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ARTICLE

Direct Enzymatic Production of Oligosaccharide Mixtures from Sugar Beet Pulp: Experimental Evaluation and Mathematical Modeling

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The potential of sugar beet pulp as raw material for neutral and acidic oligosaccharide production by direct enzymatic treatment was evaluated. The effect of the polygalacturonase to solid ratio (PGaseSR), cellulase activity to polygalacturonase activity ratio (CPGaseR), and reaction time (t) on several dependent variables (selected to quantify the mass of recovered liquors, the conversion of each polysaccharide into monosaccharides, and the conversion of each polysaccharide into oligomers) was studied. Mathematical models suitable for reproducing and predicting the experimental results were also developed. Operational conditions leading to a maximum oligomer production were calculated from models being PGaseSR = 10 U/g, CPGaseR = 0.725 filter paper units/U, and t = 12.82 h. Under these conditions, the models predicted that 906 kg of liquors containing 26.7 kg of oligosaccharides can be obtained from 100 kg of SBP, the distribution being as follows: 5.9 kg of glucooligosaccharides, 2.4 kg of galactooligosaccharides, 9.7 kg of arabinooligosaccharides, and 8.7 kg of oligogalacturonides. Therefore, this study demonstrated that pectic oligomers (for which prebiotic properties have been reported) can be obtained from SBP at high yield by direct enzymatic hydrolysis using mixtures of cellulases and pectinases.

KEYWORDS: Arabinooligosaccharides; oligogalacturonides; sugar beet pulp; prebiotics; cellulases; pectinases

INTRODUCTION

A prebiotic is a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota (*1*). This definition does not emphasize a specific bacterial group, but it is assumed that a prebiotic should increase the number and/or activity of bifidobacteria and/or lactic acid bacteria, as these groups of microorganisms are claimed to cause several beneficial effects on the host.

The interest in the use of nondigestible oligosaccharides (NDO) as functional food components targeted at gut health has increased during recent years (*2*). Some NDO are selectively fermented in the human colon and can be described as prebiotics (*3*). This context fosters interest in the manufacture of new compounds from largely available, renewable carbohydrate sources.

Pectin-derived oligosaccharides (POS) are promising candidates for prebiotic properties. Sugar beet pulp (SBP) is a byproduct of the sugar industry abundant in Europe, Japan, and the United States (*4*). In Spain, 1 million metric tons of SBP were produced in 2003, which were mainly employed for feed formulation. SBP has a high content of pectin, a complex polysaccharide mainly made up of three structural polymers: homogalacturonan (HG) and rhamnogalacturonans I and II (RGI and RGII). HG, the most abundant pectic polysaccharide, is composed of (1, 4)-linked α-D-Galp residues that can be partly methyl-esterified at C-6 and possibly partly acetyl-esterified at O-2 and/or O-3 (*5*). The second most abundant polymer in pectins is RGI, made up of chains with alternate units of galacturonic acid and rhamnose, having branched arabinan, galactan, or even arabinogalactan chains. In this polymer, the galacturonic acid units can be acetylated or methyl esterified. RGII is a polysaccharide made up of galacturonic acid, rhamnose, galactose, and unusual neutral sugars.

The composition of SBP suggests that it could be a suitable feedstock for obtaining a variety of NDO with biological activity, such as oligogalacturonides (OGaU), arabinooligosaccharides (AOS), and galactooligosaccharides (GaOS).

Most food-grade oligosaccharides are produced by enzymatic transglycosylation from simple sugars or by controlled enzymatic degradation of plant polysaccharides. Pectin can be hydrolyzed without saccharide degradation through the simultaneous action of different enzyme activities (*6*−*8*).

The use of enzymes for the recovery of valuable byproducts has been reported in the literature: for example, processing of bergamot peel with pectinolytic and cellulolytic enzymes led to the simultaneous solubilization of carbohydrates (with the formation of monosaccharides and oligosaccharides in relative

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amounts dependent on the operational conditions) and low molecular weight flavonoids (*9*). Olano Martin et al. (*10*) studied the continuous production of pectic-oligosaccharides by pectinolytic enzymes, whereas Iwasaki et al. (*11*) considered the enzymatic production of produce pectate oligosaccharides by immobilized endopoligalacturonase in a continuous stirred tank reactor.

The objectives of this work were (i) to assess the potential of SBP as a raw material for pectic oligosaccharide production by direct enzymatic hydrolysis, (ii) to develop empirical models able to reproduce and predict the compositions of hydrolysis media as a function of the operational conditions (polygalacturonase to solid ratio in the enzymatic hydrolysis, cellulase activity to polygalacturonase activity ratio and reaction time), and (iii) to select the optimal operational conditions, based on the model predictions.

MATERIALS AND METHODS

Raw Material. SBP was provided by a local factory (Azucarera Ebro, Spain), homogenized in a single lot to avoid compositional differences among aliquots, and stored in polyethylene bags at −18 °C until use.

