Effect of the combined use of β-amylase and pullulanase on the carbohydrate composition of maltose syrups

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Abstract

Introduction. The aim of this study was to investigate the effect of a complex of saccharifying amylolytic enzyme preparations of β-amylase and pullulanase on the carbohydrate composition of maltose syrups.

Materials and methods. Bacterial α-amylase, β-amylase and pullulanase, corn starch, starch hydrolysates and maltose syrups were used in the present research. The process of hydrolysis of starch has been carried out in two successive stages: liquefaction and saccharification. Physicochemical methods of analysis have been used. The carbohydrate composition of the hydrolysates has been determined by chromatographic method.

Results and discussion. The highest value of dextrose equivalent (DE) at the stage of saccharification to maltose is achieved at the optimal degree of dilution of the starch suspension, which corresponds to the value of dextrose equivalent of 10–15%. Hydrolysates with higher DE values have more short starch molecules, which are very slowly saccharified by β-amylase; tetrasaccharides are even more resistant to its action, and trisaccharides are not broken down at all, because this maltogenic enzyme has a high affinity for macromolecular substrates. Hydrolysates with lower DE values have a high viscosity, adhere to the walls of the equipment and undergo retrogradation, which leads to losses.

The most effective way to obtain maltose syrups is to use a complex of enzyme preparations (EP) of β-amylase and pullulanase at the rate of 0.03 units of β-amylase activity/g of starch and 0.0006 units of pullulanase activity/g of starch, respectively. It has been determined that with a duration of saccharification of 24 hours, the content of maltose in the hydrolysates reaches 60–62% by weight of reducing substances (RS) with a low glucose content (about 2.7% by weight of RS). Low concentration of glucose is the main indicator of the quality of syrups in the production of caramel and many other food products.

Conclusions. Kinetic regularities have been established to determine the optimal conditions for the process of liquefaction and saccharification using a complex of saccharifying enzyme preparations of β-amylase and pullulanase for the production of syrups with high concentration of maltose.

Introduction

The trend in the application of enzymes in the food industry is a relevant modern area of research. Microbial enzymes have long been used in food production, but every year they find new areas of application (Ivanov et al., 2021). Enzymatic conversion of starch to obtain syrups with a high content of maltose (more than 50% by weight of reducing sugars) are widely used for the production of ice cream, marmalade, jams, juices, beverages, beer, as well as to replace sugar in the production of lollipop caramel. High resistance to spontaneous crystallization, lower viscosity and hygroscopicity compared to glucose syrups (dextrose equivalent 38–42%) characterizes these syrups. In addition, they contain a large amount of fermentable saccharides (Baks et al., 2008; Souza et al., 2019).

In the production of maltose syrups, it is important to transfer starch grains in suspension to a soluble state by temperature gelatinization in the presence of a catalyst. Gelatinized starch responds to the action of amylolytic enzymes much faster than the native one (Porras et al., 2018; Souza et al., 2019). To achieve minimum starch losses and obtain a syrup with good filtration properties, it is necessary to fully gelatinize all starch grains, including small ones. The gelatinization temperature of small starch grains is 120–140 °C (Souza et al., 2019). The process of enzymatic conversion of starch is carried out in two stages: liquefaction and saccharification. In the process of gelatinization, the viscosity increases significantly, which makes it difficult to carry out the liquefaction process. It is also necessary to prepare the substrate for saccharification. The maltogenic enzyme β-amylase, like other saccharifying enzymes, cannot effectively catalyze the hydrolysis of gelatinized starch due to its high viscosity. β-amylase is capable of cleaving the maltose molecule only from the non-reducing edge formed at the site of the α-1,4-glucoside bond rupture (Porras et al., 2018; Souza et al., 2019). Therefore, in order to reduce the viscosity and avoid the phenomenon of retrogradation, it is necessary that the gelatinization of starch will be accompanied by its liquefaction. In the obtained low viscosity hydrolyzate it is easier to further break the starch molecule for final products of saccharification.

The degree of liquefaction of starch has a significant effect on the quality of maltose hydrolysates after saccharification. It is known (Lin et al., 2013; Radovanovic et al., 2018; Saini et al., 2017) that short starch molecules are attacked by the enzyme β-amylase very slowly, tetrasaccharides are even more resistant to its action, and trisaccharides are not broken down at all because this maltogenic enzyme shows a high affinity for high molecular weight substrates. Therefore, for maltose syrups to be obtained, in the saccharification stage it is desirable to use hydrolyzed starch with the lowest dextrose equivalent after liquefaction and a minimum amount of glucose. However, such a hydrolysate has a high viscosity (more than 2 Pa·s) and adheres to the walls of equipment and pipelines (Gebremariam et al., 2013). Therefore, it is necessary to establish the optimal value of DE hydrolysates after enzymatic liquefaction for effective saccharification of starch hydrolysates to maltose.

