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Study the influence of temperature on activity of fructosyltransferases by strain *Leuconostoc mesenteroides* Lm 17

ABSTRACT

In the present work we studied the enzyme reaction of extracellular and cell-associated fructosyltransferase from *Leuconostoc mesenteroides* Lm 17 at different temperatures in order to estimate their influence on the enzyme activity. We measured the enzyme activity at a temperature range from 30°C to 45°C in the presence of 10% raffinose as a specific substrate for fructosyltransferases (FTFs). The highest extracellular and cell-associated FTF activities were detected at different temperatures – 0,29 U/mg at 30°C and 0.72 U/mg at 40°C, respectively. We did not detect extracellular FTF activity at 35°C. At 45°C a strain Lm 17 retained only 10% of the maximum enzyme activity detected at 40°C. We found that the studied strain produces mainly cell-associated FTFs which were 71% of the total FTF activity and are more stable at higher temperature. The received results were compared to detected extracellular and cell-associated FTF activities for the referent strain NRRL B-1149.

In order to compare the type of produced FTFs from Lm 17 with referent strains *Leuconostoc mesenteroides* NRRL B-1149, NRRL B-512F and ATCC 8293, which are known as FTF producers, we performed *in situ* analysis after SDS-PAGE of extracellular and cell-associated enzyme fractions. The *in situ* analysis after Periodic Acid - Schiff's staining procedure showed that all strains produce comparable FTFs with molecular weights corresponding to 120 kDa and 86 kDa.

Key words: fructosyltransferases, *Leuconostoc mesenteroides*, raffinose

Introduction

Fructosyltransferases (FTFs) are enzymes belonging to Glycoside Hydrolases family 68 (GH68) (Pons et al., 2000). FTFs catalyze the transfer of fructosyl unit of sucrose to a growing fructan polymer chain (transferase activity) or to water (hydrolytic activity). According to the chemical nature of the linkages between fructosyl units in the synthesized products these enzymes are divided to levansucrases (EC 2.4.1.10) synthesizing levan with β-(2,6) linkages and inulosucrases (EC 2.4.1.9) synthesizing inulin with β-(2,1) linkages in the main chain. The degree of branching depends on the nature of the enzyme (Moral et al., 2008). FTFs produced by Gram-positive bacteria *Bacillus*, *Streptococcus*, *Lactobacillus* and *Leuconostoc spp.* have been studied extensively. Different strains from genera *Streptococcus*, *Lactobacillus* and *Leuconostoc spp.* produce different glycosyltransferases in a complex – fructosyltransferases and glucosyltransferases (GTFs) (Morales-Arrieta et al., 2006). There are reports describing levansucrase activity with low expression levels as a minor contaminant of dextransucrase preparations (Robyt & Walseth, 1979; Miller & Robyt, 1986; Smith & Zahnley, 1999). Robyt & Walseth, 1979 reported the presence of levansucrase in dextransucrase preparations, obtained from the industrial strain *Leuconostoc mesenteroides* NRRL B-512F. Morales-Arrieta et al., 2006 identified and characterized *levS* gene encoding levansucrase from *L. mesenteroides* NRRL B-512F with a molecular weight of 113 kDa. It has been isolated two genes encoding two FTFs in *Leuconostoc mesenteroides* ATCC 8293 with molecular weights of 112 and 113 kDa (Olvera et al., 2007). Smith & Zahnley, 1999 reported the production of FTF by *Leuconostoc mesenteroides* NRRL B-1149 with an average molecular weight of 130 kDa. Cell-associated inulosucrase

RESEARCH ARTICLE

(IslA) with molecular weight of 165 kDa, produced by *Leuconostoc citreum* CW28 has been characterized as well (Moral et al., 2008). FTFs as GTFs from *Lactobacillus* and *Leuconostoc spp.* are implicated in the synthesis of oligosaccharides important for human health because of their prebiotic properties associated with antitumor, antibacterial and immunomodulating effects (Korakli et al., 2002; Iliev et al., 2006, Patel & Goyal, 2011). The isolation, molecular and biochemical characterization of FTFs will give information about the properties of these enzymes, related to their industrial application for the production of novel functional oligosaccharides and polymers.