Analysis of the Raw Material. Aliquots from the homogenized SBP lot were dried at 50 °C, milled to a particle size of < 0.5 mm, and subjected to quantitative acid hydrolysis with 72% sulfuric acid followed by quantitative poshydrolysis [TAPPI T13m method (Technical Association of the Pulp and Paper Industry)]. A sample of the liquid phase was assayed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex instrument (Dionex, Sunnyvale, CA). Separation of carbohydrates was carried out with a CarboPac PA-1 (4 mm × 250 mm) in combination with a CarboPac PA-1 guard column (4 mm × 50 mm) maintained at 30 °C and pulsed amperometric detection. The mobile phases were degassed with helium. Analyses were performed using a gradient of deionized water (eluent A), 200 mM sodium hydroxide (eluent B), and 2 M sodium acetate in 200 mM sodium hydroxide (eluent C). The total analysis time was 45 min. This method allowed the determination of glucose, galactose, xylose, rhamnose, and arabinose. For simplicity, the results are reported as glucan, galactan, xylan, rhamnosyl substituents, mannan, and arabinan. Another sample of liquors was assayed for acetic acid using an Agilent HPLC fitted with a refractive index detector and an Aminex HPX-87H column (supplied by Bio-Rad). The mobile phase (0.005 N H_2SO_4) was eluted at a flow rate of 0.6 mL/min at 60 °C (*12*). The oven-dried weight of the solid phase from quantitative acid hydrolysis measured the content of Klason lignin. Uronic acids were determined according to the method of Blumenkrantz and Asboe-Hansen (*13*) using galacturonic acid as a standard for quantification. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined according to the methods of Goering and Van Soest (*14*). Elemental nitrogen was determined with a Thermo Finnegan Flash EATM 1112 analyzer, using 130 and 100 mL/min of He and O_2 and an oven temperature of 50 °C. Protein content was obtained by multiplying the elemental N content by 6.25. All determinations were made in triplicate.

Moisture and ash were determined according to methods ISO 638 and ISO 776, respectively.

Enzymatic Hydrolysis of Sugar Beet Pulp. Enzymatic hydrolyses of sugar beet pulp were carried out in Erlenmeyer flasks (working volume, 100 mL) placed in orbital shakers (150 rpm) using commercial enzymes under different operational conditions (see below). Experiments were performed at 37 °C and pH 5 in media containing sodium acetate buffer (50 mM) (*9*). Enzyme concentrates (Celluclast 1.5L cellulases from *Trichoderma reesei* and Viscozyme 1.5L endopolygalacturonases from *Aspergillus aculeatus*) were kindly provided by Novo Nordisk Bioindustrial (Madrid, Spain).

The cellulase activity of Celluclast 1.5L was determined by the filter paper assay (*15*). In this analytical procedure, the enzyme is incubated at 50 °C for 60 min using Whatman no. 1 filter paper strips (1 cm × 6 cm) suspended in 0.05 M citrate buffer (pH 4.85). The reducing sugars generated are then measured by the dinitrosalicylic acid (DNS) method. One unit of filter oaper activity (FPU) is the amount of enzyme that released 1 μmol of glucose min^{-1}. The polygalacturonase activity of Viscozyme 1.5L was determined by measuring the amount of D-galacturonic acid formation from 0.5% w/v polygalacturonic acid in 50 mM sodium acetate buffer (pH 5) according to the DNS method. One unit of enzymatic activity (U) is defined as the amount of enzyme catalyzing the formation of 1 μmol of D-galacturonic acid per minute at 37 °C and pH 5. The experiments were performed at liquor to solid ratio (LSR) of 12 g/g, an endopolygalacturonase to solid ratio in the range of 10−50 U/g, a cellulase to endopolygalacturonase ratio in the range of 0−1 FPU/U, and reaction time in the range of 4−16 h.

Before enzyme supplementation, samples of SBP were suspended in sodium acetate buffer (50 mM) and sterilized for 15 min at 121 °C. At the end of hydrolysis, the samples were heated at 100 °C for 5 min to inactivate the enzymes. At the end of process, liquors were separated by centrifugation, quantified, and analyzed as described below.

Analysis of Enzymatic Liquors. Samples of enzymatic liquors were centrifuged and filtered through 0.45 μm cellulose acetate filter (Sartorius Biolab Products) and analyzed by HPLC for monosaccharides. Nonvolatile compounds (NVC) were measured by oven-drying at 105 °C until constant weight. AOS, GaOS, glucooligosaccharides (GOS), and OGaU present in liquors were determined by enzymatic posthydrolysis with endopolygalacturonase (Viscozyme 1.5L from *A. aculeatus*). Enzymatic assays were carried out at 37 °C for 40 h in Erlenmeyer flasks with orbital agitation (150 rpm) at an enzyme loading of 45 U/g liquor. Sodium acetate buffer (50 mM) was employed to keep the pH at 5. The contents of oligomers (AOS, GaOS, OGaU, and GOS) were calculated on the basis of the increase in each monomer concentration (arabinose, galactose, galacturonic acid, and glucose, respectively) obtained upon quantitative hydrolysis.

Fitting of Data and Modeling. The experimental data were fitted to the proposed models using commercial software (Microsoft Excel from Microsoft, USA).

Table 1. Composition of the Raw Material

fraction	wt %, oven-dry basis
glucan	20.1
galactan	5.3
xylan	1.0
rhamnosyl moieties	1.4
mannan	1.1
arabinan	17.5
acetyl groups	2.6
galacturonan	21.0
Klason lignin	4.8
neutral detergent fiber (NDF)	60.0
acid detergent fiber (ADF)	24.5
acid detergent lignin (ADL)	3.4
ash	4.5
protein (from nitrogen determination)	10.8

RESULTS AND DISCUSSION

Composition of the Raw Material. The compositional data of the SBP lot employed in experiments are listed in **Table 1**. Galacturonan, glucan, and arabinan were the major components of the raw material, followed by galactan, rhamosyl moieties, mannan, and xylan. Protein (10.8%) and ashes (4.5%) were also found in the raw material.

