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Citrus Peels: An Excellent Raw Material for the Bioconversion into Value-added Products

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ABSTRACT

Citrus by-products are the processing wastes generated after citrus juice extraction and constitute about 50% of fresh fruit weight. This solid residue is comprised of the peel (flavedo and albedo), pulp (juice sac residue), rag (membranes and cores) and seeds. The disposal of fresh peels is becoming a major problem for many factories. Usually, citrus juice industries dry the residue and it is either sold as raw material for pectin extraction or pelletized for animal feeding, though none of these processes is very profitable. This residual material is a poor animal feed supplement because of its extremely low protein content and high amount of sugar. The application of agroindustrial by-products in bioprocesses offers a wide range of alternative substrates, thus helping to solve pollution problems related to their disposal. Attempts have been made to use citrus by-products to generate several value-added products, such as enzymes, single cell protein, natural antioxidants, ethanol, organic acids, polysaccharides and prebiotics. This article reviews developments regarding processes and products that have employed citrus peels as a substrate for biotechnological applications.

Keywords: agroindustrial residues, bioproducts
Abbreviations: AG-I, arabinogalactan I; **BPF**, by-product feedstuffs; **SCP**, Single cell protein; **DCP**, dried citrus pulp; **DM**, dry matter; **FOS**, fructo-oligosaccharides; **HG**, homogalacturonan; **HR**, hairy regions; **IDF**, insoluble dietary fibre; **POS**, pectic oligosaccharides; **RG-I**, rhamnogalacturonan-I; **RG-II**, rhamnogalacturonan-II; **SDF**, soluble dietary fibre; **SHF**, separate hydrolysis and fermentation; **SSCF**, simultaneous saccharification and co-fermentation; **SmF**, submerged fermentation; **SSF** solid state fermentation

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INTRODUCTION

According to the FAO estimates of world citrus production for 2005 was 94.8 million MT (**Table 1**) (FAO 2005). The genus *Citrus* includes several important fruits (Kale and Adsule 1995), with the most important on a worldwide basis being sweet orange (*C. sinensis*: 61.1% of world citrus production), tangerine (*C. reticulata*: 19.9%), lemon and lime (*C. limon* and *C. aurantifolia*: 12.1%) and grapefruit (*C. paradisi*: 5.0%). Minor citrus genera that comprise the bulk of the remaining 2.0% include sour orange (*C. quarantium*), shaddock (*C. grandis*), and citron (*C. medica*). About 20.6% of world production of citrus is in the Mediterranean countries of Spain, Italy, Greece, Egypt, Turkey and Morocco, with Brazil (20%), China (16%) and the USA (11%) being major individual citrus producing countries (**Table 1**). Approximately, 27 million MT of the total citrus production for the year 2005 will be processed to yield juice, essential oils and other by-products (**Table 2**) (FAO 2005). Citrus by-products are the principal solid by-product of the citrus processing industry and constitute about 50% of fresh fruit weight (Garzón and Hours 1992). Large amounts of citrus wastes are produced worldwide, and being highly biodegradable its disposal represents a serious environmental prob-

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Invited Review

Table 1 World citrus production (thousand tons) (FAO 2005).

	Total	Oranges	Tangerines	Lemon and limes	Grape fruits
WORLD	94793.1	59041.4	19224.9	11681.4	4845.4
Northern Hemisphere	67565.3	37728.9	17054.3	8588.8	4193.3
USA	10498.5	8419.1	367.3	789.4	922.7
Mediterranean region	19546.5	10922.9	5207.4	2847.7	568.5
Greece	861.0	763.2	59.7	31.5	6.6
Italy	3320.9	2105.1	611.6	597.4	6.8
Spain	6181.3	2835.4	2 500.4	809.5	36.0
Israel	639.9	184.2	122.8	68.0	264.9
Algeria	542.7	390.0	111.0	40.0	
Morocco	1320.9	827.0	463.9	25.0	5.0
Tunisia	307.9	174.7	33.2	28.0	72.0
Egypt	2706.3	1759.3	612.6	331.4	3.0
Cyprus	178.7	69.5	50.4	20.9	37.9
Lebanon	339.0	200.0	42.0	83.0	
Turkey	2316.8	1040.0	500.0	670.0	106.8
Others	831.1	721.6	99.8	143.0	
Portugal	335.7	250.3	65.6	12.3	
Japan	1341.0	88.0	1249.0		
Costa Rica	367.0	367.0			
El Salvador	46.0				
Guatemala	106.0				
Honduras	308.6				
Mexico	6910.0	4300.0	360.0	1890.0	360.0
Belize	269.4	213.4			
Cuba	216.0	200.0			7.0
Iran	3037.0	1900.0		1100.0	
China	15227.9	4462.0	8695.0		1903.0
India	4662.0	3100.0		1420.0	142.0
Pakistan	504.5				
Indonesia	1311.7	1311.7			
Korea Rep	594.0		594.0		
Viet Nam	572.5				
Others	1711.0	2194.5	516.0	529.4	290.1
Southern Hemisphere	27227.8	21312.5	2170.5	3092.6	652.1
Argentina	2670.0	770.0	430.0	1300.0	170.0
Brazil	18902.5	16565.0	1270.0	1000.0	67.5
Chile	312.0			170.0	
Colombia	330.0				
Ecuador	305.1			38.3	
Paraguay	289.6	205.7		16.4	43.3
Peru	754.0	315.0	172.0	220.0	47.0
Uruguay	242.2	124.1	77.3	33.5	
Venezuela	379.1	370.0			
Australia	716.1	571.0		35.0	15.1
South Africa	1543.0	1113.0		180.0	250.0
Others	784.2	1278.7	221.2	99.4	59.2

lem.

Over the past few decades, an increasing trend toward efficient utilization of natural resources has been observed around the world. The direct disposal of agroindustrial residues as a waste on the environment represents an important loss of biomass, which could be bioconverted into different metabolites, with a higher commercial value (Thomsen 2005). Many researchers, looking for value-added products, have proposed the use of citrus peels for the production of enzymes (Larios *et al.* 1989; Garzón and Hours 1992; Ismail 1996; Martins *et al.* 2002; Silva *et al*. 2002; Dhillon *et al.* 2004; Mamma *et al*. 2008) bioethanol (Grohman *et al.* 1994b, 1998; Wilkins *et al.* 2007), citric acid (Aravantinos-Zafiris *et al.* 1994; Rivas *et al.* 2008), xanthan gum (Bilanovic *et al.* 1994; Green *et al.* 1994; Stredansky and Conti 1999) single cell protein (de Gregorio *et al.* 2002), prebiotics (Olano-Martin *et al.* 2001; Hotchkiss *et al.* 2003; Mandalari *et al.* 2007) natural antioxidants (Li *et al*. 2006; Mandalari *et al*. 2006), among many others.

Table 2 Total citrus utilization for processing (thousand tons)(FAO 2005).