It has been shown (Porras et al., 2018) that optimal parameters for the liquefaction process are: the concentration of the corn starch suspension – 30%; using of thermostable bacterial enzyme preparation of α-amylase Alphasin T7L – 4 units of amylolytic activity per gram of dry matter of starch. The degree of liquefaction of the starch suspension should correspond to the DE value of 18–20%. However, these studies focused on the effect of the degree of starch liquefaction on the subsequent saccharification of hydrolysates to glucose, where glucoamylase was used as a saccharifying enzyme (Trisnaputri et al., 2018; Yadav et al., 2017). To obtain syrups with a high maltose content at the saccharification stage, we used maltogenic enzyme preparations. In order to clarify the results of previous studies, it was necessary to establish the effect of the degree of starch liquefaction on the kinetics of further

saccharification of hydrolysates to maltose. As it is known (Baks et al., 2008), for the production of maltose syrups with an amount of maltose of about 50%, a preparation of the maltogenic enzyme β-amylase is used as a biocatalyst for the hydrolysis reaction. The subsequent accumulation of maltose is prevented by β-limit dextrins, which are formed in an amount of about 23% under the action of β-amylase on rarefied starch (Baks et al., 2008; Lin et al., 2013). To make the substrate more accessible for the action of β-amylase, it is necessary to use an enzyme that breaks the branches in the amylopectin chains that are not affected by the enzyme preparation of β-amylase (EP 0294093 A2).

A promising direction for expanding the range of maltose syrups is the search for new enzyme preparations and their combinations to increase the accumulation of maltose in hydrolysates. One of these enzyme is a pullulanase preparation (amylo-1,6-glucosidase), which hydrolyzes α-1,6-glucosidic bonds at the branch points of the starch molecule. The ability to break α-1,6-bonds is significantly increased if they are surrounded on all sides by α-1,4-bonds. As a result of its action, linear dextrins are formed with non-reducing residues of molecules that can be hydrolyzed by the enzyme preparation of β-amylase. The use of pullulanase in the composition with β-amylase will change the carbohydrate composition of the hydrolysates in the direction of the accumulation of maltose (Gangadharan et al., 2009; Rittenauer et al., 2021; Souza et al., 2019).

The aim of the work was to determine the effect of a complex of saccharifying amylolytic enzyme preparations of β-amylase and pullulanase on the carbohydrate composition of maltose syrups.

Materials and methods

Materials

For the purposes of our research, corn starch and maltose hydrolysates of various degrees of saccharification were used.

Chemical reagents and enzymes

Hydrochloric acid (1 N concentration).

Amylolytic enzyme preparations: (1) Thermostable bacterial α-amylase Alphasin T7L from Genencor International B.V. (A Danisco Division), activity – 1240 units of α-amylase activity/g of enzyme preparation (units AA/g of EP), pH 5.5–7.0, optimal pH 5.8, temperature 60–110 °C, optimal temperature 108 °C (retains activity for 1 hour at a temperature of 105–108 °C); (2) β-amylase Optimalt BBA from Genencor International B.V. (A Danisco Division), activity – 69 units of β-amylase (saccharifying) activity/g of enzyme preparation (units SA/g of EP), pH 4.8–6.0, optimum pH 5.2–5.5, temperature 55–60 °C, optimum temperature 57 °C (retains activity for 6 hours at a temperature of 60–63 °C; inactivated in 15 minutes at a temperature of 75 °C); (3) Pullulanase Gamylozym KPU-L Gammazym (Germany), activity – 0.8 units of pullulanase activity/g of enzyme preparation (units PA/g of EP), pH 4.3–7.5, optimal pH 5.0–6.3, temperature 45– 68 °C, optimum temperature 55 °C (inactivated in 10 minutes at a temperature of 95 °C).

Research methods

The dry substances (DS) content, % by weight, in the hydrolysates and the finished product was determined refractometrically, while in the raw material it was determined by drying to constant weight (Nielsen, 2010).

The pH was determined potentiometrically (Manso et al., 2017).

Liquefaction of the starch suspension was performed under the influence of an ultrahigh frequency electromagnetic field or in a special thermostat under a slight excess pressure at a temperature of 110 °C (Nawaz et al., 2020) [JPH06102032B2].

The total content of reducing substances (% by weight of DS) – dextrose equivalent (DE) – was determined by the iodometric method of Wilshtetter and Schudl (Nielsen, 2010).