To gain more information, the raw material was also subjected to fiber analysis techniques (see results in **Table 1**). These results are in agreement with the ones reported by Bertin et al. (*16*) and Devaux et al. (*17*).

Study of the Enzymatic Hydrolysis. Table 2 summarizes the experimental plan, including the fixed variables and their values and the independent variables [polygalacturonase to solid ratio (PGaseSR), cellulase activity to polygalacturonase activity ratio (CPGaseR), and reaction time (*t*)] and their variation ranges as well as the dependent variables (selected to quantify the mass of

Table 2. Experimental Variables Involved in the Study

variable	definition and units	nomenclature	value or range
independent	polygalacturonase to solid ratio in the enzymatic hydrolysis (U/g)	PGaseSR	10−50
	cellulase activity to polygalacturonase activity ratio (FPU/U)	CPGaseR	0−1
	reaction time in the enzymatic hydrolysis (h)	t	4−16
dependent	solid yield (%)	y_1	
	cllulose conversion into glucose (%)	y_2	
	cellulose conversion into glucooligosaccharides (%)	y_3	
	galactan conversion into galactose (%)	y_4	
	galactan conversion into galactooligosaccharides (%)	y_5	
	arabinan conversion into arabinose (%)	y_6	
	arabinan conversion into arabinooligosaccharides (%)	y_7	
	galacturonan conversion into galacturonic acid (%)	y_8	
	galacturonan conversion into oligogalacturonides (%)	y_9	
	mass of recovered liquors (g)	y_{10}	

Table 3. Operational Conditions Assayed (Expressed in Terms of Dimensional Variables and Dimensionless, Normalized, Independent Variables)

	dimensional independent variables			dimensionless normalized independent variables		
expt	PGaseSR (U/g)	CPGaseR (FPU/U)	t (h)	x_1	x_2	x_3
1	10	0	10	−1	−1	0
2	10	0.5	4	−1	0	−1
3	10	0.5	16	−1	0	1
4	10	1	10	−1	1	0
5	30	0	4	0	−1	−1
6	30	0	16	0	−1	1
7	30	0.5	10	0	0	0
8	30	0.5	10	0	0	0
9	30	0.5	10	0	0	0
10	30	1	4	0	1	−1
11	30	1	16	0	1	1
12	50	0	10	1	−1	0
13	50	0.5	4	1	0	−1
14	50	0.5	16	1	0	1
15	50	1	10	1	1	0

recovered liquors, the conversion of each polysaccharide into monosaccharides, and the conversion of each polysaccharide into oligomers).

A systematic study of the effects of every independent variable along its variation range would involve a great deal of work, which can be reduced to a reasonable extent using the response surface methodology (*18*). For this purpose, a factorial, incomplete, centered, second-order experimental design was selected, which allowed the calculation of the dependent variables under defined operational conditions by means of the generalized equation

$$y_j = b_{0j} + \sum_{i=1}^{3} b_{ij} x_i + \sum_{i=1}^{3} \sum_{k=1}^{3} b_{ikj} x_i x_k \qquad (1)$$

where y_j are the dependent variables ($j = 1-10$), b_{0j}, b_{ij}, and b_{ikj} are the regression coefficients calculated from the experimental results by the least-squares method, and x_i and x_k ($k \geq i$) are the dimensionless, normalized independent variables, with variation ranges from −1 to 1.

The dimensionless, normalized independent variables are linearly related to the dimensional independent variables through the equation

$$x_i = \frac{2[\mathrm{VI}_i - \mathrm{VI}_{i\mathrm{me}}]}{\mathrm{VI}_{i\mathrm{max}} - \mathrm{VI}_{i\mathrm{min}}} \qquad (2)$$

where VIi represents the value of independent variable i, VIi_{me} represents the mean value of independent variable i, and VIi_{max} and VIi_{min} represent the maximum and minimum values of the variation range of independent variable i.

In our case, VI_1 is the polygalacturonase to solid ratio variable (PGaseSR), VI_2 is the cellulase activity to polygalacturonase activity ratio variable (CPGaseR), and VI_3 is the reaction time variable (t).

Table 3 shows the set of experiments carried out that corresponded to a Box−Benhken (*18*) optimized design. This methodology has been previously employed to assess the enzymatic hydrolysis of a variety of substrates (*19*−*21*).

The values for the experimental dependent variables, which allow quantification of the polysaccharide conversions into monomers and oligomers (y_j, $j = 2-9$), were calculated using the equation

$$y_{j(j=2-9)\mathrm{exp}} = \frac{\mathrm{HPC} \times y_{10\mathrm{exp}}}{\rho \times m \times CP_{\mathrm{SBP}}} \times 10000 \qquad (3)$$

where HCP is the volumetric concentration of each hydrolysis product in liquors (g/L), $y_{10\mathrm{exp}}$ is the mass of recovered liquors in each experiment (see **Table 4**), ρ is the average density of liquors (1025 g/L), m is the mass of SBP subjected to treatment in all of the experiments (8.33 g), and $\mathrm{CP}_{\mathrm{SBP}}$ is the weight percent of the considered polysaccharide SBP (see **Table 1**).

