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Physiological studies of *Leuconostoc mesenteroides* strain NRRL B-1149 during cultivation on glucose and fructose media

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ABSTRACT

Glycosyltransferases are extracellular and cell-associated sucrase enzymes produced mainly by lactic acid bacteria *Leuconostoc mesenteroides*, oral *Streptococcus* species and also *Lactobacillus* species. According to the synthesized polymer (glucan or fructan) in the presence of sucrose, these enzymes are divided into two groups: glucosyltransferases (GTFs) and fructosyltransferases (FTFs). Only *Streptococcus*, *Lactobacillus* and *Leuconostoc* strains are known as producers of both GTFs and FTFs. The enzymes from *Lactobacillus* and *Leuconostoc spp.* are implicated in the synthesis of polymers and oligosaccharides (OS) important for human health because of their prebiotic properties and immunomodulating activity. In the present work, we studied the production of extracellular and cell-associated glycosyltransferases by *Leuconostoc mesenteroides* strain NRRL B-1149 during its growth on media containing glucose or fructose as a main carbon source. The enzyme activities, pH and biomass formation were measured and compared during the cultivation. We have shown that glucose and fructose have not an equal role for enzyme production. The highest extracellular activity was detected at the 4th hour during the cultivation of the strain in medium with fructose – 5.45 U/mg. When the strain was cultivated in medium with glucose, the maximum of extracellular enzyme activity was detected at the 5th hour of the cultivation but the measured activity was about 9 times lower compared to these, obtained after cultivation in fructose medium. The studied strain produced mainly extracellular glycosyltransferases in glucose or fructose medium, which were 92.4% and 97.1% of the total enzyme activity, respectively. In order to characterize the produced enzymes, cell-associated and extracellular enzymes were determined using SDS-PAGE and *in situ* Periodic Acid Schiff's staining after incubation with 10% sucrose. When the investigated strain was grown in media with sucrose, glucose or fructose, several types of glycosyltransferases were detected – dextransucrase with molecular weight 180 kDa and two fructosyltransferases, corresponding to 120 kDa and 86 kDa molecular weights.

Key words: carbon source, glycosyltransferases, *Leuconostoc mesenteroides*

Introduction

Lactic acid bacteria belonging to the genera *Leuconostoc*, *Lactobacillus* and *Streptococcus* produce glucosyltransferases (GTFs) and fructosyltransferases (FTFs) (Mooser, 1992). GTFs are enzymes that synthesize glucans from sucrose by transferring glucosyl units to a nascent polymer chain. According to the chemical nature of the linkages between glucosyl units in the synthesized products, these enzymes are divided to dextransucrase (EC 2.4.1.5) synthesizing dextran with α-(1,6) linkages, mutansucrase (EC 2.4.1.5) synthesizing mutan with α-(1,3) linkages, reuteransucrase (EC 2.4.1.5) synthesizing reuteran with α-(1,4) linkages and alternansucrase (EC 2.4.1.140) synthesizing alternan with alternating α-(1,6) and α-(1,3) linkages in the main chain (Monsan et al., 2001). FTFs are

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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.

Leuconostoc mesenteroides NRRL B-1149 is known to produce branched glucan with 52% α-(1,6) and 40% α-(1,3) linkages, while dextran from *L. mesenteroides* NRRL B-512F contains about 95% of α-(1,6) and 5% α-(1,3) linkages (Jeanes et al., 1954). Low dextran solubility in water is normally associated with the presence of large numbers of α-(1,3) linkages (Jeanes et al., 1954). Recently, we reported an efficient method for purification of dextransucrase from *L. mesenteroides* NRRL B-1149 using polyethylene glycol (Shukla et al., 2010). *L. mesenteroides* NRRL B-1149 produced soluble glucan with linear structure and insoluble glucan with branched structure, which consist a high percent of α-(1,3) linkages, and additionally α-(1,2) and α-(1,4) linkages (Shukla et al., 2011).