Activity of enzyme preparations

Amylolytic activity of bacterial α-amylase. Determination of amylolytic activity of bacterial α-amylase was performed by colorimetric method. The method is based on the hydrolysis of starch by enzymes of the amylolytic complex to dextrins of different molecular weight (Iskakova et al., 2017).

Amylolytic activity of α-amylase (AA) characterizes the ability of amylolytic enzymes to catalyze the hydrolysis of starch to dextrins of different molecular weight and is expressed by the number of unit's activity of these enzymes in 1 g of the enzyme preparation.

One unit of amylolytic activity is the ability of the enzyme at certain values of temperature, pH and time of action to catalyze the hydrolysis of 1 g of starch to dextrins of different molecular weight.

Amylolytic activity of thermostable EP was determined at a temperature of 50 °C. Hydrolysis of starch under these conditions occurred at 25–30%.

Saccharification activity of β-amylase. Determination of the saccharification activity of β-amylase (SA) was performed by the polarimetric method. The polarimetric method is based on determining the rate of the enzymatic reaction of starch hydrolysis, which is set by decreasing the angle of rotation of the polarization plane. This reduction is due to the formation of low molecular weight carbohydrates due to the action of enzymes on starch (Manisha et al., 2016).

One unit of saccharification activity is defined as the amount of enzyme which in strictly defined conditions (pH 4.7–4.9, temperature 50 °C, reaction time 30 min) catalyzes the hydrolysis of 1 g of starch (which is 25–30%) to low molecular weight carbohydrates.

To determine the saccharification activity, 1% of the starch solution is hydrolyzed and the polarization of the reaction mixture before and after the action of the enzyme is determined, thus establishing a decrease in the angle of rotation of the substrate polarization plane (ΔP) under the action of enzymes.

Pullulanase activity. Pullulanase activity (PA) is determined by the amount of reducing carbohydrates formed from the substrate (phosphodestrin) during the enzymatic reaction. The amount of reducing substances is determined by iodometric method (Meile et al., 2018). The number of units of activity of the enzyme taken for analysis is calculated according to the number of reducing carbohydrates that have been formed.

One unit of pullulanase activity is the amount of enzyme which in defined conditions (temperature 30 °C, pH 4.7–4.9, reaction time 60 min) catalyzes 30% hydrolysis of α-1,6-glucoside bonds in 1 g of phosphodiesters to reducing carbohydrates.

Carbohydrate composition of hydrolysates. The carbohydrate composition of the hydrolysates was determined by chromatographic analysis on a liquid chromatograph «Agilent 1100» (manufacturer – Agilent, USA). Concentration of working solutions was 3–5% DS; water served as eluent; eluent flow rate was 0.5 ml / min. Analytical columns were SupelCogel Ca (manufacturer – SupelCo, USA) and HPH 87C (manufacturer – Bio-Rad) size 300 × 7.8 mm for the separation of carbohydrates and alcohols. Adsorbent analytical column (filler, gel filtration resin) was styrene-divinylbenzene polymer in grafted calcium form with a particle diameter of 5 μm.

Sample injection was performed using a syringe and injector with a dosing loop of 10 μl. The column was thermostated in a thermostat maintaining a column temperature of 80 °C (±0.1 °C). To protect the column, a safety cartridge was used, which, in addition to cleaning the sample from impurities, also provides its demineralization from salts (primarily from sodium chloride).

Experiment 1. Determination of the optimal degree of starch suspension liquefaction with bacterial α-amylase for further saccharification of hydrolysates to maltose

To carry out the liquefaction in one-step, we used a thermostable enzyme preparation of bacterial α-amylase as a biocatalyst, which does not lose activity at high temperatures required for gelatinization of small starch grains (Gebremariam et al., 2013; Lin et al., 2013). This will significantly increase the rate of starch liquefaction. From a wide range of enzyme preparations of α-amylase of bacterial origin, Alphasin T7L was selected, which is a thermostable enzyme preparation with the highest optimal temperature 108 ºC, and the pH of 5.5–7.0.

The method of single-stage hydrolysis, which allows obtaining a hydrolyzate with residues of starch molecules of average size and has a number of advantages, was used. The use of thermostable enzyme preparation allows one not to lower the temperature below 95 ºC, which prevents retrogradation. This method of liquefaction also enables one to reduce the dosage of the enzyme preparation.

During the study, a suspension of corn starch with a mass fraction of 30% DS was prepared. For enzymatic liquefaction, the enzyme preparation of thermostable bacterial α-amylase Alphasin T7L was used, which was added in the recommended amount of 4 units AA/g DS of corn starch (Lin et al., 2013). To detect the maximum activity of the enzyme preparation, the optimal pH value is 5.8, which was achieved by adding 1 N HCl solution to the substrate. Thinning of the suspension was carried out in one stage under a slight excess pressure in a special thermostat at a temperature of 110 °C to different values of DE 10, 15, 20, 25%.