Table 4 lists the experimental results determined for the dependent variables, and **Table 5** shows the regression coefficients, their significance (based on a Student's t test), and the parameters measuring the correlation (R^2) and significance (Fisher's F test) of the models.

Solid Yield. The values of the coefficients determined for variable y_1 (solid yield) show that PGaseSR and CPGaseR were the most influential variables (see coefficients in **Table 5**). **Figure 1** shows the calculated dependence of y_1 on both variables for experiments lasting 10 h. Variable y_1 decreased steadily with PGaseSR and CPGaseR to reach a minimum value (24%) defined by PGaseSR = 48 U/g and CPGaseR = 0.85 FPU/U, operational conditions of high severity (high enzyme charges and prolonged reaction time).

Cellulose Conversion into Glucose and Glucooligosaccharides. **Table 4** shows the experimental results obtained for both variables y_2 (cellulose conversion into glucose) and y_3 (cellulose conversion into GOS). As can be seen, when no cellulase was added to the medium (experiments 1, 5, 6, and 12), a low glucose generation was observed. In addition, it can be inferred that PGaseSR caused more pronounced effects on y_2 when CPGaseR increased. Moreover, the values of the coefficients listed in **Table 5** describing the behavior of variable y_2 (cellulose conversion into glucose) show that both the polygalacturonase to solid ratio and the cellulase to polygalacturonase ratio were influential variables. This variation pattern is in agreement with the expected synergistic effects between both kinds of enzyme activities (*17*, *22*). When CPGaseR was increased, the cellulase and β-glucosidase to solid ratios were also increased, promoting cellulose hydrolysis. The cellulose conversion into glucose increased steadily with PGaseSR and CPGaseR to reach its maximum value (59%) when the highest enzyme loadings were employed.

On the basis of the values of the regression coefficients calculated for variable y_3 (see **Table 5**), it can be inferred that the most influential variable on y_3 was CPGaseR, which was significantly affected by both the linear and quadratic terms. **Figure 2** shows the predicted dependence of y_3 on PGaseSR and CPGaseR for experiments lasting 10 h. It can be observed that the cellulose conversion into GOS increased sharply with CPGaseR and showed a limited dependence on PGaseSR. Operation at CPGaseR > 0.70 FPU/U resulted in decreased cellulose conversion into GOS, a behavior ascribed to the decomposition of GOS into glucose under these conditions. The effects caused by PGaseR depended on the CPGaseR considered: at low CPGaseR, increased PGaseSR resulted in higher production of GOS, whereas CPGaseR > 0.5 FPU/U resulted in decreased production of GOS owing to the importance of decomposition reactions. With operation at PGaseSR = 10 U/g and CPGaseR = 0.75 FPU/U for 10 h, about 28% cellulose conversion into GOS was achieved.

Galactan Conversion into Galactose and Galactooligosaccharides. The experimental results obtained for both variables y_4 (galactan conversion into galactose) and y_5 (galactan conversion into GaOS) are shown in **Table 4**. As can be observed, variable y_4 varies in the range of 14.15−43.09%, increasing with the enzyme charges and reaction time, as a consequence of the increased hydrolysis of both hemicelluloses and pectin, which contain galactose as structural units. According to the coefficients (see **Table 5**), all of the independent variables affected significantly the galactan to galactose conversion (y_4), with major effects associated with PGaseSR and t.

On the other hand, variable y_5 (galactan conversion into galactooligosaccharides) was mainly affected by CPGaseR, whereas PGaseSR and reaction time caused limited effects (see coefficients in **Table 5**). **Figure 3** shows the calculated dependence of y_5 on the CPGaseR and PGaseSR for experiments lasting 10 h (x_3 = 0). The model predicted a maximum value for y_5 (30.6%) under conditions defined by PGaseSR = 26 U/g and CPGaseR = 0.8 FPU/U. Operating at higher enzymes charges and/or longer reaction times, the conversion of galactan into GaOS decreased as a consequence of oligosaccharide hydrolysis, a fact that is in agreement with the increase observed in the galactose content of liquors.

Table 4. Results Obtained in Experiments 1−15

expt	y_1 (%)	y_2 (%)	y_3 (%)	y_4 (%)	y_5 (%)	y_6 (%)	y_7 (%)	y_8 (%)	y_9 (%)	y_{10} (g)
1	60.70	5.75	4.73	19.33	12.72	0.75	35.89	11.61	31.78	55
2	57.07	7.01	15.38	14.15	21.93	0.59	29.20	4.57	32.73	55
3	41.54	19.16	27.22	31.14	23.21	2.55	54.79	20.61	38.97	76
4	38.93	20.78	30.04	25.78	31.16	1.19	53.20	15.44	42.24	76
5	59.27	6.06	6.76	17.68	19.29	0.67	29.26	11.23	23.49	54
6	48.93	10.63	5.76	33.12	12.39	3.69	51.13	30.94	22.83	63
7	30.61	26.62	23.70	34.00	26.53	3.49	59.32	26.19	33.54	81
8	31.76	26.06	23.56	33.26	28.59	3.04	61.41	26.40	33.89	80
9	32.18	26.45	23.98	33.61	30.90	2.95	61.14	26.97	33.53	81
10	37.29	30.11	21.53	24.08	24.77	2.71	47.42	15.80	33.57	75
11	26.45	53.93	14.79	41.28	22.29	10.52	55.76	41.99	20.04	86
12	47.09	11.33	3.85	34.68	8.20	3.26	48.38	33.59	20.29	64
13	34.45	27.58	27.81	29.81	19.86	5.70	48.86	23.87	30.39	78
14	26.02	49.38	23.31	43.09	23.63	10.53	61.75	44.34	19.91	84
15	26.62	59.04	17.35	39.57	25.89	13.92	63.48	44.83	20.77	85

