Purification and Characterization of Levansucrases from *Bacillus amylo liquefaciens* in Intra- and Extracellular Forms Useful for the Synthesis of Levan and Fructooligosaccharides

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The intra- and extracellular levansucrase (LS) activities produced by *Bacillus amylo liquefaciens* were promoted by supplementing the sucrose medium with yeast and peptone as nitrogen sources. These activities were purified by polyethylene glycol (PEG) fractionation for the first time. PEGs of low molecular weight selectively fractionated the intracellular LS activity rather than the extracellular LS activity. Contrary to other LSs, *B. amylo liquefaciens* LSs exhibited high levan-forming activity over a wide range of sucrose concentrations. The optimum temperatures for the intra- (25–30°C) and extracellular (40°C) LS transfructosylation activities were lower than those for the hydrolytic activities (45–50°C; 50°C). In addition, the catalytic efficiency for the transfructosylation activity of intracellular LS was higher than that of extracellular LS. These differences between intra- and extracellular LSs reveal the occurrence of certain conformational changes to LS upon protein secretion and/or purification. This study is the first to highlight that *B. amylo liquefaciens* LSs synthesized a variety of FOSs from various saccharides, with lactose and maltose being the best fructosyl acceptors.

**Key words:** *Bacillus amylo liquefaciens*; levansucrase; polyethylene glycol; fructooligosaccharide; levan

Levan-type fructooligosaccharides (FOSs) and β-(2-6)-levan are of increasing interest because of their potential health benefits to selectively support the intestinal health. Indeed, β-(2-6)-FOSs and neoFOSs have demonstrated prebiotic effects that surpassed those of β-(2-1)-FOSs available for human consumption. In addition, levan polymers have a variety of potential applications in the food and pharmaceutical fields because of their physical properties and their biological functions as antitumor and immune cell-activating agents. Levansucrases (LSs), which are fructosyltransferases belonging to family 68 of glycoside hydrolases, catalyze the synthesis of β-(2-6)-levan by transferring the fructosyl group of non-activated sucrose into the fructan chain. Interestingly, the formation of levan can be quantitatively replaced by the formation of homo- and hetero-β-(2-6)-FOSs in the presence of various acceptors. Most of the research reported to date has been carried out on a few LSs from *Zymomonas mobilis* and *Bacillus subtilis* and, to a lesser extent, on LSs from *Lactobacillus reuteri*, *Gluconacetobacter diazotrophicus* and *Bacillus megaterium*.

LSs described so far differ widely with respect to their kinetic and biochemical properties. Only a few LSs have been fully characterized with respect to their transfructosylation product spectra and their acceptor/donor specificity. The tridimensional structures of LSs from *B. subtilis* and *G. diazotrophicus* have recently become available. In spite of having a similar active site conformation, LS from *B. subtilis* was found to predominantly catalyze the synthesis of levan, whereas that from *G. diazotrophicus* mainly synthesized the short FOSs. Although some hypotheses have been presented, there is still no clear understanding, which structural elements of LS determine the poly/oligomerization ratio and the outcome of the transfructosylation reaction.

The LS-catalyzed transfructosylation reaction has been recognized as a relevant synthetic route for the synthesis of novel β-(2-6)-FOSs and levan. However, this attractive approach is still limited by the poor availability of LSs and their low stability. The investigation of LSs with improved properties from selected microbial sources is of great interest. *Bacillus amylo liquefaciens*, having the “generally recognized GRAS” status, is one of the dominant bacterial workhorses with high potential for enzyme production. *B. amylo liquefaciens* cultivated on media containing sucrose (3) and xylose (4) has been reported to produce extracellular LS. Little information about the catalytic properties and the donor/acceptor specificity of LS from *B. amylo liquefaciens* is available. Intra- and extracellular *B. amylo liquefaciens* LS forms were pre-purified in the present study by fractionation with polyethylene glycols (PEGs), and their catalytic properties were characterized. The donor and acceptor specificities of LSs are also described.

**Materials and Methods**

**Levansucrase production.** The strain of *B. amylo liquefaciens* (ATCC 23350) was maintained on potato dextrose agar (39 g/L). After 24 h of pre-culture in a nutrient broth (8 g/L), 4 mL was transferred into a 1-L baffled Erlenmeyer flask containing 400 mL of the culture medium to reach an initial absorbance of 0.2 at 600 nm. LS was produced by using a succinate-containing medium and modified mineral salt medium, each supplemented with sucrose (10 g/L) as an
The modified succinate-containing medium consisted of (in g/100 mL) sodium succinate (10), K2HPO4 (0.7), KH2PO4 (0.3), (NH4)2SO4 (0.2), FeSO4·7H2O (0.005), MnCl2·6H2O (0.005) and MgSO4·7H2O (0.025), while the modified mineral salt medium comprised (in g/100 mL) Na2HPO4·2H2O (0.267), KH2PO4 (0.136), (NH4)2SO4 (0.05), FeSO4·7H2O (0.0005), MnSO4·H2O (0.00018), Na2MoO4·2H2O (0.00025), Ca(NO3)2·2H2O (0.001) and MgSO4·7H2O (0.02). These two media were supplemented with yeast extract (NH25 g glucose resulting from transferring fructose.

