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Treatment with Glucanhydrolase from *Lipomyces starkeyi* for Removal of Soluble Polysaccharides in Sugar Processing

LEE, JIN-HA[1], GHAHYUN KIM[2], SEUNG-HEUK KIM[3], DONG LYUN CHO[1,4], DO-WON KIM[5], DONAL F. DAY[6], AND DOMAN KIM[1,7]*

[1]*Engineering Research Institute, Chonnam National University, Gwang-Ju 500-757, Korea*
[2]*Korean Minjok Leadership Academy, Gwangwondo 225-823, Korea*
[3]*Lifenza Co., Ltd., Seoul 135-514, Korea*
[4]*Faculty of Applied Chemical Engineering, Chonnam National University, Gwang-Ju 500-757, Korea*
[5]*Department of Physics, Kangnung National University, Kangnung 210-702, Korea*
[6]*Audubon Sugar Institute, Louisiana State University Agricultural Center, St. Gabriel, LA, U.S.A.*
[7]*School of Biological Sciences and Technology and Research Institute for Catalysis and Institute of Bioindustrial Technology, Chonnam National University, Gwang-Ju 500-757, Korea*

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Abstract The sole use of the glucanhydrolase (exhibiting both dextranase and amylase activities) from *Lipomyces starkeyi* hydrolyzed the soluble polysaccharides in sugar syrup more efficiently than a mixed treatment using both commercial dextranase and amylase. The glucanhydrolase treatment of stale sugar cane juice resulted in a yield of square, light-colored sugar crystals.

Key words: *Lipomyces starkeyi*, dextranase, amylase, glucanhydrolase, dextran, sugar crystal

Dextrans are high-molecular-weight poly glucans, characterized by an α-1→6 linked main chain, and variable numbers of α-1→2, α-1→3, or α-1→4 linked branch chains. These compounds can be enzymatically synthesized from sucrose by the dextransucrases, glucansucrases, or glucosyltransferases produced by *Leuconostoc* or *Streptococci* species [1–6]. Microbial action on sugar juices has been shown to result in dextran contamination within sugar processing streams.

The first processing step in the production of raw cane sugar is the shredding of the sugar cane, usually by knives and/or shredders [7], followed by milling to extract the juice. To purify the extracted sugar juice, milk of lime is added to produce a neutral pH, followed by flash heating to denature the proteins, fats, waxes, and gums. Soluble phosphates and dicalcium phosphate or polymer flocculants are also often employed to clarify the juice, which is then concentrated via vacuum evaporation. The resulting clarified juice contains approximately 85% water, and is then concentrated to 65% solids and 35% water syrup. Crystallization is the final stage of the process.

A variety of soluble polysaccharides are contained in sugar juice, including pectin and starch, both of which are products of the metabolic activities of the growing plant, and dextran and levan (β-2→6-linked polyfructosan), which are formed as the result of the activities of microorganisms growing in the plant sap, either during its life, or at some subsequent processing stage [8]. Delays between harvest and grinding, as well as damage to the sugarcane inflicted by freezing and subsequent shipping delays, allow more time for infection and deterioration of the cane. This can also result in a buildup of microorganisms, and an increase in dextran production [8]. As such, the level of dextran in the factory mixed juice is the sum of three sources: (1) the dextran in the cane at the time of harvesting, (2) the dextran formed between the time of harvesting and grinding, *i.e.*, during shipment and while waiting in the cane yard, and (3) the dextran formed during the milling process. The principal deleterious effects of soluble polysaccharides on sugar processing include (1) the production of an excessive juice viscosity, resulting in poor clarification and filtration, (2) a reduction in the rate of sucrose crystallization, (3) elongation of the *c* axis in the sucrose crystal, impacting the separation and purging by centrifugation, and (4) an overall reduction in the economic efficiency of the mill and subsequent refining [9–11].

Dextranase can be economically utilized to alleviate many of the production problems associated with dextran.

*Corresponding author
Phone: 82-62-530-1844; Fax: 82-62-530-0874;
E-mail: dmkim@chonnam.ac.kr