	Total	Oranges	Tangerines	Lemon and limes	Grape fruits
WORLD	26635.0	21815.9	1836.6	2119.4	863.1
Northern Hemisphere	12226.1	9180.8	1455.5	929.3	660.5
USA	6968.0	6278.0	109.0	228.0	353.0
Mediterranean region	3243.6	2087.1	589.2	383.7	183.6
Greece	265.6	263.6		0.1	0.7
Italy	1274.5	903.4	200.6	170.5	
Spain	1011.9	547.6	315.3	146.8	2.2
Israel	296.0	86.0	47.0	6.0	157.0
Morocco	28.3	26.7	1.6		
Egypt	83.6	68.8			
Cyprus	54.2	26.9		4.4	10.4
Turkey	188.7	128.0	11.0	40.0	9.7
Others	40.8	36.3	13.7	15.9	3.6
Japan	149.0		143.0		
Mexico	1079.0	650.0		317.0	112.0
Cuba	111.0	110.0			1.0
China	538.0	28.0	510.0		
Others	137.5	27.7	104.3		
Southern Hemisphere	14408.9	12635.1	381.1	1190.1	202.6
Argentina	1170.0	170.0	45.0	865.0	90.0
Brazil	12621.9	11995.0	317.5		59.4
Uruguay	46.7		8.5		
Australia	235.0	24.8	10.1	11.2	7.6
South Africa	330.0	235.0		50.0	45.0
Others	5.3	210.3			

PRODUCTION AND COMPOSITION OF CITRUS BY-PRODUCTS

Citrus fruits are principally consumed by humans as fresh fruit or processed juice, either fresh chilled or concentrated. After juice is extracted from the fruit, there remains a residue (**Table 3**) comprised of peel (flavedo and albedo), pulp (juice sac residue), rag (membranes and cores) and seeds. These components, either individually or in various combinations, are the source materials from which citrus by-product feedstuffs (BPF) are produced (Sinclair 1984; Ensminger *et al.* 1990). The main citrus BPF from citrus processing (**Fig. 1**) are fresh citrus pulp which is the whole residue after extraction of juice, representing between 492 and 692 g/kg of fresh citrus fruit with 600–650 g dry matter (DM)/kg peel, 300–350 g/kg pulp and 0–100 g/kg seeds (Martínez-Pascual and Fernández-Carmona 1980), and dried citrus pulp (DCP) which is formed by shedding, liming, pressing and drying the peel, pulp and seed residues to about 80 g/kg moisture, and citrus meal and fines which is formed and separated during the drying process. A typical processing plant produces these BPF in a ratio of about 850 g/kg DCP, 140 g/kg citrus meal and 10 g/kg citrus fines. Other citrus BPF include citrus molasses, made by concentrating the press liquor from the citrus peel residue, which has a bitter taste and contains about 100–150 g/kg solubles of which 500–700 g/kg consists of sugar (Ensminger *et al*. 1990), citrus peel liquor, which is similar to citrus molasses, but not as concentrated, and citrus activated sludge which is produced from liquid wastes from citrus processing plants. Other minor BPF from citrus include cull or excess fruit (Madrid *et al*. 1996).

The composition of citrus fruit is affected by factors such as growing conditions, maturity, rootstock, variety and climate (Kale and Adsule 1995). Citrus fruits contain N (1–2 g/kg on a wet basis), lipids (oleic, linoleic, linolenic, palmitic, stearic acids, glycerol, and a phytosterol), sugars (glucose, fructose, sucrose), acids (primarily citric and malic, but also tartaric, benzoic, oxalic, and succinic), insoluble carbohydrates (cellulose, pectin), enzymes (pectinesterase, phosphatase, peroxidase), flavonoids (hesperidin, na-

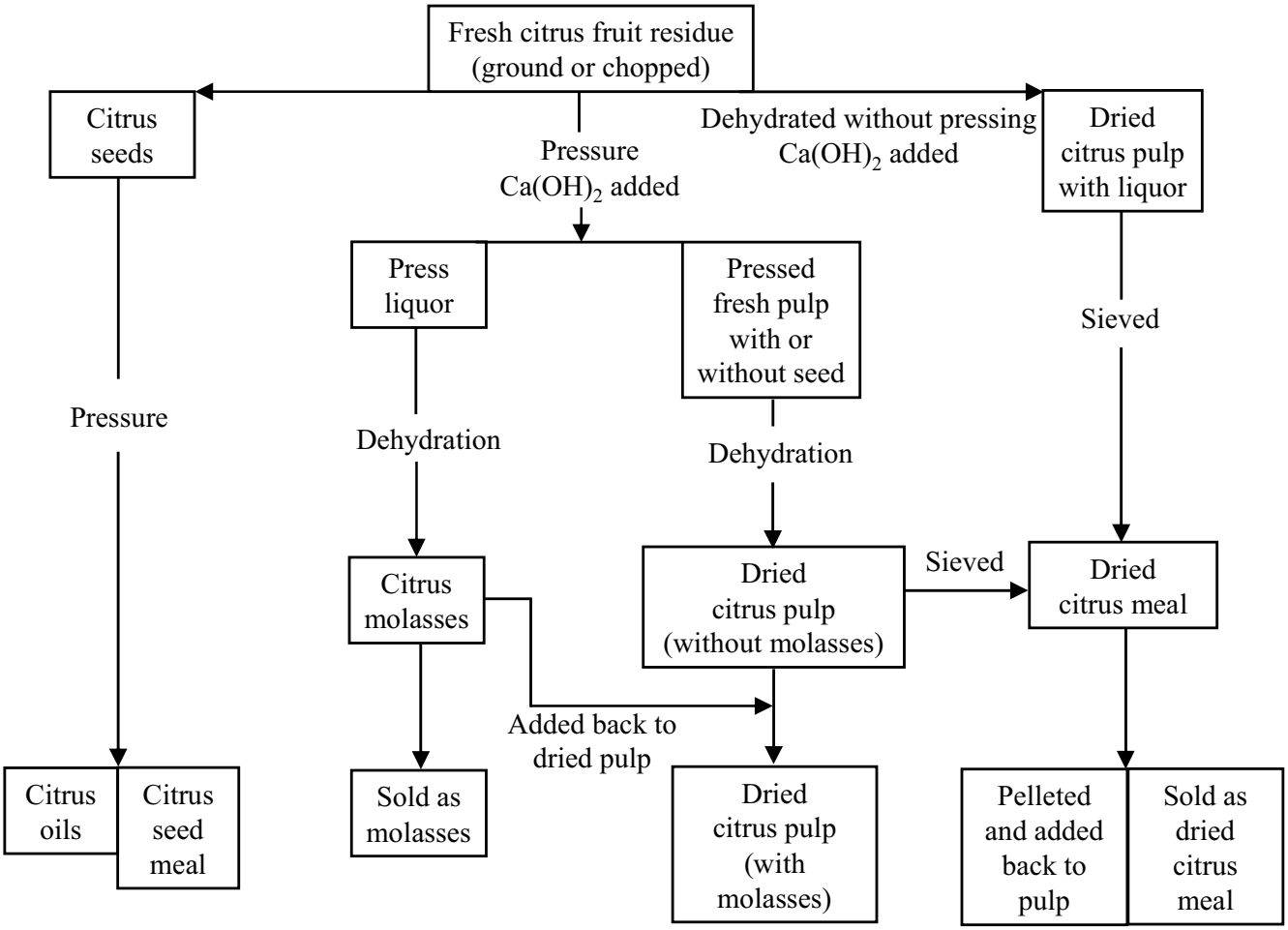

Fig. 1 Schematic presentation of citrus by-product production. (Adapted from Bampidis and Robinson (2006)).

