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Biotechnological Production of Xylitol from Agro-Industrial Wastes

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1. Introduction

Xylitol is a polyalcohol of five carbon atoms which is widely used in the food and chemical industry. Its interest is due to the sweetening power similar to that of sucrose. However, it has been shown that the use of xylitol as a sweetener is better due to its anticariogenecity, tooth rehardening and remineralization properties. It is suitable as sugar substitute for diabetics, and it limits the tendency to obesity when is continuously supplied in diet (Salgado et al., 2010a). Table 1 shows the properties of the food additives that are used as sweeteners and are permitted in the European Union (EU).

Xylitol can be found naturally in various fruits and vegetables such as strawberries, raspberries, yellow plum, lettuce and cauliflower (Prakasham et al., 2009). The extraction of xylitol from these products is low, for this reason is not a good source. It is produced mainly by chemical processes. This process is the hydrogenation of the five-carbon sugar D-xylose in the presence of nickel catalyst at elevated temperature and pressure (Prakasham et al., 2009). The chemical process has some drawbacks such as high cost of purification processes. To avoid aggressive stages of the chemical processes, biotechnological processes were studied for xylitol production. This alternative production is bioconversion of D-xylose to xylitol by microorganisms. To reduce costs and environmental problems renewable biomass from agro-industrial waste can be used as source of D-xylose.

The food, agricultural and forestry industries produce large volumes of wastes annually worldwide, causing a serious disposal problem (Rodríguez-Couto, 2008). In the EU waste hierarchy and legislation, prevention and minimization of waste is given the highest priority (Staniskis & Stasiskiene, 2005). The List of Wastes (formerly the European Waste Catalogue), is a catalogue of all waste types generated in the EU. This list of wastes is periodically reviewed on the basis of new knowledge and, in particular, of research results, and if necessary revised in accordance with Article 18 of Waste Framework Directive (75/442/EEC). The list of wastes has been published in the Spanish Official Gazette of 19th February 2002 by Order MAM/304/2002 in conformity with the Commission Decision 2000/532/EC of 3 May 2000, replacing Decision 94/3/EC establishing a list of wastes pursuant to Article 1(a) of Council Directive 75/442/EEC on waste and Council Decision 94/904/EC establishing a list of hazardous waste pursuant to Article 1(4) of Council Directive 91/689/EEC on hazardous waste. The List of Waste has been amended by Commission Decisions 2001/118/EU,

2001/119/EU and 2001/573/EU. The different types of waste in the List are fully defined by a six-digit code, with two digits each for chapter, sub-chapter and waste type. The List is used to categorize items and substances when they become waste, but does not itself define items and substances as waste.

Food additives	Code UE	Sweetness (% SP)	Calorie content (kcal · g^{-1})	Glycemic index	ADI (mg · kg^{-1} body weigh)	Approved In EU
Sorbitol	E 420	60	3.5	<10	Acceptable	Directive 94/35/EC
Mannitol	E 421	50	2.4	0	Acceptable	Directive 94/35/EC
Acesulfame K	E 950	200	0	0	0 - 15	Directive 94/35/EC
Aspartame	E 951	160 - 220	4	0	0 - 40	Directive 94/35/EC
Cyclamic acids and its Na and Ca salts	E 952	30	0	<10	0 - 7	Directive 94/35/EC
Isomalt	E 953	40	2.4	<10	Acceptable	Directive 94/35/EC
Saccharin and its Na, K and Ca salts	E 954	300	0	0	0 - 15	Directive 94/35/EC
Sucralose	E 955	500	0	0	0 - 5	Directive 2003/115/EC
Thaumatin	E 957	1400 - 2200	4	0	Acceptable	Directive 94/35/EC
Neohesperidine DC	E 959	1500	4	0	0 - 5	Directive 94/35/EC
Neotame	E 961	8000 - 13000	4	0	0 - 18	Directive 2009/163/EU
Salt of aspartame-acesulfame 1	E 962		4	0	0 - 40	Directive 2003/115/EC
Maltitol	E 965	90	2.4	36	Acceptable	Directive 94/35/EC
Lactitol	E 966	30 - 40	2.4	<10	Acceptable	Directive 94/35/EC
Xylitol	E 967	100	2.4	<10	Acceptable	Directive 94/35/EC
Erythritol	E 968	60 - 70	0.2	0	Acceptable	Directive 2006/52/EC

SP: Sweetener power respect to sucrose (100)
ADI: Acceptable daily intake established by the Scientific Committee on Food (SCF)

Table 1. Sweeteners permitted for food use in the European Union

The wastes studied in the current work, listed in Table 2, are included in paragraph 2 and 20 of the European List of Waste "Wastes from agricultural, horticultural, hunting, fishing and aquacultural primary production, food preparation and processing" and "Municipal wastes and similar commercial, industrial and institutional wastes including separately collected fractions", respectively. Their reutilization, as well as other wastes, is of great interest since,

due to legislation and environmental reasons, the industry is increasingly being forced to find an alternative use for its residual matter, at the same time that the use of these wastes considerably reduces the production costs (Rodríguez-Couto, 2008).

Lignocelluloses in nature derive from wood, grass, agricultural residues, forestry wastes and municipal solid wastes (Pérez et al., 2002). They constitute a renewable resource from which many useful biological and chemical products can be derived. Accumulation of lignocellulose in large quantities in places where agricultural residues present a disposal problem results not only in deterioration of the environment but also in loss of potentially valuable material that can be used in paper manufacture, biomass fuel production, composting, human and animal feed among others (Sánchez, 2009). These lignocellulosic materials are renewable sources of energy where approximately 90 % of the dry weight of most plant materials is stored in the form of cellulose, hemicelluloses, lignin, and pectin (Kumar et al., 2009). Some authors have reported their fractionation to obtain a variety of marketable chemicals from the polymeric fractions of the raw materials (Moldes et al., 2007), including the xylitol production by *Debaryomyces hansenii*.