For saccharification, the mixture of β-amylase and pullulanase enzyme preparations was added to the obtained liquefied suspension in the amount of 0.12 units of saccharifying activity per gram of dry matter of starch (units SA/g) and 0.003 units of pullulanase activity per gram of dry matter of starch (units PA/g). Saccharifying lasted for 48 hours, during which samples were taken and the dextrose equivalent (DE) was determined.

Experiment 2. Study of saccharification kinetics depending on the dosage of saccharifying enzyme preparations of β-amylase and pullulanase

During the study, a suspension of corn starch with a dry substance content of 30% was prepared. The enzymatic liquefaction was performed as described in experiment 1.

The liquefaction time was 13 minutes before reaching the value of DE hydrolyzate in the range of 10–15%, which, according to experiment 1, was determined as optimal for further saccharification.

After liquefaction, the hydrolyzate was cooled to a temperature of 57 °C and the pH was adjusted to 5.2–5.5 by adding a solution of 1 N HCl. These parameters are optimal for the action of the enzyme preparation β-amylase Optimalt BBA and are within the optimum for pullulanase Gamylozym KPU-L (optimum effect at pH is 5.0–7.5 and temperature 45–68 °C), which also does not contradict the optimal parameters of β-amylase. Therefore, this enzyme preparation of pullulanase was chosen.

The saccharification process was performed by simultaneously adding to the substrate enzyme preparations of β-amylase and pullulanase in different amounts. Dosing of β-amylase was performed in all samples at the rate of 0.03 units of saccharifying activity per g of dry substances (units SA/g DS) of starch (Sabadash et al., 2015). Pullulanase was dosed in amounts of 0.00005; 0.0002; 0.0004; 0.0006; 0.0008; 0.005; 0.01; 0.02; 0.03 units of pullulanase activity per g of dry substances (units PA/g DS) of starch (Figs. 3, 4). Pullulanase was not added to the control sample. Enzymatic saccharification of hydrolysates was performed for 48 h in an EL-20R thermostat at a temperature of 57 °C with constant stirring. Samples of hydrolysates taken in the saccharification process at 1, 4, 8, 12, 16, 20, 24 and 48 hours were kept in a boiling water bath for 15 minutes to inactivate the enzyme. In chilled samples, the DE index was determined by the iodometric method of Wilshtetter and Schudl.

Results and discussion

Experiment 1. Influence of the degree of liquefaction of starch on the kinetics of subsequent saccharification of hydrolysates by enzymatic preparations of β-amylase and pullulanase

