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# Optimization of levansucrase/endo-inulinase bi-enzymatic system for the production of fructooligosaccharides and oligolevans from sucrose

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ABSTRACT

A bi-enzymatic system based on the combined use of levansucrase (LS) from *Bacillus amyloliquefaciens* and endo-inulinase from *Aspergillus niger* in a one-step reaction was investigated for the synthesis of fructooligosaccharides (FOSs) and oligolevans using sucrose as the sole substrate. Sucrose concentration was the most important independent variable, whilst LS to endo-inulinase ratio exhibited significant effects on the end-product profiles. The interaction between sucrose concentration and reaction time exhibited significant effect on all responses. At the initial stage of time course, short chain FOSs (scFOSs, 1-kestose, nystose, $1^F$-fructosylnystose) were the major products, whilst 6-kestose, medium chain fructooligosaccharides (mcFOSs, levanohexaose, levanopentaose) and oligolevans became the dominant ones at the late stage. The optimal conditions leading to a high yield of scFOSs (1:1 ratio, 0.5 h, 0.6 M) were different from those resulting in a high yield of mcFOSs and oligolevans (1.85:1 ratio, 1.77 h, 0.6 M). The bi-enzymatic system has a great potential for the production of FOSs and oligolevans at a large scale because of its high yield (57–65%, w/w) and productivity (65.8–266.8 g/L h), and its uses of low temperature (35 °C) and low concentration of sucrose. To the best of our knowledge, this is the first study on the optimization of a LS/endo-inulinase bi-enzymatic system.



## 1. Introduction

Fructooligosaccharides (FOSs) constitute a class of bioactive molecules whose potential health benefits in terms of supporting intestinal health and reducing the risk of cancers are increasingly being recognized. In addition, FOSs have been recognized by their low caloric value and non-carcinogenic properties [1,2]. Enzymatic strategies for the synthesis of FOSs are generally based on the transfructosylation of sucrose or the hydrolysis of inulin [3]. The hydrolysis strategy is limited by the seasonal availability of inulin. However, the production of FOSs from sucrose, by the action of fructofuranosidase or fructosyl-transferase through transfructosylation reaction, is a more cost effective and convenient route [4].

Levansucrases (LSs) (E.C.2.4.1.10), a subclass of fructosyltransferases, have recently gained more interest, because of their ability to directly use the free energy of cleavage of non-activated sucrose to transfer the fructosyl group to a variety of acceptors including mono- (exchange), oligosaccharides (FOS synthesis) or the growing fructan chain (polymer synthesis) [5]. In fact, β-(2→6)-levan-type-FOSs, obtained through LS-catalyzed transfructosylation, have shown beneficial prebiotic effects that surpass current commercial β-(2→1)-inulin-type FOSs [6]. In addition to their excellent water-holding capacity and protecting effect, β-(2→6)-levan polymers have shown antitumor and antidiabetic activities [7–9]. Hence, microbial LSs are of high interest as biocatalysts for the catalytic synthesis of novel type FOS prebiotics as well as levan for food, cosmetics, and pharmaceutical fields [10,11].

LSs differ widely with respect to their reaction specificity (hydrolysis/transfructosylation) and oligo-/polymerization ratio, and thus result in different product spectrum (i.e. levan or FOSs) [12–16]. Recently, some hypotheses and structural features have been put forward to describe the polymerization/oligomerization ratio [12,13,17,18]. In our previous studies, a bi-enzymatic system, based on the synergistic actions of LS from *Bacillus amyloliquefaciens* and endo-inulinase from *Aspergillus niger*, was, for the first time, investigated for the synthesis of FOSs and oligolevans using sucrose as an abundant substrate [19]. LS catalyzes the synthesis of levan from sucrose, whilst the endo-inulinase hydrolyses levan into FOSs and oligolevans. These transfructosylation products may be further hydrolyzed by endo-inulinase or serve as fructosyl acceptors for LS. The bi-enzymatic system showed higher yield and productivity of FOSs and oligolevans (67%, w/w; 96 g/L h) than the LS enzymatic system (3.0%, w/w; 0.8 g/L h) alone. As compared to other biocatalytic systems [20–22], the developed

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bi-enzymatic system showed superior FOSs productivity and a broad product spectrum, whilst it used lower temperature and sucrose concentration. It has also been shown that the synthesis of transfructosylation products (FOSs and oligolevans) can be regulated by modulating the substrates and acceptors availabilities and the equilibrium of the involved reactions. Indeed, the contribution of endo-inulinase to the formation of FOSs and oligolevans through its hydrolytic activity was higher than that of LS and endo-inulinase through their acceptor reactions. However, the production of intermediate levans with appropriate molecular weight (MW) by LS was prerequisite for the production of FOSs and oligolevans. Investigation of the interactive effects of reaction parameters of bi-enzymatic system is, therefore, needed for better understanding of the synergistic interaction and the thermodynamic relationships between LS and endo-inulinase. As part of the ongoing research work in our laboratory, the specific objectives of the present study were (1) the investigation of the interactive effects of selected reaction parameters of bi-enzymatic system on the production of total transfructosylation products and, in particular, of short chain fructooligosaccharides (scFOSs: 1-kestose, 6-kestose, nystose and $1^F$-fructosylnystose), medium chain ones (mcFOSs: levanopentaose, levanohexaose) and oligolevans using response surface methodology (RSM), (2) the development of mathematical models to produce transfructosylation products with targeted yield, specific end-product profile and structural properties, and (3) the determination of the conditions for the optimum production of scFOSs, mcFOSs and oligolevans and the comparison of the efficiency of bi-enzymatic system with current available ones. To the best of our knowledge, there is no report on the optimization of LS/endo-inulinase bi-enzymatic system. RSM is an effective tool for modeling and optimizing any complex process that is affected by the levels of more than one factor [23]. The advantage of RSM is the low number of experimental trials required to study the linear or quadratic effects of the factors and also their cross product effects.

