestose + nystose produced per minute per gram dry weight. The incubation was performed at pH 8.0 and 60°C

As shown in Fig. 6 the enzyme activity (calculated after incubation for 1 h) was reduced to 50% when about 40% of the reaction mixture was made up with the specified end-product. The inhibition of the initial activity is likely to be due to 1-kestose and nystose present in the reaction mixture.

Table 2. Production of fructo-oligosaccharides under optimal conditions

Incubation (h)	Fructose (mM)	Glucose (mM)	Sucrose (mM)	1-Kestose (mM)	Nystose (mM)
1	14	41	1935	89	<10
4	41	430	1132	451	30
8	49	728	617	644	99
12	76	867	402	644	167
18	76	999	267	595	219

Production under optimal conditions

On basis of the above results the following optimal reaction conditions were used for fructo-oligosaccharide production by mycelium of *A. phoenicis* CBS 294.80: 2191 mM sucrose, 0.1 mM potassium phosphate buffer, pH 8.0, 0.18 g dry mycelium (per 100 ml reaction volume) and 55°C. The results of this incubation are summarized in Table 2. After 12 h the concentration of 1-kestose was 644 mM (about 325 g l⁻¹). The total yield (w/w) of fructo-oligosaccharides from sucrose was about 60%. During this incubation the FT/I ratio was 10–15.

Discussion

The SFT of *A. phoenicis* CBS 294.80 exhibits several favourable characteristics. The high thermostability of this enzyme in the intact mycelium makes commercial production of 1-kestose at high temperatures (50–60°C) attainable. Other SFTs sometimes lack such thermostability. Moreover, high temperatures in combination with high substrate concentrations (2191 mM: 750 g l⁻¹) prevent microbial contamination of the reaction mixture.

In contrast with other SFTs (Hirayama et al. 1989; Hayaishi et al. 1990; Shimura and Itoh 1985), the optimum pH of the SFT of *A. phoenicis* is rather high (pH 7.5–9.0). The invertase activity on the other hand is maximal at pH values below 6.0.

Preliminary enzyme purification experiments (results to be published) have indicated that at least two different enzymes are involved in the conversion of sucrose, one being an SFT with an intrinsic invertase enzyme activity (optimum pH in the alkaline region) and the other enzyme being an invertase with a more acidic pH optimum.

In order to obtain a low-caloric product invertase activity should be minimized. There are several other ways to suppress invertase activity. One of them is the use of specific inhibitors. Reagents which combine with carbonyl groups, e.g. aniline, *p*-toluidine and phenylhydrazine, inhibit invertase non-competitively, presumably by way of formation of a Schiff's base. Also with our enzyme preparation, inhibition of invertase activity by various chemicals was observed (results not shown). However, the main drawbacks in using these agents are

toxicity and the high concentrations needed for inhibition. Another way of suppressing invertase activity is to take advantage of the different thermostabilities of both enzymes. Heat-pretreatment (30 min, 65°C) of the fungal mycelium of *A. phoenicis* diminishes invertase activity by about 80% while the SFT activity was almost unaffected.

However, none of the forementioned options is needed in view of the high FT/I ratio obtained at pH values around 8.0. Under such conditions invertase activity is almost negligible ($\leq 10\%$ of the SFT activity) as a result of which high amounts of 1-kestose and nystose can be produced.

Using the enzyme preparation of *A. phoenicis* CBS 294.80 no tetrasaccharides are formed; this is in contrast to the product sold nowadays by Meiji Seika Kaisha, which is, to the best of our knowledge, made by a fructosyltransferase from either *A. niger* or *Aureobasidium pullulans* (Meiji Seika 1981). Recently the latter company has also patented the formation of fructo-oligosaccharides from inulin by means of endoinulinases (Meiji Seika 1987). Given the potential applications of these non-caloric, non-cariogenic, health-stimulating fructo-oligosaccharides, research towards production of such oligosaccharide mixtures is very likely to be intensified in the near future.

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