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A thin layer chromatographic comparison of raw and soluble starch hydrolysis patterns of some α-amylases from *Bacillus* sp. isolated in Serbia

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Abstract: Several natural isolates of *Bacillus* strains namely 5B, 12B, 16B, 18 and 24B were grown at two different temperatures in submerged fermentation for the production of raw-starch-digesting α-amylases. All strains except *Bacillus* sp. 18 produced more α-amylase at 37 °C. The hydrolysis of raw cornstarch followed the same pattern. Efficient hydrolysis was obtained with α-amylases from *Bacillus* sp. 5B, 12B, 16B and 24B grown at 37 °C and *Bacillus* sp. 18 grown at 50 °C. Zymography after isoelectric focusing showed that α-amylases were produced in multiple forms, from 2 to 6, depending on the strain when they were growing at 37 °C, while growth at 50 °C induced only 1 or 2 isoforms. Thin layer chromatography (TLC) analysis of the hydrolysis products of raw corn and soluble starch by α-amylases revealed the production of various mixtures of oligosaccharides. In most cases, G3 was the most dominant product from soluble starch while G2, G3 and G5 were the main products of raw starch hydrolysis. This indicates that the obtained α-amylases could be used for starch liquidification or short-chain-oligosaccharide formation, depending on the type of starch (raw or soluble) used for the hydrolysis.

Keywords: bacterial amylase; raw starch digestion; TLC; zymogram.

INTRODUCTION

Starch is the dominant carbohydrate reserve material of higher plants, being found in leaf chloroplasts and in the amyloplasts of storage organs such as seeds and tubers.[1] It constitutes an inexpensive source for the production of syrups containing glucose, fructose or maltose, which are widely used in the food, sweetener and ethanol industries.[2,3] Starch is a mixture of two polysaccharides,

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amylose and amylopectin, and contains small amounts of non-carbohydrate constituents, such as lipids, phosphates and proteins.[4] Starch granules from different botanical sources are of different sizes, shapes and physical properties.[5] Starch is insoluble in cold water and often resistant to chemical and enzymatic treatments.[6]

Conventionally, conversion of starch to glucose is a high temperature, liquid-phase enzymatic hydrolysis process that requires a high-energy input, resulting in increased production costs of starch-based products.[7] In view of energy costs, effective utilization of natural resources and viscosity problems, direct hydrolysis of starch below its gelatinization temperature is desirable.[2,6,7]

α-Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of the internal α-1,4-*O*-glycosidic bonds in polysaccharides with retention of the α-anomeric configuration in the products.[8] They are typical endoamylases and are often divided into two categories according to the degree of hydrolysis of the substrate. Saccharifying α-amylases hydrolyze 50 to 60 % while liquefying α-amylases cleave about 30 to 40 % of the glycosidic linkages of starch.[9] The end products of α-amylase action are oligosaccharides of varying length with the α-configuration and α-limit dextrins, which constitute branched oligosaccharides.[9] Amylases account for about 30 % of the world's enzyme production, with potential applications in starch liquefaction, the manufacture of maltose, high fructose-containing syrups, maltotetraose syrups, in the removal of starch from textiles, direct fermentation of starch to ethanol and in the treatment of starch processing water.[10] α-Amylases can be produced by different species of microorganisms, but for commercial applications, α-amylase is mainly derived from the genus *Bacillus*. *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications.[11] α-Amylase with suitable properties can be very useful in a specific industry; thus, it has become essential to characterize all available microbial strains for their productivity. Since almost all microorganisms of the *Bacillus* genus synthesize α-amylase, this genus has the potential to dominate the enzyme industry.[12] Various factors affect the efficiency of raw starch hydrolysis by amylases. They include granule dimensions and shape, amylose content, lipid and phosphate content, architecture of starch granules and the amylase source.[13]

Quantitative and qualitative estimation of malto-oligosaccharides produced by the action of α-amylases can be achieved by thin layer chromatography (TLC).[14] This method is very convenient for the characterization of amylase types defined by their mode of action that can be derived from the starch hydrolysis patterns. Based on the products from the hydrolysis visualized by TLC, selection of the amylase for application in a specified industry, such as production of ethanol or sweeteners, can be achieved.