Table 3 Products and by-products of various tissues of citrus fruits (Sinclair *et al.* 1984; Bambidis and Robinson 2006).

Whole peel or rind (pericarp)	Consists of flavedo (exterior yellow peel, epicarp) and albedo (interior whity spongy peel, mesocarp). Albedo is rich in pectin. The whole peel combined with the pulp residue (rag) and/or molasses can become a feed for animals. It is also used for production of human foods and food supplements.
Pulp (principal edible portion, endocarp)	Used mainly to produce raw juice for human nutrition, after mechanical extraction and screening. The material screened from the raw juice is also called pulp and is usually combined with other residues to produce by-products used in animal nutrition.
Pulp residue (called rag in the industry)	Consists of the fraction screened from the pulp, being cores, segment walls or membranes, juice vesicles and seeds. The pulp residue is usually combined with peel residue to manufacture by-products feeds. From the lime-treated mass peel and pulp residues, citrus processors produce such by-products as press liquor, citrus molasses, citrus pulp, citrus meal and feed yeast. It is also used for production of human foods and food supplements.
Seeds	Sometimes separated from the rag to produce seed oils, seed meals and dried seed pressed cake.
Waste waters (aqueous effluent emulsions from processing plants)	Have potential uses for production of such products as activated sludge and yeasts. It is also used for production of human foods and food supplements

ringin), bitter principles (limonin, isolimonin), peel oil (D-limonene), volatile constituents (alcohols, aldehydes, ketones, esters, hydrocarbons, acids), pigments (carotenes, xanthophylls), vitamins (ascorbic acid, Vitamin B complex, carotenoids), and minerals (primarily calcium and potassium) (Bambidis and Robinson 2006). The nutrient content of citrus BPF is influenced by factors that include the source of the fruit and type of processing (Ammerman and Henry 1991).

BIOPROCESSES INVOLVING CITRUS PEELS

Different biotechnological applications applying citrus by-products as substrate are listed in **Table 4**.

Enzyme production

The most important area of citrus peels utilization is the production of enzymes, especially pectinolytic ones. Pectinolytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances. Pectinases are classified under three headings according to the following criteria: whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate, whether pectinases act by *trans*-elimination or hydrolysis and whether the cleavage is random (endo-, liquefying of depolymerizing enzymes) or endwise (exo- or saccharifying enzymes). The three major types of pectinases are: pectinesterases, depolymerising enzymes (enzymes that either hydrolyze glycosidic linkages or cleave α-1,4-glycosidic linkages by *trans*-elimination) and protopectinase (Jayani *et al.* 2005). Pectinolytic enzymes are of significant importance in the

Table 4 Bioprocesses using citrus peels as substrate.

Application	Microorganism or enzyme used	Process	Reference
Enzyme production			
Pectinases	*Aspergillus foetidus* ATCC 16878	SSF[a]	Garzón and Hours 1992
	A. niger A-20	SmF	Ismail 1996
Alkaline pectinase	*Bacillus* sp. MG-cp-2	SmF	Kapoor *et al.* 2000
Polygalacturonase	*Aspergillus* sp.	SmF	Maldonado *et al.* 1986
	Fusarium oxysporum F3, *A. niger* BTL, *Neurospora crassa* DSM 1129; *Penicillium decumbens*	SSF	Mamma *et al*. 2008
	A. niger, *Trichoderma viride*	SLSF[c]	de Gregorio *et al.* 2002
	Tubercularia vulgaris	SmF	Fonseca and Said 1994
	Thermoascus aurantiacus 179-5	SSF	Martins *et al.* 2002
	P. vericatum Rfc 3	SSF	Silva *et al.* 2002
Endo-polygalacturonase	*Aspergillus* sp. CH-Y-1043	SmF	Larios *et al.* 1989
	Aureobasidiurn pulIulans	SmF	Federici and Petruccioli 1985
	A. niger MTCC 281	SmF[b]	Dhillon *et al.* 2004
Pectinesterase	*Aspergillus* sp.	SmF	Maldonado *et al.* 1986
Polygalacturonase lyase	*B. subtilis* strain 11089	SmF-CC[d]	Mahmood *et al*. 1998
Pectate lyase	*F. oxysporum* F3, *A. niger* BTL, *N. crassa* DSM 1129, *P. decumbens*	SSF	Mamma *et al*. 2008
Pectin lyase	*P. vericatum* Rfc 3	SSF	Silva *et al.* 2002
α-Amylase	*A. niger* A-20	SmF	Ismail 1996
	B. subtilis strain 11089	SmF-CC	Mahmood *et al.* 1998
Xylanase	*A. niger* A-20	SmF	Ismail 1996
	F. oxysporum F3, *A. niger* BTL, *N. crassa* DSM 1129, *P. decumbens*	SSF	Mamma *et al*. 2008
Cellulase	*A. niger* A-20	SmF	Ismail 1996
	F. oxysporum F3, *A. niger* BTL, *N. crassa* DSM 1129, *P. decumbens*	SSF	Mamma *et al*. 2008
Protease	*B. subtilis* strain 11089	SmF-CC	Mahmood *et al.* 1998
Bioethanol production			
	Erwinia chrysanthemi EC16, *E. carotovora* SR38	SmF	Grohman *et al.* 1998
	Escherichia coli K011	SmF	Grohman *et al.* 1994a, 1995a
	Saccharomyces cerevisiae	SmF	Grohman *et al*. 1994b; Wilkins *et al*. 2007c
Xanthan production			
	Xanthomonas campestris	SmF	Bilanovic *et al.* 1994; Green *et al.* 1994
	X. campestris	SSF	Stredansky and Conti 1999
Citric acid production			
	A. niger	SmF	Aravantinos-Zafiris *et al.* 1994; Rivas *et al.* 2008
	A. niger	SSF	Zhang 1988; Kang *et al.* 1989
Nutritional enrichment			
Protein enrichment	*Penicillium* spp	SSF	Scerra *et al.* 1999
Single Cell Protein	*A. niger*, *T. viride*	SLSF	de Gregorio *et al.* 2002
Enzymatic pectin extraction			
	Endopolygalacturonase, pectin methyl esterase	-	Massiot *et al.* 1989; Renard *et al.* 1991a; Donaghy and McKay 1994
	Protopectinase	-	Sakai and Ozaki 1988; Nakamura *et al.* 1995; Sakamoto *et al.* 1995
	Pectinase 62L, Pectinase 690L, Cellulase CO13P	-	Mandalari *et al.* 2006
Prebiotic oligosaccharides			
	Pectinase 62L	-	Mandalari *et al.* 2007
	Endo-polygalacturonase	EMR[e]	Olano-Martin *et al.* 2001
Enzymatic extraction of flavonoids			
	Cellulase® MX, Cellulase® CL, Kleerase® AFP	-	Li *et al.* 2006
	Pectinase 690L, Pectinase 62L, Cellulase C013P	-	Mandalari *et al*. 2006

[a]SSF: Solid State Fermentation, [b]SmF: Submerged Fermentation, [c]SLSF: Slurry State Fermentation, [d]CC: Continuous Culture, [e]EMR: Enzyme Membrane Reactor

current biotechnological era with their all embracing applications in fruit juice extraction and its clarification, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, in poultry feed additives and in the alcoholic beverages and food industries (Kashyap *et al.* 2001; Hoondall *et al.* 2002; Jayani *et al.* 2005).