The bacterial strain was grown at 37 °C with continuous agitation at 120rpm by an orbital shaker (Lab-Line 3527 Orbit Environ-Shaker). Growth was assessed by reading the absorbance at 600nm with a DU 800 UV/Visible spectrophotometer (Beckman). Aliquots of 2 mL were withdrawn in parallel to evaluate the LS activity of the cell-free growth media (extracellular) and the cell lysate (intracellular).

Preparation of the levansucrase extracts. The cells and the supernatants were recovered after 10–11h of incubating the culture by centrifugation at 8000 rpm for 40 min at 4 °C. The cells were resuspended in a 50 mM potassium phosphate buffer (pH 6.0) containing Triton X-100 (1%) and then disrupted by ultrasonication (2 kHz, 30/50 s cycle). The resulting suspension was shaken at 4 °C with continuous agitation at 120 rpm by an orbital shaker (Lab-Line 3527 Orbit Environ-Shaker). Growth was assessed by reading the absorbance at 600 nm with a DU 800 UV/Visible spectrophotometer (Beckman). Aliquots of 2 mL were withdrawn in parallel to evaluate the LS activity of the cell-free growth media (extracellular) and the cell lysate (intracellular).

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Purification of the levansucrases by PEG fractionation. Both intra- and extracellular LSs were purified by PEG fractionation. Low-molecular-weight PEG-200, PEG-350 and PEG-400 were each added to 100 mL of the intra- and extracellular LS extracts to obtain final concentrations of 10, 20, 30 and 40% (v/v). Stock solutions of high-molecular-weight PEG-2000, PEG-3500 and PEG-4000 were each added to the parental LS extract to achieve the same final PEG concentrations of 10, 20, 30 and 40% (v/v). Each mixture was incubated for 2h at 4 °C under mild stirring. The pellet containing precipitated LS was recovered by centrifugation at 8000 rpm for 45 min at 4 °C. The pre-purified LS extracts were dialyzed through a membrane with a cutoff of 5–6 kDa and then analyzed for their LS activity and protein concentration.

Levansucrase activity assays. The LS activities of total LS, levan-forming, hydrolytic and transfructosylation were assayed by using sucrose as a substrate. One unit of total LS activity is defined as the amount of the biocatalyst to liberate 1 µmol of the reducing sugars (glucose and fructose) from sucrose per min under the standard assay conditions. One unit of levan-forming activity is defined as the amount of the biocatalyst required to form 1 µmol of levan per min, and one transfructosylation unit of LS is defined as the amount of the biocatalyst to release 1 µmol of glucose per min as a result of transferring fructose. Subtracting the total amount of fructose from that of glucose provides the amount of glucose resulting from transferring fructose.

The enzymatic reactions were initiated by adding 0.25 mL (2.5–25 µg of protein) of the appropriate diluted LS extract suspension to 0.25 mL of the sucrose substrate solution (0.9 M) that had been prepared in a potassium phosphate buffer (50 mM at pH 6.0). Each reaction mixture was incubated in a water-bath shaker at 30 °C for 20 min. Two blank assays, without the substrate or without the enzyme, were conducted in tandem for the trials. All assays were run in triplicate. The concentration of the released reducing sugars (glucose and fructose) was measured as dinitrosalicylic acid (DNS).17 After adding 0.75 mL of a 1% (w/v) dinitrosalicylate reagent, which had been prepared in 1.6% (w/v) NaOH, each reaction mixture was placed in a boiling-water bath for 5 min to develop the reducing sugar color. A potassium sodium tartrate solution (0.25 mL, 50%, w/v) was then added to the mixture. The absorbance of the resulting mixture was measured spectrophotometrically at 540 nm against the reagent blank.

The amount of released glucose sugars was then determined by constructing a standard curve that had been constructed with the standard solutions of glucose (328.11 M/cm). The synthesis of levans was monitored by following the increase in turbidity at 600 nm, using a molar extinction coefficient of 772.61 M/cm.

The hydrolytic and transfructosylation activities were measured by quantifying glucose and fructose with a high-pressure anion-exchange chromatograph equipped with a pulsed amperometric detector (HPAEC-PAD, Dionex), using Chromatography software and a CarboPac PA20 column (3 × 150 mm) set at a temperature of 32 °C. Isocratic elution was performed with 10 mM NaOH as the mobile phase at a flow rate of 1 mL/min. Prior to injection to the HPAEC-PAD instrument, methanol was added at a ratio of 1:1 (v/v) to precipitate the proteins and levan, each reaction mixture being centrifuged at 10,000 × g for 10 min. The concentration of each product was estimated by constructing standard curves for glucose and fructose.

Effect of pH on the levansucrase activity. The effect of pH on the total intra- and extracellular LS activities was carried out at pH values ranging from 4.0 to 9.0 by using different buffers (50 mM) of sodium citrate (pH 3.0–3.5), sodium acetate (pH 4.0–5.5), potassium phosphate (pH 6.0–7.0) and Tris–HCl (pH 7.5–9.0). The LS assays were carried out using sucrose as a substrate at 30 °C as already described.

