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LEVAN SYNTHESIS DURING ASSOCIATED ACTION OF LEVANSUCRASE AND *CANDIDA CACAOI* DSM 2226 YEAST

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Levan – as a fructose homopolymer – because of its physicochemical properties as well as physiological effect on the human organism, has been the subject of numerous research. The aim of this study was to investigate the course of the synthesis of this polymer with the addition of crude enzymatic preparation isolated from the cell-free culture liquid of the *Bacillus subtilis* DSM 347 bacterium. Saccharose was the donor of fructofuranosyl residues. The effects of some metal ions during synthesis were determined. The innovation in these experiments was the usage of *Candida cacaoi* DSM 2226 yeasts which were introduced into the dialysing membrane (situated in reaction mixture). The yeasts selectively removed glucose from the reaction media because this mono-sugar is considered as an inhibitor of levansucrase. It was found that the decrease of glucose concentration in the medium by 16-19% resulted in the increased degree of levan polymerisation (by 6 to 9%) and efficiency of levan synthesis (by 9 to 11%). The isolated preparation of levansucrase showed high stability during 140 h synthesis at 37°C which allowed to achieve a compromise between the quantity of released glucose in time and the possibilities of *Candida cacaoi* DSM 2226 yeast to utilise that free glucose. The Mn^{2+} ions (at a concentration of 2.5 mmol/L) activated levansucrase (twice increased the initial transferase rate of enzyme) and in reaction medium supplemented with that metal ions the efficiency of levan synthesis increased up to 39%. Associated action of levansucrase and yeasts in the presence of Mn^{2+} effected in the increase of levan synthesis efficiency to 64%.

INTRODUCTION

Levansucrase (EC 2.4.1.10) from *Bacillus subtilis* catalyses the transfer of fructosyl moieties from donors to acceptors (D-fructosyl donors, water, D-glucose, sucrose and levans), as a result of which a polymer is created which is made up of 60,000 D-fructofuranosyl residues linked through $\beta(2\rightarrow6)$ bonds in the main linear chain, while branching results from $\beta(2\rightarrow1)$ bonds [LeBrun & Van Rapenbusch, 1980]. This enzyme is a single polypeptide whose chain is deprived of cysteine [Chambert *et al*., 1974]. Investigations on the kinetics of the levansucrase-catalysed reaction indicate that the transfructosylation reaction occurs with the retention of the configuration of the fructose transferred by this enzyme, a mechanism of the levan synthesis referred to as *ping-pong*. The specificity of the enzyme reaction process is based on the fructosyl-enzyme intermediate [Chambert *et al*., 1974] isolated as a covalently linked complex from a reaction mixture of the enzyme and sucrose [Chambert & Gonzy-Teboul, 1976]. Levansucrase is an extracellular enzyme which is synthesised by *Bacillus subtilis* during the exponential growth phase and its secretion occurs in two stages [Petit-Glatron *et al*., 1987; Elisashvili, 1984]. Levans are also synthesised by numerous microorganisms, *e.g. Zymomonas mobilis* [Muro *et al*., 2000], *Bacillus circulans, Bacillus polmyxa, Erwinia amylovora, Erwinia herbicola, Seraria* sp. [Oseguera *et al*., 1996], *Rahnella aquatilis* [Kim *et al*., 2000], *Pseudomonas syringae* [Hettwer *et al*., 1995], *Acetobacter xylinum* NCI 1005 [Tajima *et al*., 1998], and their properties depend, to a considerable extent, on the molecular weight as well as their structure.

In recent years, intensive investigations and experiments have been done with the aim to find food constituents which exert a beneficial impact on the human organism, especially in the context of prebiotic properties. That is why studies are being done concerning oligosaccharides of desirable physiological activity which are also low-caloric, non-carcinogenic and, in addition, will support the growth of bifidobacteria in the food microflora [Monsan & Paul, 1995]. These experiments include also levans isolated from different sources and are carried out at different aspects.

Bacillus subtilis bacterium is the most intensive studied microorganism producing levansucrase. Investigations are connected with the synthesis conditions, their influence on gene expression, including *sacB* gen (coding levansucrase) [Kunst & Rapoport, 1999] and secretion of levansucrase as affected by the concentration of H^+, Ca^{2+} ions on the external side of the cytoplasmic membrane [Chambert *et al*., 1995]. The kinetics studies of levansucrase and its prop-

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erties [Chambert & Petit-Glatron, 1990] as well as its precursor [Scotti *et al*., 1995] have been carried out as well. The impact of the ionic strength on the enzyme hydrolytic activity was investigated too [Yamamoto *et al*., 1985].

Investigations were also undertaken to study the effect of amphiphilic compounds on the secretion of levansucrase by *Zymomonas mobilis*, which demonstrated the increase of the levansucrase activity and total amount of protein in the medium during incubation [Zikmanis *et al*., 2005]. The enzyme obtained from the *Zymomonas mobilis* batch cultures was subjected to immobilisation on hydroxyapatite and its activity was compared with the native form of levansucrase depending on the effect of pH, metal ions and detergents [Jang *et al*., 2000]. In addition, the response of levansucrase activity to sodium chloride was determined [Vigants *et al*., 1998].