Dextranase is an enzyme that specifically degrades high-molecular-weight dextrans into smaller dextrans, thereby reducing the viscosity of juices, massecuites, and molasses [12, 13]. As such, the use of dextranase can prevent elongation of the grain, shorten the boiling time, and allow the product to flow through the boiling house more smoothly, owing to a reduction in viscosity. Of further concern, the presence of dextran at concentrations above 250 ppm results in deleterious effects with regard to sugar refining [14, 15]. Two kinds of dextranases are primarily used for dextran hydrolysis; Dextranex, which is generated by *Chaetomium* (Miles, Dextranex, fungal dextranex), and a NOVO enzyme (Novo, dextranase Novo 25L) that can be prepared from *Penicillium* [12, 13, 16]. At present, 90% of sugar mills worldwide use a combination of dextranase and amylase to treat soluble polysaccharide problems, including difficulties associated with dextran and starch. Meanwhile, the remaining 10% that do not use commercial dextranases are all located in the U.S.A. Since commercial dextranase-producing strains are all fungi and also known to produce toxic materials in addition to dextranase, the U.S. F.D.A. requirements forbid the use of the crude enzymes in food-related applications. Furthermore, these dextranases specifically hydrolyze the α-1,6-glucosyl linkage, resulting in the requirement of additional treatment with amylase [16].

Lipomyces starkeyi, an ascosporogenous yeast, produces an endo-dextranase (an enzyme that cleaves the α-1→6-D-glucopyranosyl linkages in dextran) and/or α-amylase [7–20]. This yeast has already been used in food-related applications, and is not known to produce antibiotics or toxic metabolites [21, 22]. With the exception of a few bacterial dextranases, microbial dextranases are generally inducible [19, 23]. Kim and Day [18, 24, 25] reported on the isolation of a derepressed and partial constitutive mutant for dextranase and amylase, designated as *L. starkeyi* ATCC 74054. They characterized the dextranase and amylase activities of this strain, and reported on its use for the production of small-size dextran using sucrose and/or starch. Subsequently, a new strain, a constitutive and glucanhydrolase (incorporating both dextranase and amylase activities) hyperproducing strain (*L. starkeyi* KSM 22), was developed, which exhibited both dextranase and amylase abilities [26, 27]. Furthermore the present authors demonstrated that the *L. starkeyi* glucanhydrolase included an enzyme exhibiting both dextranase and amylase activities in a single 100 kDa protein [25], where competition studies, using different amounts of dextran and starch as substrates, also yielded a competition plot consistent with the hypothesis that the hydrolysis of dextran and starch occurs at two independent active sites, specific for starch or dextran, respectively. Furthermore, the *L. starkeyi* glucanhydrolase also hydrolyzed levan (β-2→6-linked polyfructosan) and mutan (α-1→3-linked glucans), as well as dextran and starch. As such, the multifunctional carbohydrolase activities of this enzyme may be useful with regard to the removal of a variety of polysaccharides associated with the sugar process. In addition, the use of a single enzyme is clearly preferable to the use of two enzymes in most situations.

Accordingly, the current study analyzed the effects of the *L. starkeyi* glucanhydrolase with regard to the hydrolysis of dextran and starch in raw sugar syrup. As a result, the quality of the raw sugar was improved, plus many of the deleterious effects associated with the contamination of raw sugar by dextrans were prevented. Furthermore, the use of a single enzyme makes the entire process far more convenient.

The *L. starkeyi* KDM1, a carbohydrolase hyperproducing strain obtained from *L. starkeyi* ATCC 74054 after ethylmethane sulfonate mutagenesis [25], was maintained on a slant of an LW medium containing 1% (w/v) soluble starch and 0.05% (w/v) 2-deoxy-D-glucose. The LW medium consisted of 0.3% (w/v) yeast extract and 0.3% (w/v) KH_2PO_4, and its pH was adjusted to 4.5 using HCl [26]. The glucanhydrolase was produced in a 10-l fermentor (Han-Il Co., Korea) using 8.0 l of the LW medium, containing 1% (w/v) starch. The pH was maintained at 4.0 through the addition of 3.0 M NaOH. The aeration rate, temperature, and stirring rate were 3.0 vvm, 28°C, and 250 rpm, respectively. The inoculum was 1.5% (v/v) culture, grown for 48 h in the LW medium containing 1% (w/v) starch, as previously described. One unit of dextranase or amylase was defined as the amount of enzyme required to liberate 1 μmole of isomaltose or maltose equivalents in one minute at 37°C when using 2% (w/v) dextran (Sigma Chemical Co. D5376, U.S.A.) or soluble starch (Yakuri Pure Chemical Co., Ltd., Japan) as the enzyme substrate. The reducing value was determined using the copper-bicinchoninate method [27]. The culture supernatant was concentrated from 8 l to 500 ml using a 50 K cutoff microfiltration module (Pall, Japan). The final dextranase and amylase activities were measured to be 0.73 U/ml and 9.7 U/ml, respectively.