The aim of the present work is to study the enzyme activity of crude extracellular and cell-associated FTFs by strain *Leuconostoc mesenteroides* Lm 17 at different temperatures.

Materials and Methods

Bacterial strains and culture media

Leuconostoc mesenteroides Lm17 was obtained from the bacterial culture collection of the Department of General and Industrial Microbiology, Sofia University. As referent strains were used *Leuconostoc mesenteroides* ATCC 8293, NRRL B-512F, NRRL B-1149. For the production of glycosyltransferases the strains were cultivated 6-8 h in culture media containing 4% (w/v) sucrose at 27°C on a rotary shaker (200 rpm) (Iliev et al., 2008).

Biomass measurements

Bacterial growth was measured by a turbidimetric method at 620 nm and calibrated against cell dry-weight measurements as previously described (Iliev et al., 2003).

Concentration of glycosyltransferases

The culture medium after sucrose cultivation was first centrifuged for 20 min at 7000g and 4°C for cell separation. The supernatant was then filtered with a Sartorius membrane (0.2 μm cutoff) to ensure the total absence of cells in the supernatant. The glycosyltransferases were separated from the supernatants and concentrated by using of PEG-1500 to final concentration of 20% (w/v) (Paul & Monsan, 1984). The glycosyltransferases were separated by centrifugation at 7000g for 20 min at 4°C, collected in the pellet, and diluted in 20 mM sodium acetate buffer, pH 5.4.

Fructosyltransferase assay

FTF activity was determined in 20 mM sodium acetate buffer, pH 5.4, with 100 g of raffinose per liter, 0.05 g of $CaCl_2$ per liter, and 1 g of NaN_3 per liter. The enzyme activity was assayed at 30°C, 35°C, 40°C and 45°C. The hydrolytic activity was determined by release fructose (μmol) per minute. Fructose concentration was determined with hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and phosphoglucose isomerase (EC 5.3.1.9) (commercial available kit, Cat. No. K-FRUGL, Megazyme International Ireland Limited).

Protein determination

Protein concentration was determined according to the procedure of Bradford (Bradford, 1976) using bovine serum albumin as a standard.

Electrophoresis analysis

SDS-PAGE (70x80 mm slab gels, 5% acrilamide) was conducted by the method of Laemmli (Laemmli, 1970). The proteins were stained with Coomassie Brilliant Blue R 250 (Sigma Chemical Co.). The glycosyltransferase activities were detected by incubating the gels in 10% sucrose, and for specific detection of FTFs the gels were incubated in 2% raffinose overnight, followed by staining of polysaccharides according to a Periodic acid - Schiff's procedure (Miller & Robyt, 1986). As a protein standards were used Precision Plus Protein^{tm} (Bio-Rad).

Results and Discussion

In our previous studies, we showed that a strain *Leuconostoc mesenteroides* Lm 17 produces two types of glycosyltransferases – GTF and FTF (Vasileva et al., 2009). In the present work, we studied the enzyme activity of extracellular and cell-associated FTF fractions from *L. mesenteroides* Lm 17 at different temperatures in order to estimate their influence on the enzyme activity. The enzyme activity was measured at 30°C, 35°C, 40°C and 45°C. As a specific substrate for FTFs we used raffinose at a concentration 10%. The obtained results were compared to these received for a referent strain NRRL B-1149 which is known producer of FTF (Smith & Zahnley, 1999). We detected an extracellular FTF activity for Lm 17 only at 30°C – 0.29 U/mg which is comparable to measured activity for a referent strain NRRL B-1149 at the same temperature – 0.34 U/mg (Figure 1, Figure 2). In contrast, cell-associated FTF activity for both strains was detected at all tested temperatures, with highest values at 40°C – 0.72 U/mg for Lm 17 and 0.94 U/mg for NRRL B-1149, respectively

RESEARCH ARTICLE

(Figure 1, Figure 2). At 30°C and 35°C the enzyme from Lm 17 showed 62.5% and 91.7% of the maximum activity, detected at 40°C. At 45°C the studied enzyme retained only 10.4% of the maximum activity. Significant decrease of enzyme activity also was detected at 45°C for the enzyme from NRRL B-1149 which retained only 7.9% of the maximum activity. The received results showed that Lm 17 produces mainly cell-associated FTFs which were 71.3% of the total FTF activity and were comparable to the ratio between extracellular and cell-associated FTF activity for the referent strain NRRL B-1149 (26.6:73.4%). The cell-associated FTFs from Lm 17 are more stable at temperatures over 30°C than extracellular ones.