The issues undertaken by the authors of this study are aimed at determining the effect of the environmental conditions, especially the concentration of glucose liberated in the course of the transfructosylation reaction, on the efficiency of the process of levan synthesis.

MATERIAL AND METHODS

MICROORGANISMS

***Bacillus subtilis*, growth medium.** *Bacillus subtilis* DSM 347 bacteria were obtained from the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The strain was stored at a temperature of 4°C on agar slants that contained: bovine meat extract – 1.0 g/L, yeast extract – 2.0 g/L, peptone – 5.0 g/L, NaCl – 5.0 g/L and agar – 15.0 g/L and the pH was maintained at the level of 7.4 [Online catalogue – http://www.dsmz.de/]. The strain was systematically passaged onto fresh slants every month and incubated at a temperature of 28°C for the period of 48 h.

The cultures were conducted on the medium with the following composition: saccharose – 150.0 g/L, citric acid – 11.7 g/L, sodium sulphate – 4.0 g/L, ammonium hydrogen phosphate – 4.2 g/L, yeast extract – 5.0 g/L and 100 mL/L of salt solution (with the following composition: KCl – 7.62 g/L, $MgCl_2$ – 4.18 g/L, $MnCl_2$ – 5.43 g/L, $FeCl_3$ – 0.49 g/L, $ZnCl_2$ – 0.21 g/L) [Szwengiel *et al*., 2004]. The pH of culture medium was adjusted to 6.8 using 25% ammonia solution. All media were autoclaved for 20 min at 121°C prior to inoculation.

Cultivation conditions. Cultures were carried out in Erlenmeyer flasks. The inoculum was prepared by the incubation of the culture medium with the bacterium from the slant and then shaking cultures were conducted for 16 h at a temperature of 37°C at 150 rpm. The 16 h-old seed culture (2×10^8 cells/1 mL) prepared in this way was used to prepare the main culture in the flasks (10% pre-culture was added to the culture medium). The culture was carried out in aerobic conditions for 16 h. The number of yeast cells was determined in the Thom counting chamber.

***Candida cacaoi* DSM 2226, culture medium and cultivation conditions**. Yeasts were stored at a temperature of 4°C and were systematically passaged onto fresh slants every two weeks. Medium 186 – a Universal Medium for Yeasts (YM) of the following composition was applied: yeast extract – 3.0 g/L, malt extract – 3.0 g/L, peptone from soybean – 5.0 g/L, glucose – 10.0 g/L, agar – 15.0 g/L [Online catalogue – http://www.dsmz.de/]. The yeast biomass used in the experiment was obtained from the liquid medium of the same composition (the solidifying factor – agar – was eliminated). The culture media were inoculated by washing the yeast culture from the slant by distilled water. Cultures were carried out in Erlenmeyer flasks in aerobic conditions as shaking cultures for 16 h at 37°C at 150 rpm. During the period of 16 h, the number of 10^7 cells/1 mL was obtained. The biomass was centrifuged, rinsed with bidestilled sterile water, centrifuged again and then introduced onto the dialysing membrane in the form of suspension.

ENZYME

Preparation of the crude levansucrase precipitate. Levansucrase was obtained from the batch cultures of *Bacillus subtilis* DSM 347 bacterium at the end of the second phase of the exponential growth. Investigations were made on a crude enzymatic preparation obtained by the ethanol precipitation of the supernatant collected after the centrifugation of the bacterial biomass (earlier pH was corrected to 4.2); the final ethanol concentration was 48% (v/v). All stages of levansucrase harvesting were conducted at 4°C, the colloidal protein fraction was centrifuged (7500 × g for 15 min) and the obtained ethanol precipitate was suspended in 0.05 mol/L phosphate buffer, pH 6.0 [Dedonder, 1966]. Crude levansucrase preparation was portioned and stored at -18°C. The method of obtaining levansucrase using ethanol precipitation with different modifications was used by many authors [LeBrun & Van Rapenbusch, 1980; Chambert *et al*., 1974; Chambert & Gonzy-Teboul, 1976].

