Final report of TNN_16-123305
Investigation of complex utilisation of agricultural and agro-industrial by-products by biotechnological methods

I. Selection and investigation of the potential raw materials

In the first period of the project different agro-industrial residues were investigated regarding their composition, availability in Hungary (and Thailand), and potential of value-added utilisation in a biorefinery process. In the first round, wheat bran and rice straw were selected as potential raw materials to be investigated in the project. Wheat bran is a cheap agro-industrial by-product deriving from the milling process of wheat, and it is widely available in Hungary. Rice straw has a very promising composition for biorefining and it is widely available in the collaborating country (Thailand). Latter, further biomasses were included in the investigations, such as brewer's spent grain and corn fibre. Both biomasses are produced in Hungary as poorly-valorised by-products in large quantities, thus they could also be potential raw materials for high-value valorisation in an appropriate biorefinery process. Wheat bran, brewer's spent grain and corn fibre were kindly donated by Hungarian producers, while rice straw was received from the collaborating partner (from Thailand). Composition of the selected raw materials was determined using the method of National Renewable Energy Laboratory (NREL) with minor modifications, and by using laboratory protocols developed by our research group. The composition of the different raw materials is shown in Table 1.

Table 1. The composition of different raw materials

	Wheat bran I.	Brewers spent grain	Corn fibre	Wheat bran II.	Rice straw
	Percentage of dry matter (%)				
Glucan	22.2	17.9	36.0	26.6	38.8
Starch	8.7	3.2	16.0	15.8	n.m
Xylan	20.7	15.1	15.0	16.8	19.4
Arabinan	11.4	7.4	8.0	9.5	3.8
Protein	n.m.	32.6	15.7	17.4	3.7
Klason-lignin	11.9	19.0	8.0	6.4	14.0
Ash	7.4	4.0	8.6	5.7	13.2
Other	26.4	4.0	8.7	17.6	7.1

n.m. – not measured

During the experiments, high amount of wheat bran was processed, thus a new batch from this raw material was needed. The first and second batches are named as wheat bran I and II, respectively. The composition of the two batches was different, since it can be influenced by several factors, such as wheat variety, harvest time and processing method.

The composition of the different raw materials differed significantly, thus it was necessary to develop and optimise fractionation methods for all different raw materials. The total carbohydrate content of the selected raw materials was high (around 40-60%) with considerable amount of arabinose. Thus, an extensive literature research was accomplished in the field of novel biotechnological applications and productions of L-arabinose form agro-industrial residues, and the selective removal of arabinose during the planned fractionation processes was addressed [1]. Arabinose can be a high value product for the food industry or a high-value intermediate molecule for the pharmaceutical industry.

II. Development and optimisation of acid-based fractionation processes

Fractionation processes of the different raw materials were examined in order to produce arabinose- and xylose-rich or arabinoxylo-oligosaccharide (AXOS)-rich liquid fractions and cellulose-rich solid fractions. The developed and optimised fractionation methods included acid-catalysed hydrolytic process steps.

Acidic-fractionation of wheat bran [2]

A two-step acidic fractionation of wheat bran I was developed by using sulfuric acid. The aim of the first step of the acidic fractionation was to produce AXOS-rich liquid fraction or arabinose-rich hydrolysate. The optimization of the first step was carried out according to a full factorial orthogonal design of experiments with quadruplicate in the centre point, in which the independent parameters were the concentration of sulphuric acid and the reaction time. The design space (ranges of acid concentration and reaction time), the reaction temperature and the dry matter content of the reaction mixtures were determined based on preliminary experiments. The designed experiments were carried out at 90°C and 10% dry matter. The sulphuric acid concentration and reaction time were set to be 0.25%, 1.25%, 2.25% and 25, 75, 125 minutes. The obtained liquid fractions were analysed for monomer sugars (xylose, arabinose, glucose) and oligosaccharides. Oligosaccharides were determined as monomer sugars after an acidic treatment decomposing oligosaccharides into monomers. The results of the designed experiments were evaluated by StatisticaTM v.13 (Statsoft®, Tulsa, USA) software. Statistica software was used to fit a second-order polynomial model for the measured data, and to enable the analysis of variance. The goodness of the fitted model was also checked. In order to determine the optimum condition in terms of two different response variables simultaneously, a desirability function approach was applied. The selected response variables were the followings: monomer glucose yield, monomer xylose yield, monomer arabinose yield, yield of solubilised xylose as part of oligosaccharides, yield of solubilised arabinose as part of oligosaccharides, yield of solubilised glucose as part of oligosaccharides. According to the statistical optimisation, the best conditions to solubilise high amount of AXOS but low amount of monomer sugars are 1.75% sulphuric acid concentration and 25 minutes reaction time. However, even in that case the monomer sugar concentration was higher than the concentration of AXOS. In order to maximise the amount of solubilised arabinose and minimize the amount of solubilised xylose, the best conditions proposed by the fitted model were the followings: 1.16% sulphuric acid and 50 minutes reaction time or 1.61% sulphuric acid and 47 minutes reaction time. In the case of 1.16% sulphuric acid and 50 minutes reaction time, the achieved arabinose, xylose and glucose yields were 63%, 42% and 49%, respectively. In the case of 1.61% sulphuric acid and 47 minutes reaction time, the achieved arabinose, xylose and glucose yields were 79%, 50% and 53%, respectively. The solubilised sugars occurred mainly in the form of oligosaccharides hence an oligomer hydrolysis step was required to obtain arabinose-rich hydrolysate. The oligomer hydrolysis was achieved by a treatment at 120°C and 1 hour reaction time. The proposed yields, hence the fitted models, were verified by independent measurements. According to these results, mild acidic treatment of wheat bran was appropriate to produce a liquid fraction rich in arabinose (and glucose) while the main part of the xylan remained in the solid residue. The obtained solid residue was treated in the second-acidic step of the fractionation process in order to obtain xylose-rich hydrolysate. For this, a 1 w/w% sulfuric acid-treatment was applied at 120°C for 30 minutes by using 7.5 w/w% dry matter content, based on preliminary experiments. As a result, the following hydrolysates were obtained in

the case of solid residues after the 1.16 w/w% and 1.61 w/w% sulphuric acid treatment: 0.9 g/L glucose 22.2 g/L xylose, 9.9 g/L arabinose and 0.9 g/L glucose 21.3 g/L xylose 7.7 g/L arabinose, respectively. These results showed that xylose-rich hydrolysates were successfully produced in the second acidic step. Compositional analysis of these solid residues of the second acidic step showed that the glucan contents were increased up to 50 w/w% of dry matter. Hence, those solid fractions can be called as cellulose-rich solid residues.