Table 5. Regression Coefficients and Significance (Based on a *t* Test) and Statistical Parameters Measuring the Correlation and Significance of Models Obtained for Variables y_1-y_{10}, in Experiments 1−15

coefficient	y_1 (%)	y_2 (%)	y_3 (%)	y_4 (%)	y_5 (%)	y_6 (%)	y_7 (%)	y_8 (%)	y_9 (%)	y_{10} (g)
					Regression Coefficients and Significance					
b_{0j}	31.52*	26.38*	23.75*	33.62*	28.67*	3.16*	60.62*	26.52*	33.65*	80.67*
b_{1j}	−8.01*	11.83*	−0.63	7.09*	−1.43	3.54*	6.17*	11.80*	−6.80*	6.13*
b_{2j}	−10.84*	16.26*	7.83*	3.24*	6.44*	2.50*	6.90*	3.84*	2.28**	10.75*
b_{3j}	−5.64*	7.79*	−0.05	7.86*	−0.54	2.20*	8.59*	10.30*	−2.30**	5.88*
b_{12j}	0.32	8.17*	−2.95	−0.39	−0.19	2.55*	−0.55	1.85	−2.49	0.00
b_{13j}	1.77	2.41**	−4.08	−0.93**	0.62	0.72	−3.18**	1.11	−4.18**	−3.75**
b_{23j}	−0.12	4.81*	−1.44	0.44	1.11	1.20**	−3.38**	1.62	−3.22**	0.50
b_{11j}	4.30*	−0.78	0.73	−1.64*	−3.35	1.03***	−3.82**	−0.90	0.32	−3.46**
b_{22j}	7.52*	−1.38	−10.49*	−2.15*	−5.83***	0.59	−6.57*	0.74	−5.20*	−7.21*
b_{33j}	3.95**	0.18	−1.05	−2.44*	−3.16	0.65	−8.16*	−2.27***	−3.47**	−3.96**
					Statistical Parameters Measuring Correlation and Significance of Models					
R^2	0.989	0.997	0.896	0.997	0.846	0.985	0.986	0.991	0.959	0.985
F-exp	52	180	4.80	222	3	37.4	40	59	13.1	36
significance level (%)	>99	>99	>95	>99	>88	>99	>99	>99	>99	>99

* Significant coefficients at the 99% confidence level. ** Significant coefficients at the 95% confidence level. *** Significant coefficients at the 90% confidence level.

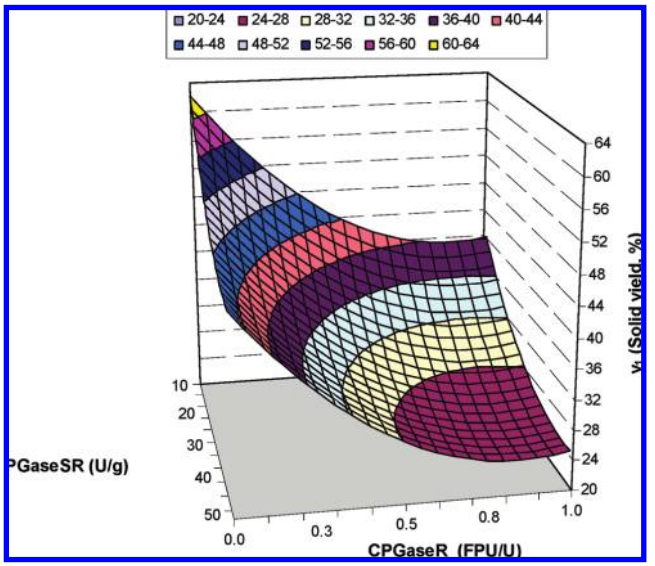

Figure 1. Calculated dependence of the solid yield (variable y_1) on the polygalacturonase to solid ratio (PGaseSR) and the cellulase activity to polygalacturonase activity ratio (CPGaseR) for experiments lasting 10 h.

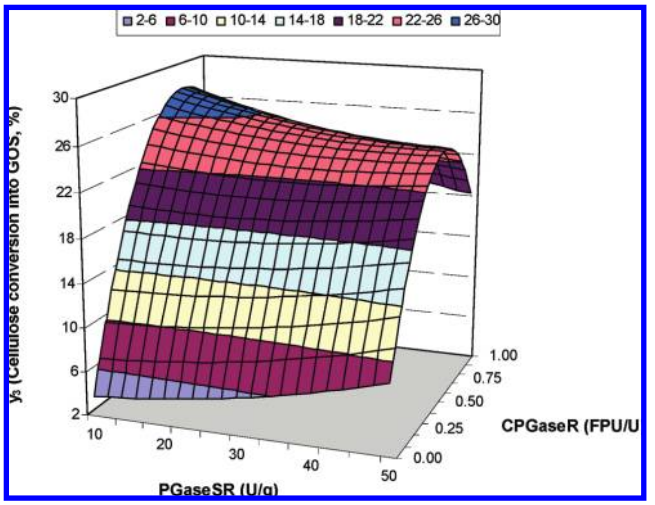

Figure 2. Calculated dependence of cellulose conversion into glucooligosaccharides (variable y_3) on the polygalacturonase to solid ratio (PGaseSR) and the cellulase activity to polygalacturonase activity ratio (CPGaseR) for experiments lasting 10 h.