Levansucrase activity. A unit of levansucrase activity is defined as the amount of the enzyme that catalyses the formation of one micromole of glucose per minute at 37°C in 1 mol/L concentration of saccharose in the phosphate buffer (pH 6.0; 0.05 mol/L) [Euzenat *et al*., 1997]. Samples from the reaction mixture were collected every 5 min in the period of 30 min and were heated in a boiling water bath to inactivate the enzyme. The total activity of the crude enzymatic preparation was calculated on the basis of the initial reaction rate of glucose released in the reaction mixture. The amount of glucose (in total) released into the medium (Glc_T) was determined enzymatically using a glucose oxidase – peroxidase mixture (reagent - GLUKOZA, Aqua-med, Poland). The concentration of reducing sugars ($Glc_T + Fru_h$) (converted into reducing sugars, *i.e.* glucose and fructose) was determined in the reaction with the DNS reagent [Miller, 1959]. On the basis of the difference in the amount of reducing sugars and glucose, it was possible to assume the concentration of free fructose (Fru_h) and this information was used for the determination of hydrolytic activity. Transferase activity was calculated on the basis of the amount of fructose which underwent the hydrolytic process of transfructosylation ($Fru_t = Glc_T - Fru_h$). Assays were performed in accordance with the methodology presented by Oseguera *et al.* [1996] in modification (fructose (Fru_h) was measured by enzymatic test (Boehringer Mannheim) in the original work). The quantitative determination of protein

was carried out with the method of Lowry *et al.* [1951] using bovine serum albumin (BSA) as a standard.

EXPERIMENTAL DESIGN

Synthesis. The enzymatic crude preparation of levansucrase was suspended in 300 mL of sterile 15% saccharose solution (0.45 U/L g saccharose) in a citrate buffer (see media below) after sterilisation (Millex GP Filter Unit, 0.22 μm; Millipore, France). In the flask with the above medium, the authors placed a magnetic stirrer as well as a dialyzing membrane (Nadir – Nalo Cellulose, MWCO 10000 – 20000 Da; Carl-Roth) with the yeast biomass earlier suspended inside the dialyzing membrane. The synthesis was conducted in Erlenmeyer flasks at 37°C and mixer rotations of 100 rpm. Throughout the period of synthesis (140 h), samples were taken periodically and next assessed for glucose concentration.

Three media (M) containing saccharose (150.0 g/L) dissolved in 0.05 mol/L citrate buffer of pH 6.0 [M(Suc)] were used for the performed investigations. Two of these media were additionally supplemented with $MnCl_2$ (0.315 g/L) – [M(Suc+Mn^{2+})], the optimal concentration of Mn^{2+} ions was determined experimentally, or with a mixture of ions in the form of salts: $MnCl_2$ (0.315 g/L), concentrations of the remaining chlorides were identical as in the case of the growth medium for the *Bacillus subtilis* DSM 347 bacteria: KCl (7.62 g/L), Mg Cl_2 (4.18 g/L), $FeCl_3$ (0.49 g/L), $ZnCl_2$ (0.21 g/L) – [M(Suc+all salts)]. The medium without metal ions was treated as a reference sample. The following two experimental variants were employed: the synthesis was carried out only with the enzyme or, alternatively, yeasts (Y) were placed in the membrane and the so-called coupled levan synthesis was conducted with the simultaneous utilisation of glucose [M, Y(Suc+Mn^{2+}); M, Y(Suc+all salts)].

EVALUATION OF THE SYNTHESIS

Analysis of the reaction medium. Samples were collected periodically during the synthesis and the concentration of glucose was determined using the enzymatic test (Aquamed, Poland) and this information was used to infer the amount of saccharose which underwent reaction. In the 140th h of synthesis, 50 mL samples were collected and levan was precipitated with ethanol [Szwengiel *et al*., 2004]. The sample was then analysed with gel chromatography (GPC). The mean number of dead yeast cells inside the membrane was also monitored carrying out microscopic observations in the presence of an aqueous solution of methylene blue (1:10000) [Burbianka *et al*., 1983].

Molecular weight and polydispersity of levan. The gel permeation chromatography (GPC) technique was employed to determine molecular weights of the obtained levans (after the termination of synthesis). Three Ultrahydrogel™ columns arranged in a series were used. Analyses were carried out using the Waters Company apparatus (Alliance HPLC System 2695) equipped with a refractometric detector (RI) Waters 2414. The obtained data were processed using the Empower Pro software in the GPC option. The following parameters were applied during the chromatographic analysis: temperatures of the injector – 25°C, column – 40°C, measuring cell – 35°C; and flow rate of the solvent (deionised water) – 0.700 mL/min. The calibration curve was obtained using dextran standards (Polymer Standards Service-USA, Inc.). Polydispersity of individual peaks was determined as the ratio of Mw:Mn (weight-average molecular weight/number-average molecular weight) [Calazans *et al*., 2000; Vatanasuchart *et al*., 2005].

Statistical analysis. The obtained results were analysed statistically with the Microsoft Excel 2000 spread sheet together with one-way ANOVA and multiple comparison test – LSD test (Least Significant Difference test) was done. The functional correlation – Gompertz model treated as the Sigmoidal Family was also fitted into measuring points. The equation coefficients were determined with CurveExpert Version 1.38 (comprehensive curve fitting system for Windows which employs a large number of regression models, both linear and nonlinear).