A one-step acid hydrolysis was also investigated to produce a cellulose-rich solid fraction from wheat bran. One-step acidic hydrolysis was investigated for two reasons: preliminary techno-economical evaluations suggested that a one-step process might have better potential regarding the economic viability of a biorefinery process valorising wheat bran; the one-step process allows a more efficient production of cellulose-rich fraction that is needed for the collaborating partner. Wheat bran was treated with different sulphuric acid concentrations (1.2; 1.5; 1.75%) at 121°C for 30 minutes with 10% dry matter content. The yield of the hemicellulosic sugars was more than 90% and the starch was totally solubilised. By increasing the time of the treatment at 1.5% sulphuric acid concentration, the hemicellulose fraction could totally solubilise resulting in a xylose-rich hydrolysate and a cellulose-rich solid residue with 41-45% of cellulose. The cellulose-rich solid fractions were sent to the collaborating partner to investigate its enzymatic digestibility and the production of 2,3-butanediol.

For the prebiotic investigations an AXOS-rich liquid fraction was required. In order to produce AXOS-rich liquid fractions by acidic treatments of wheat bran, desirability function approach was used to determine the appropriate process conditions based on the previously developed models. Two settings were selected to produce AXOS-rich fractions: 1.75% sulphuric acid treatment for 25 min and 0.25% sulphuric acid treatment for 25 min.

Acid fractionation of brewer's spent grain [3]

Based on the composition of brewer's spent grain, it could also be a potential raw material for the two-step acid hydrolysis to separate the arabinose and xylose fractions. A similar two-step acidic fractionation method was developed and optimized that is in the case of wheat bran. During the optimisation of the first acidic hydrolysis aiming the solubilisation of arabinose, the sulfuric acid concentrations were 0.5, 1.25 and 2.0 w/w% and the reaction times were 10, 30 and 50 minutes, based on preliminary experiments. Optimisation and investigation of the interactions between the process parameters were also accomplished by Statistica software. Second-polynomial models were fitted and were validated experimentally. The first acid hydrolysis was also optimised by using desirability function approach. The following conditions were determined as optimal conditions for the first acidic treatment of brewer's spent grain: 1.85 w/w% sulfuric acid concentration and 19.5 minutes reaction time. The arabinose enriched hydrolysate contained 0.4 g/L xylose, 6.2 g/L arabinose, and 6.6 g/L AXOS. To obtain xylose-rich hydrolysate, second acidic hydrolysis was performed at 10% dry matter content under the same conditions which was used for wheat bran. The second step of the fractionation resulted in a xylose-rich liquid fraction and a cellulose-rich solid fraction containing 14.5 g/L xylose and 32.2% cellulose, respectively. Thus a two-step fractionation process was also successfully developed for brewer's spent grain.

Acid fractionation of rice straw [4]

The acid fractionation process was also optimised for rice straw to obtain a xylose-rich hydrolysate and a cellulose-rich solid residue. In this case, separation of arabinose was not investigated, since the rice straw had very low arabinose content (3.8%). Two types of acidic treatments were tested to solubilize the xylose. One was a phosphoric acid treatment suggested by the collaborating partner. The hydrolysis was performed by soaking the rice straw a 2 N phosphoric acid for 4 hours followed by autoclaving (121°C, 30 min). The initial dry matter content was 10 w/v%. In the other case, a sulfuric acid treatment with 1.5 w/w% sulfuric acid was applied for 30 minutes in an autoclave, based on preliminary experiments. The initial dry matter content was 10 w/w%. The experiments were also performed with ground and fine-ground rice straw. According to the statistical evaluation the particle size did not cause significant differences in the xylose yields. However, there was a significant difference between the xylose yields obtained by the two acidic treatments. The xylose yields of sulfuric acid treatment (91–94%) were much higher than that of the phosphoric acid treatment (68–69%). Thus, sulfuric acid treatment was found to be more suitable to produce a xylose-rich hydrolysate. The sugar concentrations of the sulfuric acid hydrolysate were the followings: 5.5 g/L glucose 20.6 g/L xylose and 3.9 g/L arabinose. The solid residue after the sulphuric acid treatment contained 54.4% glucan, 8.4% xylan, 1.3% arabinan and 18.1 Klason-lignin. Hence a cellulose-rich solid fraction remained after the acidic hydrolysis step.

Enzymatic fractionation of corn fibre [5]

Acidic fractionation of corn fibre was investigated in a previous research project (OTKA PD-1080389) by our research group. Within the actual research project, an enzymatic fractionation process using subsequent hemicellulase and cellulase hydrolyses on soaking in aqueous ammonia-treated corn fibre was investigated and compared with the previously developed acidic fractionation. The main goal was to compare the two methods in terms of their applicability to produce a glucose-rich liquid fraction for bioethanol production. Acidic fraction was found to be favourable in terms of bioethanol production, however, enzymatic fractionation could be useful for other biorefinery valorisations.

Enzymatic fractionation of other lignocellulosic biomass [6]

The investigations of the applicability of enzymatic fractionation processes were extended by using other plant-derived materials (such as pollen grains) for specific applications. Selective removal of the cellulosic intine wall of pollen grains by using chemical pre-treatment and enzymatic hydrolysis steps was investigated and developed in order to obtain purified sporopollenin exine shells. Purified exin shells have many possible and promising applications in the pharmaceutical and food industry. Thus an environmental friendly and cost effective purification method based on enzymatic fractionation is of great importance.