Arabinan Conversion into Arabinose and Arabinooligosaccharides. Table 4 shows the experimental results obtained for both variables y_6 (arabinan conversion into arabinose) and y_7 (arabinan conversion into AOS). From the results obtained for variable y_6, it can be deduced that the variation pattern is similar to the one observed for cellulose conversion into glucose, and it can be interpreted on the basis of the synergistic action of cellulolytic and pectinolytic enzymes.

The values of the coefficients concerning variable y_6 (listed in **Table 5**) show that all of the independent variables were statistically significant. As expected, high enzyme loadings resulted in increased arabinan conversion into arabinose as a result of the increased hydrolysis rate of hemicellulosic polysaccharides and reaction intermediates. It is also observed that the effects of CPGaseR are negligible when low PGase charges are employed. Oppositely, the effects of cellulase were more pronounced operating with increased values of PGase. Arabinase activity was detected in both Celluclast 1.5 L and Viscozyme preparations by Spagnuolo et al. (*22*), who measured their hemicellulase activities

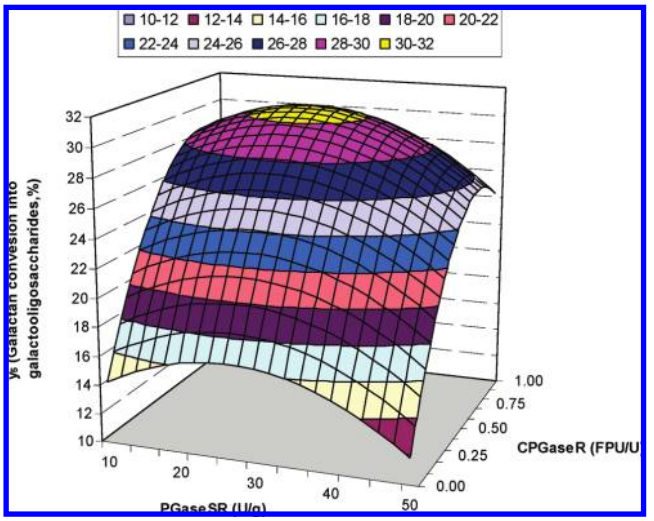

Figure 3. Calculated dependence of galactan conversion into galactooligosaccharides (variable y_5) on the polygalacturonase to solid ratio and the cellulase activity to polygalacturonase activity ratio for experiments lasting 10 h.

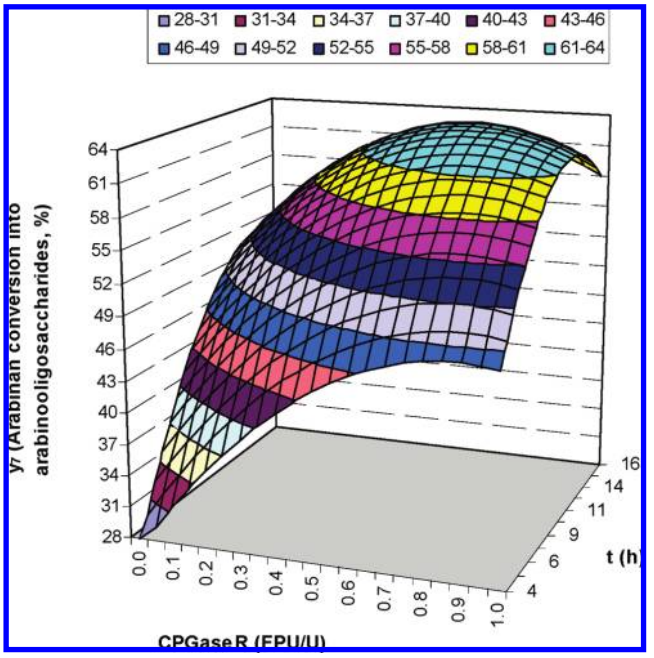

Figure 4. Calculated dependence of arabinan conversion into arabinooligosaccharides (variable y_7) on the cellulase activity to polygalacturonase activity ratio and the reaction time for experiments carried out at CPGaseR = 30 FPU/U.

by arabinose determination after enzymatic hydrolysis of arabinogalactan.