The aim of this work was to compare raw corn and soluble starch hydrolysis patterns of some Serbian *Bacillus* sp. α-amylases in order to elucidate their potential industrial application.

EXPERIMENTAL

Chemicals

All reagents and solvents were of the highest available purity and at least of analytical grade. They were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. Raw cornstarch was isolated in our laboratory according to standard recommended procedure.

Production of α-amylase

Different soil and milk samples were taken from various regions of Serbia as a source of microorganisms that were identified as *Bacillus* sp.[15] according to the methods described in Bergey's Manual of Systematic Bacteriology.[16] α-Amylase was produced from different strains of *Bacillus* using a submerged fermentation described previously.[7] The strains were grown at 37 °C and 50 °C for the comparison of the effect of each temperature on the α-amylase production. Fermentation mediums containing α-amylase were used for the experiments.

α-Amylase activity assay

The α-amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis. The reaction mixture containing 0.05 mL of appropriately diluted enzyme and 0.45 mL of 1.0 % (w/v) soluble starch (Merck) in 50 mM phosphate buffer (pH 6.5) was incubated at 65 °C for 30 min. The amount of liberated reducing sugar was determined by the dinitrosalicylic acid (DNSA) method.[17] One unit of α-amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per min at 65 °C. Maltose was used to construct a standard curve.

Isoelectric focusing of α-amylase isoforms

Analytical isoelectric focusing was performed using Multiphor II Electrophoresis system (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's instruction. Focusing was realized on a 7.5 % acrylamide gel with ampholytes in the pH range 3.0–10.0, at 7 W constant power for 1.5 h at 10 °C. After isoelectric focusing, the α-amylases were detected by zymogram detection with I_2/KI staining solution according to a previously published method.[18] The α-amylase activity appeared as clear bands on a dark background.

Hydrolysis of raw corn and commercial soluble starch

The raw starch digestion ability of crude α-amylase extracts (10 % v/v) was investigated by measuring the hydrolysis of 1 % raw cornstarch granules in a short time period of 6 h at pH 6.5 and 65 °C. The degradation of the raw starch was monitored as previously described using the DNSA method, with maltose as the standard.[7] At appropriate time intervals, the hydrolysis reactions were stopped by centrifugation at 14000 rpm for 1 min in order to separate the non-hydrolyzed raw starch from the solution. Aliquots for TLC analysis were withdrawn and kept at –20 °C. Hydrolysis of the soluble starch was performed in the same way as the hydrolysis of raw starch, except that the hydrolysis lasted for 2 h since this was the time when the percent of hydrolysis was comparable to that obtained for the raw starch. After this time, the starches were no longer significantly hydrolyzed. The reaction of soluble starch hydrolysis was stopped by mixing aliquots with DNSA and further monitored as for the raw starch degradation.[7] Aliquots for TLC analysis were withdrawn and kept at –20 °C.

TLC analysis

Oligosaccharide separation was performed by horizontal thin layer chromatography on silica plates, 10 cm×10 cm (Silica Gel 60 F_{254}, Merck, Darmstadt, Germany), using a Camag horizontal HPTLC development chamber in the tank configuration. All plates were pre-washed with a mixture of methanol and water (7/1, v/v). Standard solution of the oligosaccharides (0.33 mg/mL each) was prepared in deionized water. Standard solutions and samples in appropriate dilution (20 μL) were applied in bands using an autosampler (Linomat 5, Camag). The employed mobile phase was mixture of *n*-butanol, ethanol and 0.1 % water solution of boric acid (5/4/3, v/v/v). All separations were realized at ambient temperature (22±2 °C). After drying, the plates were sprayed with diphenylamine–aniline–phosphoric acid reagent and then heated at 110 °C for 10 min.[19]

RESULTS AND DISCUSSION

The most commonly used *Bacillus* strains, such as *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus*, are reported to produce α-amylase usually at temperatures between 37 °C and 60 °C depending on the strain.[20–22] For this reason, it was necessary to test the growth of different wild type *Bacillus* sp. strains as well as the production of different α-amylase isoforms at 37 °C and 50 °C. The results showed that the production of α-amylases was dependant on the growth temperature for all tested strains, Fig. 1. When strains were grown at 37 °C, the highest enzyme activity was detected in a *Bacillus* sp. 16B fermentation broth. On the other hand, when the strains were grown at 50 °C, the highest enzyme activity was detected in the fermentation medium of *Bacillus* sp. 18. Moreover, all strains, except *Bacillus* sp. 18, produced more α-amylase when grown at 37 °C than when grown at 50 °C. The differences in α-amylase production between the strains regarding the temperatures used for their growth were expected. Different strains have different optimal conditions for the growth and enzyme production and individual optimization of cultural conditions is important for maximum production of the microbial strains.[12]