The kinetics of starch saccharification to maltose by a complex of β-amylase and pullulanase enzyme preparations was studied. The effect of DE after liquefaction on the saccharification of hydrolysates to maltose was determined. The duration of the experiment was 48 hours before reaching the maximum content of maltose in the hydrolyzate. The graphs of the kinetics of the saccharification process for the first twelve hours of the experiment for different values of DE, which were achieved during liquefaction, are presented in Figure 1. Figure 2 shows the effect of the degree of liquefaction on the glucose equivalent after 24 and 48 hours of saccharification.
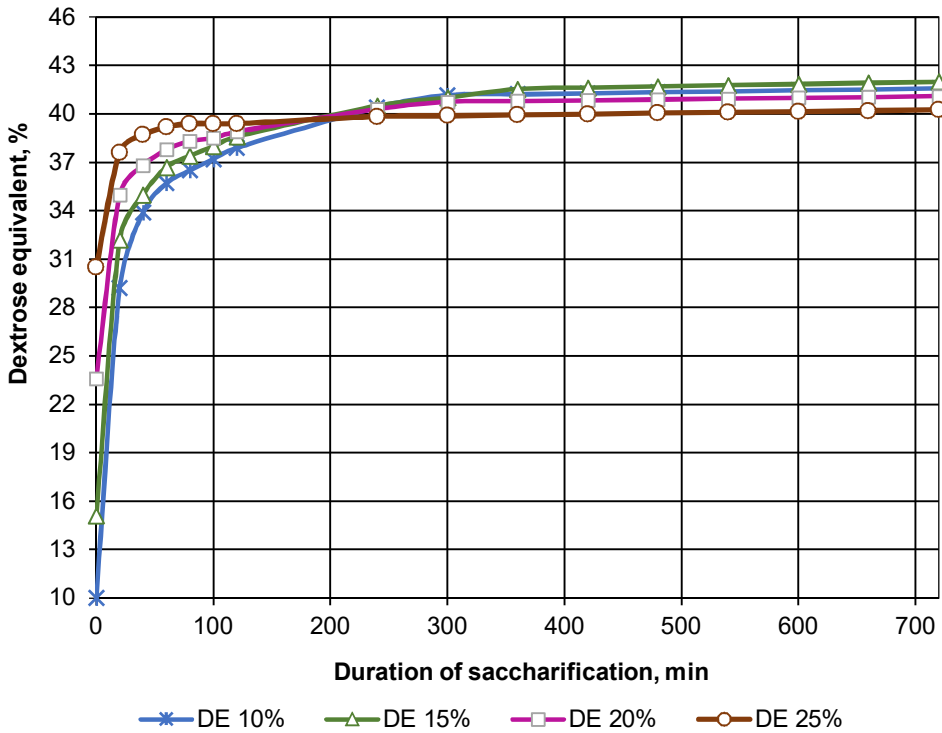
Figure 1. Kinetics of the process of enzymatic saccharification of liquefied starch hydrolysates at different DE values after liquefaction: 10; 15; 20; 25%
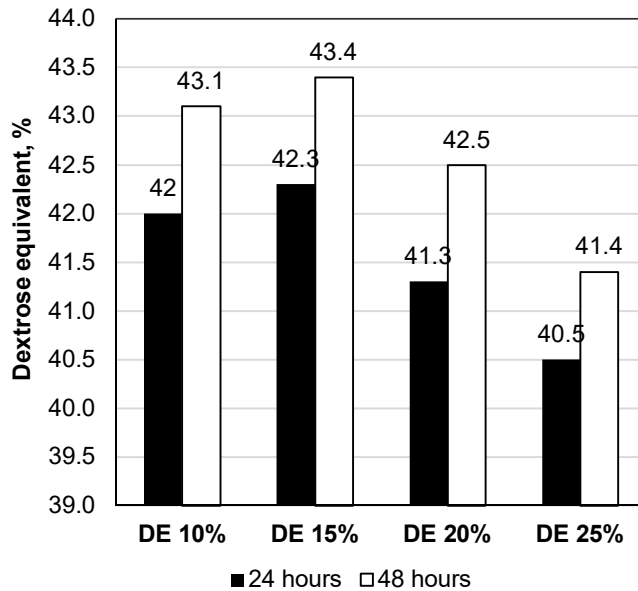

Figure 2. Effect of the degree of liquefaction on glucose equivalent after saccharification after 24 and 48 hours

At the beginning of the saccharification process, the increase in DE for all four samples occurs at different speeds, but after 3–4 hours from the beginning of the process the values of DE almost coincide (Figure 1). Continuation of the saccharification process leads to a gradual increase in the DE in the hydrolysates that had lower DE values after liquefaction (DE 10; 15%). That is, with a decrease in the degree of polymerization of hydrolyzate molecules after liquefaction (DE 20; 25%) there is a decrease in the rate of their cleavage during saccharification to maltose, because β-amylase has an affinity for high molecular weight substrate. In addition, the ability of pullulanase to cleave α-1,6-bonds is significantly increased if they are surrounded on all sides by α-1,4-bonds, which is possible with a low degree of liquefaction (DE 10; 15%). Low DE after liquefaction (DE 10; 15%) enables the formation of linear dextrins that are hydrolyzed by the maltogenic enzyme preparation of β-amylase (Souza et al., 2019; Gangadharan et al., 2009; Rittenauer et al., 2021), which allows to obtain hydrolysates with higher DE after saccharification (Figure 2).

However, samples with low DE values after liquefaction (10%) have a high viscosity of the suspension (Baks et al., 2008), which impairs its contact with the enzyme (Rittenauer et al., 2021) and complicates the saccharification process (Rittenauer et al., 2021). Also, the saccharification of high-molecular substrate (GE 10%) is complicated by its tendency to retrogradation (Souza et al., 2019).

For saccharification in the production of maltose syrups, it is desirable to use a liquefied hydrolyzate containing cleavage products of medium size. The degree of pre-dilution of starch has a significant effect on the liquefaction of hydrolysates to maltose, even minor changes in carbohydrate composition after liquefaction could slow down the saccharification process. Due to the presence of unwanted by-substances in the hydrolyzate, the quality of final maltose syrups deteriorates, their yield and maltose content decrease, as it is impossible to achieve sufficient complete saccharification of starch (Baks et al., 2008; Rittenauer et al., 2021). Therefore, the enzymatic liquefaction of the suspension should be carried out to the value of GE hydrolyzate 15% (Figure 2), which is optimal for further saccharification to maltose. The viscosity of such hydrolysates is significantly reduced (Souza et al., 2019), and the degree of polymerization of polysaccharide molecules remains high (Rittenauer et al., 2021).