It is known that the production of glycosyltransferases from *Streptococcus spp*. is constitutive, while the production of these enzymes from *Leuconostoc spp*. is inducible by sucrose used as a main carbon source in the medium (Robyt, 1996; Dols et al., 1998a). In the presence of sucrose, the produced glycosyltransferases are in complex with the synthesized polymers, which leads to increased viscosity of the culture medium and make difficult the enzyme purification (Majumder et al., 2007). There are reports for production of glycosyltransferases in media with a carbon source different from sucrose (Dols et al., 1997a; 1998b). These authors reported for a production of GTF by strain *Leuconostoc mesenteroides* NRRL B-1299 in media with glucose or fructose. The measured activity of GTF produced by this strain after cultivation in glucose or fructose media was significantly lower than the reported enzyme activity measured after cultivation on sucrose medium. In addition, the authors have reported a higher GTF activity after cultivation of *L. mesenteroides* NRRL B-1299 in medium with fructose instead of glucose (Dols et al., 1998b). Smith & Zahnley (1999) also reported low, but detectable GTF and FTF activities after cultivation of *L. mesenteroides* strains in media with glucose, maltose or melibiose. In media with sugars other than sucrose, high efficient glycosyltransferase production was achieved using constitutive mutants of *L. mesenteroides* (Robyt et al., 1995; Kitaoka & Robyt, 1998; Kim & Robyt, 1995; Vasileva et al., 2010).

The production of glycosyltransferases in the absence of polymers also could greatly facilitate the study of their properties and mechanism of action, which is essential for the synthesis of polymers and oligosaccharides with prebiotic properties and immunomodulating activity (Korakli et al., 2002; Iliev et al., 2008; Patel & Goyal, 2011).

The aim of the present work was to study the production of glycosyltransferases from a strain *L. mesenteroides* NRRL B-1149 grown on media containing glucose or fructose and to analyze the type of produced extracellular and cell-associated enzymes. Additionally, we have determined the ratio between soluble and insoluble glucan produced on fructose medium.

Materials and Methods

Bacterial strains, culture media and biomass measurements

Leuconostoc mesenteroides NRRL B-1149 was obtained from Department of Biotechnology, Indian Institute of Technology Guwahati, Assam, India. For the production of glycosyltransferases the strain was cultivated 6-8 h on culture media containing 4% (w/v) glucose or fructose at 27°C on a rotary shaker (200 rpm) (Iliev et al., 2008).

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

After cultivation the culture medium 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. Then, the filtered supernatants of glucose and fructose cultures were concentrated 10-fold using Spin-X^{R} UF concentrators MW 30,000 (Corning^{R}, Corning, NY, USA).

Enzyme activity assay

One unit of glucosyltransferase is defined as the amount of enzyme that catalyzes the production of 1 μmol of fructose per min. at 30°C in 20 mM sodium acetate buffer, pH 5.4, with 100 g of sucrose per liter, 0.05 g of $CaCl_2$ per liter, and 1 g of NaN_3 per liter. It was ascertained that the reducing sugar measured by DNS assay was due to glucosyltransferase and not to levansucrase, inveratase, or sucrose phosphorylase activity as described by Dols et al. (1997b, 1998a).

Glucose and protein determination

Glucose was determined by UV-method, enzymatically with hexokinase (EC 2.7.1.1), glucose-6P dehydrogenase (EC 1.1.1.49) and phospho-glucose isomerase (EC 5.3.1.9) (commercial available kit, Cat. No. K-FRUGL, Megazyme

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International Ireland Ltd, Wicklow, Ireland).

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 R250 (Sigma Chemical Co.). The glycosyltransferase activities were detected by incubating the gel in 10% sucrose 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, Cat. No. 161-0374).

Production and purification of soluble and insoluble glucan

The production and purification of soluble and insoluble glucan were performed according to Shukla et al. (2011). The content of α-(1,6) linkages in the two glucan forms was determined by treatment with dextranase from *Penicillium spp*. (EC 2.4.1.2) (Sigma), 0.5 U per ml for 24 h at 28°C.