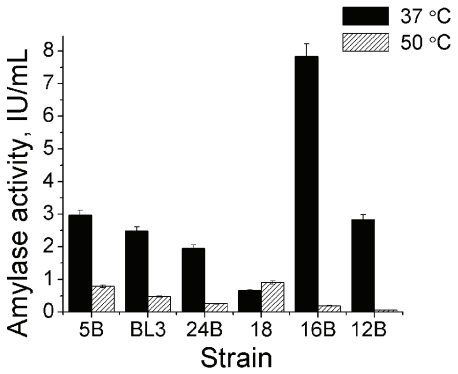

Fig. 1. Extracellular α-amylase activities of different *Bacillus* sp. strains. Each data point represents the mean of three independent assays.

The production of α-amylase isoforms by the specified strains were analyzed using zymography after isoelectric focusing. As could be seen from Fig. 2, diffe-

rent *Bacillus* sp. strains produced different isoforms of α-amylase. All the tested strains produced multiple forms of α-amylase, from 2 to 6, depending on the strain when growing at 37 °C, while only 1 or 2 isoforms were induced when growing at 50 °C.

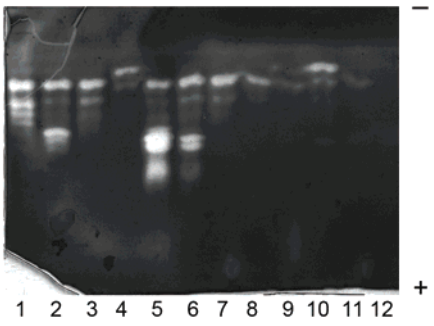

Fig. 2. Zymogram of α-amylases of different *Bacillus* sp. strains obtained after isoelectric focusing. Lanes 1–6: strains grown at 37 °C, lanes 7–12: strains grown at 50 °C. Lanes 1 and 7: *Bacillus* sp. 5B, lanes 2 and 8: *Bacillus* sp. BL3, lanes 3 and 9: *Bacillus* sp. 24B, lanes 4 and 10: *Bacillus* sp. 18, lanes 5 and 11: *Bacillus* sp. 16B and lanes 6 and 12: *Bacillus* sp. 12B.

The production of multiple isoforms of α-amylase by *Bacillus* sp., such as two extracellular α-amylase isoenzymes produced by *Bacillus* species WN11;[23] three extracellular α-amylase isoenzymes produced by *Bacillus* species B-3881[24] or six extracellular α-amylase isoenzymes produced by *B. licheniformis* ATCC 9945a,[7] was reported previously. The present study concerning *Bacillus* sp. α-amylase isoforms showed that isoforms were also detected when the strains were grown at different temperatures in solid-state fermentation on triticale (results not shown). Moreover, same strains produced different isoforms even at the same growth temperature due to different fermentation conditions. All these results are important from the viewpoint of the ability of specific isoforms to hydrolyze raw starch in comparison to the crude α-amylase activities obtained from the fermentation broth.

Many factors contribute to the efficiency of raw starch hydrolysis and one of them is the source of the employed enzyme.[13] The amount of hydrolysis of raw cornstarch by α-amylase produced by specified strains after 6 h at 65 °C are shown in Fig. 3. As expected, more starch was hydrolyzed by α-amylases produced by strains that were grown at 37 °C, since their α-amylases had higher activities. The exception was again *Bacillus* sp. 18 grown at 50 °C, when the α-amylase was more efficient in the hydrolysis of cornstarch than that obtained when the strain was grown at 37 °C.