The optimum level of dextranase and amylase activities with the glucanhydrolase occurred at a pH of 5.0, whereas the optimum pH stability (the pH range at which 80% of the original activity could be maintained) for the amylase equivalent activity was pH 3.0–7.5, and pH 4.0–7.0 for the dextranase equivalent activity. The optimum temperature for the dextranase and amylase equivalent activities was 50°C and 60°C, respectively, and the temperature at which 80% of the original activity was retained was 50°C for the dextranase equivalent activity and 80°C for the amylase equivalent activity.

An attempt was also made to characterize the effects of the glucanhydrolase as regards the removal of soluble polysaccharides from the sugar syrup. The sugar syrup was generously supplied by the Midland Co. (Kansas City,

U.S.A.). The final polysaccharide concentration in the membrane-filtered and concentrated sugar syrup was 13.3%. To prepare a syrup polysaccharide without monosaccharide or sucrose contamination, 200 ml of the sugar syrup was treated with 500 ml of 100% ethyl alcohol to precipitate all the polysaccharides present in the sample. The precipitant was then redissolved with distilled water (5 ml) and reprecipitated using 20 ml of ethanol. This process was repeated three times, and finally the precipitate was dissolved in 1 ml of distilled water. To remove the dextran, the sample was treated with dextranase [commercial dextranase, Midland Co. (Kansas City, U.S.A.)], 5.8 U/(ml reaction digest), and 7.5 U/(ml reaction digest) of amylase applied to hydrolyze the starch [commercial amylase, Midland Co. (Kansas City, U.S.A.)]. For the combined enzyme treatment, 5.8 U of dextranase and 7.5 U of amylase/(ml reaction digest) were simultaneously applied to the same sample. Then, for the glucanhydrolase treatment, the sample was treated with the equivalent of 5.8 U of dextranase (and the equivalent of 7.5 U of amylase). The final pH of the enzyme reaction mixture was 5.0, and the mixture was incubated for 4 h at 37°C. After 48 h of hydrolysis reactions, the end products were identified using thin-layer chromatography, as previously described [29].

The remaining polysaccharide levels after treating the sugar juice with the commercial dextranase, commercial amylase, combined commercial dextranase and amylase, and glucanhydrolase were as follows: 116, 102, 66, and 30 μg/ml of the original polysaccharides, respectively (Fig. 1). The mixed activities of the glucanhydrolase proved

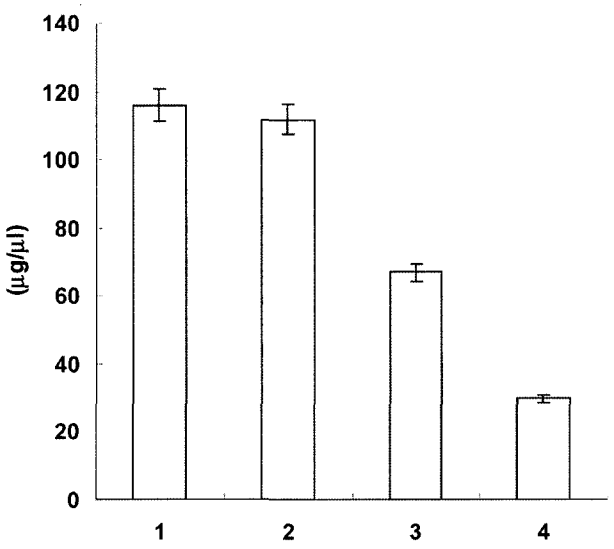

Fig. 1. Amount of remaining polysaccharides after enzyme treatment of sugar syrup polysaccharides.
1, Commercial dextranase treatment; 2, Commercial amylase treatment; 3, Combined dextranase and amylase (commercial) treatment; 4, *L. starkeyi* glucanhydrolase treatment.