Results and Discussion

It is known that *L. mesenteroides* strain NRRL B-1149 produces dextransucrase and fructosyltransferase when grown on media with sucrose. Additianally, the dextransucrase from this strain synthesizes soluble and insoluble glucan polymers (Shukla et al., 2011). In order to study the constitutive production of glycosyltransferases, this strain was cultivated on media with 4% glucose or 4% fructose, respectively. The enzyme activity, pH and biomass formation were measured and compared during the cultivation (Figure 1 and Figure 2).

When the strain NRRL B-1149 was cultivated on glucose medium, extracellular glycosyltransferase activity was detected at the 4th hour and reached highest level (0.73 U/mg) at the 5th hour from the beginning of the cultivation (Figure 1). When the studied strain was cultivated on fructose medium, extracellular glycosyltransferase activity was detected at the 2nd hour from the beginning of the cultivation (Figure 2). The maximum of extracellular glycosyltransferase activity was detected at the 5th hour (5.75 U/mg) of the cultivation on fructose medium. The highest growth rate was reached to 11.7 g/l at the end of cultivation on medium with fructose. The final biomass concentration in media with glucose was lower (9.1 g/l) than that obtained on fructose medium. During the cultivation pH gradually decreased due to heterofermentative production of lactic and acetic acid, and finally the cultivation was stopped at pH 4.33 on medium with glucose and at pH 3.96 on fructose medium.

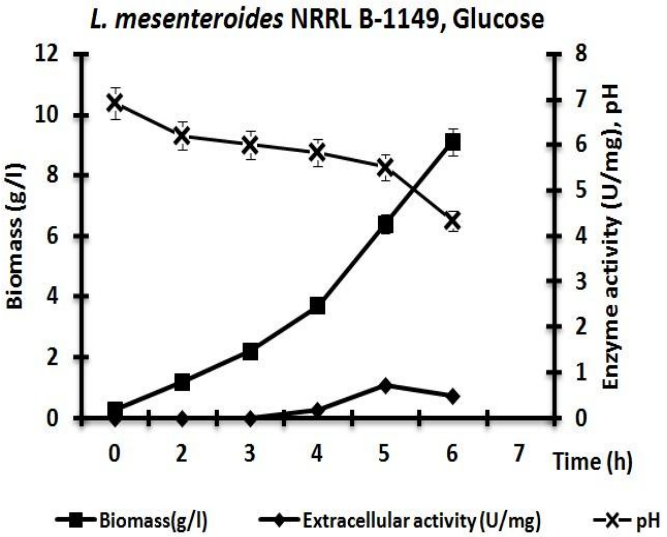

Figure 1. *Measure of extracellular glycosyltransferase activity, pH and biomass production during cultivation of Leuconostoc mesenteroides NRRL B-1149 on medium with glucose.*

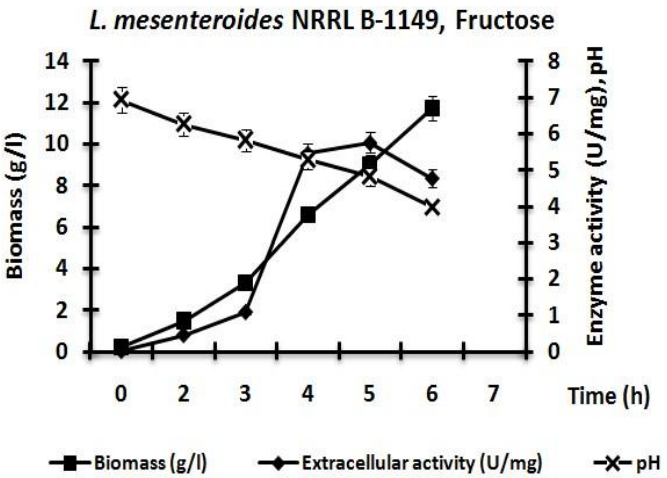

Figure 2. *Measure of extracellular glycosyltransferase activity, pH and biomass production during cultivation of Leuconostoc mesenteroides NRRL B-1149 on medium with fructose.*

According to Miller et al. (1986) glycosyltransferases are active at a pH range between 4.8 and 6.2. An important loss of activity occurred when the pH fall to values lower than 5.0

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(Miller et al., 1986). Our results demonstrated that loss of enzyme activity (34%) was detected at pH below 5 during the cultivation on glucose medium (Figure 1). We detected about 20% loss of enzyme activity at pH 3.96 at the end of cultivation on medium with fructose (Figure 2). The detected glycosyltransferase activities are lower than the enzyme activity detected when the studied strain was cultivated on medium with sucrose (14.64 U/mg). This could result from rapid denaturation of the enzymes in the absence of polymers, which is known to stabilize glycosyltransferase enzymes (Willemot et al., 1988; Dols et al., 1998b).