It is known that the fructosyltransferases from different bacterial strains show a maximum enzyme activity at different temperatures (Ammar et al., 2002; Olivares-Illana et al., 2002; Ozimek et al., 2005; Tieking et al., 2005). Olivares-Illana et al. (2002) have studied the properties of extracellular and cell-associated inulosucrase (IslA) from *L. citreum* CW28. They have found that the optimum temperature for a cell-associated IslA (45°C) is higher than the optimum found for the soluble form (35°C). The enzyme activity in both extracellular and cell-associated fractions decrease rapidly at temperatures above 45°C (Olivares-Illana et al., 2002). Higher optimal temperatures also have been reported for FTFs from *Lactobacillus reuteri* (50°C) (Ozimek et al., 2005), *Lactobacillus sanfranciscensis* (35 – 45°C) (Tieking et al., 2005), and *Bacillus spp.* (60°C) (Ammar et al., 2002).

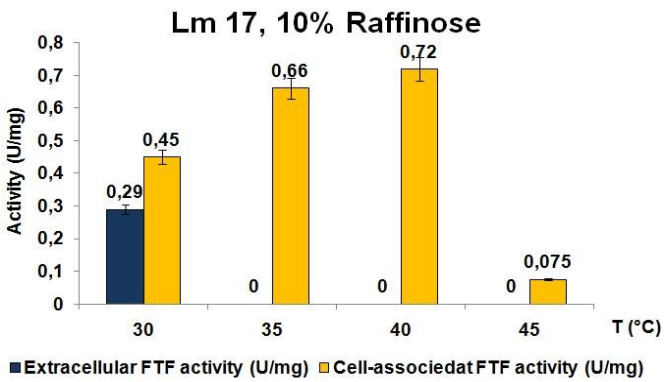

Figure 1. *Influence of temperature on extracellular and cell-associated FTFs activity produced by L. mesenteroides Lm 17.*

In order to compare the type of the produced FTFs from Lm 17 with the enzymes from referent strains *Leuconostoc mesenteroides* NRRL B-1149, NRRL B-512F and ATCC 8293, we performed *in situ* analysis after SDS-PAGE of extracellular and cell-associated enzyme fractions after cultivation in medium with sucrose. We used the referent strains as a control because they are known as FTF producers (Smith & Zahnley, 1999; Morales-Arrieta et al., 2006; Olvera et al., 2007). After SDS-PAGE, the gels were extensively washed to remove residual SDS and incubated in the presence of 10% sucrose, in order to observe the bands of activity as a result of polymer synthesis (Figure 3).

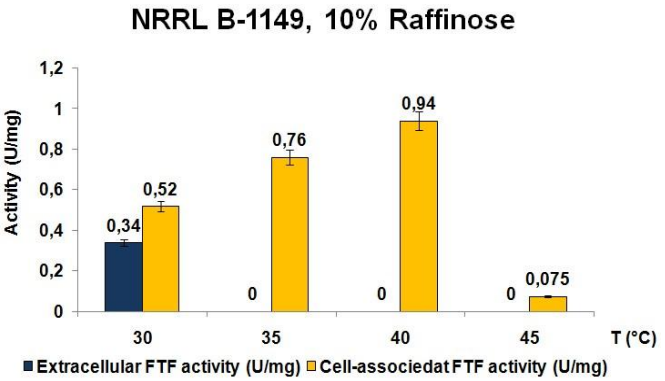

Figure 2. *Influence of temperature on extracellular and cell-associated FTFs activity produced by L. mesenteroides NRRL B-1149.*

The *in situ* analysis after Periodic Acid - Schiff's staining procedure showed that all strains produced several types of glycosyltranserases with molecular weights: 180 kDa, 120 kDa and 86 kDa, corresponding to dextransucrase (Robyt & Walseth, 1979; Smith & Zahnley, 1999) and fructosyltransferases, respectively (Morales-Arrieta et al., 2006; Olvera et al., 2007). In addition to the common activity bands, a strain *Leuconostoc mesenteroides* ATCC 8293 showed an additional extracellular and cell-associated activity band with molecular weight about 300 kDa, corresponding to GTF (Olvera et al., 2007; Bounaix et al., 2010).