III. Optimization of xylitol fermentation by using *Candida boidinii* NCAIM Y.01308 strain

Optimisation of xylitol fermentation on semi-defined medium: investigation the effects of aeration and initial xylose concentration [4]

The wild strain of *Candida boidinii* NCAIM Y.01308 was investigated in xylitol fermentation experiments. The efficiency of xylitol fermentation highly depends on the aeration and initial

xylose concentration. Hence, the effect of the oxygen transfer rate (OTR) (or specific oxygen uptake rate) and initial xylose concentration on the maximum xylitol yield, xylitol yield after 24 hours and xylitol volumetric productivity were investigated through a full factorial orthogonal design evaluated by statistical methods. The levels of OTR and initial xylose concentration were 1.1, 2.1 and 3.1 mmolO_2/(L×h) and 30, 55 and 85 g/L, respectively. The experiments were carried out in 100 mL Erlenmeyer flasks on semi-defined xylose medium, where the OTR was influenced by the working volume (35, 50 and 65 mL). The results of the designed experiments were also evaluated by StatisticaTM v.13 (Statsoft®, Tulsa, USA) software. Investigating the maximum xylitol yield, the low xylose concentrations as well as the low aeration levels were favourable for achieving high xylose yields. Similar results were observed in the case of the xylitol yield after 24 hours. It is confirmed that microaerobic condition is advantageous for xylitol fermentation and the high initial xylose concentration has a negative effect on the xylitol yield. Investigating the maximum xylitol productivity, the statistical analysis predicted a maximum point at 71 g/L initial xylose concentration and 2.6 mmol O_2/(L×h) OTR. The statistical evaluation of the maximum xylitol productivity also showed that the aeration and initial xylose concentration were not independent from each other; there was an interaction between the two factors. During the xylitol fermentation, *Candida boidinii* NCAIM Y.01308 also produced ethanol; however, it was totally consumed by the end of the fermentations.
To check the reliability of the obtained and fitted models, xylitol fermentations were performed at two independent points on semi-defined xylose medium. Firstly at the point of 30 g/L of initial xylose concentration and 2.1 mmol O_2/(L×h) OTR value. Since the experimental design has already contained this point, the reproducibility of xylitol fermentation experiments was also investigated at this point. The other selected point was at 71 g/L initial xylose concentration and 2.1 mmol O_2/(L×h) OTR to verify the obtained models. The experimental results were in the prediction intervals in all of the cases which were determined by the fitted models. Hence, xylitol yields and productivities were proved to be predictable and reproducible within the design space.

On the other hand, scaling-up experiments were not successful in bench-scale bioreactor on semi-defined xylose medium. Several experiments were performed with 1-3 mmol O_2/(L×h) OTR and 30 g/L initial xylose concentration at 300 mL working volume. However, the maximum xylitol yields were only between 30-40%. One of the fermentation profiles of xylitol fermentation in bioreactor is shown in Figure 1. The results, which were obtained in bioreactor, have not published yet.

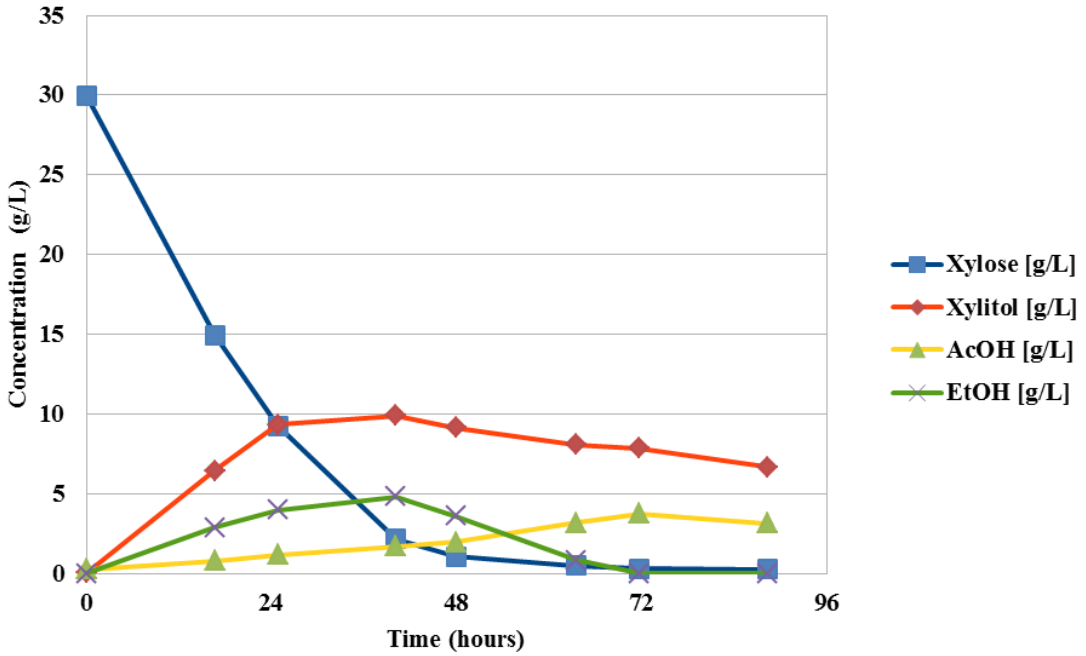


Figure 1. The fermentation profile of xylitol production in bioreactor (30 g/L initial xylose concentration 1.1 mmol O2/(L×h) OTR)

Investigation the efficiency of xylitol production on xylose-rich lignocellulose-based hydrolysates [2,4]

Xylitol fermentations were also performed on xylose-rich hydrolysates in order to investigate the efficiency of the xylitol fermentation. Firstly, the investigation of xylitol fermentation on wheat bran hydrolysates was performed. Fermentations were carried out by using *Candida boidinii* NCAIM Y.01308, however, *Ogataea zsoltii* NCAIM Y.01540 was also included in the project, since preliminary experiments have revealed that it has a very good ability to produce xylitol on lignocellulose-based hydrolysates. The maximum xylitol yields and productivities were 56-57% and 0.34-0.35 g/(L×h) on xylose-rich wheat bran hydrolysates by using *O. zsoltii*. Xylitol fermentations by *C. boidinii* were performed on the hydrolysate obtained from 1 w/w% sulfuric acid-treatment of the solid residue of wheat bran hydrolysis with 1.16 w/w% sulfuric acid. This wheat bran hydrolysate was chosen, because its initial xylose concentration was higher than the others. The OTR value of the hydrolysate was measured in order to investigate the applicability of the previously established models on wheat bran hydrolysate, which was 1.6 mmol O_2/(L×h). During the fermentation, the highest xylitol yield was reached after 24 hours, resulting in 60% xylitol yield (14.2 g/L xylitol concentration). The maximum xylitol productivity was 0.58 g/(L×h). Compared to *O. zsoltii* strain, *C. boidnii* NCAIM Y.01308 showed better results under the same conditions. The obtained experimental results were also compared to the values predicted by the models. The predicted values were in accord with the experimentally obtained values. Hence, the models implemented on semi-defined medium could be used with a good reliability for fermentations on wheat bran hydrolysate, and the efficiency of the fermentation (yield, productivity) could be predicted.