2. Wastes from agricultural, horticultural, hunting, fishing and aquacultural primary production, food preparation and processing			
Code	Origin	Type of waste	Characterized waste
02 01 03	Primary production wastes	Plant tissue waste	Leaf fruit Vine leaf
02 03 04	Wastes from fruit, vegetables, cereals, edible oils, cocoa, coffee and tobacco preparation and processing; tobacco processing; conserve production	Materials unsuitable for consumption or processing	Pistachio shells Chesnut shells Nut shells Hazelnut shells
02 06 01	Wastes from the baking and confectionery industry	Materials unsuitable for consumption or processing	Wheat bran leaves
02 07 02		Waste from spirits distillation	Distilled bagasse
02 07 04		Materials unsuitable for consumption or processing	White and red grape stems
20. Municipal wastes and similar commercial, industrial and institutional wastes including separately collected fractions.			
Code	Origen	Type of waste	Characterized waste
20 02 01	Garden and park wastes (including cemetery waste)	Compostable waste	Grass

Table 2. Classification of the characterized wastes according to the European List of Waste

This chapter deals with the study of different agroindustrial wastes through their characterization, fractionation by acid pre-hydrolysis and further analysis of the hemicellulosic fraction. The pre-hydrolysis of pistachio hulls was optimized; and finally, the liquors obtained under the optimal conditions, after supplementation, where assayed to carry out the xylose to xylitol bioconversion by *D. hansenii*.

2. Materials and methods

2.1 Quantitative acid hydrolysis

Aliquots from the homogenized lot were submitted to moisture determination and to quantitative hydrolysis in a two-stage acid treatment (the first stage with 72 wt % sulfuric acid at 30 ^{o}C for 1 h, the second stage after dilution of the media to 4 wt % sulfuric acid at 121 ^{o}C for 1 h) (Bustos et al., 2004). The solid residue after hydrolysis was considered as Klason lignin meanwhile hydrolyzates were analyzed by HPLC as described bellow.

2.2 Acid hydrolysis

Hydrolyzates were obtained in autoclave at 130 °C with 3 % H_2SO_4 solutions during 30 min, using a liquid/solid ratio of 8 g g^{-1} (Portilla-Rivera et al., 2007). The liquid phase from the acid hydrolysis was neutralized with $CaCO_3$ to a final pH of 5.8-6.0, and the $CaSO_4$ precipitated was separated from the supernatant by filtration.

2.3 Charcoal adsorption

Powdered charcoal (Probus, Madrid, Spain) was activated with hot water and dried at room temperature. Charcoal detoxification of hydrolyzates was carried out by contacting hydrolyzates and charcoal (mass ratio: 10 g g^{-1}) at room temperature under stirring for one hour (Rivas et al., 2002). The liquid phase was recovered by filtration and used for making culture media.

2.4 Experimental design and statistical analysis of the pistachio shells prehydrolysis

Pistachio shells were treated with solutions containing 1-3 % H_2SO_4 during 15–45 min. at 130 ^{o}C, according to a statistical experimental design (Design Expert version 5.0, Stat-Ease Inc., Minneapolis, USA), to optimize the prehydrolysis stage, and evaluated by the Response Surface Methodology using Statistica version 5.0 (Statsoft, USA) software considering the SS residual to evaluate the significance of the effects and the model. The influence of two operational variables (concentration of catalyser and reaction time) was tested on three levels in a 3**(2-0) full factorial design. All experiments were carried out in duplicate in randomized run order. Liquors were separated from the solid fraction by vacuum filtration through common laboratory paper filters and analyzed the concentration of xylose, glucose, arabinose, acetic acid, formic acid and furfural, which were the dependent variables.

This design allowed the estimation of the significance of the parameters and their interaction using Student's *t*-test. The interrelationship between dependent and operational variables was established by Eq.1, a model including linear, interaction and quadratic terms:

$$y = b_0 + b_1 \cdot x_1 + b_{11} \cdot x_1^2 + b_2 \cdot x_2 + b_{22} \cdot x_2^2 + b_{12} \cdot x_1 \cdot x_2 \quad (1)$$

where y represents the dependent variables, b denotes the regression coefficients (calculated from experimental data by multiple regression using the least-squares method), and x denotes the independent variables.

2.5 Microorganism and culture conditions

Debaryomyces hansenii NRRL Y-7426 was kindly provided by the National Center for Agricultural Utilization Research (Peoria, Illinois, USA). Freeze-dried cells were grown on a basal medium containing 30 g L^{-1} commercial xylose, 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, and 5 g L^{-1} peptone. The microorganism was maintained in agar slant tubes containing a medium formulated with the same components and concentrations as the previous one plus 20 g L^{-1} agar. Inocula were prepared by solubilization of cells with sterile water and underwent growth during 24 hours in the previous medium without agar. Biomass in inocula was measured by optical density at 600 nm and adjusted by dilution with water, and added to fermentation broth to reach a final concentration in the vicinity of 0.04 g L^{-1}.

Shake flask fermentation experiments were carried out under microaerophilic conditions in 250 mL Erlenmeyer flasks containing 100 mL of culture media (sterilized in autoclave at 100 ^{o}C during 60 min) prepared with 85 mL hydrolyzates and 10 mL of the nutrients indicated in Table 3.

Fermentation	Hydrolyzate	Nutrients
1	Raw	Liquid vinasses + stream B (conc.)
2		Corn steep liquor (30 g/L)
3		YE (3 g/L), ME (3 g/L), Peptone (5 g/L)
4		Without nutrients
5	Detoxified	YE (3 g/L), ME (3 g/L), Peptone (5 g/L)

YE: Yeast Extract; ME: Malt Extract.