Effect of temperature on the levansucrase activity. The optimum temperature, and hydrolytic and transfructosylation activities of the intra- and extracellular LS forms were determined by assays over a wide range of temperature (25–70 °C) in a 50 mM potassium phosphate buffer at the optimum pH value. These assays were carried out by using sucrose as a substrate at 30 °C as already described.

Determination of the kinetic parameters. The levan-forming, hydrolytic and transfructosylation activities were measured at a substrate concentration ranging from 10 mM to 1.3 mM under the standard assay conditions. Lineweaver-Burk plots (1/V = 1/Vmax + (Km/Vmax) × 1/[S]) enabled the apparent Michaelis-Menten constant (Km) and maximum velocity (Vmax) for both extra- and intracellular LSs to be estimated by using Sigma Plot software (Jandel Scientific, Germany).

Nuclear magnetic resonance (NMR) characterization of levan. Levan was recovered by overnight precipitation with ethanol (1:1, v/v). After centrifugation at 8,000 × g for 45 min at 4 °C, the levan in the pellet was dialyzed against water for three consecutive days at 4 °C. The dialyzed levan was lyophilized, and the glycosidic linkages were examined by an NMR characterization. The sample was dissolved in D2O, and the spectra were obtained at room temperature on a Varian VNMRS-500 instrument operated at 100.5 MHz for 13C and 499.9 MHz for 1H. The 13C spectrum represents the accumulation of 448 transients with a 45° pulse width, acquisition time of 1.3 s and recycle delay of 1 s. Lorentzian broadening of 1.0 Hz was applied before Fourier transformation. The 1H spectrum represents the accumulation of 4 transients with a 45° pulse width, acquisition time of 2.0 s and recycle delay of 1 s.

Characterization of the product spectrum. The enzymatic reaction was carried out with 0.4 M sucrose and 3–5 enzymatic units/mL of purified LSs in a potassium phosphate buffer (50 mM, pH 6.0) at 30 °C for up to 24 h. The protein and levan polymers were precipitated with methanol (1:1, v/v) and separated by centrifugation. The product spectrum was analyzed by HPAEC-PAD, using a CarboPac PA20 column. The reaction components were eluted by using a linear gradient of 0–100% of 200 mM sodium acetate in 100 mM NaOH for 20 min. The elution of the oligosaccharides was monitored by pulsed amperometric detection. 1-Kestose, nystose, 6-kestose and FOSs from chichory inulin were used as internal standards to identify the peaks.
Table 1. Effect of Nitrogen (N) Source on the Intra- and Extracellular Levansucrase (LS) Activities Produced by *B. amyloliquefaciens*

<table>
<thead>
<tr>
<th>Growth (Ab600)</th>
<th>Intracellular LS activity* (U/L)</th>
<th>Extracellular LS activity* (U/L)</th>
<th>Total LS activity* (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate-containing medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without organic N source</td>
<td>1.55±0.01</td>
<td>250.0±30.2</td>
<td>162.8</td>
</tr>
<tr>
<td>Peptone</td>
<td>1.41±0.04</td>
<td>400.1±41.0</td>
<td>554.7</td>
</tr>
<tr>
<td>Yeast</td>
<td>1.35±0.01</td>
<td>740.5±70.8</td>
<td>670.3</td>
</tr>
<tr>
<td>Mineral-containing medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without organic N source</td>
<td>1.06±0.04</td>
<td>245.5±21.1</td>
<td>316.5</td>
</tr>
<tr>
<td>Peptone</td>
<td>1.41±0.04</td>
<td>372.5±10.1</td>
<td>529.5</td>
</tr>
<tr>
<td>Yeast</td>
<td>1.44±0.02</td>
<td>380.1±22.2</td>
<td>674.7</td>
</tr>
</tbody>
</table>

*The cell lyase obtained after sonication was used to measure the intracellular LS activity.

*The cell-free growth medium was used to measure the extracellular LS activity.

*One unit of total LS activity is defined as the amount of the biocatalyst that liberates 1 µmol of the reducing sugars (glucose and fructose) from sucrose per min under the standard assay conditions.

*Standard deviation was calculated from triplicate samples.

**Results and Discussion**

**Production and preparation of the levansucrase enzymatic extracts**

*B. amyloliquefaciens* was cultivated on the succinate- and mineral-based media in the presence and the absence of sucrose as an LS inducer. No significant LS production was achieved in the absence of sucrose (data not shown). Contrary to *B. subtilis*, *B. amyloliquefaciens* produces more inducible LS than the constitutive type. Supplementation of the selected media with yeast or peptone resulted in a significant increase in LS production; however, the use of yeast led to a higher ratio of total LS activity to bacterial growth (Ab600) (Table 1). Euzanat et al. have similarly reported that the use of yeast as a nitrogen source produced 2-fold more LS by *B. subtilis* than the use of peptone. The results also indicate that *B. amyloliquefaciens* cells expressed LS activity in both the extra- and intracellular forms. In comparison with the succinate-containing medium, the mineral-containing medium favored the secretion of *B. amyloliquefaciens*. The secretion process for constitutive membrane-bound LS remains unclear, although some hypotheses have been put forward.