Xylitol fermentation experiments were also performed on xylose-rich rice straw hydrolysate. Ground rice straw was hydrolysed by 1.5 w/w% sulfuric acid, and the supernatant was used for xylitol production. The maximum xylitol yield was 20% of theoretical and it was obtained after 72 hours. The xylitol yield after 24 hours was almost the same as the maximum xylose

yield (19%), but in this case 8 g/L xylose remained in the hydrolysate. The maximum xylitol productivity also showed very low value: 0.24 g/(L×h). These results were far from the values predicted by the models. It is also important to note that a significant amount of ethanol was formed during the fermentations (8.4 g/L), which was much higher than the achieved xylitol concentration (3.8 g/L). The initial xylose concentrations were almost the same in both rice straw and wheat bran hydrolysates. Concentrations of other components which could have inhibitory effects on xylitol fermentation (acetic acid, formic acid, furfural, HMF, phenols) and the protein contents were also determined. The amounts of inhibitors were significantly higher in the case of rice straw hydrolysate. There was also a significant difference in the protein concentrations of the hydrolysates. Only 0.3 g/L protein was analysed in the rice straw hydrolysate, which could lead to decreased xylitol production. Thus, xylitol fermentation on rice straw hydrolysate was repeated by nitrogen source supplementation and detoxification. Due to this, xylitol yield and productivity were successfully increased compared to the untreated rice straw hydrolysate. The maximum xylitol yield, the xylitol yield after 24 hours and the maximum productivity were 30%, 29% and 0.19 g/ (L×h), respectively. Unfortunately, these results were still far from the expected and predicted results by the models.

Similar results were obtained on BSG hydrolysate than on rice straw hydrolysate. During xylitol fermentation on BSG hydrolysate, maximum xylitol yield of 19.8% was achieved, which was far from the expected value. BSG hydrolysate also contained high amount of inhibitory compounds. Thus, detoxification of the BSG hydrolysate prior xylitol fermentation was examined, and it resulted in an increase in the maximum xylitol yield (26.9%). However, this increased yield was still far from the ones achieved on wheat bran hydrolysates or xylose-containing semi-synthetic media.

Improvement the xylitol production of *Candida boidinii* NCAIM Y.01308

Candida boidinii NCAIM Y.01308 was handed over to the collaborating partner, where its ability to utilize high concentration of xylose and produce xylitol was increased by metabolic evolution method. Fermentation experiments were carried out by the modified strain (labelled as *Candida boidinii* TF45) at 100 g/L initial xylose concentration. Compared to the wild-type strain, it was found that the modified strain assimilated the xylose more rapidly, however, some xylose remained in the broth after 168 h. The xylose yield corrected with the remained xylose was 0.94 g/g for the modified strain and 0.7 g/g for the wild-type strain. The fermentation profiles of the fermentations are shown in Figure 2. The modified strain was handed over by the collaborating partner; however, a bit smaller xylitol yields were achieved in Hungary compared to that reported by the collaborating partner. New batches of the modified strain were requested to reaffirm the improved xylitol production by the modified strain in our laboratory. Results achieved by the modified strain of *C. boidinii* have not been published yet however a manuscript is under preparation.

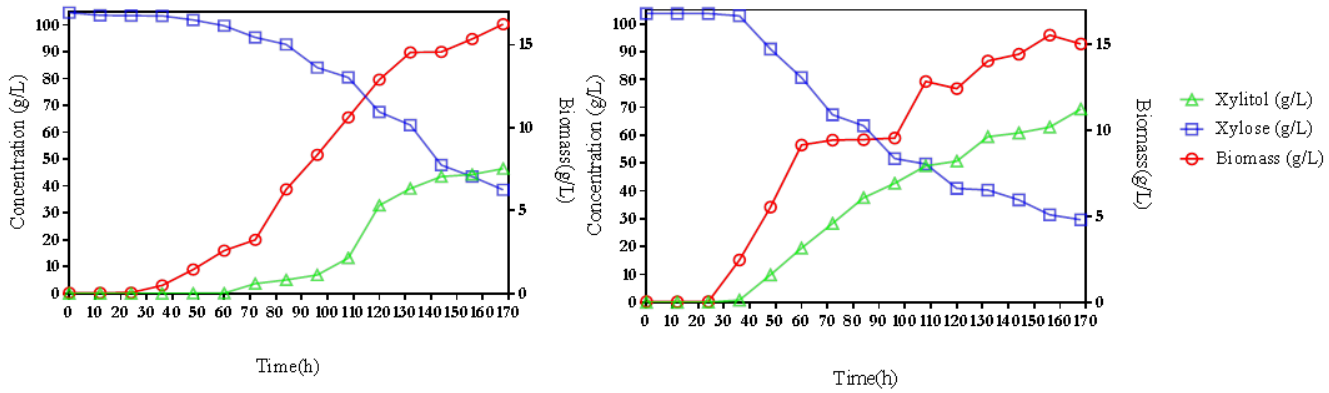


Figure 2. Batch fermentation profiles of wild type *C. boidinii* (left) and *C. boidinii* TF45 (right) for xylitol production on semi-defined medium containing 100 g/L xylose.

Investigation of other strains of *C. boidinii* for xylitol fermentation

Other strains of *C. boidinii* (NCAIM Y.01709, Y.01640, Y.01058, Y.00869, Y.01637, Y.01641) were also tested in xylitol fermentation experiments using xylose-containing semi-synthetic medium. Although xylitol production was observed in most of the cases, the achieved xylitol yields were much lower compared to the strain of NCAIM Y.01308.