Experiment 2. Study of the saccharification process depending on the dosage of enzyme preparations of β-amylase and pullulanase

According to experimental data, kinetic curves of the process of saccharification of liquefied starch with a complex of enzyme preparations of β-amylase and pullulanase depending on their dosage were constructed (Figure 3). Pullulanase was not added to the control sample. The research results are presented in Figures 3 and 4. The carbohydrate composition, in particular the maltose content in the obtained hydrolysates, was determined by the method of chromatographic analysis on a liquid chromatograph «Agilent 1100» (Figure 5).
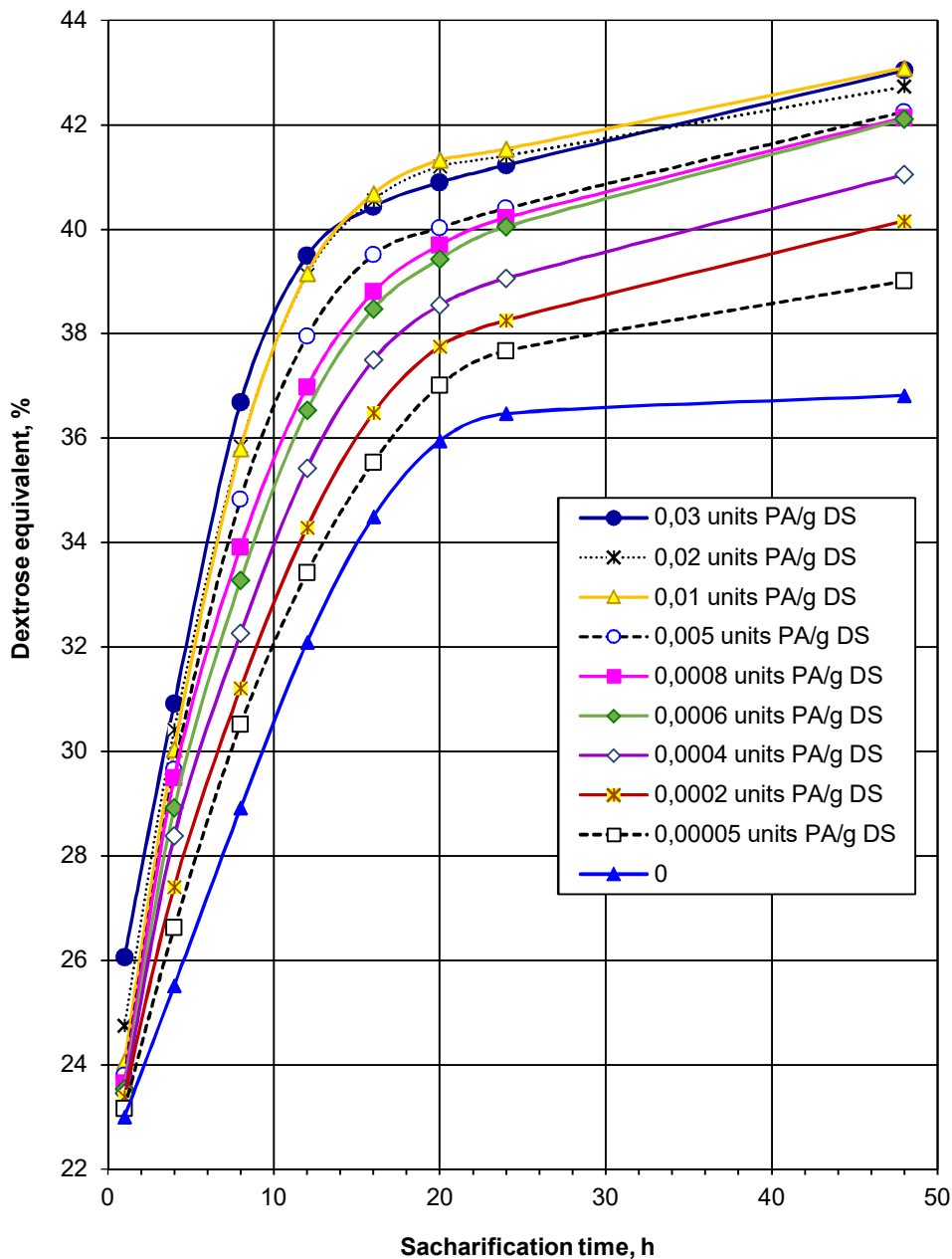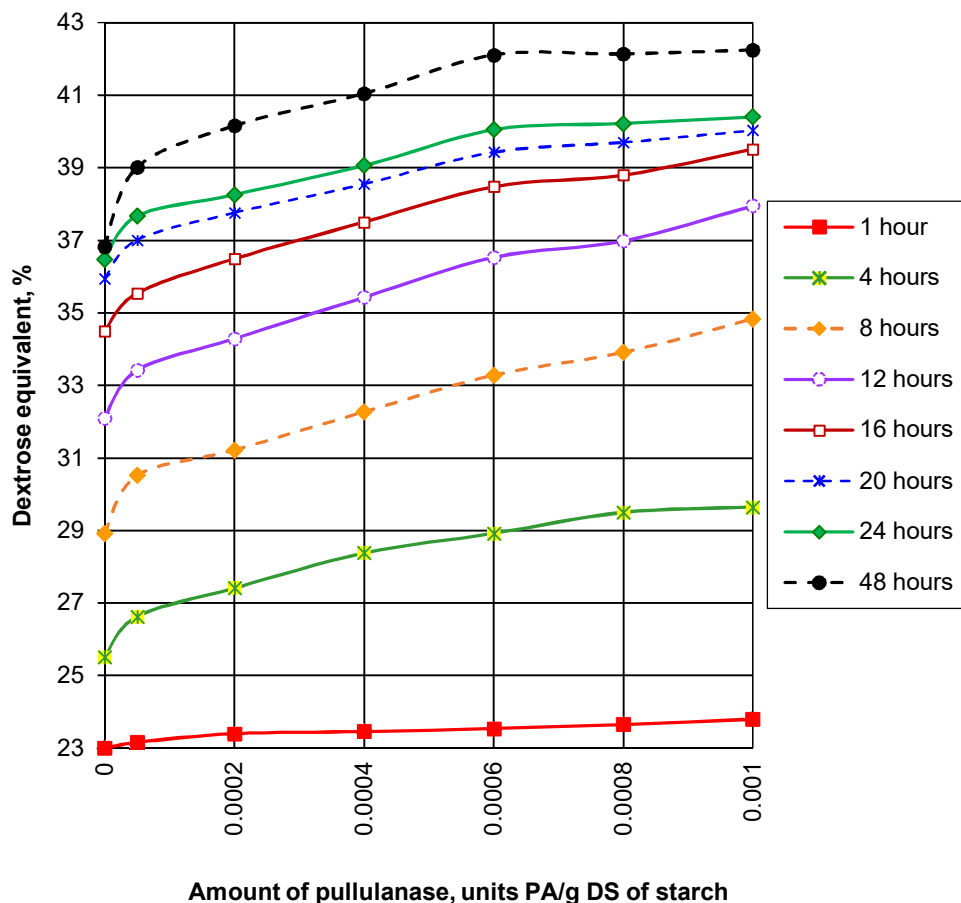
Figure 3. Kinetics of enzymatic saccharification of liquefied starch under the conditions of using a complex of enzymatic preparations of β-amylase (in the amount of 0.03 units SA/g DS of starch) and different amounts of pullulanase