On the other hand, the arabinan conversion into arabinooligosaccharides (y_7) was significantly affected by all of the independent variables (see **Table 5**), but CPGaseR and t were more influential than PGaseSR. **Figure 4** shows the calculated dependence of y_7 on the CPGaseR and t for experiments carried out at PGaseSR = 30 U/g ($x_1 = 0$). CPGaseR had a positive effect on y_7, particularly in the first half of its variation range. The maximum value of y_7 (63.9%) was predicted for conditions defined by CPGaseR = 0.70 FPU/U and t = 12.40 h. Harsher operational conditions (higher enzyme charges and/or longer reaction times) resulted in decreased production of AOS as a consequence of oligomer hydrolysis reactions. Interestingly, the

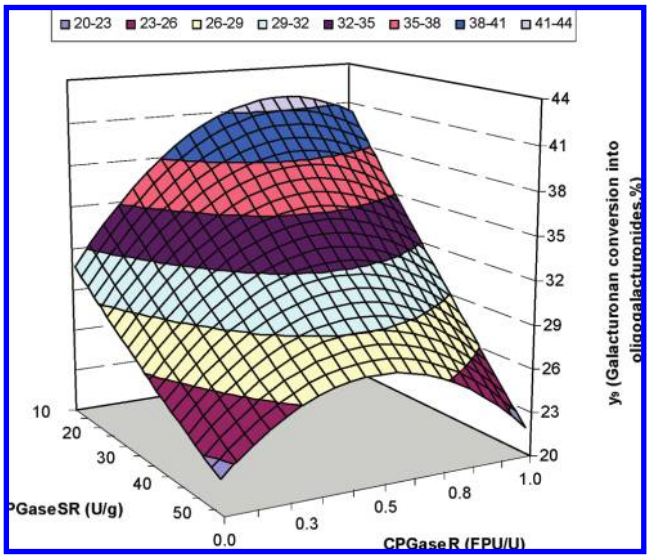

Figure 5. Calculated dependence of galacturonan conversion into oligogalacturonides (variable y_9) on the polygalacturonase to solid ratio and the cellulase activity to polygalacturonase activity ratio for experiments lasting 10 h.

model predictions confirmed that arabinan conversion into arabinooligosaccharides near 60% can be achieved using low enzyme loadings.

Galacturonan Conversion into Galacturonic Acid and Oligogalacturonides. Table 4 also shows the experimental results obtained for both variables y_8 (galacturonan conversion into galacturonic acid) and y_9 (galacturonan conversion into oligogalacturonides). As can be seen, the variable y_8 varied in the range of 4.57−44.8%, this maximum being achieved when under the highest enzyme charges as a result of intense pectin hydrolysis.

On the other hand, **Figure 5** shows the calculated dependence of galacturonan conversion into oligogalacturonides (y_9) on the independent variables PGaseSR and CPGaseR for experiments lasting 10 h ($x_3 = 0$). Increased values of PGaseSR resulted in decreased values of y_9, due to the oligogalacturonide conversion into galacturonic acid. CPGaseR showed a positive effect on the conversion of galacturonan into oligogalacturonides, mainly in the first half of its variation range. Variable y_9 varied from 21.8% (operating at PGaseSR = 50 U/g and CPGaseR = 1 FPU/U) up to 41.85% (operating at PGaseSR = 10 U/g and CPGaseR = 0.75 FPU/U).

Mass of Recovered Liquors. The values of the coefficients concerning variable y_{10} (mass of recovered liquors) listed in **Table 5** show that all of the independent variables were statistically significant. **Figure 6** shows the calculated dependence of y_{10} on PGaseSR and CPGaseR for experiments lasting 10 h, which was characterized by a steady increase of y_{10} with both independent variables a consequence of the major solubilization of the sugar beet polymers. The maximun value (87.4 g) corresponded to operation at PGaseSR = 48U/g and CPGaseR = 0.85 FPU/U.

Model Validation and Selection of Operational Conditions. Additional assays (experiments 16−18) were performed for model validation under operational conditions randomly selected (see **Table 6**). **Table 7** shows the close agreement between experimental and predicted values, which confirms the suitability of the models for reproducing and predicting the experimental results. From the results shown in this last table, it can be deduced that relative errors of $<\pm 11\%$ were found for the majority of the variables and experiments.

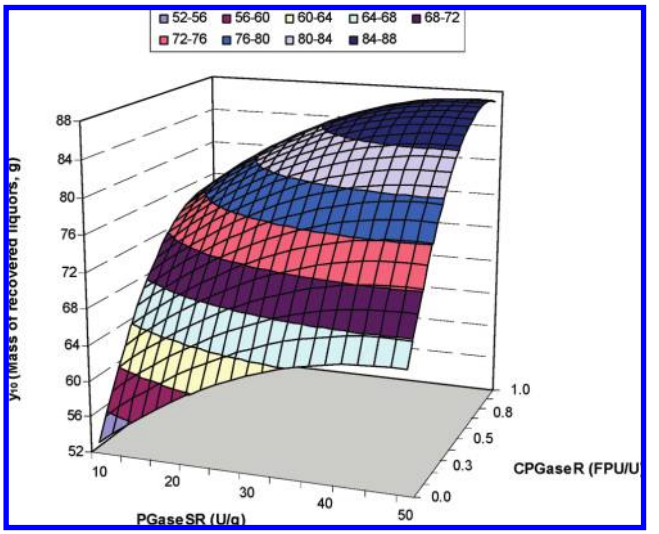

Figure 6. Calculated dependence of the mass of recovered liquors (variable y_{10}) on the polygalacturonase to solid ratio and the cellulase activity to polygalacturonase activity ratio for experiments lasting 10 h.