Investigation of ethanol production by using *Candida boidinii* NCAIM Y.01308 [5]

Ethanol production was observed during xylitol fermentation experiments in small quantities. Based on this observation, the capability of *C. boidinii* to produce ethanol from glucose under anaerobic conditions was tested. Fermentations on glucose medium proved that *C. boidinii* is also suitable for ethanol production. As a next step, ethanol fermentations were successfully demonstrated on the glucose-rich hydrolysate of corn fibre. Moreover, *C. boidinii* NCAIM Y.01308 resulted in the same ethanol yield than that of obtained by *S. cerevisiae*. Thus, *C. boidinii* is proved to be a promising cell factory for the production of multiple products on lignocellulosic hydrolysates.

IV. Investigation of the prebiotic properties of AXOS fractions obtained from fractionating agro-industrial by-products

Main part of the results achieved in this section of the research project has not been published so far, so these results are presented with more details compared to those already published.

Investigation of commercial prebiotics with different probiotic bacteria and *Escherichia coli*

As a first step regarding this part of the research project, preliminary experiments on commercial prebiotics were carried out with different probiotic strains. Two prebiotic formulations, namely Beneo Orafti HSI and Orafti P95, were selected. Orafti HSI is a fructooligosaccharide-rich inulin product that contained 10% monomeric sugar (fructose). Orafti P95 is a 95% fructooligosaccharide (FOS) product with less monomer content. A specific instrument, called BacTrac (Sy-Lab GmbH, Neupurkersdorf, Austria), was used in the prebiotic experiments. Growth of different bacteria was followed by measuring impedance in the broth by BacTrac equipment. This equipment is capable to determine the growth characteristic of the microorganism (in this case probiotic bacteria) growing on a well-defined medium. For the experiments, 2-2 *Lactobacillus* and *Bifidobacterium* strains were selected

from the National Collection of Agricultural and Industrial Microorganisms (NCAIM): *Lactobacillus casei* B.01526, *Lactobacillus paracasei* B.01357, *Bifidobacterium longum subsp. infantis* B.01821T and *Bifidobacterium adolescentis* B.01822T. The selection of probiotic bacteria was based on literature research. The experiments were performed in a working volume of 10 mL for 24 hours. MRS medium was used for *Lactobacillus* strains containing 10 g/L peptone, 10 g/L beef extract, 4 g/L yeast extract, 20 g/L carbon source, 5 g/L sodium acetate trihydrate, 1 g/L polysorbate 80 (also known as Tween 80), 2 g/L dipotassium hydrogen phosphate, 2 g/L triammonium citrate, 0.2 g/L magnesium sulphate heptahydrate, and 0.05 g/L manganese sulphate tetrahydrate. For *Bifidobacterium*, the following medium was used: 10 g/L casein peptone (tryptic digest), 5 g/L yeast extract, 5 g/L meat extract, 5 g/L Bacto soytone, 10 g/L carbon source, 2 g/L dipotassium hydrogen phosphate, 0.2 g/L magnesium sulphate heptahydrate 0.05 g/L manganese sulphate tetrahydrate, 1 mL/L Tween 80, 5 g/L sodium chloride; 0.5 g/L cysteine-HCl dehydrate, and 0.5 g/L Resazurin. In both cases the pHs were adjusted to 6.8. The carbon source in each media was Orafti HSI or P95 products. Glucose was also used as a control carbon source. BacTrac recorded the growth curve during the measurements. From the data obtained, the maximum growth rates were calculated using the general logistic equation of microbial growth. SigmaPlot v.11 software was used for accomplish the calculations. The maximum growth rates for each strain are shown in Table 2.

Table 2. The maximum growth rates of probiotic bacteria on different carbon sources

	Lactobacillus casei	***Lactobacillus paracasei***	***Bifidobacterium adolescentis***	***Bifidobacterium longum subsp. infantis***
		Maximum growth rate (1/h)		
Glucose	33	36	54	55
Orafti HSI	52	49	34	48
Orafti P95	48	51	38	60

Based on the results, maximum growth rates of *Lactobacillus* were similar on both prebiotic formulations (48-52 1/h), while the maximum growth rate obtained on glucose was lower (33-36 1/h). For *Bifidobacterium adolescentis*, this tendency was turned, the maximum growth rate obtained on glucose (54 1/h) was higher than the growth rate determined on the two prebiotic formulations (34-38 1/h). In the case of *Bifidobacterium longum subsp. infantis*, there was no big difference in maximum growth rates obtained on the three carbon sources (50-60 1/h). Besides the maximum growth rate, other useful data are obtained during the measurements, which were the detection times. The detection time shows how fast the growth of the different strains is on the applied medium. The values of the different detection times are shown in Table 3.

Table 3. Detection times of probiotic bacteria on different carbon sources in BacTrac

	Lactobacillus casei	***Lactobacillus paracasei***	***Bifidobacterium adolescentis***	***Bifidobacterium longum subsp. infantis***
		Detection time (h)		
Glucose	4.4	9.1	8.2	5.3
Orafti HSI	5.5	10.6	8.8	5.4
Orafti P95	5.2	11.4	10.2	4.8