Results in this study demonstrated that the investigated strain *L. mesenteroides* NRRL B-1149 produces glycosyltransferases constitutively when grown on media with glucose or fructose, since the enzyme activity could be detected in the concentrated supernatants. In addition, it could be concluded that the fructose is a better carbone source than glucose for the production of glycosyltransferases.

It is known that some *L. mesenteroides* strains produce extracellular and cell-associated glycosyltransferase enzymes, which synthesize different types of polymer (Robyt et al., 1995; Zahnley & Smith, 1995). We studied the ratio between extracellular and cell-associated glycosyltransferases produced by NRRL B-1149 on media containing glucose or fructose as a main carbon source (Figure 3). The studied strain produced mainly extracellular glycosyltransferases, which were 92.9% (in glucose containing media) and 97.3% (in fructose containing media) of the total enzyme activity (Figure 3).

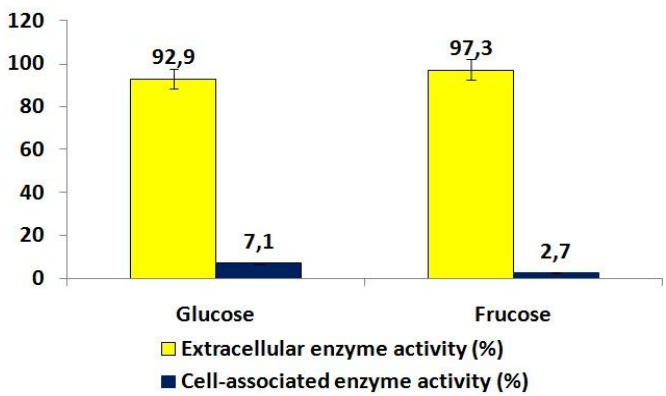

Figure 3. *Ratio between extracellular and cell-associated glylosyltransferase activity after cultivation of Leuconostoc mesenteroides NRRL B-1149 on medium with glucose or fructose.*

In order to characterize the enzymes produced by *L. mesenteroides* strain NRRL B-1149, the extracellular and cell-associated glycosyltransferases were determined using SDS-PAGE and *in situ* Periodic Acid Schiff's staining after incubation of the gel with 10% sucrose (Figure 4). *In situ* analysis of extracellular enzymes produced after cultivation on media with sucrose, glucose or fructose showed a few molecular forms corresponding to 180 kDa, 120 kDa and 86 kDa molecular sizes. The same activity bands were also detected in the cell pellets (Figure 4).

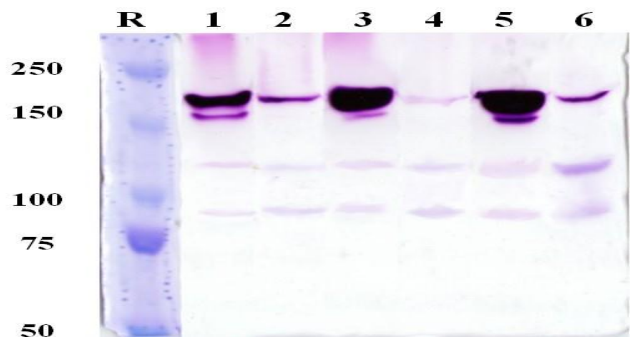

Figure 4. *SDS-PAGE and in situ analysis of extracellular and cell-associated glylosyltransferases produced by Leuconostoc mesenteroides NRRL B-1149 on medium with sucrose, glucose or fructose as a main carbon source. The gel was incubated in 10% sucrose.* ***R*** *– protein standard (Bio-Rad™);* ***1*** *and* ***2*** *– extracellular and cell-associated glycosyltransferases, sucrose medium;* ***3*** *and* ***4*** *– extracellular and cell-associated glycosyltransferases, glucose medium;* ***5*** *and* ***6*** *– extracellular and cell-associated glycosyltransferases, fructose medium.*