We performed *in situ* analysis after SDS-PAGE of extracellular and cell-associated enzymes produced by all strains using 2% raffinose as a specific fructosyl residue donor for FTFs (Figure 4A and 4B). In this figure, two activity bands with molecular weights of 120 kDa and 86 kDa were observed after analysis of extracellular and cell-associated FTF enzymes produced by Lm 17. The same

RESEARCH ARTICLE

bands were also detected for all referent strains (Figure 4A and 4B). The presence of 180 kDa dextransucrase band was not detected for all the strains after raffinose incubation of the gels. The received results from the comparative *in situ* analysis showed that a strain Lm 17 produces FTFs comparable to the control strains used in this study, for which the FTF activity is reported before (Smith & Zahnley, 1999; Morales-Arrieta et al., 2006; Olvera et al., 2007; Bounaix et al., 2010).

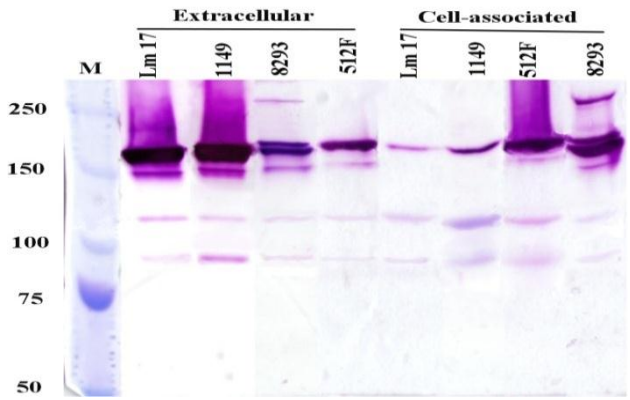

Figure 3. *In situ analysis of extracellular and cell-associated glycosyltransferases after incubation of the gel in 10% sucrose.*

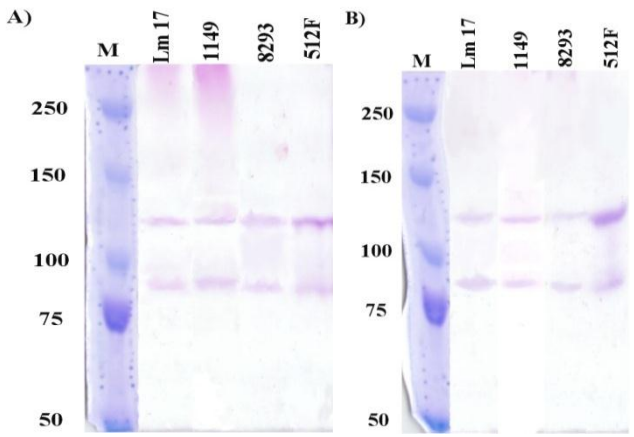

Figure 4. *In situ analysis of extracellular (A) and cell-associated (B) FTFs after incubation of the gels in 2% raffinose.*

Conclusion

We studied the influence of temperature on activity of extracellular and cell-associated FTFs produced by strain *Leuconostoc mesenteroides* Lm 17. Extracellular FTF activity was detected for Lm 17 only at 30°C – 0.29 U/mg. Cell-associated FTF activity was measured at all tested temperatures, with highest values at 40°C – 0.72 U/mg. At 30°C and 35°C the cell-associated enzyme from Lm 17 showed 62.5% and 91,7% of the maximum activity, detected at 40°C. Significant decrease of enzyme activity was detected at 45°C for cell-associated enzyme which retained only 10,4% of the maximum activity. The received results showed that Lm 17 produces mainly cell-associated FTFs which were 71.3% of the total FTF activity and are more stable at temperatures higher than 30°C. The comparative *in situ* analysis using 2% raffinose as a specific fructosyl residue donor showed that a strain Lm 17 produces the same type of extracellular and cell-associated FTFs with molecular weight of 120 kDa and 86 kDa. The obtained results give a good base for further studies of FTFs produced by strain Lm 17, including cloning of corresponding FTF gens and their recombinant expression, in order to characterize the biochemical properties of these enzymes.

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RESEARCH ARTICLE

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