RESULTS AND DISCUSSION

On the basis of a study by Euzenat *et al.* [1997], the authors put forward a thesis that the reduction of free glucose in the medium should result in increased levan synthesis accompanied by a simultaneous change in the distribution of the molecular weights of the polymer. In order to reduce the concentration of glucose, the authors applied a combined synthesis of levan utilising crude enzymatic preparation and yeasts which removed selectively free glucose from the medium.

Levansucrase

The obtained crude levansucrase preparation did not exhibit proteolytic activity (determined with the Anson method) and contained the desirable protein fraction. Catalytic activity increase of this crude preparation of levansucrase was observed during the purification process with the hydroxyapatite (data not published), which had earlier been noticed in other studies [LeBrun E & Van Rapenbusch, 1980; Chambert *et al*., 1974; Chambert &Gonzy-Teboul, 1995; Dedonder, 1966; Euzenat *et al*., 1997]. Properties of the levansucrase and usefulness of its crude preparation for industrial practice without the necessity of using frequently expensive and inefficient purification processes were determined. In the performed experiments the authors applied a crude enzymatic preparation characterised by specific activity of 4.2 U/mg protein.

Yeasts

The *Candida cacaoi* yeast form white or cream colonies, are characterised by multilateral budding, elaborate pseudohyphae and no sexual reproduction and are found in cacao. Both under aerobic and anaerobic conditions, they do not utilise, among others, saccharose, inulin and starch as a source of carbon. Moreover, they grow on media which do not contain vitamins (myo-inosytol, pantothenate, biotin, thiamin, pyridoxine, niacin, folic acid, PABA) and in a wide spectrum of temperatures (the tested temperatures ranged from 25°C to 42°C) [Barnett *et al*., 1983]. On the basis of the above data, the examined strain of yeast was accepted

as fulfilling the expected demands, *i.e.* no hydrolytic activity in relation to saccharose and growth in a wide spectrum of temperatures (from 25 to 45°C). During the first stage of experiments, the authors examined the impact of the tested media (M(Suc), M(Suc+all salts), M(Suc+Mn^{2+}) supplemented with 0.5% (w/v) glucose) on the growth of the yeast (Figure 1). Glucose was applied at a concentration of 0.5%to determine the substrate specificity of the *Candida cacaoi* DSM 2226 yeasts to utilisation of glucose as a saccharide carbon source in the presence of saccharose (Figure 2). In all of the applied media during synthesis, 0.5% glucose concentration was measured up to the 5th h; this concentration was by 16 times lower than the amount of glucose introduced into synthesis media (with saccharose). Part of the media were supplemented with a salt mixture of metal ions (all salts) and Mn^{2+} ions due to the fact that investigations on the effect of ions on the levansucrase activity indicate that both the type and concentration of ions affect the activity of levansucrase obtained from *Zymomonas mobilis* [Jang *et al*., 2000]. Levansucrases from *A. diazotrophicus* [Hernandez *et al*., 1995] and *R. aquatilis* JCM-1683 [Ohtsuku *et al*., 1992] were slightly inactivated by $FeSO_4$, whereas the enzyme obtained from *Bacillus* sp. TH4-2 [Ammar *et al*., 2002] was highly activated by Fe^{3+}; at the same concentrations the enzyme showed different values of relative activity for separate ions (in comparison with *Z. mobilis* [Jang *et al*., 2000]). In addition, our studies indicate that these interdependences cannot be transferred within one genus of bacterium and that is why the optimisation of the selection and concentration of individual ions was carried out during the preliminary investigations and the Mn^{2+} ion was selected as it ensured high total and transferase activity at relatively low hydrolytic activity. The transferase activity of levansucrase in the reaction mixture supplemented with Mn^{2+} was 100% higher than the enzyme activity measured in medium without metal ions (reference medium). The hydrolytic activity of the levansucrase was lower by 80% than the enzyme activity detected in the reference medium.

The highest number of yeast cells (Figure 1) was recorded in the medium containing the mixture of metal ions (4×10^7 cells/mL), while in the case of M(Suc)+(0.5% Glc), M(Suc+Mn^{2+})+(0.5% Glc) media, the number of cells determined during the stationary growth phase was by about 50% lower and in the first case [M(Suc+all salts)+(0.5% Glc)], in addition, no sharp point was recorded during the transition of yeasts (from the logarithmic to the stationary growth phase). No increase of the population size was observed in the M(Suc) medium. It should be emphasised that the authors did not analyse biomass increase but only the number of yeast cells which, even after the fourth day of culturing, showed considerable vitality (over 97%). Simultaneously, measurements presented in Figure 1 were accompanied by the detection of free glucose (Figure 2) during the growth of *Candida cacaoi* DSM 2226. A small amount of glucose (0.6 mg/mL) was present in the initial stage of culturing also in the M(Suc) medium (Table 1), which was the result of saccharose inversion during medium sterilisation. Additionally, the medium containing only glucose M (0.5% Glc) was introduced into the experimental system with the aim to compare to what extent the presence of saccharose (which causes increased viscosity) can affect the course of the glucose utilisation process. The analysis of the obtained data was carried out fitting the measuring points of the rectilinear regression equation. Results of this analysis are presented in Table 1. The obtained results were satisfactory; high correlation coefficients allowed the authors to make the following observations: the addition of the metal ions mixture reduced significantly the time of glucose content decrease in the medium (by 50%), the examined yeasts did not induce saccharose M(Suc) hydrolysis despite the fact that the medium did not contain an easily available source of carbon such as glucose, no pH changes were observed (the environment was buffered with citrate buffer, pH 6.0) at any time, which indicates that the *Candida cacaoi* DSM 2226 yeasts did not utilise citrate anions. The authors also carried out a correlation analysis of