The α-amylases from *Bacillus* sp. strains 5B and 18 were the most efficient in the hydrolysis of raw cornstarch when the strains were grown at 50 °C. On the other hand, the α-amylases from all *Bacillus* sp. strains grown on 37 °C were very efficient in the hydrolysis of raw starch; slightly better results were obtained for strains designated as 5B, 12B, 16B and 24B. Although the *Bacillus* sp. strain 16B produced the most active amylases at 37 °C (its activity was almost three

times higher that the activity of the amylases produced by the other strains), the degree of raw starch hydrolysis was not proportional. This is probably because the most active amylase isoforms produced by the strain 16B might not be the ones responsible for raw starch hydrolysis. In a previous study, it was found that only one isoform amongst several produced by SSF of strain 12B had the ability to hydrolyze raw starch.[15] It is very important to notice that the control strain in this study (BL3), *B. licheniformis* ATCC9945a, which was already shown to produce highly efficient α-amylase for the digestion of raw starch,[7] was less efficient than the tested wild type strains.

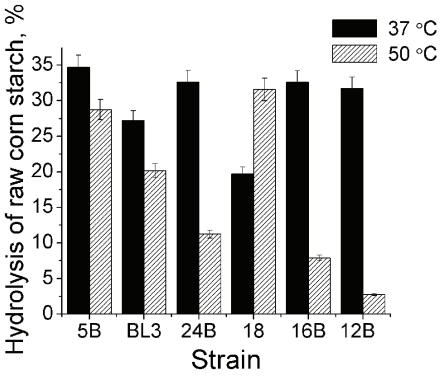

Fig. 3. Hydrolysis of raw cornstarch by α-amylases of different *Bacillus* sp. strains. Each data point represents the mean of three independent assays.

Due to the different reaction conditions used, comparison with other published results on the α-amylases from *Bacillus* sp. was difficult. However, for example, hydrolysis yields in a period of 5 h of raw cornstarch were 62 % for the α-amylase from *B. amyloliquefaciens*,[25] while after 6 h of hydrolysis at 60 °C, 15 % of cornstarch was digested by the α-amylase from *Bacillus* sp. GRE1.[26] Nevertheless, it is very important to emphasize that approximately 10–100 times lower enzyme doses were applied in the present study where 35 % of raw cornstarch was hydrolyzed after 6 h of incubation at 65 °C.

To determine hydrolysis products of soluble and raw corn starch by *Bacillus* sp. that were more efficient α-amylase producers (all strains grown at 37 °C except *Bacillus* sp. 18, for which the one grown at 50 °C was used for the analysis), distributions of the reaction products were analyzed by TLC.

From the results obtained and shown in Fig. 4, it can be seen that maltose (G2) and maltotriose (G3) were among the first produced when both starches were hydrolyzed. They were observed after only 15 minutes of hydrolysis. Other products appeared after 15 or 30 min and their concentrations only increased as the hydrolysis continued. They include oligosaccharides from G2 to G7 and minor amounts of G1 for all strains in the case of raw starch hydrolysis, while only α-amylase from *Bacillus* sp. 16B released glucose from soluble starch. As expected, the hydrolysis profiles differed between strains since they represent

different sources for the production of α-amylase. α-Amylases from *Bacillus* sp. 16B did not produce oligosaccharides > G5 regardless of the starch type (raw or soluble), while those from *Bacillus* sp. 24B produce only G2 and G3 when soluble starch was used.