Due to the chemical composition of citrus peels other enzymes could be produced resulted in multienzyme complexes. Cellulases and hemicellulases are among these enzymes (**Table 4**). Cellulases are a complex enzyme system, comprising endo-1,4-β-D-glucanase, exo-1,4-β-glucanase and β-D-glucosidase (Hildén and Johansson 2004). These enzymes are employed in feed, fuel and chemical industries for the processing of lignocellulosic materials (Pandey *et al.* 1999; Cherry and Findantsef 2003). The hemicellulolytic system carrying out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and *p*-coumaric acid) esterase. Xylanolytic enzymes have attracted a great deal of attention in the last decade, particularly because of their biotechnological potential in various industrial processes such as food, feed, and pulp and paper industries (Beg *et al.* 2001). Bacteria, yeasts, and fungi under both submerged (SmF) and solid state fermentation (SSF) conditions are able to produce these enzymes (**Table 4**).

Ismail (1996) investigated the production of multienzyme preparations containing pectinase, cellulase and xylanase using six fungal isolates, namely *Aspergillus niger* 2, *A. niger* A-20, *A. oryzae* 1911, *Memmoniella* sp. 6, *Penicillium chrysogenum* 3486 and *P. oxalicum* 7, all grown on orange peels as the sole carbon source in SmF. Of the fungal isolates tested, *A. niger* A-20 proved to be the most potent and produced highly active multienzyme systems (56.0 U/ml pectinase, 4.39 U/ml cellulase, 3.33 U/ml xylanase

and 0.01 U/ml α-amylase) after 5 days at 30°C. The multi-enzyme complexe obtained from *A. niger* A-20 exhibited optimum enzymic activities at 45-50°C and pH 4-5.

Maldonado *et al.* (1986) tested *Aspergillus* sp. from decaying lemons for extracellular pectinase production using differently pretreated lemon peel as the carbon source instead of pectin. It was found that the production of extracellular polygalacturonase was about the same and that of pectinesterase substantially higher when unwashed fresh lemon peel was used instead of pectin. The culture filtrate obtained showed a clarifying capacity similar to that of a commercial pectinase preparation, but the vitamin C of the juice was less affected by the treatment.

Pectinase production by *Tubercularia vulgaris* using orange-pulp pellets or citrus pectin as carbohydrate sources was investigated by Fonseca and Said (1994). The highest levels of extracellular polygalacturonase were detected with OPP as inducing substrate. High levels of endo-polygalacturonase were produced by *Aureobasidiurn pullulans* on orange-peel waste (Federici and Petruccioli 1985). Polygalacturonase and pectin lyase production by *P. viridicatum* strain Rfc3 was carried out by means of solid state fermentation using orange bagasse, corn tegument, wheat bran and mango and banana peels as carbon sources. The maximal activity value of polygalacturonase (30 U/g) was obtained using wheat bran as carbon source while maximal pectin lyase (2000 U/g) activity value was obtained in medium composed of orange bagasse. The mixture of orange bagasse and wheat bran (50%) increased the production of polygalacturonase and pectin lyase to 55 U/g and 3540 U/g respectively (Silva *et al.* 2002).

Larios *et al.* (1989) studied the endo-polygalacturonase production by *Aspergillus* sp. CH-Y-1043 using untreated lemon peel and citrus pectin as carbon sources. Untreated lemon peel proved to be a better substrate, while *Aspergillus* sp. CH-Y-1043 produced more endo-polygalacturonase at 37°C than at 29°C. Endo-polygalacturonase production was very sensitive to pH changes. Optimization of the culture medium as far as the nitrogen source, concentration of phosphates and initial culture pH resulted 65.2 U/ml endo-polygalactorunase activity.

The production of endo-polygalacturonase by the fungus *A. niger* MTCC 281 using powdered citrus peel was studied by Dhillon *et al.* (2004). Maximum enzyme activity (0.940 µM/ml/min) was observed with 15% substrate semi-solid substrate, when incubated at 30°C for 120 h, using 5% inoculum.

Mahmood *at al*. (1998) found that *Bacillus* sp. 11089 was capable of growth in continuous culture on orange substrate as carbon-energy source in a mineral salts basal medium and produced α-amylase, neutral and alkaline proteases, and polygalacturonate-lyase. The activities obtained were 19.0 U/ml α-amylase, 20 U/ml neutral protease, 7.5 U/ml alkaline protease and 2.0 Units/ml polygalacturonate lyase. The enzyme production was similar better than that produced by glucose used at the equivalent weight-volume concentrations

A. foetidus ATCC 16878, when grown under solid-state fermentation using citrus waste as carbon source, produced pectic enzyme activities up to 1600-1700 U/g after 36 h of culture, as reported by Garzón and Hours (1992). Yield of pectinases was 25% higher than that achieved with the same fungal strain and culture conditions using apple pomace as a substrate.

Bacillus sp. MG-cp-2, isolated from the outer covering of seeds of *Celastrus paniculatus*, produced 140.1 U/ml of an alkaline and thermostable polygalacturonase when grown on orange peels as carbon source. The partially purified polygalacturonase was optimally active at 60°C at pH 10.0 with half-lives of 120, 118 and 20 min at 60, 70 and 80°C, respectively. The enzyme was 100% stable at 50°C for more than 12 h. Polygalacturonase was stable in a broad alkaline pH range 7.0–12.0 for more than 24 h at room temperature, retaining more than 80% of its activity (Kappor *et al.* 2000).

Martins *et al.* (2002) reported the pectin lyase and polygalacturonase production by the thermophilic fungus *Thermoascus aurantiacus* 179-5 under solid state fermentation using orange bagasse as carbon source. *T. aurantiacus* cultivation provided 43 and 19320 U/g polygalacturonase and pectin lyase activity respectively, while time course of enzymes production revealed sequential induction of them. Polygalacturonase and pectin lyase had optimum activity at pH 5.0 and 10.5–11.0, respectively. Maximal activity of the enzymes were determined at 65°C.