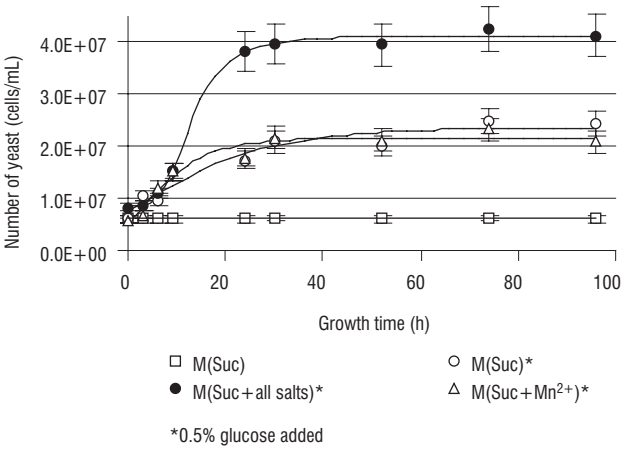

FIGURE 1. Comparison of changes in the number cells of *Candida cacaoi* DSM 2226 yeast inside the membrane during growth on different media (□ – 15% saccharose solution, ○ – 15% saccharose solution with 0.5% glucose content, – 15% saccharose solution containing all salts and 0.5% glucose, △ – 15% saccharose solution containing Mn^{2+} ions and 0.5% glucose).

TABLE 1. Comparison of changes in glucose concentration during the growth of *Candida cacaoi* DSM 2226 yeasts on the basis of the mathematical analysis of plots presented in Figure 2.

Medium	Simple regression equation	Determination coefficient	Initial value of glucose (mg/mL) ±SD	Time of glucose content reduction (by 50% in the medium (h)*	Velocity of glucose content reduction in the medium (mg Glc/h)**
M(0.5% Glc)	y = -1.3576x + 3.519	R^2 = 0.9824	5.6±0.6	32	0.07
M(Suc)	y = -3.1213x + 81.12	R^2 = 0.8324	0.6±0.2	10	0.06
M(Suc)+(0.5% Glc)	y = -0.3838x + 7.725	R^2 = 0.8335	5.5±0.5	98	0.02
M(Suc+all salts)+(0.5% Glc)	y = -0.5183x + 7.621	R^2 = 0.8861	5.6±0.6	72	0.03
M(Suc+Mn^{2+})+(0.5% Glc)	y = -0.4022x + 9.425	R^2 = 0.8599	5.6±0.6	98	0.02

*calculated on the basis of the regression equation; ** on the basis of the directional coefficient of the simple regression equation

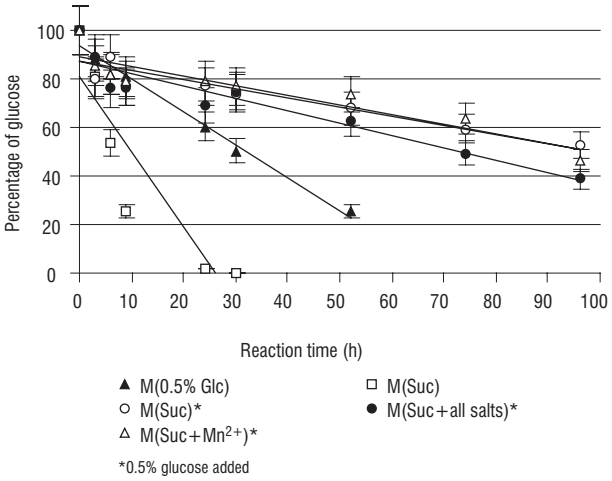

FIGURE 2. Changes in glucose concentration during the growth of *Candida cacaoi* DSM 2226 yeast (inside membrane) on different media: □ – 15% saccharose solution, ○ – 15% saccharose solution with 0.5% glucose content, – 15% saccharose solution containing all salts and 0.5% glucose, △ – 15% saccharose solution containing Mn^{2+} ions and 0.5% glucose, ▲ – solution with 0.5% glucose content.