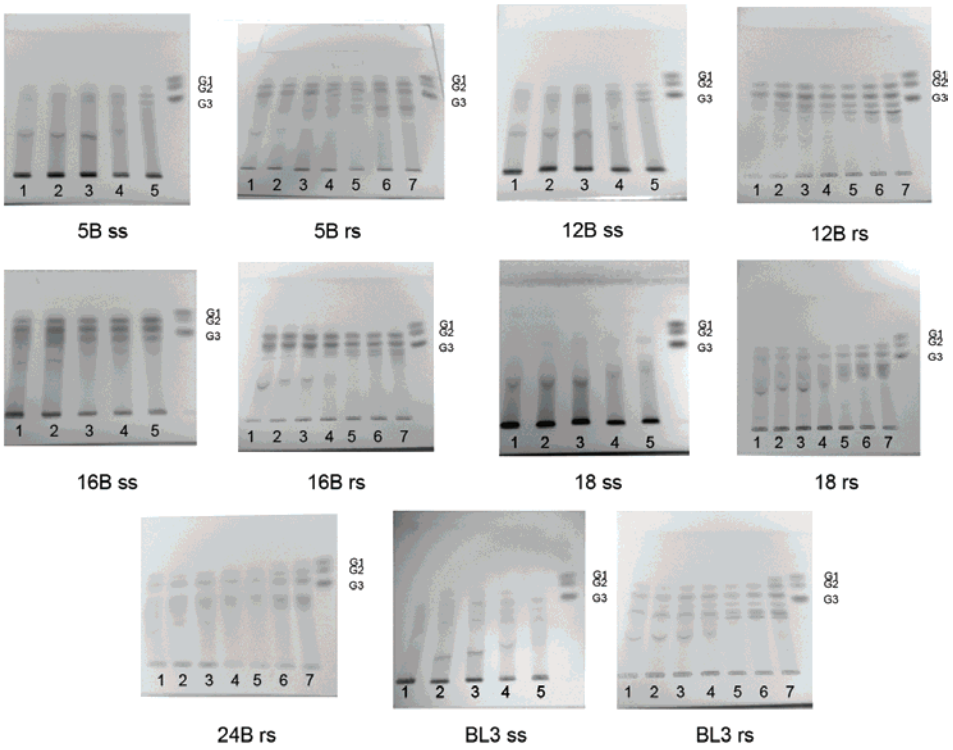

Fig. 4.TLC analysis of the time course of the hydrolytic products of soluble starch (ss) and raw starch (rs) by α-amylases of different *Bacillus* sp. strains at 37 °C and *Bacillus* sp. 18 at 50 °C. Lane 1: 15, lane 2: 30, lane 3: 45, lane 4: 60, lane 5: 120, lane 6: 240 and lane 7: 360 min. G1, G2, G3 represent standards. The curve-shaped bands visible at the area of slower mobility originated from the enzyme solutions, which was confirmed by TLC analysis of the fermentation broths alone (results not shown). The time course the hydrolytic products of soluble starch by α-amylases of *Bacillus* sp. 24B is not presented due to the very high background with hydrolysis product clearly visible only after 2 h of hydrolysis as shown in Fig. 5.

In general, less G4 was produced when the raw starch was hydrolyzed by the different amylases, Fig. 5. The end product profiles of the hydrolysis of soluble and raw starch by all the tested strains showed the formation of malto-oligosaccharides, Fig. 5, which indicates that the endo-mode of action was operative for all of the tested α-amylases on both raw and soluble starch. This random mode of action is typical for α-amylases.[27,28] Endo-amylases produce oligosaccharides of

different length, which was observed in this work and is in agreement with other published results.[27,29–31] The hydrolysis products ranged from G1 to G7 and these products are typical for endoamylolytic hydrolysis.[32] First products to appear were G2 and G3 for both raw and soluble starch. This was also observed for the α-amylase from *Geobacillus thermoleovorans*.[31] However, in the present study, the concentrations of all other products increased as the hydrolysis proceeded, especially in the hydrolysis of raw starch when finally G5 became dominant with G2 and G3 for all the strains, except for *Bacillus* sp. 16B. This observation was similar to those of others,[14,33] where G3 and G6 were the main products of hydrolysis of native cornstarch by α-amylase from *B. subtilis*. On the other hand, the hydrolysis patterns for soluble starch revealed that G3 was the most dominant product for all the strains, except for *Bacillus* sp. 16B where G2 and G3 were dominant.