Seyis and Aksoz (2005) investigated the use of apple pomace, orange pomace, orange peel, lemon pomace, lemon peel, pear peel, banana peel, melon peel, and hazelnut shell as substrate for xylanase production using *Trichoderma harzianum*. The maximum enzyme activity was observed when melon peel was used as the substrate for SSF, followed by the apple pomace and hazelnut shell. *Sporotrichum thermophile* produces a thermostable polygalacturonase under submerged culture and citrus peel as carbon source (Kaur *et al.* 2004).

Single cell protein (SCP) and crude pectinolytic enzymes production from citrus pulps was reported by De Gregorio *et al.* (2002). SCP and enzymes were produced by slurry-state flask cultivation of *A. niger* and *T. viride*. *A. niger* showed a significant enzyme production starting with the 7th incubation day while *T. viride* showed later production. For the former mould the maximum polygalacturonase production was recorded about the 14th incubation day while in *T. viride* it was detected later, at 25th incubation day, when the observed Units (9.01 U/ml) were about seven times more than in *A. niger* (1.27 U/ml).

Mamma *et al.* (2008) reported the production of multi-enzyme preparations containing pectinolytic, cellulolytic and xylanolytic enzymes by the mesophilic fungi *A. niger* BTL, *Fusarium oxysporum* F3, *Neurospora crassa* DSM 1129 and *P. decumbens* under solid state fermentation on dry orange peels. Under optimal initial culture pH and moisture conditions *A. niger* BTL was by far the most potent strain in polygalacturonase (135.7 U/g) and pectate lyase (130.8 U/g), production followed by *F. oxysporum* F3, *N. crassa* DSM 1129 and *P. decumbens*. *N. crassa* DSM 1129 (138.5 U/g) produced the highest endoglucanase activity and *P. decumbens* (45.5 U/g) the lowest one. Comparison of xylanase production revealed that *A. niger* BTL (77.1 U/g) produced the highest activity followed by *N. crassa* DSM 1129, *P. decumbens* and *F. oxysporum* F3. *N. crassa* DSM 1129 and *P. decumbens* did not produce any β-xylosidase activity, while *A. niger* BTL (1.04 U/g) produced approximately 10 times more β-xylosidase than *F. oxysporum* F3. The highest invertase activity was produced by *A. niger* BTL (72.5 U/g) while the lowest ones by *F. oxysporum* F3 and *P. decumbens*.

Bioethanol production

With the inevitable depletion of the world's petroleum supply and due to increased prices for oil, there has been an increasing worldwide interest in alternative, non-petroleum-based sources of energy (Kerr 1998; Wheals *et al.* 1999; Aristidou and Penttila 2000; Jeffries and Jin 2000; Zaldivar *et al.* 2001; IEA 2004). Ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels (Cardona and Sánchez 2007).

Mature technologies for ethanol production are crop-based, utilizing substrates such as sugar cane juice and cornstarch. Since the cost of raw materials can be as high as 40% of the bioethanol cost (von Sivers *et al.* 1994; Wyman 1999), recent efforts have concentrated on utilizing lingocellulosic biomass. This natural and potentially cheap and abundant polymer is found as agricultural waste (wheat straw, corn stalks, soybean residues, sugar cane bagasse), industrial waste (pulp and paper industry), forestry residues, municipal solid waste, etc. (Wiselogel *et al.* 1996). It has been estimated that lignocellulose accounts for about 50% of the biomass in the world (10–50 billion tons according to

Claasen *et al.* 1999).

Biomass is seen as an interesting energy source for several reasons. The main reason is that bioenergy can contribute to sustainable development (Monique *et al.* 2003). Resources are often locally available, and conversion into secondary energy carriers is feasible without high capital investments. Moreover, biomass energy can play an important role in reducing greenhouse gas emissions; since CO_2 that arises from biomass wastes would originally have been absorbed from the air, the use of biomass for energy offsets fossil fuel greenhouse gas emissions (Lynd 1996). In addition, application of agro-industrial residues in bioprocesses not only provides alternative substrates but also helps solve their disposal problem.

Overall fuel ethanol production from lignocellulosic biomass includes five main steps: biomass pretreatment, cellulose hydrolysis, fermentation of hexoses, separation and effluent treatment Furthermore, detoxification and fermentation of pentoses released during the pre-treatment step can be carried out (Cardona and Sánchez 2007). The sequential configuration employed to obtain cellulosic ethanol implies that the solid fraction of pretreated lignocellulosic material undergoes hydrolysis (saccharification); this fraction contains the cellulose in an accessible to acids or enzymes form. Once hydrolysis is completed, the resulting cellulose hydrolyzate is fermented and converted into ethanol. This process is called separate hydrolysis and fermentation (SHF) (Hahn-Hägerdal *et al.* 2006).

The above mentioned two process steps can be performed together in so-called simultaneous saccharification and co-fermentation (SSCF), which has been shown to have several advantages over performing the steps separately (Hahn-Hägerdal *et al.* 2006; Lin and Tanaka 2006; Cardona and Sánchez 2007). The SSCF process alleviates end-product inhibition of the enzymes, and is also less capital intensive than SHF (Wingren *et al.* 2003). Furthermore, SSCF has been shown to be superior to SHF in terms of overall ethanol yield (Söderström *et al.* 2005).

Several researchers have successfully hydrolyzed both orange and grapefruit peel waste using commercial cellulase and pectinase enzymes to glucose, galactose, fructose, arabinose, xylose, rhamnose, and galacturonic acid (Grohmann and Baldwin 1992; Grohmann *et al.* 1994a, 1995a; Wilkins *et al*. 2007a). According to Grohmann *et al.* (1994a), glucose, fructose and galactose from hydrolyzed citrus peel waste can be fermented to ethanol by *Saccharomyces cerevisiae* yeast. Galacturonic acid from pectin hydrolysis, arabinose, and xylose as well as the sugars mentioned above can be fermented by *Escherichia coli* K011 to produce ethanol and acetic acid (Grohmann *et al.* 1994b, 1995b). *E. coli* KO11 is a recombinant bacterial strain developed to ferment arabinose and xylose as well as hexoses to ethanol (Beall *et al*. 1991). However in order to ferment these sugars, orange peel oil concentration in the hydrolysate must be reduced prior to fermentation (Grohmann *et al.* 1994a). The inhibitory effect on yeast growth due to orange peel oil and/or D-limonene, a monoterpene that makes up more than 90% of orange and grapefruit peel oils has been observed by several researchers (Winniczuk and Parish 1997; Wilkins *et al*. 2007b, 2007c). The mechanisms by which limonene and other monoterpenes similar in structure to limonene inhibit yeast function and growth have been the subject of several studies (Uribe *et al.* 1990; Uribe and Pena 1990).

Wilkins *et al*. (2007b) reported that ethanol produced by *S. cerevisiae* and *Kluyveromyces marxianus* during fermentation of a solution modelling hydrolyzed orange peels waste was 37.1 g/l and 40.9 g/l respectively (80% and 88.3% theoretical yield, respectively) in the absence of limonene, while in the presence of 0.2% limonene ethanol production reduced at 23.3 g/l and 13.1 g/l respectively (50.3% and 28.3% theoretical yield, respectively). It also should be noted that limonene concentrations tested by Wilkins *et al.* (2007b) were less than concentrations observed in commercial citrus peel, which have been reported as 1.8% (w/w) for orange peel waste. Grohman *et al.* (1994b) reported that the recombinant bacterium *E. coli* KO11 produced 27.6 g/l ethanol from approximately 66.6 g/l sugars in orange peels hydrolysate. The hydrolysate contained 18.6 g/l galacturonic acid which could efficiently fermented to ethanol by the bacterium.