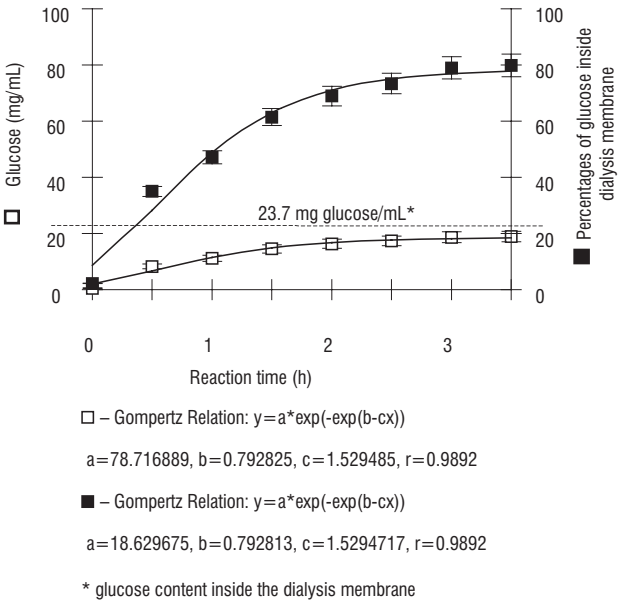

FIGURE 3. Changes in glucose content inside the dialysis membrane, initial glucose gradient was 23.7 mg/mL.

changes in the number of yeasts cells as a function of glucose content of the medium. The results of analyses are presented in Table 2. High negative correlation coefficients provide evidence of a close correlation between the number of yeast cells and the change of glucose content (with the increase of yeasts, glucose was gradually assimilated).

Investigation of membrane porosity

The examination of membrane porosity was done to control exchange of constituents – glucose – between the external medium and the medium inside the membrane (Figure 3). In this investigation glucose gradient was prepared – 23.7 mg glucose/mL – which corresponded to 30% of saccharose transformed with the assistance of transfructosylation into the levan assuming 100% efficiency of the synthesis (a similar efficiency was obtained during syntheses *in vivo* [Szwengiel *et al*., 2004]). A mathematical function (described by the Gompertz Relation) was fitted to measurement points. So already after three and a half hours the system attained the state of equilibrium and glucose concentration inside the membrane reached 18.6 mg glucose/mL, *i.e.* 79% of the initial concentration outside the porous membrane. The nanofiltration techniques was not used for fractionation of saccharides in those experiments because the ultrafiltration processes are unfeasible on account of the poor selectivity of membranes employed [Aydogan *et al*., 1998]. That is why, the authors applied in this study a physico-microbiological arrangement, *i.e.* a combination of a porous membrane of high molecular weight cut-off (MWCO: 10,000–20,000 Da) and yeasts which are able to remove free glucose from the reaction medium.

Levan synthesis

After the termination of the initial but necessary investigations aimed at characterising the experimental system, the authors began the main part of the experiment – the synthesis of levan. The system presented in Figure 4 shows results of synthesis (medium M(Suc) which was the reference for all the performed syntheses; at the same time it provides a graphic example of changes in the analysed media. Results together with the statistical analysis were presented in Table 3. In each case, the analysis was carried out on the basis of the Gompertz Relation which depicts changes in the glucose content as a function of time. It should be stated the *a* coefficient from this equation corresponds the asymptote value, the *b* coefficient – to the rate of changes on the curve section of constant velocity course, and the coefficient *c* is here a proportionality constant. Very high correlation coefficients were obtained

TABLE 2. List of correlation coefficients (α=0.05) depending on changes in the number of *Candida cacaoi* DSM 2226 yeast and glucose content.

Number of yeasts in / Content of glucose in	M(Suc)	M(Suc) +(0.5% Glc)	M(Suc+all salts)+(0.5% Glc)	M(Suc+Mn^{2+}) +(0.5% Glc)
	Correlation coefficients			
M(Suc)	-0.92341			
M(Suc)+(0.5% Glc)		-0.94217		
M(Suc+all salts)+(0.5% Glc)			-0.80414	
M(Suc+Mn^{2+}) +(0.5% Glc)				-0.76557

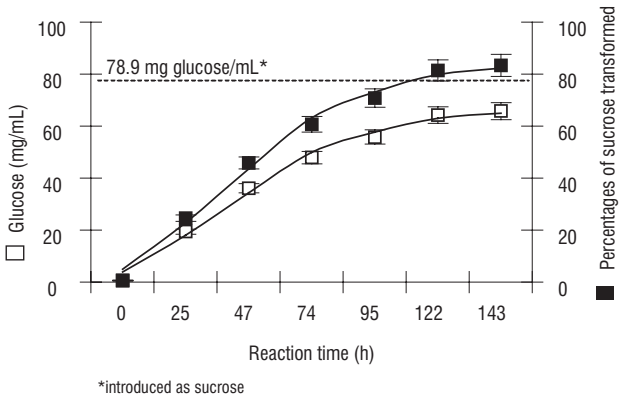

FIGURE 4. Changes in glucose content during levan synthesis in the M(Suc) medium; curves were plotted on the basis of the Gompertz Model; the list of equation coefficients is presented in Table 3.