## 2. Materials and methods

### 2.1. Chemicals and materials

D-(−)-fructose, D-(+)-glucose, D-(+)-raffinose, and sucrose were purchased from Sigma–Aldrich (St-Louis, MO). Carbohydrate standards 1-kestose, nystose, and $1^F$-fructosylnystose were purchased from Wako Pure Chemical (Japan). Chemical reagents, including, 3,5-dinitrosalicylic acid (DNS), $K_2HPO_4$, $KH_2PO_4$, NaOH, polyethylene glycol (PEG) 200, and triton X-100 were also obtained from Sigma–Aldrich (St-Louis, MO). Endo-inulinase (EC 3.2.1.7) from *A. niger* was purchased from Sigma–Aldrich (St-Louis, MO).

### 2.2. Levansucrase preparation

*B. amyloliquefaciens* (ATCC 23350) was obtained from American type culture collection (Manassas, USA). *B. amyloliquefaciens* was grown aerobically at 150 rpm and 35 °C for 11 h in a mineral based medium supplemented with yeast extract (10 g/L) and sucrose (10 g/L) as described previously by Tian et al. [16]. Intracellular LS was recovered by ultrasonication (2 kHz, cycle 25/50 s, 550 Sonic Dismembrator, Fisher Scientific) of cell suspension in 50 mM potassium phosphate buffer (pH 6.0) containing Triton X-100 (1%). After centrifugation (9800 × *g*, 20 min) to remove cell debris, the intracellular LS extract was further purified by PEG 200 fractionation (30%, v/v) [24]. The partially purified LS extract was dialyzed against 5 mM potassium phosphate buffer (pH 6.0) through a membrane with a cutoff of 5–6 kDa at 4 °C and then lyophilized. PEG-200 precipitation selectively purified LS with a purification factor of 76-fold and a high yield of 57% [24].

### 2.3. Levansucrase activity assay

LS activity was assayed using sucrose as substrate as described previously [24]. The assay was initiated by adding 0.25 mL of LS extract (6–25 μg) to 0.25 mL of sucrose solution (1.8 M), prepared in potassium phosphate buffer (50 mM, pH 6.0). The reaction mixtures were incubated at 30 °C for 20 min, and then heated in 100 °C water bath for 5 min to stop the reaction. The concentrations of glucose and fructose were quantified using high-pressure-anionic-exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD, Dionex), the Chromeleon Software and a CarboPac PA20 column (3 mm × 150 mm) set at a temperature of 32 °C. Isocratic elution was applied with 10 mM NaOH as the mobile phase at a flow rate of 0.5 mL/min. Subtracting the total amount of fructose from that of glucose provides the amount of glucose resulting from transferring fructose. One transfructosylation unit of LS is defined as the amount of the biocatalyst that releases 1 μmol of glucose as a result of transferring fructose, per min. Specific activity was expressed as the transfructosylation units per mg of protein.

### 2.4. Endo-inulinase activity assay

The hydrolytic activity of endo-inulinase was investigated using low MW levan (5.5 ± 0.5 kDa) as substrate, which was prepared as previously described through LS-catalyzed transfructosylation reaction [19]. Only very minor activity of exo-inulinase (<8%) was detected in the investigated endo-inulinase product. The enzymatic assay consisted of 0.25 mL endo-inulinase solution (0.36–0.52 mg proteins) and 0.25 mL of 2% (w/v) levan as substrate in potassium phosphate buffer (50 mM, pH 6.0). The reaction mixtures were incubated at 30 °C for 20 min, and then heated in 100 °C water bath for 5 min to deactivate the enzyme. The reducing fructose end-groups of FOSs were quantified using the dinitrosalicylic acid method. After adding 0.75 mL of 1% (w/v) dinitrosalicylate reagent, prepared in 1.6% (w/v) NaOH, the reaction mixtures were then placed in a boiling water-bath for 5 min, for the development of reducing ends color. 0.25 mL of potassium sodium tartrate solution (50%, w/v) was, thereafter, added to the mixtures. The absorbance of the resulting mixture was measured spectrophotometrically at 540 nm, against reagent blank. The amount of the released reducing fructose end-groups was determined from the standard curve, constructed with fructose. One unit of endo-inulinase was estimated as the amount of the biocatalyst that released 1 μmol of reducing fructose end-groups per min of reaction.

### 2.5. Bi-enzymatic system

The combined use of LS and endo-inulinase in one-step bi-enzymatic system was investigated using an initial sucrose concentration of 0.4 M and an enzymatic ratio of 1:1 (0.6 U/ml:0.6 U/ml). The reactions were carried out at 35 °C in 0.1 M potassium phosphate buffer (pH 6.0) and at 70 rpm using an orbital incubator shaker (New Brunswick Scientific Co, Inc, Edison, NJ). At selected reaction times, aliquots were withdrawn, and methanol was added at a ratio of 1:1 (v/v) followed by boiling for 5 min. The analysis of the reaction components was carried out by HPAEC and high performance size exclusion chromatography (HPSEC).

### 2.6. Identification and characterization of product spectrum

The product spectrum of the bi-enzymatic system was characterized by HPAEC using a Dionex (ICS-3000) system equipped with pulsed amperometric detector (PAD), the Chromeleon Software, and a CarboPac PA200 (3 mm × 250 mm) column set at 32 °C. The elution of the reaction components was carried out at 0.5 mL/min using a linear gradient of sodium acetate from 0 to