Table 3. Nutrients employed in fermentations

Fermentation 1 contains the optimal amount of economic nutrients described by Salgado et al. (2010b) consisting in 50 mL liquid vinasses and 25 mL stream B but concentrated 7.5 times in rotavapor at 50 ^{o}C to reach a final volume of 10 mL. Fermentation 2 contains another economic nutrient, corn steep liquor. Fermentation 4 was carry out using non supplemented hydrolyzates, meanwhile Fermentations 3 and 5 contains commercial nutrients using raw or dextoxified hyrolyzates.

After inoculation (5 mL), fermentations were carried out in orbital shakers (New Brunswick, Edison, NJ, USA) at 100 rpm and 31 ^{o}C for 96 hours. Samples (2 mL) were taken at given fermentation times and centrifuged at 6,000 rpm for 3 min. The supernatants were stored for analyses.

2.6 Analytical methods

Glucose, xylose, arabinose, xylitol, glycerol, acetic acid, ethanol, furfural, and hydroxymethylfurfural (HMF) were measured by a high-performance liquid chromatographic system (Agilent, model 1200, Palo Alto, CA) equipped with a refractive

index detector and an Aminex HPX-87H ion exclusion column (Bio Rad) eluted with 0.003 M sulfuric acid at a flow rate of 0.6 mL min^{-1} at 50 ^{o}C. Biomass concentration in experiments was measured by centrifugation, washing of the cells, and oven-drying to constant weight at 100 ^{o}C.

3. Results and discussion

3.1 Characterization of different agro-industrial wastes

Lignocellulosic materials consists of three types of polymers – cellulose, hemicelluloses, and lignin – that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages. Cellulose and hemicelluloses are macromolecules from different sugars; whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors (Pérez et al., 2002). They also contain smaller amounts of proteins, pectin, acids, salts, minerals, ashes and extractives, including soluble non-structural sugars, nitrogenous material, chlorophyll, and waxes.

Cellulose is a linear polymer of D-glucose subunits linked to each other by β-(1,4)-glucosidic bonds with fibrous structure with a degree of polymerization of up to 10,000 or higher (Jørgensen et al., 2007). The long-chain cellulose polymers are linked together by hydrogen and van der Waals bonds, which cause the cellulose to be packed into microfibrils. Hemicelluloses and lignin cover the microfibrils (Kumar et al., 2009). Cellulose can appear in crystalline form (crystalline cellulose) and, in smaller percentage, in non-organized cellulose chains (amorphous cellulose) (Pérez et al., 2002).

Hemicelluloses is a complex carbohydrate polymer consisting of short highly branched chains of different five-carbon sugars (xylose, rhamnose and arabinose) and six-carbon sugars (glucose, galactose and mannose) and smaller amounts of non-sugars, mainly acetyl groups, but also uronic acids such as 4-o-methylglucuronic, D-glucuronic, and D-galactouronic acids. The backbone of hemicelluloses is either a homopolymer or a heteropolymer with short branches linked by β-(1,4)-glycosidic bonds and occasionally β-(1,3)-glycosidic bonds, and also can have some degree of acetylation. These polymers do not aggregate, even when they cocrystallize with cellulose chains (Kumar et al., 2009). Glucuronoxylan is the principal component of hardwood hemicelluloses whereas glucomannan is predominant in softwood (Pérez et al., 2002). The degree of polymerization is below 200 (Jørgensen et al., 2007).

Finally, lignin is a complex network formed by polymerization of phenyl propane units and constitutes the most abundant non-polysaccharide fraction in lignocellulose (Jørgensen et al., 2007). Lignin is an amorphous heteropolymer complex. The large molecular structure contains cross-linked polymers of phenolic monomers linked by alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds (Kumar et al., 2009). Lignin encrusts the cell walls and cements the cells together (Hamelinck et al., 2005), conferring structural support, impermeability, and resistance against microbial attack and oxidative stress (Pérez et al., 2002).

Table 4 summarizes the quantitative acid hydrolysis of different agroindustrial wastes. Cellulose changes widely from only 10.1 % in vine leaf up to 80-95 % in cotton seed hairs, and 85-99 % in papers, which is an almost exclusively cellulosic material. Furthermore, the composition varies no only among species but also within a single plant depending on

	Cellulose	Hemicelluloses				Lignin	Extractives	Reference
		Total	Xylan	Arabinan	Acetyl groups			
Almond shell	50.7	28.9				20.4	2.5	Demirbas, 2003
	26.8	32.5	26.1	2.4	04.0	27.4	5.0	Nabarlatz et al., 2005
Apple fiber	20.8	10.0-28.4				12.1		Claye et al., 1996
Barley bran husks	23.0	34.3	26.6	6.1	1.6	21.4		Cruz et al., 2000
Chestnut shells	21.1	13.9	10.5	1.4	2.0	46.5	18.6	*
Coastal Bermuda grass	25.0	35.7				6.4		Kumar et al., 2009
Corncob	31.7	38.1	30.9	3.8	3.4	20.3		Cruz et al., 2000
	52.0	32.0				15.1		Demirbas, 2003
	41.2	36.0				6.1		Domínguez et al., 1997
	45	35				15		Kumar et al., 2009
Corn fiber	14.3	16.8				8.4		Mosier et al., 2005
Corn leaves	37.6	37.7	30.3	4.2	3.2	12.6		Cruz et al., 2000
Corn stover	51.2	30.7				14.4		Demirbas, 2003
	37.5	22.4				17.6		Mosier et al., 200517
Cotton seed hairs	80-95	5-20				0		Kumar et al., 2009
Distilled grape marc	10.8	11.2	7.5	2.2	1.6	50.9	14.7	Portilla-Rivera et al., 2008
Flax	34.9	23.6				22.3		Fan et al. 1987
Grasses	25-40	35-50				10-30		Kumar et al., 2009
Hardwood stems	40-55	24-40				18-25		Kumar et al., 2009
Hazelnut kernel shells	29.6	15.7				53.0		Demirbas, 2003
Hazelnut shell	25.9	29.9				42.5	3.3	Demirbas, 2003
	23.7	24.4	19.6	0.7	4.1	40.8	11.1	Portilla-Rivera et al., 2008
Leaf fruit	11.1	14.7	9.2	1.2	4.3	20.5	53.8	*
Leaves	15-20	80-85				0		Kumar et al., 2009
Newspaper	61.3	9.8				12.0		Kim & Moon, 2003
	40-55	25-40				18-30		Kumar et al., 2009
Nut shells	25-30	25-30				30-40		Kumar et al., 2009
Oats	26.6	30.2				21.4		Claye et al., 1996
Oat fiber	26.6	17.0-21.3				21.4		Claye et al., 1996
Oat straw	39.4	27.1				17.5		Fan et al., 1987