TABLE 4. The effect of reaction media on levan synthesis yield and fructose transformation into levan (Y – syntheses with yeast).

Medium	Reaction time (h)	Levan yield (%) LSD = 3.84*	Fructose transformation into levan (%) LSD = 4.02
M(Suc)	140	21[a]	44[a]
M(Suc+all salts)	140	22[a]	46[a]
M, Y(Suc+all salts)	140	26[b]	55[b]
M(Suc+Mn^{2+})	140	29[b]	61[c]
M, Y(Suc+Mn^{2+})	140	34[c]	72[d]
in vivo synthesis	33	33[c]	70[d]

in the analysed experimental systems, justifying the prediction of the glucose concentration in the analysed time interval. The highest glucose concentration during the final stage of synthesis was determined in the medium containing Mn^{2+} ions (94.9% of the glucose introduced into the system). The sucrose transformation into levan was between 44 and 70% and efficiency of levan synthesis with yeasts was higher than without *Candida* (Table 4). The experiment with yeasts inside the membrane resulted in the reduction of glucose content by 18.9% in the identical period of time. Consequently, it also led to a slight but significant change in the degree of polymerisation of the isolated polymer. In addition, Mn^{2+} ions had an effect on the change in the degree of saccharose conversion into levan as well as on the change in the degree of its polymerisation in relation to the M(Suc) medium (Table 3). Moreover, this positive effect intensifies the reduction in glucose concentration during the synthesis. However, the selection of the appropriate ion and its concentration is crucial, as could be observed during the synthesis of levan in the medium containing a mixture of metal ions M(Suc+all salts), where no significant difference was recorded in the percentage glucose content in relation to the reference medium M(Suc), with the exception of a slight increase in the amount of fructose monomer subunits in the fructan chain. When yeasts (Y) were used during the synthesis M, Y (Suc+all salts) the content of glucose was reduced by 16.1%, which resulted in the increased polymerisation (by 4 subunits of fructose) (Table 3). Similar results were observed in the medium containing the Mn^{2+} ions (M, Y(Suc+Mn^{2+})). Significant differences were found between the degree of polymerisation of levan obtained from medium M(Suc) and M, Y(Suc+Mn^{2+}). The chain of levan obtained from medium M,Y (Suc+Mn^{2+}) was 19 subunits of fructose longer than the polymer precipitated from medium M (Suc). The levan synthesis yield increased significantly when the actions of levansucrase crude preparation and yeast cells were associated (Table 4). Fructose transformation yield into levan was higher during syntheses with *Candida cacaoi* DSM 2226 yeast (Table 4). The kinetic constants determined from Lineweaver-Burk plots (not shown) in accordance with different concentrations of glucose indicated the existence of allosteric inhibition. The increase in the degree of polymerisation as well as the measured Michaelis constant and the maximum velocity corroborated that glucose acts as an inhibitor of the transfructosylation reaction.

TABLE 3. Degree of levan polymerization and list of correlation coefficients used to determine the content of glucose in the 143rd h of levan synthesis.

Medium	Gompertz Relation y=a*exp(-exp(b-cx)) for changing glucose			Content of glucose in 143rd h of synthesis (%)* LSD = 3.28**	Degree of polymerization of isolated levan
	Coefficient data	Standard error	Correlation coefficient		
M(Suc)	a = 67.4046 b = 1.43188 c = 0.03056	2.5729	0.9962	85.4[c]	46
M(Suc+all salts)	a = 67.27598 b = 0.971261 c = 0.030018	3.9889	0.9907	85.2[c]	50
M, Y(Suc+all salts)	a =54.54700 b =0.822519 c =0.028819	4.8312	0.9781	69.1[a]	54
M(Suc+Mn^{2+})	a = 74.93137 b = 1.146905 c = 0.036731	2.3071	0.9977	94.9[d]	61
M, Y(Suc+Mn^{2+})	a =59.93395 b =1.089558 c =0.039779	2.0394	0.9972	76.0[b]	65

* values were estimated on the basis of free glucose in the medium to the glucose contained in sucrose at the beginning; **the superscripts from *a* to *e* are results of the LSD test (α = 0.05)

TABLE 5. GPC analyses of levans isolated from two media in the 140th h.