The time-course characteristics for *B. amyloliquefaciens* growth and LS production were investigated by using succinate- and mineral-based media supplemented with sucrose as an LS inducer and yeast as a nitrogen source. Absorbance at 600 nm, Intra- (■) and Extracellular (□) LS Activity.

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*Substrate specificity.* Selected fructosyl-donors (sucrose and raffinose) and saccharide acceptors (galactose, glucose, lactose, maltose and raffinose) were used to investigate the acceptor/donor specificity of *B. amyloliquefaciens* LS. Enzymatic reactions consisted of 0.9 M of sucrose and a saccharide acceptor and were initiated by adding 5–7 LS units per mL of the reaction mixture. The reaction of each mixture was run under the optimal reaction conditions. Aliquots of 30 µL were withdrawn at different times and analyzed by thin-layer chromatography (TLC) which was performed on Silicagel 60 plates according to the modified method of Park et al. Aliquots of 5 µL of each reaction mixture were spotted on the Silicagel 60 plate, the developing solvent consisting of a mixture of butanol/acetic acid/deionized water (5:4:1, v/v/v). The fructose-containing compounds were detected by first spraying the TLC plate with a resorcinol solution prepared in acetic acid (0.1% w/v resorcinol and 0.25% w/v thiourea acid). After drying, the resulting plate was sprayed with a sulfuric acid solution prepared in methanol (2%, v/v), before heating at 100°C for 2 h. The fructose-containing spots were detected under visible light, and the TLC plates were scanned by using the Bio-Rad ChemiDoc XRS imaging system operated by Image Lab software. The migration distance of selected fructose-containing glycosides was calculated by using the Image Lab software.
Membrane-bound LS (intracellular) was extracted by suspending the sonicated cell debris fraction in a potassium phosphate buffer (50 mM at pH 6.0) containing non-ionic Triton X-100 detergent at different concentrations (0–3% v/v) before sonication for the recovery of endocellular LS. The presence of Triton X-100 was added to the suspended cells at different concentrations (%, v/v) before sonication for the recovery of the endocellular LS. Triton X-100 was added to the suspended cells at different concentrations (%, v/v) before sonication for the recovery of the endocellular LS.

The effects of PEGs with selected molecular weights (200, 350, 400, 2000 and 4000 Da) at different concentrations (10, 20, 30 and 40%, w/v or v/v) on the fractionation yield of the intra- and the extracellular LS activities are shown in Fig. 2. The overall findings show that the intra- and extracellular LS activities responded differently to the variations in molecular weight and concentration of PEG. However, the highest fractionation yield of both activities of 73% was achieved by using the lowest molecular weight of PEG-200 at a concentration of 30% (v/v). Increasing the concentration of PEG-200 to 40% resulted in a significant decrease in the respective fractionated intra- and extracellular LS activity yields to 55% and 35%. As the molecular weight of PEG was increased, the maximum fractionation yield of both LS activities likewise decreased. The results (Fig. 2) also show that varying the concentrations of PEG-350 and PEG-400 of low molecular weights resulted in similar extracellular LS activity profiles to that obtained with PEG-200; however, the maximum fractionation yield of extracellular LS activity at a concentration of 30% (v/v) was higher with PEG-350 (56%) than that with PEG-400 (26%). In contrast, the profiles of the variation in intracellular LS activity yield with increasing concentrations of PEG-350 and PEG-400 were different from that obtained with PEG-200; indeed, the maximum fractionation yields of the intracellular LS activity of 32% and 33% were respectively achieved at a lower concentration of 20% (v/v) PEG-350 and 10% (v/v) PEG-400. PEG-2000 of higher molecular weight led to low fractionation yields (<27%) for both the intra- and extracellular LS activities at all the investigated concentrations. A higher intracellular LS activity yield of

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**Table 2. Recovery and Preparation of the Crude Enzymatic Extracts of Intra- and Extracellular Levansucrase (LS) from *B. amyloliquefaciens***

<table>
<thead>
<tr>
<th>Method</th>
<th>LS Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Residual Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular LS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triton®</td>
<td>0.22(±0.01)</td>
<td>0.72(±0.02)</td>
<td>46.1(±1.5)</td>
<td>40.4</td>
</tr>
<tr>
<td></td>
<td>0.47(±0.12)</td>
<td>1.57(±0.41)</td>
<td>100.0(±2.2)</td>
<td>72.2</td>
</tr>
<tr>
<td></td>
<td>0.42(±0.03)</td>
<td>1.39(±0.12)</td>
<td>88.7(±7.6)</td>
<td>80.4</td>
</tr>
<tr>
<td></td>
<td>0.45(±0.09)</td>
<td>1.52(±0.32)</td>
<td>96.6(±3.5)</td>
<td>74.4</td>
</tr>
<tr>
<td>Extracellular LS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.70(±0.03)</td>
<td>37.83(±1.74)</td>
<td>100.0(±4.6)</td>
<td></td>
</tr>
<tr>
<td>Ultracentrifugation®</td>
<td>1.31(±0.07)</td>
<td>56.82(±3.02)</td>
<td>61.1(±3.3)</td>
<td></td>
</tr>
<tr>
<td>Freeze-drying®</td>
<td>154.40(±8.33)</td>
<td>52.06(±0.45)</td>
<td>38.0(±2.0)</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 2. Effect of Polyethylene Glycol (PEG) of Different Molecular Weights on the Fractionation of Levansucrase (LS) Activity in the Extra- (A) and Intra-Cellular (B) Forms: PEG-200 (△), PEG-350 (□), PEG-400 (△), PEG-2000 (■), PEG-4000 (×).**
Properties of Levansucrases from *Bacillus amyloliquefaciens* 1933