Figure 4. Dependence of the glucose equivalent of maltose syrup on the amount of enzyme preparation of pullulanase, the amount of added β-amylase 0.03 units SA/g DS of starch

The data obtained (Figures 3, 4) show that the use of a complex of enzyme preparations of β-amylase and pullulanase improves the saccharification process, compared with the use of β-amylase only (control sample), and leads to increased maltose content in hydrolysates. This is due to the hydrolysis of dextrins to maltose (Table 1) (Lin et al., 2013). Depending on the consumption of pullulanase, DE increases by 1– 5% in 24 hours of saccharification (Figure 4). During the second day, there is an increase in DE by another 1.3– 2%, which does not happen in the control sample (Figure 4). It is most effective to use a complex of enzyme preparations of β-amylase and pullulanase at the rate of 0.03 SA/g and 0.0006 PA/g DS of starch, respectively. At the same time, after 24 hours of saccharification a DE value of 40% can be achieved. It is optimal to carry out the saccharification process for 24 hours.
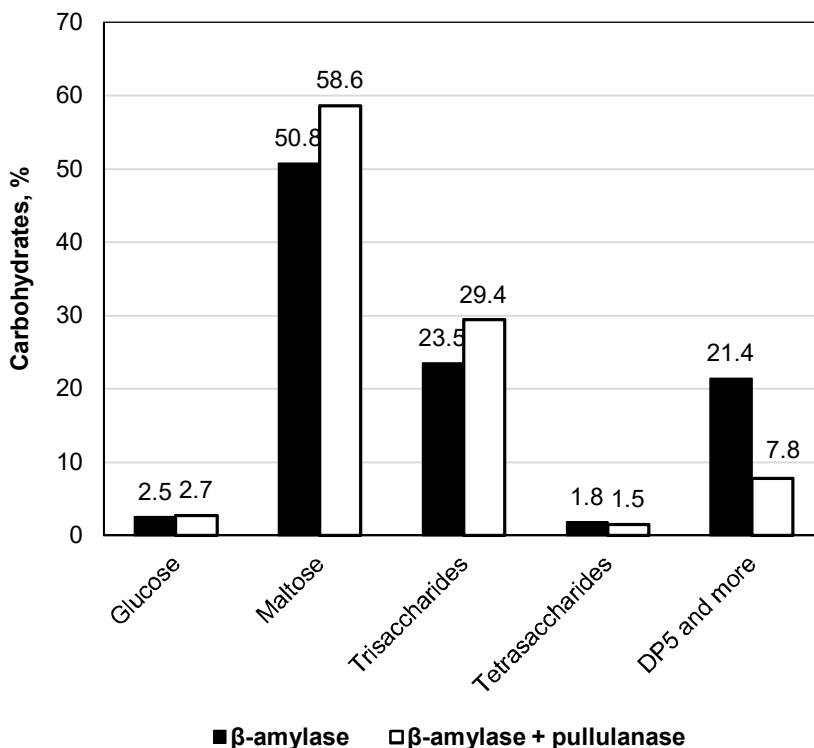
Table 1 shows the results of chromatographic studies of starch hydrolyzate obtained at the optimal dosage of β-amylase (control sample) and at the optimal dosage of the complex of enzyme preparations of β-amylase and pullulanase after 24 hours of saccharification.