Shukla et al. (2010, 2011) reported the production of extracellular dextransucrase of 180 kDa during cultivation of a strain NRRL B-1149 on sucrose medium. The activity band of 180 kDa also corresponds to dextransucrase produced by strain *L. mesenteroides* NRRL B-512F (Robyt & Walseth, 1979; Willemot et al., 1988; Goyal & Katiyar, 1994; Dols et al., 1997b; Smith & Zahnley, 1999). The bands of 120 kDa and 86 kDa correspond to the two molecular forms of FTF described before by Shukla et al. (2011) for NRRL B-1149 cultivated on sucrose medium. The received results showed that when grown on glucose or fructose media the studied strain produces constitutively the same type of extracellular and cell-associated glycosyltransferases as on sucrose medium.

It is known that dextransucrase produced by *L. mesenteroides* NRRL B-1149 on sucrose medium catalyzes

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the synthesis of soluble and insoluble glucan (Shukla et al., 2011). In order to determine if the enzyme produced on fructose medium synthesized the same polymers, we analyzed the ratio between soluble and insoluble glucan synthesized by crude extracellular dextransucrase preparation from *L. mesenteroides* NRRL B-1149. The synthesis and purification of soluble and insoluble glucan were performed according to Shukla et al. (2011). In order to determine the content of α-(1,6) linkages, we hydrolyzed the purified polymers with dextranase from *Penicillium spp.* (EC 2.4.1.2). We have found that the extracellular dextransucrase produced by strain NRRL B-1149 on fructose medium synthesize soluble and insoluble glucan in a ratio (%) 70:30. On the basis of the released glucose measured after dextranase treatment, we supposed the type of the synthesized polymers (Table 1). We have found that the content of α-(1,6) linkages in insoluble glucan is 51.7%. The high content of linkages different from α-(1,6) is associated with low polymer solubility. In contrast, the content of α-(1,6) linkages in soluble glucan is 86.2% which is associated with its solubility.

Table 1. *Dextranase hydrolysis of soluble and insoluble glucan synthesized with dextransucrase produced by L. mesenteroides NRRL B-1149 on fructose medium.*

Polymer	Released glucose (mmol/l)	α-(1,6) linkages (%)	Other linkages (%)
Soluble glucan	8.62	86.2	13.8
Insoluble glucan	5.17	51.7	48.3

According to the received results, it could be concluded that dextransucrase produced by *L. mesenteroides* strain NRRL B-1149 on fructose medium synthesizes two types of glucan – soluble and insoluble, and the relation between the content of α-(1,6) linkages and polymer solubility is in agreement with the results obtained by Shukla et al. (2011) when the enzyme is produced on sucrose medium.

Conclusions

We showed that *L. mesenteroides* strain NRRL B-1149 produces detectable glycosyltransferases when glucose or fructose replace sucrose as a main carbon source. This confirms that the studied strain is constitutive for glycosyltransferase production. The highest extracellular activity (5.75 U/mg) was detected at the 5th hour during the cultivation of the strain on medium with fructose. On glucose or fructose medium *L. mesenteroides* NRRL B-1149 produces mainly extracellular glycosyltransferases. *In situ* analysis of the extracellular and cell-associated enzymes from *L. mesenteroides* NRRL B-1149 after cultivation on media with sucrose, glucose or fructose showed the same enzyme molecular forms of 180 kDa, 120 kDa and 86 kDa. The dextransucrase produced by *L. mesenteroides* NRRL B-1149 on fructose medium synthesize two types of glucan – soluble and insoluble in a ratio 70% : 30%. The lower content of α-(1,6) glycosidic linkages in insoluble glucan corresponds to its low solubility and is associated with a potential application in various directions, including medicine and nutrition.

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