### Table 3. Effect of Polyethylene Glycol (PEG) on the Fractionation Yield and the Purification Factor of Intra- and Extracellular Levansucrases (LSs) from *B. amyloliquefaciens*

<table>
<thead>
<tr>
<th>Concentration*</th>
<th>Specific activity*</th>
<th>Purification factor*</th>
<th>Yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>30%</td>
<td>30%</td>
<td>40.7(±5.8)</td>
</tr>
<tr>
<td>350</td>
<td>20%</td>
<td>30%</td>
<td>31.2(±0.9)</td>
</tr>
<tr>
<td>400</td>
<td>10%</td>
<td>30%</td>
<td>105.9(±22.7)</td>
</tr>
<tr>
<td>2000</td>
<td>10%</td>
<td>40%</td>
<td>46.0(±15.2)</td>
</tr>
<tr>
<td>4000</td>
<td>40%</td>
<td>30%</td>
<td>37.0(±0.5)</td>
</tr>
</tbody>
</table>

*Concentration of PEGs is expressed in v/v or w/v.

Specific activity is expressed as the umole of reducing sugars released per min of reaction per mg of proteins.

Purification factor was calculated as the specific activity of the pre-purified enzymatic extract over that of the initial extract, multiplied by 100.

Yield was calculated as the total recovered enzymatic units over the initial enzymatic units, multiplied by 100.

53% was achieved with PEG-4000 at a concentration of 40% (v/v) than that of 34% obtained for the extracellular LS extract at a concentration of 30%.

The fractionation ability and efficiency of PEG have been primarily attributed to the excluded volume mechanism, whereby proteins can be selectively precipitated according to their molecular weight, intrinsic solubility and preferential binding parameters. The fact that lower molecular weight PEG favored the effective fractionation of LSs from *B. amyloliquefaciens* may suggest that LSs neither exist in the aggregated form in a solution nor in the associated form with a levan polymer. Indeed, PEGs of high molecular weight have been used for purifying biocatalysts associated with the polymer. Moreover, the differences between the intra- and extracellular LSs in response to variations in the molecular weight and concentration of PEG may reveal some differences in their form, structural conformation and steric hindrance. To assess the selectivity of PEG to fractionate LSs from *B. amyloliquefaciens*, the specific activity of the pre-purified LS extracts and their purification factor were determined by using the optimal PEG concentrations (Fig. 2). The overall results (Table 3) indicate that all investigated PEGs selectively fractionated *B. amyloliquefaciens* intracellular LS, resulting in a purification factor varying from 14.9 to 38.8, while only a very low purification factor of 0.6 to 1.8 was obtained upon the fractionation of extracellular LS by the selected PEGs. These results may have been due to differences in the extract composition and/or in conformational changes of LSs in the intra- and extracellular forms which could have occurred during the protein secretion and purification. Although the use of PEG-400 resulted in a lower intracellular LS activity yield, it led to the highest purification factor of 38.8. These results (Table 3) suggest that intracellular LS exhibited higher interaction affinity toward PEG-400 than to the other contaminating proteins in the enzymatic extract. However, the extent of purification of the intracellular LS activity by the other investigated PEGs of high and low molecular weights was comparable with a purification factor in the range of 11.6 to 17.2. Contrary to the intracellular LS activity, the extracellular LS activity was not selectively fractionated by the non-ionic hydrophilic polymer PEGs, although a significant decrease in the extracellular LS specific activity of 33% and 40% was respectively apparent upon fractionation by PEG-400 and PEG-2000. The direct effect of PEGs on the enzyme itself, by binding either in or near its active site, may explain this decrease in the extracellular LS activity. The literature indicates that, in addition to their fractionation ability, the non-ionic PEGs may also have an activating or deactivating effect as well as a stabilizing effect on the catalytic activity. Timasheff has reported that the presence of PEGs may favor the stability of native proteins through preferential hydration and/or steric exclusion. Shulgin and Ruckensteim have also provided evidence for the preferential steric exclusion mechanism of PEGs by applying the Kirkwood–Buff theory. As far as the authors are aware, no study has been reported on optimizing the purification of an LS biocatalyst by fractionating with PEGs. One study did however use PEG-1500 for purifying LS from *Bacillus circulans* and reported an eight-fold purification factor with an apparent yield of the recovered activity of 150%. The PEG-purified LSs obtained upon fractionation with PEG-200 were analyzed by SDS–PAGE electrophoresis to assess their purity. The results confirm the higher degree of purification of intracellular LS than extracellular LS from *B. amyloliquefaciens* by the non-ionic hydrophilic polymer PEG of low molecular weight. However, both the PEG-purified intra- and extra-cellular LS fractions showed an abundant single band corresponding to a 54 kDa molecular mass by SDS–PAGE (data not shown). A molecular mass of 49–52 kDa has been similarly reported for LS from *B. amyloliquefaciens*. The molecular weight of *B. amyloliquefaciens* LS falls within the range reported for other LSs from *Bacillus* species of 52–55 kDa and for LSs from gram-negative bacteria, including *Z. mobilis* and *G. diazotrophicus* varying from 48 to 58 kDa.