It should be noted that due to the high amounts of citrus wastes available in the US researchers of the US Department of Agriculture worked with commercial enzymes to hydrolyze pectin, cellulose and hemicellulose economically from citrus peel wastes. The goal was to optimize the process and develop a model refinery that would also extract marketable by products (Widmer and Stewart 2006; Predd 2006). FPL Energy LLC planned to develop a commercial scale cellulosic ethanol plant that can produce ethanol using waste citrus peel as feedstocks (O'Sullivan and Stewart 2007), while the southeast Biofuels LLC subsidiary has filed an application with the Florida Department of Agriculture and Consumer Services for a $500.000 grant in concerting citrus peel waste to ethanol (Ames 2008).

Xanthan gum production

Manufacture of high-molecular-weight compounds with thickener properties has been traditionally related to plants, seeds and seaweeds. These compounds have been named gums. The rheological properties of their solutions of their solutions show important alterations depending on uncontrolled variables such as weather and their natural-collection labor cost can often influence their market price (García-Ochoa *et al.* 1999).

Production of molecules with thickener properties from microorganisms was an important advance. This production is made under control and the polymer has constant properties. Xanthan gum is one of these biopolymers first commercialized in the 1960's, and since then has played an important role in industrial gum applications (Kang and Pettitt 1993).

It is a hetero-polysaccharide produced by *Xanthomonas campestris*. Xanthan gum is the most important microbial polysaccharide from the commercial point of view, with a worldwide production of about 30000 tons per year. It has widespread commercial applications as a viscosity enhancer and stabilizer in the food, pharmaceutical and petrochemical industries (Margaritis and Pace 1985; Galdino 1994).

Xanthan molecules show very high molecular weights of several millions of Daltons. The acetyl and pyruvyl contents can change depending on culture conditions and microorganism used (Kennedy and Bradshaw 1984). Therefore, the polymer solutions show different rheological behaviour, depending on molecular weight and composition. Xanthan with a high pyruvate content (4-4.8%) shows a greater thickener behaviour than that with low pyruvate content (2.5-5%) (Kang and Pettitt 1993). Pyruvate free xanthan is employed in enhanced oil recovery (EOR) because microgels are not formed, although in other applications this is not so important.

Most bacteria of the *Xanthomonas* genus produce extracellular polysaccharides as bacterial capsules (Bradbury 1984). The type of xanthan gum produced is quite different depending on the *Xanthomonas* species used, since the different composition of the gums is related to their contents of glucose, glucoronic acid, mannose, pyruvate and acetate, and another sugar, galactose is introduced into the molecule by some species (Kennedy and Bradshaw 1984). Further, media composition seems to influence the puryvate content of the biopolymer and operational conditions (such as temperature, pH, dissolved oxygen and so on) employed in the fermentation influence the molecular weight of the product obtained (Shu and Yang 1990; García-Ochoa *et al.* 1992, 1997).

The cost of the fermentation medium represents another critical aspect of the commercial production of xanthan. The use of cheap substrates instead of the commonly used glucose or sucrose, might result in a lower cost of the final product. For example the construction of genetically modi-

fied lactose-utilizing *X. campestris* enabled the use of whey as a cheap fermentation medium (Papoutsopoulou *et al.* 1994).

In studies by Bilanovic *et al.* (1994) and Green *et al.* (1994) four different fractions of citrus waste were compared as substrates for xanthan fermentation: whole citrus waste, pectic, hemicellulosic and cellulosic extracts, in submerged culture. The whole waste was found to be a good substitute for glucose media for xanthan production, since the production was 37% higher than that of on standard glucose medium. *X. campestris* ATCC 13951 utilized both simple and complex carbon compounds originating from citrus wastes. Substrate utilization in the medium based on pectin extract was similar to that in the medium based on a whole citrus waste and the pectic extract yielded the same amount of xanthan as the whole waste. This indicated that water soluble substances in citrus waste such as pectins, organic acids and simple carbohydrates were readily converted into xanthan and that they were the main contributor to xanthan production from the whole waste. The biodegradability of the hemicellulose and cellulose extracts of citrus waste was found to be much lower than that of the pectic extract. Substrate utilization and its conversion to xanthan in the hemicellulosic extract was 36% lower, and in the cellulosic extract 60% lower, than those in the whole citrus waste.

In submerged culture, the excretion of the polysaccharide during fermentation results in a highly viscous and shear thinning broth. The rheological behaviour of the fermentation broth causes serious problems of mixing, heat transfer, and oxygen supply, thus limiting the maximum gum concentration achievable as well as the product quality (Petres *et al.* 1989; Wecker and Onken 1991).

Stredansky and Conti (1999) studied the solid state fermentation as an alternative strategy for the production of xanthan by *X. campestris*. The choice was based on the observation that solid substrates reproduce the natural habitat of this phytopathogenic bacterium (Brown *et al.* 1993; Pierce *et al*. 1993). This technique allows problems connected with broth viscosity to be overcome and utilizes cheap substrates. Citrus peels and apple pomace based substrates were employed. The conversion efficiency of citrus peel into xanthan was 24.5%, lower than that obtained with apple pomace (28–32%) but comparable to that reported in the aforementioned study (Bilanovic *et al*. 1994) for xanthan production in submerged cultivation.

Citric acid production

Citric acid is widely used in the food, beverage, pharmaceutical, and cosmetic industries and finds applications in a variety of other industries, from textiles to electroplating (Bodie *et al*. 1994). In addition, production of citric acid could offset the disposal costs of the wastes (Tran *et al.* 1998).

Aravantinos-Zafiris *et al.* (1994) reported citric acid fermentation from orange processing wastes having sugar content of 55 g/l, in submerged culture, using *A. niger* and 40g/l methanol. Yields obtained were citric acid concentration 30 g/L, productivity [Q_P=0.104 g/(L·h)] and yield $Y_{P/S}$=0.63 g/g. *A. niger* does not assimilate methanol, and, although its exact role in the stimulation of citric acid production is not yet known, it is believed that methanol increases the permeability of the microorganism cell membrane, thereby making the excretion of citric acid easier (Hang *et al.* 1987; Navaratnam *et al.* 1998).

Rivas *et al.* (2008) reported that a treatment of autohydrolysis at 130°C and liquid/solid ratio of 8.0 g/g, a novel technology for orange peel, had a beneficial effect on its hydrolysis, producing liquors rich in soluble sugars, mainly sucrose, glucose, and fructose, which could be utilized for citric acid production by *A. niger* ATCC 9142. The highest values of citric acid concentration (9.2 g/L), product yield on consumed sugars ($Y_{P/S}$=0.53 g/g), and productivity [Q_P=0.128 g/(L·h)] were achieved within 3 days from initial sugar content of 25.5 g/l and in the presence of $CaCO_3$ and 40 ml/kg methanol.