Table 4. (continues on next page) Quantitative acid hydrolysis (in %) of agro-industrial wastes

Office paper	68.6	12.4				11.3		Mosier et al., 2005
Olive husk	24.0	23.6				48.4		Demirbas, 2003
Paper	85-99	0				0-15		Kumar et al., 2009
Pistachio shells	15.2	38.5	33.1	0.0	5.4	29.4	17.0	*
Red grape stem	13.3	8.47	6.7	0.77	1.0	35.9	42.4	*
Rice bran	24.4	7.6-24.0				18.4		Claye et al., 1996
Rice hulls	35.6	11.96				15.4		Saha et al., 2005
Softwood stems	45-50	25-35				25-35		Kumar et al., 2009
Solid cattle manure	1.6-4.7	1.4-3.3				2.7-5.7		Kumar et al., 2009
Sorted refuse	60.0	20				20		Kumar et al., 2009
Soybean stems	34.5	24.8				19.8		Fan et al., 1987
Sugarcane bagasse	35.0	35.8				16.1		Sasaki et al., 2003
Sunflower shell	48.4	34.6				17.0	2.7	Demirbas, 2003
Swine waste	6.0	28				-		Kumar et al., 2009
Switchgrass	32.0	25.2	21.1	2.8		18.1	17.5	Hamelinck et al., 2005
	45.0	31.4				12		Kumar et al., 2009
	31.0	20.4				17.6		Mosier et al., 2005
	22.2	13.9	10.4	3.5	0.0	18.0	46.0	*
Thistle	31.1	12.2				22.1		Jiménez & López, 1993
Tomato fiber	19.7	13.2-23.3				13.8		Claye et al., 1996
Vine leaf	10.1	8.37	5.9	1.8	0.67	44.4	37.1	*
Vineshoots	34.1	19.0	12.8	0.90	5.3	27.1	7.1	Bustos et al. 2004
Walnut shell	25.6	22.1				52.3	3.3	Demirbas, 2003
	23.0	21.0	16.0	1.2	3.8	37.4	18.6	Portilla-Rivera et al., 2008
Waste papers from chemical pulps	60-70	10-20				5-10		Kumar et al., 2009
Wheat bran	32.2	16-28				5.2		Claye et al., 1996
Wheat bran leaves	26.2	24.3	15.8	8.5	0.0	7.9	41.5	*
Wheat straw	28.8	39.1				18.6		Demirbas, 2003
	30.0	50				15		Kumar et al., 2009
	38.2	21.2				23.4		Mosier et al., 2005
	48.6	27.7						Saha et al., 2005
White grape stem	20.7	11.37	10.4	0.00	0.97	31.3	36.7	

*Current work.

Table 4. (continued) Quantitative acid hydrolysis (in %) of agro-industrial wastes

several factors such as age, location or stage of growth. For instance, we obtained a percentage of 22.2 % in switchgrass, but Kumar et al. (2009) reported a percentage of 45 %. Meanwhile, hemicelluloses are in general in smaller amounts, although in some particular cases such as leaves can account for values as high as 80-85 % (Kumar et al., 2009), being xylan the main constituent in all the cases indicated. Regarding lignin, Demirbas (2003) reported the highest percentage of lignin (53.0 %) in hazelnut kernel shells, although this percentage varies widely depending on the material. Papers and herbaceous plants show in general the lowest contents; meanwhile softwoods and nuts have the highest contents (see Table 4).

3.2 Prehydrolysis of lignocellulosic materials

Due to the robust structure of lignocellulosic biomass, pretreatment is a prerequisite for hydrolysis into fermentable sugars to be completed within an industrially acceptable time frame (Jørgensen et al., 2007). Although fermentable D-glucose could be produced from cellulose through the action of either acid or enzymes breaking the β-(1,4)- glycosidic linkages (Kumar et al., 2009), the structure of cellulose along with the intermolecular hydrogen bonds gives cellulose high tensile strength, makes it insoluble in most solvents and is partly responsible for the resistance of cellulose against microbial degradation (Ward & Moo-Young 1989). On the contrary, hemicelluloses, because of their branched, amorphous nature, are relatively easy to hydrolyze (Hamelinck et al., 2005), particularly, in contrast to cellulose, the polymers present in hemicelluloses are easily hydrolyzable (Kumar et al., 2009).

Over the years a number of different technologies have been successfully developed for pretreatment of lignocellulose (Jørgensen et al., 2007), including concentrated or dilute-acid hydrolysis. Concentrated acids such as H_2SO_4 and HCl have been used to treat lignocellulosic materials. Nevertheless, although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive, hazardous, and thus require reactors that are resistant to corrosion, which makes the pretreatment process very expensive. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Kumar et al., 2009).