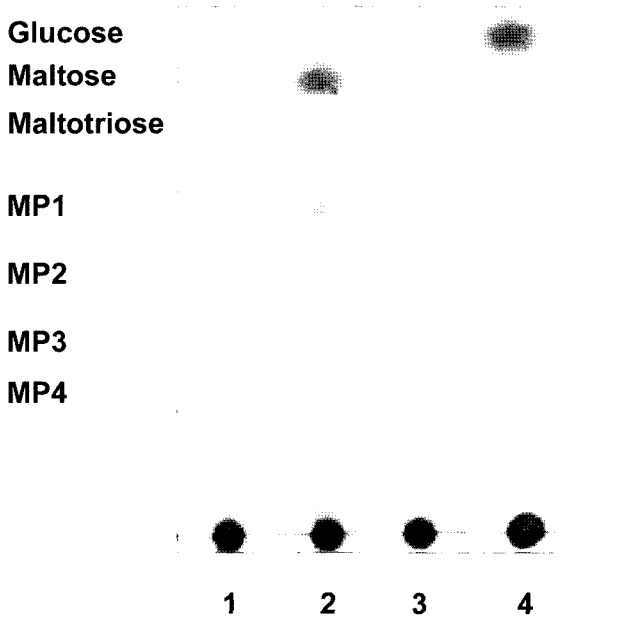

Fig. 2. Thin layer chromatogram of enzyme reaction digests of sugar syrup polysaccharides.
1, Commercial dextranase treatment; 2, Commercial amylase treatment; 3, Combined dextranase and amylase (commercial) treatment; 4, *L. starkeyi* glucanhydrolase treatment. MP1, panose; MP2, 6^2-α-isomaltosylmaltose; MP3, 6^2-α-isomaltotriosylmaltose; MP4, 6^2-α-isomaltotetraosylmaltose.

more efficient in the hydrolysis of the soluble polysaccharides than the other methods. The use of a single glucanhydrolase also rendered the process both more convenient and more economical. In addition, an analysis of the hydrolysis products generated by the commercial dextranase or amylase treatment, as well as the combined enzyme treatment, revealed that these hydrolyses predominantly generated oligosaccharides. In contrast, the glucanhydrolase treatment primarily generated glucose as a reaction product (Fig. 2) under the current reaction conditions. Both dextran and oligosaccharides are known to stimulate the elongation of sugar crystals [30]. Thus, the current commercial enzyme treatment is clearly a suboptimal solution to the dextran and starch problems, whereas the *L. starkeyi* glucanhydrolase produces monosaccharides, which may possibly reduce the degree to which dextran is formed in sugar. The sugar crystals acquired from the carbohydrolase-untreated stale sugar syrup were dark in color, and malformed. The glucanhydrolase-treated sugar crystals were square and light in color (Fig. 3).

With dextran concentrations of 250 ppm or higher, a factory grinding 6,000 tons of cane per day while using dextranase could potentially realize an additional sugar recovery of between $800–$2,500 because of the resulting alteration in the molasses purity. Thus, the cost of the dextranase would be more than justified by the increased sugar recovery. Other potential savings include an efficient production flow and energy savings, both of which can

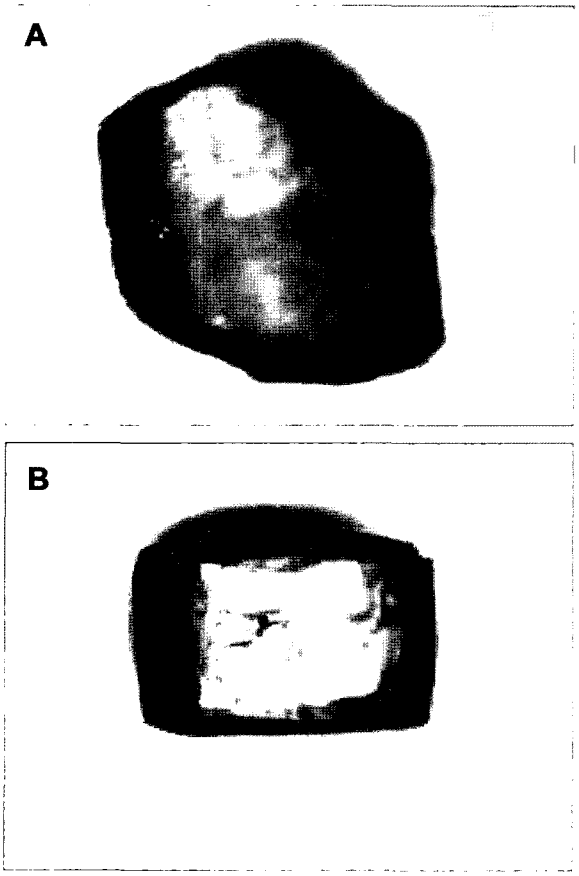

Fig. 3. Sugar crystals acquired from untreated sugar syrup (**A**) and *L. starkeyi* glucanhydrolase-treated sugar syrup (**B**).

significantly add to the overall savings. Of additional importance is the fact that the raw sugar producer can avoid the problems associated with excessive levels of dextran.

The optimum temperature for the dextranase activity of the glucanhydrolase was found to be 10°C lower than the optimal temperatures reported for the commercial Miles Dextranex and Novo DN 25L enzymes. Although this low optimum temperature associated with the *Lipomyces* glucanhydrolase is not a problem in the treatment of cane juice prior to clarification, where the juice rarely exceeds a temperature of 40°C, it could present a problem with regard to the treatment of the syrup, where the temperature usually exceeds 70°C. Therefore, the development of a glucanhydrolase with an increased thermostability and a pilot scale experiment using glucanhydrolase treatment at a sugar mill are both currently underway.

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