**Effect of sucrose concentration on the levan production**

Although LSs have been reported to essentially behave as transferases, they possess hydrolytic activity that is regarded as transfer of the fructosyl group to water. The levan-forming activity of intra- and extra-cellular LSs from *B. amyloliquefaciens*, and the conversion yield of sucrose to levan and fructose were determined at different sucrose concentrations ranging from 15 mM to 1.3 M to assess the extent of the transfructosylation and hydrolysis reaction. Fructose can only result from sucrose hydrolysis, whereas levan formation occurs from the transfructosylation reaction with the release of free glucose. Since the degree of turbidity produced was proportional to the rate of
glucose accumulation, measuring the turbidity reflected the true levan formation. The results (Fig. 3A) show respective increases in the levan-forming activity of intra- and extra-cellular LSs to 135.9 and 99.0 nmol of produced levan/min·mg of protein, when the initial sucrose concentration was increased to 0.2 M. A further increase in the sucrose concentration to 1.2–1.3 M resulted in a subsequent respective decreases in the levan-forming activity of the intra- and extracellular LSs to 52.1 and 45.4 nmol of produced levan/min·mg of protein. These decreases may be attributable to classical substrate inhibition and/or to unfavorable thermodynamic reaction equilibrium. Oseguer "et al." have similarly reported a decrease in the levan-forming activity of LSs from both Pseudomonas syringae and B. circulans by excess substrate inhibition.

Figure 3B indicates that the transfructosylation reaction (reflected by the levan formation) was favored even at very low sucrose concentrations, and that the hydrolysis reaction (reflected by the fructose accumulation) was not significant over the investigated range of sucrose concentration. However, the yield of levan produced by the LS-catalyzed transfructosylation reaction decreased inversely with the sucrose concentration (Fig. 3B). These results can be attributed to LS inhibition by an excess of the substrate, accumulation of the glucose product and/or to oligosaccharide formation at a high concentrations of sucrose, as reported by Belghith et al.

The first part of the levan-forming activity versus sucrose concentration curves did not follow the Michaelis-Menten model. However, the inset to Fig. 3A indicates that the levan-forming reaction catalyzed by LSs fitted well to the Hill model \((V = V_m[S]^n / (K_m + [S]^n); R^2 of 98.4–99.5)\) with Hill coefficient \(n_H\) of 2.0. These results suggest that B. amyloliquefaciens LSs may have exhibited cooperative binding of the substrate in forming levan. Anderson et al. have similarly reported that LS from Z. mobilis catalyzing the polymerization of the fructose moiety from raffinose obeyed the Hill type of kinetics with an \(n_H\) value of 1.61. Effect of pH on the levansucrase activity

The effect of pH on the B. amyloliquefaciens intra- and extra-cellular LS activities was investigated (data not shown). These LS activities were displayed over a broad pH range (4.0–9.0), but remained highly active in the neutral region. Both intra- and extra-cellular LSs exhibited their maximum activity in the pH range of 6.0–6.5. However, in the pH range of 5.0–7.5, intra-cellular LS retained more than 62% of its maximum activity, whereas extracellular LS maintained more than 75% of its maximum activity. The extracellular LS activity remained higher than the intracellular LS activity at higher pH values of 8.0 and 9.0, while similar LS activity was obtained for the intra- and extracellular LSs at the acidic pH value of 4.0. The overall pH activity profiles of intra- and extra-cellular LSs showed only a slight difference. Mantsala and Puntala have similarly reported an optimum pH of 6.0–6.2 for extracellular LS from B. amyloliquefaciens. The pH activity profile of B. amyloliquefaciens LSs also showed features common to those of other LSs from bacillus species with an optimum pH in the range of 6.0–6.6, with the exception of LS from B. circulans which exhibited optimum activity in the pH range of 5.7–6.3. The pH optima reported for LSs from such gram-negative bacteria as G. diazotrophicus and Rahnella aquatilis were a little more towards the acidic region (pH 5.0–6.0).