Among the chemichal hydrolysis methods, dilute-acid hydrolysis is probably the most commonly applied (Taherzadeh & Karimi, 2007). Therefore, dilute-acid hydrolysis (prehydrolysis) where a mineral acid is added to the reaction media to break down polymers, can be a possible first processing step for an integral use of biomass, since hemicelluloses can be almost completely solubilized, whereas little alteration is caused in both lignin and cellulose, which are recovered in the solid phase (Moldes et al., 2007). Among them, sulfuric acid has been of the most interest in such studies as it is inexpensive and effective (Kumar et al., 2009).

The lignocellulosic materials were subjected to prehydrolysis treatments under the same conditions (3 % H_2SO_4, 130 ^oC, 30 min., liquid to solid ratio = 8 g/g) previously reported by other authors (Portilla-Rivera et al., 2007). Table 5 reports the sugars content and other compounds solubilized during the treatment. Xylose was the sugar released in highest concentration, reaching a maximal xylose content of 41.8 g L^{-1} in pistachio shells. Additionally, glucose and arabinose appear in the lowest amounts of 0.88 g L^{-1} and 0.67 g L^{-1},

respectively, showing a xylose to glucose ratio of 47.6. Nevertheless, some possible toxins of further fermentable broths were generated, including acetate from the deacetylation of xylan and furan dehydration products (furfural from xylose and hydroxymethylfurfural from glucose). Using pistachio shells, 8.0 g L^{-1} of acetic acid and 1.7 g L^{-1} of furfural were released, meanwhile the highest amount of HMF, 1.2 g L^{-1}, was observed after the acid hydrolysis of white grape stems. Other compounds were also present in hydrolyzates, including citric, tartaric and lactic acids, and glycerol. Tartaric acid concentrations were relatively elevated in viticulture wastes (white and red grape stems and vine leafs) with concentrations up to 9.8 g L^{-1}. Nevertheless, these acids are not toxic for further fermentations stages.

Agroindustrial wastes	Citric acid	Tartaric acid	Glucose	Xylose	Xylose/ Glucose ratio	Arabinose	Lactic acid	Glycerol	Acetic acid	HMF	Furfural
Chestnut shells	0.0	6.9	2.6	13.3	5.0	3.2	0.0	0.0	2.8	0.0	0.0
Grass	1.7	1.0	8.4	12.4	1.5	3.4	0.0	0.0	1.5	0.51	0.51
Leaf fruit	0.0	1.4	5.8	9.1	1.6	5.5	0.0	0.0	2.2	0.47	0.0
Pistachio shells	0.0	1.5	0.88	41.8	47.6	0.67	0.0	0.0	8.0	0.0	1.7
Red grape stem	0.79	9.8	5.3	12.6	2.4	2.1	0.0	1.1	2.7	0.0	0.0
Vine leaf	0.0	9.4	4.4	6.8	1.6	3.7	0.0	0.0	1.6	0.0	0.0
Wheat bran leaves	0.0	0.0	23.8	22.5	0.95	9.9	0.0	0.0	0.71	0.69	0.88
White grape stem	1.2	8.2	12.1	13.5	1.1	2.0	0.98	3.5	2.6	1.2	0.0

Table 5. Composition (g/L) of hydrolyzates after prehydrolysis at 130 °C with 3 % H_2SO_4 during 30 min. using a liquid/solid ratio = 8 g g^{-1}

3.3 Optimization of the prehydrolysis of pistachio shells

Considering that pistachio shells provides the highest amount of xylose, the acid hydrolysis of this material was optimized using a 3**(2-0) full factorial design, to produce the highest level of hemicelluloses hydrolysis with the lowest level of degradation of the cellulosic fraction. The use of experimental designs to optimize the hydrolysis conditions is a technology widely used in bibliography (Bustos et al., 2005). In the present work, the independent variables considered and their variation ranges were: H_2SO_4 concentration (1-3 %) and duration of treatments (15-45 min). The standardized (coded) adimensional variables employed, having variations limits (-1,1), were defined as x_1 (coded H_2SO_4 concentration) and x_2 (coded time). The correspondence between coded and uncoded variables was established by linear equations deduced from their respective variation limits:

$$x_1 = ([H_2SO_4] - 2) \quad (2)$$

$$x_2 = (t - 30)/15 \quad (3)$$

The composition of liquors was measured by dependent variables: y_1 (glucose, g L^{-1}), y_2 (xylose, g L^{-1}), y_3 (arabinose, g L^{-1}), y_4 (acetic acid, g L^{-1}), y_5 (formic acid, g L^{-1}) and y_6 (furfural, g L^{-1}). Table 6 shows the set of experimental conditions assayed expressed in terms of coded variables, as well as the experimental data obtained for variables y_1 to y_6. The sequence for the experimental work was randomly established to limit the influence of systematic errors on the interpretation of results.

Operational conditions			Experimental results					
Experiment	x_1	x_2	y_1	y_2	y_3	y_4	y_5	y_6
1	-1	-1	0.44	34.4	0.62	7.2	0.79	0.21
2	-1	0	0.66	37.9	0.83	7.5	0.75	1.3
3	-1	1	0.62	37.8	0.92	7.9	0.82	1.6
4	0	-1	0.56	39.0	0.60	8.1	0.90	0.65
5	0	0	0.74	41.1	0.64	8.1	0.84	1.3
6	0	1	0.88	38.8	0.65	8.3	1.1	1.9
7	1	-1	0.67	40.6	0.65	8.1	1.2	1.1
8	1	0	0.98	41.8	0.64	8.1	1.1	2.2
9	1	1	1.1	38.0	0.67	8.5	1.3	3.0

Table 6. Operational conditions considered in this study (expressed in terms of the coded independent variables dimensionless H_2SO_4 concentration x_1 and dimensionless time x_2 and experimental results achieved for the dependent variables y_1 (glucose concentration, g/L); y_2 (xylose concentration, g/L); y_3 (arabinose concentration, g/L); y_4 (acetic acid concentration, g/L); y_5 (formic acid concentration, g/L) and y_6 (furfural concentration, g/L). Results are the average of two independent experiments. Standard deviations were in the range of 1-3 % of the mean

Table 7 lists the regression coefficients and their statistical significance (based on a t-test) as well as the statistical parameters (R^2, R^2 adjusted, F and the significance level based on the F test) measuring the correlation and the statistical significance of the models, respectively. It can be noted that all the models showed good statistical parameters for correlation and significance allowing a close reproduction of experimental data. In relation with the influence of independent variables, H_2SO_4 concentration (x_1) caused the strongest effect on the variation of dependent variables considered, as it can be seen from the absolute value of the corresponding coefficients.