Table 1

Results of chromatographic studies of starch hydrolysate obtained after 24 hours of saccharification

Carbohydrate composition of hydrolysates	Saccharification with β-amylase (at the rate of 0.03 units SA/g DS of starch) (control sample), % by weight of RS	Saccharification with a complex of enzymatic preparations of β-amylase (at the rate of 0.03 units SA/g DS of starch) and pullulanase (at the rate of 0.0006 units PA/g DS of starch) % by weight of RS
Glucose	2.5	2.7
Maltose	50.8	58.6
Trisaccharides	23.5	29.4
Tetrasaccharides	1.8	1.5
DP5 and more	21.4	7.8

According to the results of chromatographic analysis, the carbohydrate composition of maltose syrups was calculated, which is presented in the diagram (Figure 5).

Figure 5. Carbohydrate composition of maltose syrups obtained under optimal conditions and amounts of added saccharifying enzyme preparations: β-amylase (control sample) and a complex of β-amylase and pullulanase

These data make it possible to compare the carbohydrate composition of maltose syrups obtained using different saccharifying enzyme preparations: β-amylase (control sample) and mixture of β-amylase and pullulanase.

The results of chromatographic analysis allow to compare the carbohydrate composition of maltose hydrolysates obtained at the optimal dosage of the complex of enzyme preparations of β-amylase and pullulanase, with the control sample, in which saccharification occurred only with maltogenic enzyme preparation of β-amylase (Table 1). Figure 5 shows that the composition of starch hydrolysates after saccharification differs significantly depending on the combinations of saccharifying enzyme preparations. The introduction of pullulanase makes it possible to obtain syrups with a higher content of maltose (58.6%, compared with 50.8% in the absence of pullulanase). The accumulation of maltose occurs due to the rupture of α-1,6 glucoside bonds in amylopectin chains, which are not affected by the enzyme preparation of β-amylase (EP 0294093 A2). The hydrolysates obtained using pullulanase have a lower content of β-limiting dextrins (7.8%, compared with 21.4% in the absence of pullulanase). These dextrins are a source of additional maltose accumulation, as the substrate becomes available for β-amylase action in the absence of branching (Baks et al., 2008; Lin et al., 2013).

Based on the research results, the optimal parameters of the saccharification process of liquefied starch with a complex of enzyme preparations of β-amylase and pullulanase were determined. Using a complex of enzyme preparations it is possible to obtain maltose syrups with a maltose content of 60–62% and a DE value of 40–42% (Figure 3 and Table 1).

Conclusion

The kinetics of the process of enzymatic saccharification of starch hydrolysates depending on the obtained values of dextrose equivalent after liquefaction has been studied. It has been established that the highest value of dextrose equivalent in the process of saccharification to maltose is achieved at the optimal degree of liquefaction of the starch suspension, which corresponds to the value of glucose equivalent of 10–15%.

The kinetics of the process of enzymatic saccharification of liquefied starch by a complex of enzymatic preparations of β-amylase and pullulanase has been examined. It has been found out that these enzyme preparations are best used in quantities of 0.03 units SA/g DS and 0.0006 units PA/g DS of starch, respectively. It has been determined that with the 24 hours' duration of saccharification the content of maltose in the hydrolysates reaches 60–62% by weight of RS, with the content of glucose being insignificant (about 2.7% by weight of RS). Low glucose is a key indicator of the quality of syrups in the production of caramel and many other foods.

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