Based on this, slightly different results were obtained compared to the maximum growth rates. In the case of *Lactobacillus casei*, the fastest detection time was obtained on glucose (4.4 h), while it was almost the same on the two other carbon sources (5.2–5.5 h). In the case of *Lactobacillus paracasei*, it was also observed that the fastest detection time was achieved on glucose, followed by the inulin-based prebiotic and finally the Orafti P95. The detection time of *Lactobacillus* strains was inconsistent with the maximum growth rates. A similar trend was observed for *Bifidobacterium adolescentis* as for *Lactobacillus paracasei*. The detection time of *Bifidobacterium longum subsp. infantis,* on glucose and Orafti HSI showed similar results (5.3-5.4 h). In this case of Orafti P95, the detection time showed the lowest value (4.8 h). This result is consistent with the maximum growth rate, where the highest value was obtained on Orafti P95. Different organic acids (lactic acid, propionic acid, acetic acid, butyric acid) were also measured during the fermentations by HPLC. Both *Lactobacillus* strains produced the same amount of lactic acid (10-12 g/L) as the main organic acid on each carbon source. In addition, acetic acid (~3 g/L) and propionic acid (~0.8 g/L) were also present, but their amounts were low compared to lactic acid. *Lactobacillus* strains were able to utilize the carbon sources efficiently, which were monitored by the depletion of glucose and fructose. In the case of *Bifidobacterium*, the amount of organic acids was much lower compared to *Lactobacillus*. Lactic acid (~2-3 g/L) was present in the highest concentration followed by acetic acid (~0.4-0.6 g/L). The propionic acid concentration was almost the same (~0.6 g/L) as in the case of *Lactobacillus*. Small amount of butyric acid (0.3 g/L) was also formed in this case. It was observed that most of the organic acid was produced on glucose medium, followed by inulin (Orafti HSI), while the least organic acid was obtained by using FOS (Orafti P95). The experiments were also monitored by pH measurement. The pH decreased for all carbon sources from near neutral pH (6.8) to pH 4.2 in all cases of *Lactobacillus* fermentation. *Bifidobacterium* showed different pH values depending on which carbon source was used. The lowest pH obtained on glucose (pH = 4.7), while the highest was on medium containing Orafti P95 (pH = 5.22). One of the criteria of the prebiotic products is to inhibit the growth of harmful microorganisms in the intestinal flora. Thus, measurements by using *Escherichia coli* as a harmful bacterium were also performed. The growing medium (M9) conatained 33.9 g/L disodium-hydrogen phosphate, 15 g/L potassium dihydrogen phosphate, 5 g/L ammonium chloride, 2.5 g/L sodium chloride, and 10 g/L carbon source. In the case of *E. coli,* the detection times were very high, even in the case of glucose. Small differences were observed in the pH between the carbon sources (glucose: 4.88; inulin: 4.89; FOS: 4.98). The HPLC results showed that the concentration of organic acids was very low in each carbon source. Investigating the depletion of carbon sources, it was observed that glucose was utilized by *E.coli*, while in the case of the other two carbon sources, no change was observed during the experiments. As a summary, *Lactobacillus* strains were able to utilize the commercial prebiotics and *Bifidobacterium* strains too, but with lower efficiency. Utilization of prebiotics by *E.coli* was the worst, which confirmed the prebiotic effects of these products.

Investigation of acidic and enzymatic hydrolyses on wheat bran to produce potential prebiotic fractions

Wheat bran was used as a raw material for the preparation of potential prebiotic product, since this raw material contained the highest amount of arabinoxylan, making it suitable to obtain a fraction rich in arabinoxylo-oligosaccharides (AXOSs). The experimental design for the separation of arabinose from wheat bran showed that at high acid concentration and reaction time, large number of monomers was present in the hydrolysate, which is not favourable for prebiotic production. To reduce the monomer yields and increase the yield of solubilised AXOS, a new experimental design was performed where the design space was changed. The

acid concentration was 0.25; 0.75; 1.25 w/w% while the reaction time was 5, 15 and 25 minutes. The reaction was performed under the same conditions as in the previous experimental design: 10 w/w% initial dry matter content at 90°C. The experimental design carried out by wheat bran II. The total glucose yield ranged from 55 to 75% depending on the treatment parameters, and the yield of monomers was also high (10%). This large amount of released glucose monomer may have been caused by the hydrolysis of starch due to the dilute acid treatment. The wheat bran II. had a higher starch content than wheat bran I. In order to reduce the amount of glucose monomers, starch removal was performed on wheat bran. Wheat bran was treated with thermostable α-amylase (5 g/kg dry matter enzyme dose) for 3 hours at 90°C to eliminate the starch from the wheat bran. The starch content decreased to 0.33% after the treatment. Hence the starch removal of wheat bran was successful. After this process, the composition of the carbohydrates of the wheat bran was the following: 26.3% xylan, 20.8% non-starch glucan, and 16.8% arabinan. The Klason-lignin content was 14.7%. The experimental design repeated in this wheat bran and the monomer and oligomer and yields were investigated. The results are shown in Table 4.

Table 4. Results of the experimental design (3^2) to produce AXOSs

		Monomer yield (%)			**Oligomer yield (%)**		
	Acid (%)/ Time (min)	0.25	0.75	1.25	0.25	0.75	1.25
Glucose	5	0	0	0	4.95	8.48	11.66
	15	0	1.93	1.94	6.22	12.53	16.3
	25	1.92	1.94	1.95	6.79	13.99	15.02
Xylose	5	1.55	1.72	1.87	6.31	19.54	23.33
	15	1.58	1.85	2.35	12.21	27.38	27.32
	25	1.62	2.13	3.48	14.54	27.11	34.74
Arabinose	5	4.64	13.17	27.05	6.26	13.58	11.75
	15	6.54	25.17	38.33	9.96	20.8	16.08
	25	8.1	34.9	52	11.12	13.55	16.1

The glucose monomer was very low in all of the cases (<2%), which significantly reduced compared to the treatment with wheat bran containing starch. The glucose oligomer yields were also very low. In the xylose yields, it was observed that the monomer and oligomer yields were increased by increasing reaction time and acid concentration. The monomer yield of xylose was not considerable in either case, and even the harshest treatment showed a yield of 3.5%. However, investigating the monomeric and oligomeric yields of arabinose, significant amount of arabinose was already present in monomeric form (>10%), except at 0.25 w/w% sulfuric acid treatments. Desirability function approach was used in order to obtain an optimal treatment for potential prebiotic production. The criterion was the following: arabinose and xylose had to be in oligomeric form in the solution, besides minimizing the amount of monomeric sugars. As a result, the following treatment was obtained as the optimum: 1.25 w/w % sulfuric acid concentration and a treatment time of 25 minutes. By repeating the experiment under these conditions, 37.8% of xylose oligomer yield was obtained. However, it worth to note that arabinose was mainly present in monomeric form (52%).

Because of the high monomer concentration obtained in the acidic treatment, especially in the case of arabinose, enzymatic hydrolysis was also investigated to produce AXOS-rich fraction.