Fig. 1 shows a three dimensional representation of the response surface for the predicted dependence of the xylose concentration of samples (y_2) on the sulfuric acid concentration and reaction time as well as a two-dimensional contour plot generated by the model. The response surface shows a slight continuous increase in y_2 with catalyze and an optimum value at intermediate reaction times.

From the coefficients of Table 7, Eq. 4 was deduced for y_2 (xylose concentration):

$$y_2 = 41.06889 + 1.72 * x_1 - 1.21333 * (x_1)^2 - 2.16333 * (x_2)^2 - 1.4975 * x_1 * x_2 \quad (4)$$

Using the "solver" application of Microsoft Excell the maximum xylose concentration predicted for the model (y_1 = 41.8 g L^{-1}) was achieved when x_1 = 0.90 (2.9 %) and x_2 = -0.31 (25.3 min.). This value corresponds to 90.32 % of the theoretical xylose present in the raw material.

a) Regression coefficients and significance level for the dependent variables.						
Coeficientes	y_1	y_2	y_3	y_4	y_5	y_6
b_0	0.7822*	41.06889*	0.6422*	8.1289*	0.86*	1.4*
b_1	0.17*	1.72*	-0.06833**	0.3533*	0.1883*	0.5317*
b_{11}	0.01667	-1.21333*	0.091667***	-0.2933***	0.045	0.285
b_2	0.1533*	0.08	0.061667**	0.2483**	0.045***	0.75*
b_{22}	-0.08333***	-2.16333*	-0.018333	0.08167	0.105**	-0.2
b_{12}	0.06***	-1.4975*	-0.07***	-0.0775	0.015	0.1425

b) Statistical parameters (R^2 and F) measuring the correlation and significance of the models.				
Variable	R^2	corrected R^2	Fexp.	significance level (based on the F test)
y_1	0.98722	0.96592	540.715	96.69
y_2	0.99356	0.98283	1080.1478	97.66
y_3	0.93434	0.82491	99.6115	92.30
y_4	0.95926	0.89137	164.8324	94.01
y_5	0.97965	0.94574	337.0255	95.81
y_6	0.98035	0.94761	349.3056	95.88

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

Table 7. Regression coefficients, significance level for dependent variables and statistical parameters measuring the correlation and significance of the models

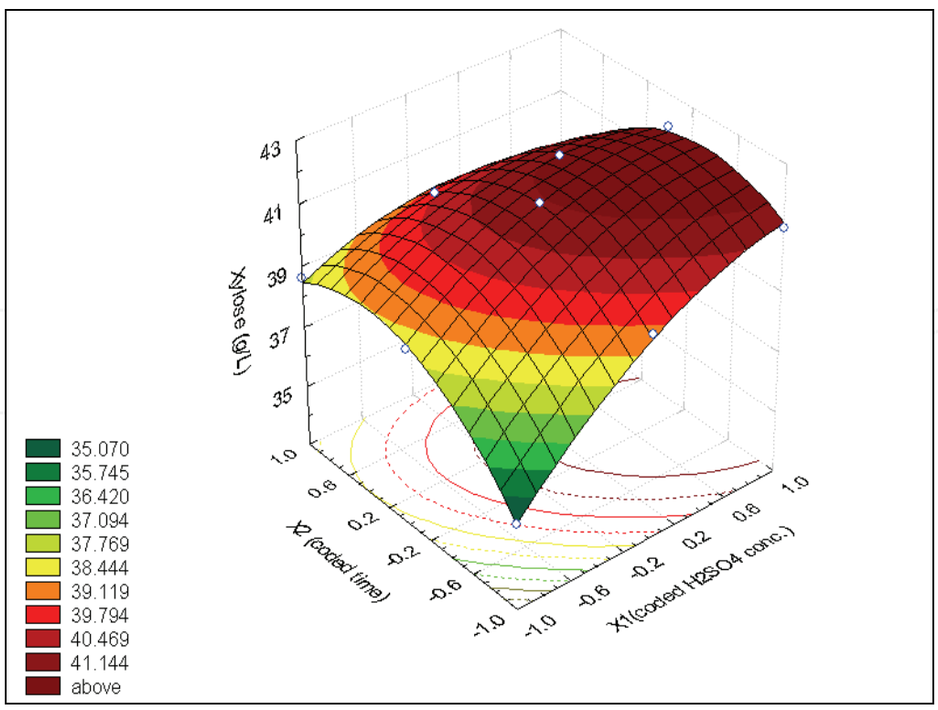

Fig. 1. Response surface and contour plot for the dependence of xylose concentration (y_2) on H_2SO_4 conc. (x_1) and reaction time (x_2)

3.4 Fermentation under the optimal hydrolysis conditions of pistachio shells

Diluted acids were used under the optimal conditions predicted by the solver application to obtain hydrolyzates with high content of xylose. Fig. 2 and Table 8 show the effect of nutrient supplementation on xylitol production by *D. hansenii*. Under all the conditions assayed, a 24h lag phase was observed. Xylose started to be consumed after glucose was firstly depleted within 24h. Conversely to other works with the same yeast (Carvalheiro et al., 2007), it is interesting to note the pronounced diauxic growth pattern obtained for the