Usually less methanol is required to stimulate citric acid release in solid-state fermentation. Hang *et al.* (1987) reported optimal methanol concentration of only 20 ml/kg in solid-state fermentation of kiwifruit peel by *A. niger* ATCC 9142, obtaining 82 g/L of citric acid after 5 days from 168 g/l of initial sugars [Q_P=0.683 g/(L·h), $Y_{P/S}$=0.60 g/g]. Similar results were reported by Zhang (1988) for the solid residue of an orange juice factory and by Kang *et al.* (1989) for tangerine peel.

Production of SCP

Although most citrus by-products have a low nitrogen content, processing can raise their nutritive value. Taiwo *et al.* (1995) reported that unfermented citrus pulp (910 g/kg DM) had relatively high levels of glucose and low levels of other nutrients. However, fermentation of citrus pulp without or with 100 g/kg molasses for 61 days resulted in production of primarily lactic and acetic acid (Taiwo *et al.* 1995), which enhanced citrus pulp ammonia holding capacity from 0.1 g NH_3 N/kg DM in unfermented citrus pulp to 10.6 and 16.4 g NH_3 N/kg DM in fermented citrus pulp without and with molasses, respectively. This suggests that the N content of citrus pulp can be enhanced by trapping excess ammonia generated from, for example, urea treated barley straw (Taiwo *et al.* 1995). Scerra *et al.* (1999) found that colonization of bergamot fruit peel with 10 strains of *Penicillium* spp. improved its nutritional value by increasing levels of crude protein, crude fat and structural carbohydrates *versus* untreated bergamot fruit peel.

de Gregorio *et al*. (2002) showed that by utilizing *A. niger*, but preferably *T. viride*, it was possible to extensively hydrolyse the lemon pulps, producing useful amounts of single cell protein (SCP) and a high-activity crude pectinases. The highest protein level was reached with *A. niger* after 14 days growth and later with *T. viride*, though the final amount of N was higher in *T. viride* (31.9%) than in *A. niger* (25.6%). Pectinases could be utilized in the citrus processing factories producing the wastes as well as in other fruit processing industries, while protein residues could be utilized in ruminant feeding.

Enzymatic pectin extraction

Pectin is an important component of dicotyledonous plant cell walls, besides cellulose and hemicellulose (Carpita and McCann 2000). Pectin is probably the most complex macromolecule in nature, because it can be composed of as many as 17 different monosaccharides (Ridley *et al.* 2001). Rather than making all possible combinations with these monosaccharides, mother nature has provided us a number of distinct polysaccharides which together form pectin. Three pectic polysaccharides (homogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II) have been isolated from primary cell walls and structurally characterized (O'Neill *et al.* 1990; Visser and Voragen 1996; Vincken *et al*. 2003). Homogalacturonan (HG) is a linear chain of 1,4-linked α-D-galactopyranosyluronic acid (Gal*p*A) residues. The Gal*p*A residues can be methyl-esterified at *C*-6, and carry acetyl groups on *O*-2 and *O*-3. "Smooth" regions are mainly composed of HG (Ridley *et al.* 2001; Vincken *et al*. 2003). Rhamnogalacturonan-I (RG-I) is a family of pectic polysaccharides that contain a backbone composed of as many as 100 repeats of the disaccharide [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]. The ramnosyl residues can be substitued at *O*-4 with neutral sugars. The side chains are mainly composed of galactosyl and/or arabinosyl residues. They can be single unit [β-D-Gal*p*-(1→4)], but also polymeric such as arabinogalactan I (AG-I) and arabinan (50 glycosyl residues or more) (Vincken *et al.* 2003). AG-I is composed of a 1,4-linked β-D-Gal*p* backbone; α-L-Ara*f* residues can be attached to the *O*-3 of the galactosyl residues (Ridley *et al.* 2001). The arabinans consist of a 1,5-linked α-L-Ara*f* backbone, which can be substitueted with α-L-

Ara*f*-(1→2)-, α-L-Ara*f*-(1→3)-, and/or α-L-Ara*f*-(1→3)- α-L-Ara*f*-(1→3)- side chains (Ridley *et al.* 2001; Vincken *et al.* 2003). Complexes of RG-I and AG-I and arabinan are often referred to as pectic "hairy regions" (HR), in which AG-I and arabinan are the "hairs". The abundance of HR can differ from species to species. Also, the amount and nature of the "hairs" can differ considerably among species (Renard *et al.* 1991a; Oosterveld *et al.* 2001). RG-II is not structurally related to RG-I since its backbone is composed of 1,4-linked α-D-galactopyranosyluronic acid (Gal*p*A) residues rather than the repeating disaccharide [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→] (O'Neill *et al.* 1990). A nonasaccharide and an octasaccharide are attached to *C*-2 of some of the backbone GalA residues and two structurally different disaccharides are attached to *C*-3 of the backbone (Ridley *et al.* 2001).

In food industries, pectin is used as a jellifying agent in jams and jellies. Gelation by pectin in fruit drink concentrates provides stabilization of emulsions, suspensions and foam (Sakai *et al.* 1993). In a normal western diet around 4–5 g of pectin are consumed each day (Pilnik 1990). Extracted pectin is widely used as functional food ingredient and it (or its EU code, E440) is listed among the ingredients of innumerable food products. Worldwide annual consumption is estimated at around 45 million kilograms, with a global market value of at least 400 million Euros (Savary *et al.* 2003). Pectins are used in the pharmaceutical sector as detoxifying agents, and are well known for their anti-diarrheal effects (Pilnik and Voragen 1970; Chenoweth and Leveille 1975; Bechard and McMullen 1986). Pectin is also used for the production of single cell protein in a modified 'symba' process (Fellows and Worgan 1986), as well as in cosmetics as gels and pastes (Sakai *et al*. 1993).

The chief raw materials for the industrial production of pectin are the residues from the manufacture of fruit juices, apple pomace and citrus fruits (Alkorta *et al*. 1998; Blanco *et al.* 1999; Hoondall *et al*. 2002; Willats *et al*. 2006). Pectins can be extracted from the cell walls by physical, chemical, as well as enzymatic ways. Physical methods, such as extrusion-cooking or microwave-assisted extraction, can be used (Fishman *et al*. 2000, 2003; Kratchanova *et al*. 2004; Liu *et al*. 2006).

Classically, pectin extraction is carried out by acid hydrolysis at a pH range of 2–3 for 5 h at high temperature (70–100°C). The solid to liquid ratio is normally about 1:18. The pectin extract is separated from the pomace using a hydraulic press or by centrifugation. The extract is filtered, and finally concentrated to a standard setting strength. For powdered pectin preparation, the concentrated liquor is treated with organic solvents or certain metallic salts to precipitate the polymers. This process usually results in corrosion of equipment and water pollution problems. This uncontrolled hydrolysis may also result in a reduced degree of pectin polymerization, an important characteristic of commercial pectins (Sakai *et al.* 1993). Moreover, the consumer demand for "green" products stimulates the search for alternative means of extraction.