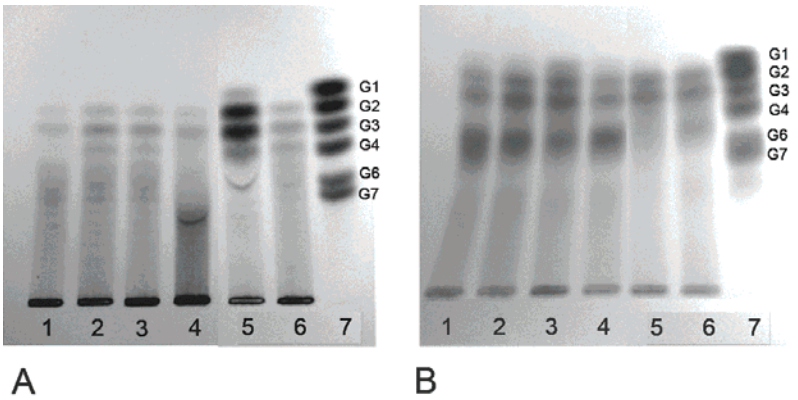

Fig. 5. TLC analysis of hydrolytic products of A) soluble starch and B) raw starch by α-amylases of different *Bacillus* sp. strains. Lane 1: *Bacillus* sp. 18 (50 °C), lane 2: *Bacillus* sp. 5B (37 °C), lane 3: *Bacillus* sp. BL3 (37 °C), lane 4: *Bacillus* sp. 24B (37 °C), lane 5: *Bacillus* sp. 16B (37 °C) and lane 6: *Bacillus* sp. 12B (37 °C). Lane 7: G1, G2, G3, G4, G6 and G7 represent standards. The curve-shaped bands visible in samples 4 and 5, panel A, originated from the enzyme solutions, which was confirmed by TLC analysis of the fermentation broths alone (results not shown).

Based on the described results, it seems that the α-amylases described in this study belong to the liquefying α-amylase type since there are many end products from hydrolysis of both types of starch. The liquefying α-amylase from *B. amyloliquefaciens* was reported to produce maltosaccharides predominantly while the saccharifying enzyme from *B. subtilis* yielded mostly glucose and maltose from starch.[34] With respect to the major products of hydrolysis, the α-amylases tested here might be useful for starch liquefaction or formation of short-chain-oligosaccharide, and specific application might be directed by the type of starch (raw or soluble) that was to be hydrolyzed.

CONCLUSIONS

The present study showed that the tested *Bacillus* sp. strains produced highly efficient raw starch digesting α-amylases able to hydrolyze raw cornstarch at a temperature below the temperature of gelatinization. TLC analysis of the hydrolyzed products showed that oligosaccharides from G2 to G7 were obtained when raw or soluble starches were used for the hydrolysis,. In most cases, G3 was preferentially produced from soluble starch while G2, G3 and G5 were the main products of raw starch hydrolysis. G1 was produced from raw starch only by the α-amylase from *Bacillus* sp. 16B. The advantages of the α-amylases from *Bacillus* sp. 5B, 12B, 16B, 18 and 24B compared to the previously reported ones are related to a high hydrolytic affinity towards raw cornstarch granules, the wide range of oligosaccharides produced by their action on starch as well as different hydrolysis pattern obtained after digestion of raw and soluble starch. This indicates that obtained α-amylases could be used for starch liquefaction and the formation of short-chain-oligosaccharides.

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ИЗВОД

ПРИМЕНА ТАНКОСЛОЈНЕ ХРОМАТОГРАФИЈЕ ЗА ПОРЕЂЕЊЕ ПРОИЗВОДА ХИДРОЛИЗЕ СИРОВОГ И РАСТВОРНОГ СКРОБА α-АМИЛАЗАМА СОЈЕВА *Bacillus* ИЗОЛОВАНИХ У СРБИЈИ

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Неколико природних изолата сојева *Bacillus* названих 5B, 12B, 16B, 18 и 24B су гајени на две различите температуре течном ферментацијом ради продукције α-амилаза које хидролизују сирови скроб. Сви сојеви осим *Bacillus* sp. 18 су продуковали више α-амилазе на 37 °C. Хидролиза сировог скроба је пратила исту шему. Ефикасна хидролиза је остварена са α-амилазама из *Bacillus* sp. 5B, 12B, 16B и 24B који су гајени на 37 °C и *Bacillus* sp. 18 гајен на 50 °C. Зимограмска детекција након изоелектричног фокусирања је показала да су α-амилазе продуковане у више изоформи, од 2 до 6, зависно од соја када су гајени на 37 °C, док је гајење на 50 °C индуковало само 1 или 2 изоформе. TLC анализом продуката хидролизе сировог кукурузног и растворног скроба α-амилазама показана је продукција различитих смеша олигосахарида. У већини случајева G3 је био најдоминантнији продукт из растворног скроба док су G2, G3 и G5 били главни продукти хидролизе сировог скроба. Ово указује на то да се добијене α-амилазе могу користити за отечњавање скроба и продукцију кратко-ланчаних олигосахарида у зависности од тога који тип скроба (сирови или растворни) је коришћен за хидролизу.

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