Medium	Retention time (min)	Molecular weight (Da) ±SD				
		$\overline{Mn}$ [a]	$\overline{Mw}$ [b]	$\overline{MP}$ [c]	$\overline{Mz}$ [d]	$\overline{Mw}/\overline{Mn}$ [e]
M, Y(Suc+all salts)	36.487±0.101[3]	5927±178	7400±222	8824±265	8764±263	1.25±0.04
M, Y(Suc+Mn^{2+})	36.103±0.056[4]	6733±201	8546±256	10561±317	10177±305	1.27±0.04

[a]number-average molecular weight; [b] weight-average molecular weight; [c]peak average molecular weight; [d]z-average molecular weight; [e]polydyspersity

GPC analysis

Table 5 presents the distribution of molecular weights for the experimental treatments in which the synthesis occurred simultaneously. The *Bacillus subtilis* levansucrase catalysed the formation of two different types of levan – named high and low molecular levan [Dodonder & Péaud-Lenoël, 1957], two fractions were also obtained during the *in vitro* synthesis [Euzenat *et al*., 1997]. In our earlier studies on levan syntheses *in vivo* with the *Bacillus subtilis* DSM 347 strain we obtained two fractions of different molecular weight which were not observed during the *in vitro* syntheses. Analysing the presented mean molecular weights which characterise, in an objective manner, the proportion of individual homologues of a given polymer on the basis of a gravimetric-differential distribution function, suggest higher part of levan with the higher degree of polymerisation in whole mass of polymer (on the basis of the function asymmetry of which one of the measures is the sample polydispersity averaging, in both cases, 1.26).

CONCLUSIONS

Because of the low proportion of the enzyme in relation to saccharose concentration (0.45 U/L g saccharose), the time of synthesis was distinctly longer (by 4.2 times) than in the case of *in vivo* synthesis (Table 4). In our study, we applied 27 times lower enzyme concentrations (on the basis of the levansucrase activity against 1 g of saccharose) than in experiments carried out by Euzenat *et al*. [1997] and that is why the time of 95% of saccharose transformation was about 23 times longer. The same efficiencies were achieved between M, Y(Suc+Mn^{2+}) and *in vivo* synthesis (Table 4). During syntheses the authors failed to achieve the profile of the levan molecular weights appropriate for levan obtained in the *in vivo* course. It is a well-known fact that a compromise between the degree of polymerisation and the availability of levan to probiotic microflora of the gastrointestinal tract should be found. It was demonstrated that levan of high degree of polymerisation remains unavailable for bifidobacteria [McKellar *et al*., 1993; Muramatsu *et al*., 1994]. Marx and co-workers [2000] demonstrated that levan of low molecular weight (about 3200 Da) is utilised by the tested *Bifidobacterium* species. In addition, the above researchers suggested that the molecular mass and not the linkage type of the fructan is the decisive factor for the degradation. Hydrolysis of long-chain FOS may be prevented by steric hindrance at the active site of the hydrolytic enzymes [Marx *et al*., 2000]. Analysing our results in the context of levan bioavailability as a selective source of carbon for species of *Bifidobacterium*, we put forward a proposal, in accordance with results of Marx and co-workers [2000], that all our levans have too high molecular weights. An advantageous result of this syntheses was the increase of levan yield. In the light of these investigations, the optimisation of synthesis as well as application studies on levans should be continued, focusing on the origin of the levansucrase enzyme.

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SYNTEZA LEWANU PODCZAS SKOJARZONEGO DZIAŁANIA LEWANOSACHARAZY I DROŻDŻY *CANDIDA CACAOI* DSM 2226

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Lewan jako homopolimer fruktozy ze względu na swoje właściwości zarówno fizykochemiczne, jak i fizjologiczne oddziaływanie na organizm człowieka jest przedmiotem wielu rozpraw naukowych. W pracy podjęto badania nad syntezą tego polimeru przez pozakomórkową lewanosacharazę, wytwarzaną przez *Bacillus subtilis* DSM 347. W badaniach wykorzystano surowy preparat, otrzymany przez wytrącenie białka enzymatycznego etanolem. Substratem w reakcji transfruktozylacji była sacharoza. Sprawdzano wpływ jonów metali w mieszaninie reakcyjnej na wydajność syntezy lewanu. Innowacją w prowadzonych badaniach było użycie drożdży, umieszczonych w worku dializacyjnym, zanurzonym w mieszaninie reakcyjnej, których zadaniem było selektywne zużywanie glukozy, uważanej za inhibitor lewanosacharazy. Stwierdzono, że obniżenie stężenia glukozy o 16–19% powoduje 6–9% wzrost stopnia polimeryzacji oraz 9–11% wzrost wydajności syntezy lewanu (tab. 3 i 4). Izolowany preparat lewanosacharazy wykazywał wysoką stabilność w ciągu 140 godzin syntezy w 37°C, co pozwoliło osiągnąć kompromis między ilością uwalnianej w czasie glukozy, a możliwościami utylizacji jej przez drożdże *Candida cacaoi* DSM 2226 (rys. 2 i 4). Jony Mn^{2+} (w stężeniu 2.5 mmol/L) aktywowały lewanosacharazę (dwukrotnie zwiększały początkową aktywność transferyczną enzymu) i w ich obecności uzyskiwano wzrost wydajności syntezy lewanu o 39% (tab. 4). Skojarzone działanie lewanosacharazy i drożdży w obecności jonów Mn^{2+} skutkowało wzrostem wydajności syntezy lewanu o 64%.