Destarched wheat bran was used also in the enzymatic hydrolysis experiments. For enzymatic hydrolysis, industrial enzyme preparations with endo-xylanase activity were used. Endo-xylanases are endo enzymes that cleave the xylan chain, creating xylooligosaccharides with different chain lengths. The industrial enzyme preparations were NS50030, Cellic Htech and NS22083 from Novozymes A/S (Denmark). Preliminary experiments were carried out to determine which enzyme could be the most suitable for oligosaccharide production. The experiments were performed at 5% of initial dry matter content, since the mixing of the sample was inadequate and inhomogeneous at 10% initial dry matter content. The enzyme dose in each case was 2.5 g enzyme/kg dry matter. Enzymatic hydrolysis was carried out at 50°C for 72 hours in sodium acetate buffer (0.1 M, pH 4.8). After enzymatic hydrolysis, the efficiency of the enzyme was checked by thin layer chromatography (TLC). The results of thin layer chromatography are shown in Figure 3.

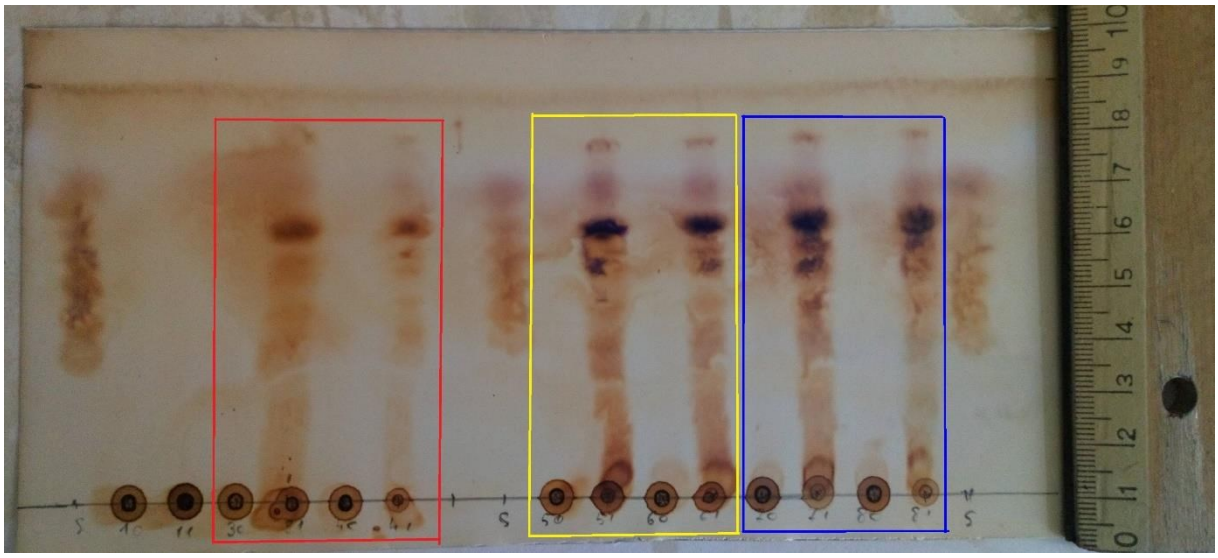


Figure 3. TLC of the enzymatic hydrolyes. NS50030 (red), HTech (yellow), NS22083 (blue) and Standard (S). The samples labelled with 0 are zero hour samples, and samples labelled with 1 are 72-hours samples.

Different amounts of xylooligomers were released by the enzymatic treatments in each case. The weakest spots were obtained in the case of NS50030 and few xylooligomers were observed. For the other two enzymes, similar results were obtained. In both cases, sharp spots were observed dominated by xylobiose. Monomeric sugars were also present during these enzymatic treatments, as shown in the Figure 4 (by the spots above the sharp xylobiose). Further experiments were performed with the enzyme NS22083, since the sharpest points appeared there. After selecting the appropriate enzyme preparation, it was still important to determine the optimal treatment time. Therefore the enzymatic hydrolysis was repeated by NS 22083 using 24-hour sampling. TLC and HPLC measurements were also performed on the samples taken. The result of the TLC showed that the optimum time for the enzymatic treatment was 24 hours, since the sugar yields were not increased significantly with longer treatment time. This is confirmed by the HPLC results. The sugar yields of the enzymatic treatment are summarized in Table 5. In xylose yields, the monomer yield was 4.3% after 24 hours, compared to 6.6% after 72 hours. The yield of monomer arabinose increased from 1.6% (24 h) to 1.8% (72 h). The glucose monomer yield was 3.9% after 24 hours and 6.3% after 72 hours. The oligomeric yields of xylose (42.1%) arabinose (20.0%) and glucose (15.3%) slightly increased after the first day of hydrolysis. Comparing the results of the two treatments (acidic and enzymatic), higher xylose oligomer yield (~45%) obtained during the

enzymatic hydrolysis, while high arabinose (21.5%) and glucose (16.40%) oligomer yield were also occurred. The amount of monomer sugars was negligible. Hence the 24-hour enzymatic treatment by NS22083 was chosen for the production of prebiotic fractions.

Since enzymatic hydrolysis was found to be more efficient in this process than acidic hydrolysis, production of appropriate enzyme mixtures mainly containing endo-xylanase activity was also added to the project. Investigations in this field had been started within a previous project funded by NRDIO of Hungary (TÉT_15-1-2016-0085). Based on the results of the previous project, different *Cellulomonas* strains were investigated on different biomass substrates to produce enzymes with high endo-xylanase activity. The most promising enzyme preparation was tested on a pre-treated agro-industrial waste biomass, and resulted in the production of high amount of xylooligosaccharides [7]. Deeper investigation of this enzyme preparation in the production of prebiotic products could be the goal of a future research projects.