Effect of temperature on the levansucrase activity

The total, transfructosylation and hydrolytic activities of intra- and extra-cellular LSs were investigated over a wide reaction temperature range from 25 to 70 °C (Fig. 4). The results show different temperature profiles for the transfructosylation and hydrolytic activities of LSs. The respective transfructosylation activity of intra- and extra-cellular LSs had maximum values at 25–30 °C and 40 °C (Fig. 4), while the respective maximum hydrolytic activity of intra and extracellular LSs was at 45–50 °C and 50 °C. The results also show that the ascending part of the temperature-activity profiles for the two LS forms were not similar, suggesting that the limiting step for the catalysis by both LSs was different. The transfructosylation activity of intracellular LS decreased significantly at higher temperatures of 45 and 50 °C, at which the enzyme respectively showed only 16% and 11% of its maximum value (Fig. 4A), whereas the extracellular LS activity still maintained 59% and 43% of its maximum value at these temperatures (Fig. 4B). These results may suggest higher thermal stability for the transfructosylation activity of extracellular LS than that of intracellular LS. These differences between intra- and extra-cellular LSs reveal the occurrence of certain conformational changes to LS upon protein secretion and/or purification.
The results shown in Fig. 4 also indicate that the ratio of the transfructosylation to hydrolytic activities was dependent on the reaction temperature. Higher ratios for the intra- (5.0–11.5) and extra-cellular (2.0) LSs were obtained in a lower temperature range of 15–30°C. The transfructosylation activity has been similarly reported to be predominant at lower temperatures for LSs from *Pseudomonas syringae* pv. (18°C), *Z. mobilis* (15°C), and *B. amyloliquefaciens* (4°C).14) The optimum temperature for *B. amyloliquefaciens* LSs of 30–40°C is similar to that reported for LSs from *M. laevaniformans*18) and *B. amyloliquefaciens* type 1.13) However, higher optimum temperature values within the range of 45–50°C have been reported for LSs from *B. subtilis*,36) *B. megaterium*,9) *Z. mobilis*,26) and *L. reuteri*,37) and a higher optimum temperature of 60°C has been reported for LSs from *Bacillus* sp.38) and *R. aquatilis*.39)

Kinetic parameters
Despite the complexity of LS simultaneously catalyzing several reactions (hydrolysis, transfructosylation, and polymerization), it was still considered worthwhile to determine and analyze the kinetic parameters. The effects of sucrose concentration on the total, transfructosylation and hydrolytic activities of intra- and extra-cellular LSs were investigated over a substrate concentration range of 2.5–1.2M under standard optimal conditions. The results shown in Fig. 5 reveal that the kinetics of *B. amyloliquefaciens* LSs followed the Michaelis-Menten model, as indicated by the linearity of the corresponding Lineweaver-Burk plots (data not shown). Figure 5 also shows that the transfructosylation and hydrolytic activities of intra and extracellular LSs were not significantly inhibited at high substrate concentrations (1.0–1.2 M). Table 4 indicates that the catalytic efficiency for the transfructosylation activity of intracellular LS (1.1 × 10⁻³ L/min·mg of protein) was higher than that of extracellular LS (8.5 × 10⁻⁵ L/min·mg of protein). These differences between intra- and extracellular LSs reveal the occurrence of certain conformational changes to LS upon protein secretion and/or purification. However, the hydrolytic activity of LSs in both the intra- and extra-cellular forms showed close catalytic efficiency (2.5–1.5 × 10⁻³ L/min·mg of protein). The *K*ₘₐₚₚ values for *B. amyloliquefaciens* LSs (Table 4) are higher than those reported for LSs from *Acetobacter diazotrophicus* (11.8 mM),10) *L. reuteri* (9.7 mM),10) and *B. megaterium* (6.6 mM),39) although lower *K*ₘₐₚₚ values have also been obtained for LSs from *B. amyloliquefaciens* (19.0 mM)13) and from *B. subtilis* (13.5–40 mM).39)

Product spectrum for *B. amyloliquefaciens* LSs
The product spectrum for the *B. amyloliquefaciens* LS-catalyzed reaction was investigated by using sucrose as the sole substrate. The reaction conditions used for determining the product spectrum favored the trans-
fructosylation (77%) reaction over hydrolysis (23%). The levan produced by LSs from *B. amyloliquefaciens* was identified by a TLC analysis using fructose-specific detection. The levan was purified and analyzed by NMR for structural characterization. The $^1$H-NMR spectrum (Fig. 6A) for levan produced from *B. amyloliquefaciens* is similar to that for levan produced by *Acetobacter xylinum* NCI 1005. $^{13}$C-NMR spectrum (Fig. 6B) shows six broad resonance signals at 104.1 (C2), 80.2 (C5), 76.2 (C3), 75.13 (C4), 63.2 (C6) and 59.8 (C1) ppm corresponding to the peak position for $\beta$(2→6)-levan. These chemical shifts are similar to those for levan synthesized corresponding to the peak position for $\beta$(2→6)-levan. These chemical shifts are similar to those for levan synthesized by *B. subtilis* (natto)$^{11}$ and *B. amyloliquefaciens*. $^{12}$ The detection of $\beta$(2→6)-glycosidic linkages confirms the formation of the polysaccharide levan. The HPAEC chromatogram (Fig. 7) shows that *B. amyloliquefaciens* LS synthesized FOSs as well as levan. 1-kestose (peak 2, $\alpha\beta$-fructofuranosyl-(2→1)-$\alpha\beta$-fructofuranosyl-(2→1)-$\alpha\beta$-fructofuranosyl-(2→1)[-D-glucopyranoside], 6-kestose (peak 5, $\alpha\beta$-fructofuranosyl-(2→6)-$\alpha\beta$-fructofuranosyl-(2→1)-$\alpha\beta$-fructofuranosyl-(2→1)-$\alpha\beta$-fructofuranosyl-(2→1)[-D-glucopyranoside] and nystose (peak 6, $\alpha\beta$-fructofuranosyl-(2→1)-$\alpha\beta$-fructofuranosyl-(2→1)-$\alpha\beta$-fructofuranosyl-(2→1)-$\alpha\beta$-fructofuranosyl-(2→1)[-D-glucopyranoside]) were identified as the main end-products; these being known acceptors for the transfer of fructosyl units. $^{13}$ Other minor unknown products as well as a wide range of higher-molecular-weight oligofructoses (peak 7) were produced in addition to those three abundant FOS products.