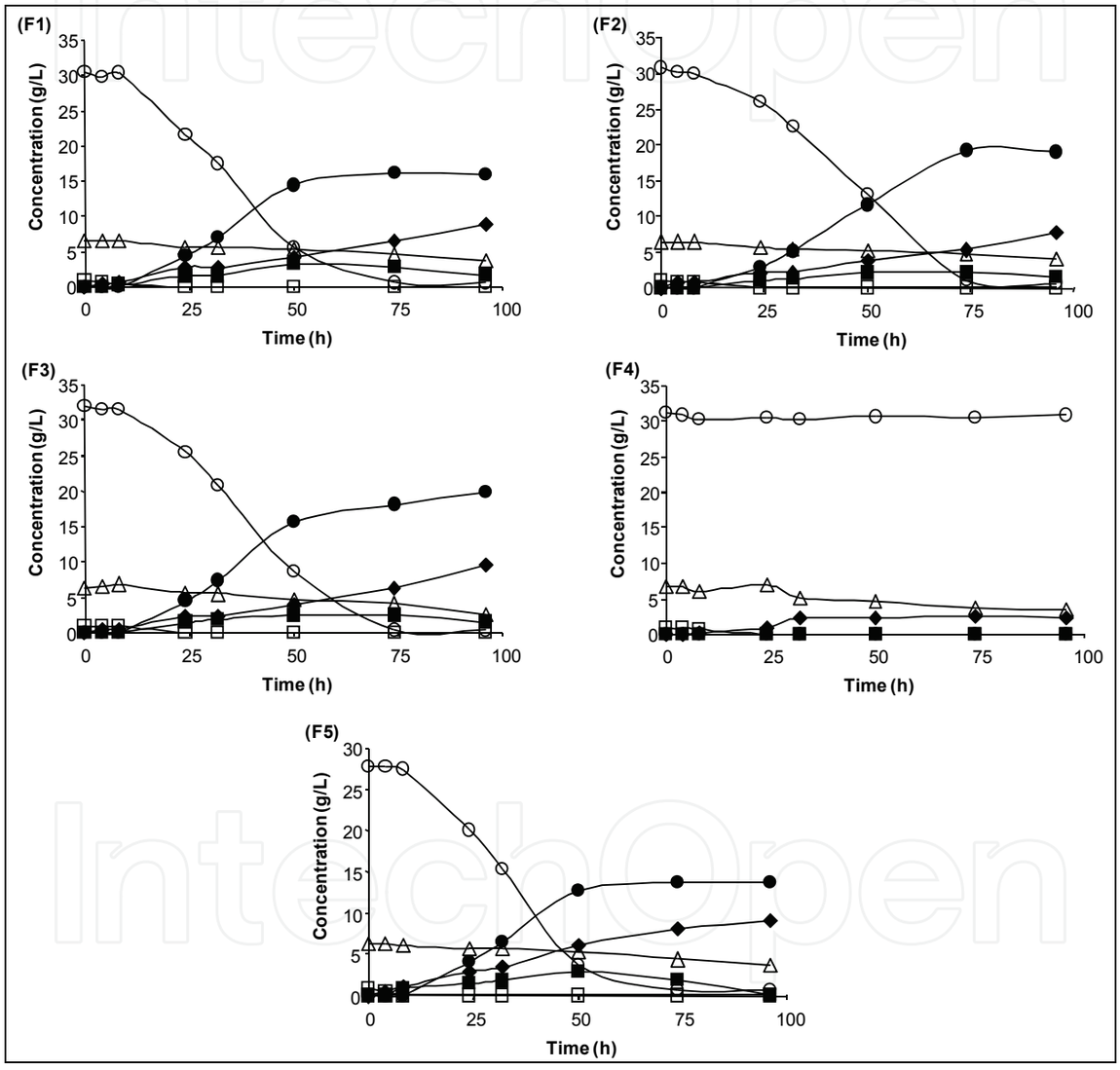

Fig. 2. Course with time for the xylose to xylitol bioconversion by *Debaryomyces hansenii* during fermentations carried out using the nutrients described in Table 3. (O) xylose, (□) glucose, (△) acetic acid, (●) xylitol, (■) ethanol, (◆) biomass. (F1) raw hydrolyzate, liquid vinasses and stream B (conc.); (F2) raw hydrolyzate, corn steep liquor (30 g/L); (F3) raw hydrolyzate, yeast extract (3 g/L), malt extract (3 g/L), peptone (5 g/L); (F4) raw hydrolyzate, without nutrients; (F5) detoxified hydrolyzate, yeast extract (3 g/L), malt extract (3 g/L), peptone (5 g/L)

SC = total sugars consumed; Q_S = volumetric rate of substrates (xylose, glucose and arabinose) consumption; $Q_{Xylitol}$ = global volumetric productivity of xylitol; $Y_{Xylitol/SC}$ = xylitol yield (xylitol produced/ total sugars consumed); Q_X = biomass volumetric productivity; $Y_{X/SC}$ = biomass yield

Table 8. Stoichiometric parameters, productivities and yields after 74h for hemicellulosic pistachio shells hydrolyzates bioconversion in assays carried out by *D. hansenii*. Results are the average of two independent experiments. Standard deviations were in the range of 2-4 % of the mean