Table 5. Investigation of the proper time of enzymatic hydrolysis

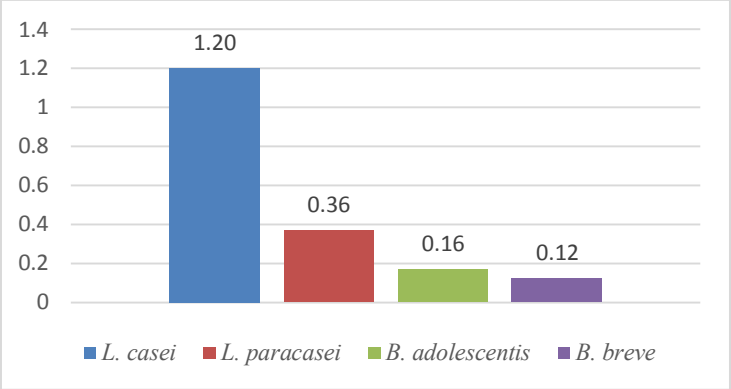
	24 hours			**48 hours**			**72 hours**		
Yield	Monomer (%)	Oligomer (%)	Total (%)	Monomer (%)	Oligomer (%)	Total (%)	Monomer (%)	Oligomer (%)	Total (%)
Xylose	4.3	42.1	46.4	5.97	44.9	50.9	6.6	44.5	51.2
Arabinose	1.6	20.0	21.6	1.75	21.4	23.2	1.8	21.4	23.3
Glucose	3.9	15.3	19.3	5.35	16.4	21.7	6.3	15.9	22.2

In order to test the prebiotic properties of the produced AXOS-rich fractions, fermentations were performed by using both *Lactobacillus* and *Bifidobacterium* strains. Instead of *Bifidobacterium longum subsp. infantis,* a similar microorganism *Bifidobacterium breve* was used due to cultivation problems. MRS medium (described above) was used in all of the cases; however the medium was supplemented by cysteine-HCL in the case of *Bifidobacterium*. The carbon sources were glucose (as control) and an AXOS-rich powder obtained by lyophilisation of the hydrolysates of wheat bran. The fermentation time was 24 hours. The fermentation was monitored by following the pH and counting the viable cells. The initial pH values of the MRS medium were between 6.6 and 6.75. After fermentation experiments, the following pH values were observed: *L. casei*: 2.5 (glucose), 1.3 (AXOS), *L. paracasei*: 2.6 (glucose), 1.4 (AXOS), *B. adolescentis*: 2.3 (glucose), 1.4 (AXOS), *B. breve*: 2.1 (glucose), 0.9 (AXOS). On the potential prebiotic AXOS fraction, the pH decreased greater compared to glucose. In terms of viable cells, it was observed that the cells grew better on glucose than on the AXOS powder. Only *L. casei* was found to grow better on the AXOS preparation than glucose. It is also observed that *Lactobacillus* grew better than *Bifidobacterium*. The experiments were also performed by *E.coli*, as a negative control, where the medium was the previously mentioned M9 medium. Unlike the other strains, *E. coli* did not grow well on either glucose or the AXOS preparation. The changes in both pH and cell number were smaller compared to the probiotic bacteria. To evaluate and quantify the suitability of the AXOS powder as a prebiotic agent, prebiotic activity values were calculated for each strain by using the following equation:

$$\text{Prebiotic activity score} = \frac{\text{Probiotic} \log \frac{\text{CFU}}{\text{ml}} \text{ on prebiotics (24 h)} - \text{Probiotic} \log \frac{\text{CFU}}{\text{ml}} \text{ on prebiotics (0 h)}}{\text{Probiotic} \log \frac{\text{CFU}}{\text{ml}} \text{ on glucose (24 h)} - \text{Probiotic} \log \frac{\text{CFU}}{\text{ml}} \text{ on glucose (0 h)}} - \frac{E.coli \log \frac{\text{CFU}}{\text{ml}} \text{ on prebiotics (24 h)} - E.coli \log \frac{\text{CFU}}{\text{ml}} \text{ on prebiotics (0 h)}}{E.coli \log \frac{\text{CFU}}{\text{ml}} \text{ on glucose (24 h)} - E.coli \log \frac{\text{CFU}}{\text{ml}} \text{ on glucose (0 h)}}$$

The results are shown in Figure 4. *Lactobacillus* strains utilized better the lyophilized hydrolysate (AXOS powder) than *Bifidobacterium* strains. The prebiotic scores were the following: *L.casei*: 1.20, *L. paracasei*: 0.36, *B. adolescentis*: 0.16, and *B. breve*: 0.12. Based on the obtained results, it can be concluded that the AXOS powder obtained from wheat bran can potentially be a prebiotic, since the prebiotic activity scores gave a positive result in all of the cases.

Figure 4. The prebiotic activity values of different probiotic strains by using AXOS powder



V Summary

Main focus of the research project contained the investigation of acidic fractionation of different agro-industrial residues to produce sugar- and oligosaccharide-containing fractions, investigation and optimisation of xylitol fermentation by wild type and modified *C. boidinii*, and investigation of the prebiotic potential of oligosaccharides obtained from lignocellulosic by-products. In addition, the originally planned research activity was extended in many directions based on the promising results obtained during the project, such as testing additional biomasses and microorganisms, examining enzymatic fractionation processes, studying the production of enzymes.

The main results of the project are summarized in the following bullet points:

- Acidic fractionation processes were developed and optimized to produce arabinose-rich and xylose-rich or AXOS-rich liquid fractions, and cellulose-rich solid residue from wheat bran, brewer's spent grain and rice straw.
- Enzymatic fractionation process of corn fibre was compared to acidic fractionation process for glucose-rich hydrolysates.
- The effects and interactions of the different parameters affecting xylitol fermentation by using *C. boidinni* were deeply investigated. The fermentation parameters were optimized.

- Xylitol fermentations by using *C. boidinni* on lignocellulosic hydrolysates were successfully demonstrated and improved.
- Xylitol fermentation by using modified *C. boidinni* with enhanced xylitol producing capability was examined.
- *C. boidinii* was also found to be appropriate for ethanol fermentation.
- Production of AXOS-rich fractions potentially having prebiotic activity was examined and an enzymatic process on wheat bran was developed.
- Prebiotic properties of the AXOS-rich fractions were deeply investigated and prebiotic effects of the produced AXOS preparations were proved.

These results could serve as a basis for the development of biorefinery processes which utilize agro-industrial by-products (e.g. wheat bran, brewer's spent grain, rice straw) to produce wide-spectrum of value-added bio-products (such as xylitol, arabinose, prebiotics, bioethanol). In addition, preliminary studies aiming the techno-economic evaluation of these kind of biorefinery processes provided very promising results in terms of their economic viability on an industrial scale.

VI References

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