### Table 4. Kinetics Parameters of Intra- and Extra-Cellular Levansucrases (LSs) from *B. amyloliquefaciens* Determined by Using Sucrose as a Substrate

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Kinetic parameters</th>
<th>Catalytic efficiency $^c$</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular LS</td>
<td>$K_{\text{napp}}$</td>
<td>$V_{\text{napp}}$</td>
<td>$V_{\text{napp}}/K_{\text{napp}}$</td>
</tr>
<tr>
<td>Hydrolytic activity</td>
<td>11.7($\pm$0.9)</td>
<td>31.5($\pm$2.2)</td>
<td>2.5 × 10$^{-3}$</td>
</tr>
<tr>
<td>Transfructosylation activity</td>
<td>322.5($\pm$12.1)</td>
<td>357.1($\pm$25.8)</td>
<td>1.1 × 10$^{-3}$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Extracellular LS</th>
<th>Hydrolytic activity</th>
<th>Transfructosylation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3($\pm$3.1)</td>
<td>11.3($\pm$1.2)</td>
<td>1.5 × 10$^{-3}$</td>
</tr>
<tr>
<td>1556.4($\pm$90.1)</td>
<td>133.3($\pm$8.2)</td>
<td>8.5 × 10$^{-5}$</td>
</tr>
</tbody>
</table>

$^a$ $K_{\text{napp}}$ is expressed in mm.

$^b$ $V_{\text{napp}}$ is expressed as mol of reducing sugars released per min of reaction per mg of proteins.

$^c$ Catalytic efficiency is expressed in L per min per mg of protein.

**Fig. 7.** Transfructosylation End-Products of the *B. amyloliquefaciens* Levansucrase (LS)-Catalyzed Reaction.

To investigate the donor/acceptor specificity of *B. amyloliquefaciens* LSs, transfructosylation reactions were conducted by using sucrose and raffinose as fructosyl donors and different saccharides (galactose, glucose, maltose and lactose) as acceptors. A TLC analysis of the product spectrum at different reaction times was carried out (Fig. 8). Table 5 gives the migration distances and RG values for separation of the fructose-containing glycosides; the results show that FOSs were well separated based on their molecular weights. The specificity for the intra- and extra-cellular LS activities from *B. amyloliquefaciens* towards the different acceptors was similar (data not shown). Figure 8 shows that, *B. amyloliquefaciens* LSs synthesized mainly levan at a very early stage of the reaction when using sucrose and raffinose as fructosyl donors; however, bands corresponding to FOSs were detected after 36 and 63 h of the reaction. *B. amyloliquefaciens* LSs also demonstrated high specificity toward the investigated saccharide acceptors (mono-, di- and tri-saccharides; furanose and pyranose rings). In contrast, LSs from *R. aquatilis* $^{18}$ and *M. laevisiformans* $^{18}$ have shown low or no acceptor specificity toward such saccharides as raffinose and xylose having a furanose ring. The results shown in Fig. 8 also indicate that the maltose and lactose disaccharides were more effective...
acceptors of the fructofuranosyl residue from sucrose than the galactose and glucose monosaccharides. Interestingly, the production of levan was also apparent, in addition to the formation of hetero-FOSs, by *B. amyloliquefaciens* LSs with lactose as an acceptor. The formation of the erlose trisaccharide by *B. amyloliquefaciens* LSs was detected at a very early stage of the reaction with maltose as the fructosyl acceptor, while no significant levan formation was apparent, indicating the inhibitory effect of maltose, as reported by Canedo et al. \[42\]

**Conclusion**

Fractionation by low-molecular-weight PEGs appeared to be an effective separation method for purifying intracellular LS; however, extracellular LS could not be purified by PEG fractionation. Both intra- and extracellular LSs showed slight differences in their catalytic properties, indicating the occurrence of certain conformational changes in LS upon protein secretion and/or purification. This investigation of the properties of *B. amyloliquefaciens* LSs reveals the potential use of these biocatalysts as tools for the synthesis of levan and FOSs. Further studies to structurally characterize fructosyl homo- and hetero-FOSs produced by *B. amyloli-

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**References**