	Glucose (g/L)		Xylose (g/L)		Arabinose (g/L)		SC (g/L)	Q_S (g/L·h)	Acetic acid (g/L)		Xylitol (g/L)		$Q_{Xylitol}$ (g/L·h)	$Y_{Xylitol/SC}$ (g/g)	Glycerc (g/L)	
	T_0	T_f	T_0	T_f	T_0	T_f			T_0	T_f	T_0	T_f			T_0	T
F1	0.89	0	30.6	0.51	0.57	0.50	31.1	0.420	6.5	4.8	0	16.2	0.219	0.52	0	0.
F2	0.93	0	30.8	1.2	1.0	0.93	30.6	0.413	6.5	4.7	0	19.3	0.260	0.63	3.8	3.
F3	0.91	0	32.0	0.45	0.64	0.61	32.5	0.439	6.3	4.2	0	18.0	0.244	0.56	2.8	3.
F4	0.78	0	31.3	30.4	0.57	0.52	1.7	0.023	6.6	1.9	0	0	0	0	0	0
F5	0.76	0	27.8	0.58	0.52	0.41	28.1	0.379	6.4	4.1	0	13.7	0.185	0.49	0	1.

mixture of glucose and xylose. A similar pattern was observed for *Candida guilliermondii* (Canilha et al., 2005), and *Candida tropicalis* (Walther et al., 2001). Arabinose and acetic acid were scarcely consumed (see Table 8). Thus, meanwhile no arabinose was consumed in fermentation 4 (raw hydrolyzates without supplementation), the consumption in fermentation 5 (detoxified hydrolyzates supplemented with commercial nutrients) only reached 37 %; whereas acetic acid consumption ranged 39.1-58.8 % regardless of supplementation or detoxification treatment. Supplementation with economic nutrients (vinasses or corn steep liquor) led to an increase in the overall sugars consumption rate in comparison with the use of commercial nutrients.

Xylitol was the main product for all media except for fermentation 4 where no production was observed, meaning that these hydrolyzates require to be supplemented with additional nutrients to stimulate the xylitol production. The necessity to supplement hydrolyzates with nutrients seems to depend on the raw material (Carvalheiro et al., 2007), and while the supplementation was shown to be beneficial for *Candida guilliermondii* during the xylose-to-xylitol bioconversion in *Eucalyptus* hydrolyzates (Canettieri et al., 2001), no addition of nutrients was necessary in rice straw hydrolyzates for the same yeast (Silva & Roberto, 1999). The highest concentration, 19.3 g L^{-1} after 74 hours, was obtained in hydrolyzates supplemented with economic wastes (vinasses and stream B), corresponding to fermentation 2 (Q_P = 0.260 g L^{-1} h^{-1}; $Y_{P/S}$ = 0.63 g g^{-1}), followed by the use of another economic nutrient in fermentation 3, corn steep liquor, where xylitol reached up to 18.0 g L^{-1} (Q_P = 0.244 g L^{-1} h^{-1}; $Y_{P/S}$ = 0.56 g g^{-1}). In fermentations supplemented with commercial nutrients (fermentations 1 and 5), conversely to the expected, the higher xylitol value, 16.2 g L^{-1}, was observed in fermentation without detoxification. Ethanol and glycerol were also produced although in small amounts.

Finally, it is important to mention the amount of biomass generated in all fermentations with the exception of fermentation 4 where only 2.3 g L^{-1} was achieved. Usually, the growth begins with a lag phase in which the microorganism adapts to the enzymatic systems in order to metabolize the new substrate, but without growing (Sánchez et al., 2008), however, the previous adaptation of *D. hansenii* to hydrolyzates minimize this phase, showing a linear growth during the total period of fermentation, producing up to 9.5 g L^{-1} after 96 hours in fermentation 3 (Q_X = 0.099 g L^{-1} h^{-1}; $Y_{X/S}$ = 0.29 g g^{-1}).

4. Conclusions

In this study, the following conclusions can be drawn related to agro industrial wastes evaluation:

1. Based on the characterization and further diluted acid hydrolysis carried out to the agro industrial wastes evaluated, it can be concluded their great potential as feedstocks for the production of industrially relevant food additives.
2. In particular, pistachio shells show the higher xylan content. Optimizing the conditions of hydrolysis by an experimental design reaches 90% of the theorical xylose present in raw material.
3. Hydrolyzate medium obtained with nutrients supplementation can be effectively employed for the xylitol production by *Debaryomyces hansenii*.

5. Acknowledgements

The authors gratefully thank the financial support from the XUNTA DE GALICIA (project 09TAL013383PR).

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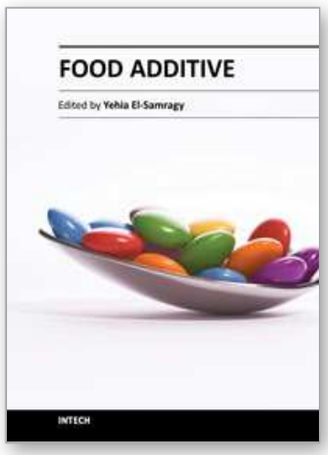

Food Additive

Edited by Prof. Yehia El-Samragy

ISBN 978-953-51-0067-6
Hard cover, 256 pages
Publisher InTech
Published online 22, February, 2012
Published in print edition February, 2012

A food additive is defined as a substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value. Food additives are natural or manufactured substances, which are added to food to restore colors lost during processing. They provide sweetness, prevent deterioration during storage and guard against food poisoning (preservatives). This book provides a review of traditional and non-traditional food preservation approaches and ingredients used as food additives. It also provides detailed knowledge for the evaluation of the agro-industrial wastes based on their great potential for the production of industrially relevant food additives. Furthermore the assessment of potential reproductive and developmental toxicity perspectives of some newly synthesized food additives on market has been covered. Finally, the identification of the areas relevant for future research has been pointed out indicating that there is more and more information needed to explore the possibility of the implementation of some other materials to be used as food additives.

How to reference

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José Manuel Domínguez, José Manuel Salgado, Noelia Rodríguez and Sandra Cortés (2012). Biotechnological Production of Xylitol from Agro-Industrial Wastes, Food Additive, Prof. Yehia El-Samragy (Ed.), ISBN: 978-953-51-0067-6, InTech, Available from: http://www.intechopen.com/books/food-additive/biotechnological-production-of-xylitol-from-agro-industrial-wastes

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