Table 6. Operational Conditions of Experiments 16−19

	dimensionless normalized independent variables		
expt	x_1	x_2	x_3
16	−0.6	−0.3	0.7
17	−0.3	0.5	0.6
18	0.7	0.2	−0.67
19	−1	0.45	0.47

Table 7. Experimental and Predicted Results Obtained in Experiments 16−19

	predicted values				exptl values			
variable	expt 16	expt 17	expt 18	expt 19	expt16	expt7	expt 18	expt 19
y_1 (%)	39.1	28.4	30.9	37.7	40.7	29.7	32.2	36.7
y_2 (%)	19	35	31.7	20.7	20.5	33.4	29.6	19.6
y_3 (%)	22	25.6	26	29.2	26.2	27.2	27.6	27.3
y_4 (%)	32.2	36.6	32.3	29.7	33	35.3	30.9	27.5
y_5 (%)	23.4	29.4	25.5	27.5	27.7	31.5	28.4	28.6
y_6 (%)	2.5	4.9	5.3	1.8	2.2	4.4	4.7	1.9
y_7 (%)	56.8	62	56.6	55.7	61.7	65.4	61.2	55.5
y_8 (%)	23.7	30.4	26.7	19	23.9	28.6	24.7	16.7
y_9 (%)	35.4	33	31.3	41.3	37.1	35.4	35.1	41.6
y_{10} (g)	75.5	85	81	78.2	79	83	80	75.5

The set of mathematical models listed above was employed to calculate the optimal operational conditions, which were assumed to lead to the maximum yield of total oligomers (OS). This parameter was calculated using the equation

$$\text{OS}\ (\%) = \frac{C_{\text{Gn}}}{100} \times y_3 + \frac{C_{\text{Gan}}}{100} \times y_5 + \frac{C_{\text{An}}}{100} \times y_7 + \frac{C_{\text{GaUn}}}{100} \times y_9 \qquad (4)$$

where C_{Gn}, C_{Gan}, C_{An}, and C_{GaUn} are the glucan, galactan (including xylan, rhamnosyl groups, and mannan), arabinan, and galacturonan contents of SBP (expressed %, oven-dry basis, see **Table 1**).

The optimal operational conditions were $x_1 = -1$, $x_2 = 0.45$, and $x_3 = 0.47$, which corresponded to PGaseSR = 10 U/g, CPGaseR = 0.725 FPU/U, and $t = 12.82$ h. Under these conditions, the models predicted that 906 kg of liquors containing

Table 8. Composition of Hydrolysates and Solids Obtained under Optimal Conditions (Experiment 19)

(a) Composition of the Final Liquid Phase	
component	content
glucose (g/L)	4.50
galactose (g/L)	2.73
arabinose (g/L)	0.37
galacturonic acid (g/L)	3.95
acetic acid (g/L)	0.48
glucooligosaccharides (g/L)	6.21
galactooligosaccharides (g/L)	2.85
arabinooligosaccharides (g/L)	10.99
oligogalacturonides (g/L)	9.90
acetyl groups (g/L)	1.55
protein (g/L)	1.60
other nonvolatile compounds (ONVC)[a]	23.28
(b) Composition of the Solid Phase	
component	content (wt % oven-dry basis)
glucan	17.8
galactan[b]	9.1
arabinan	16.7
acetyl groups	1.6
uronic acids	13.1
protein	20.4
lignin	12.9
others	8.3

[a] Expressed as kg of ONVC/100 kg of NVC. [b] Includes mannan, rhamnosyl groups, and xylan.

26.7 kg of OS can be obtained from 100 kg of SBP, the distribution being as follows: 5.9 kg of GOS, 2.4 kg of GaOS, 9.7 kg of AOS, and 8.7 kg of OGaU. In addition, 10.1 kg of monosaccharides (glucose, 3.9 kg; galactose, 2.4 kg; arabinose, 0.3 kg; and galacturonic acid, 3.5 kg) were simultaneously produced. To obtain an oligosaccharide concentrate suitable for use as a prebiotic ingredient, the monosaccharides content is expected to be reduced by application of a sequence of purification steps including membrane technologies.

To confirm the model predictions, a new experiment (expt 19) was performed under these conditions. **Table 7** lists the experimental and calculated results for the dependent variables achieved in expt 19, which were in close agreement, confirming once more the suitability of the empirical model for quantitative predictions.

Additional relevant information on the composition of both solid and hydrolysates obtained under optimal conditions is given in **Table 8**: 27.8 kg of OS [including GOS, GaOS, AOS, AcO (acetyl groups) and OGaU] with potential as prebiotic food ingredients can be obtained by enzymatic treatment of 100 kg of SBP under the selected conditions. In addition, a protein-rich solid (maybe suitable for use as animal feed or as a nutrient source for biotecnological applications) is simultaneously generated.

ABBREVIATIONS USED

AcO, acetyl groups in oligomers; ADF, acid detergent fiber; ADL acid detergent lignan; AOS, arabinooligosaccharides; An, arabinan; CPGaseR, cellulase activity to polygalacturonase activity ratio; Gan, galactan; GaUn, galacturonan; GaOS, galactooligosaccharides; GOS, glucooligosaccharides; Gn, glucan; HG, homogalacturonan; NDF, neutral detergent fiber; NDO, nondigestible ologosaccharides; NVC, nonvolatile compounds; OGaU, oligogalacturonides; ONCV, other nonvolatile compounds; OS, total oligomers; PGaseSR, polygalacturonase to solid ratio; POS, pectin-derived oligosaccharides; RGI, rhamnogalacturonan I; RGII, rhamnogalacturonan II; SBP, sugar beet pulp; *t*, reaction time.

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