Therefore, enzymes could represent, despite their potential cost, an alternative and environmentally friendly way to extract "green labeled" pectins. Two different approaches can be considered. The first one involves enzymes to degrade the pectins and isolate pectin fragments. Enzymes degrading the pectin backbone (endo-polygalacturonase together with pectin methyl esterase, or endo-pectin lyase) are able to solubilize all the galacturonic acid (Voragen *et al*. 1980). These enzymes extract high molecular mass fragments of "hairy regions", carrying many side chains, but also galacturonic acid oligomers from "smooth regions" (Renard *et al*. 1991a). The yield of extraction obtained with pectinolytic enzymes is higher than that with chemical means (Massiot *et al*. 1989; Renard *et al.* 1991a; Donaghy and McKay 1994). Some studies have also been done with various "protopectinases" (Sakai and Ozaki 1988; Nakamura *et al*. 1995; Sakamoto *et al*. 1995). According to their authors, these enzymes are a heterogeneous group that solubilizes pectins from the insoluble pectin in plant tissues (the so-called "protopectin") by restricted depolymerization. They can be active against the pectin backbone or against the pectin side chains. Their action is strongly dependent on the type of enzyme and the nature of substrate (Nakamura *et al*. 1995). Some "protopectinases" can be more efficient to extract pectin than acid, depending on the substrate. This is the case for protopectinases having a pectin lyase or an endo-polygalacturonase activity, but also an arabinanase activity (Sakamoto *et al.* 1995). However, pure arabinanase and galactanase were found to be inefficient to extract galacturonic acid-containing polymers and oligomers (Thibault *et al.* 1988; Renard *et al.* 1991b).

The second approach consists of using enzymes able to deconstruct the plant cell wall and isolate pectins. The primary plant cell wall of dicotyledons is composed of various polysaccharides (cellulose, xyloglucan, pectin) and proteins, which form entangled networks (Carpita and McCann 2000). Xyloglucan is known to associate to cellulose microfibrils, probably via hydrogen bonding (Vincken *et al*. 1995). The cellulose/xyloglucan network is embedded in a pectic matrix, together with a protein network. However, recent studies have shown the possibility to form interactions between cellulose and pectin side chains (Oeschlin *et al*. 2003; Zykwinska *et al.* 2005). Regarding pectin extraction, the combined use of cellulases and proteases could allow the isolation of pectic polysaccharides by degrading the cellulose/ xyloglucan and protein networks. Pectinolytic and cellulolytic enzymes (Pectinase 62L, Pectinase 690L, and Cellulase CO13P) were used to evaluate the solubilization of carbohydrates from bergamot peel (Mandalari *et al.* 2006). The addition of Pectinase 62L or 690L alone, or the combination of Pectinase 62L and Cellulase CO13P, was capable of solubilizing between 70 and 80% of the bergamot peel while Cellulase CO13P alone solubilized 62% of the peel. Over a 24-h time course, a rapid release of cell wall carbohydrates was observed after treatment with Pectinase 62L.

Enzymatic production of prebiotic oligosaccharides

In recent years a number of oligomers termed prebiotics have been described. These resist digestion in the upper GI tract and are able to modulate the gut microbiota by stimulating indigenous beneficial flora components while suppressing, or not affecting, less desirable bacteria, such as proteolytic bacteroides and clostridia (Tuohy *et al.* 2001). Prebiotics have also been reported to indirectly lead to a reduction in serum triglyceride levels (Williams and Jackson 2002). In addition, there is evidence showing that prebiotics may indirectly affect mineral absorption in the large bowel and show beneficial effects against inflammatory bowel diseases by stimulating butyrate production and thus accelerating the mucosal cell proliferation and healing processes (Roberfroid 2000; Bamba *et al.* 2002). Although any dietary material that enters the large intestine can be considered as potentially prebiotic, currently, the most well known prebiotics are non-digestible oligosaccharides (Gibson *et al*. 1995). Different oligosaccharides with prebiotic properties are commercially available, such as inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides and lactulose, but currently there is increasing interest in the identification and development of new prebiotic compounds, perhaps with added functionality (Menne *et al.* 2000; Rao 2001; Tuohy *et al.* 2002).

Pectic substances are hydrolysed by the action of pectinases or pectolytic enzymes that are widely distributed in higher plants and microorganisms (Jayani *et al*. 2005). Pectic oligosaccharides (POS) were manufactured from commercial pectin in an enzyme membrane reactor (Olano-Martin *et al.* 2001) and then evaluated for their prebiotic properties (Olano-Martin *et al.* 2002). These pectic oligosaccharides had a low prebiotic potential compared to FOS, although they were more selectively fermented than were the

parent pectins (Olano-Martin *et al.* 2002). Pectic oligosaccharides also protected colonocytes against *Escherichia coli* verocytotoxins (Olano-Martin *et al.* 2003a) and stimulated apoptosis in human colonic adenocarcinoma cells (Olano-Martin *et al.* 2003b). Recently, it has been demonstrated that POS from orange peel showed prebiotic properties increasing the bifidobacterial and *E. rectale* numbers (Manderson *et al.* 2005). Orange peel albedo (white part) was also a good source of pectic oligosaccharides with prebiotic properties produced by a microwave and autoclave extraction (Hotchkiss *et al.* 2003). Incubating bergamot peel for 2 h with a commercial enzyme preparation from *Aspergillus* sp. (pectinase 62L) produced a material rich in oligosaccharides. The prebiotic effect of a pectic oligosaccharide rich extract enzymatically derived from bergamot peel was studied using pure and mixed cultures of human faecal bacteria. Addition of the bergamot oligosaccharides (BOS) resulted in a high increase in the number of bifidobacteria and lactobacilli, whereas the clostridial population decreased. A prebiotic index (PI) was calculated for both FOS and BOS after 10 and 24 h incubation. Generally, higher PI scores were obtained after 10 h incubation, with BOS showing a greater value (6.90) than FOS (6.12) (Mandalari *et al*. 2007).

Natural antioxidants in citrus by-products

Phenolic compounds are important for their sensory and nutritional qualities that impart the colours, flavours and tastes of many plants. Of them, flavonoids (Gorinstein *et al.* 2001) have shown to have beneficial implications in human health (Voragen *et al*. 1995; Vincken *et al*. 2003), due to their antioxidant activities and free radical scavenging abilities.

Citrus processing by-products potentially represent a rich source of natural flavonoids (e.g., hesperidin, diosmin, naringin, and tangeretin), owing to the large amount of peel produced, and that citrus peels contain a high concentration of phenolic compounds (Rouseff *et al*. 1987; Bocco *et al*. 1998; Manthey and Grohmann 2001; Moure *et al.* 2001; Wolfe *et al.* 2003).

Flavonoids have been found to have health-related properties, including anticancer, antiviral, and anti-inflammatory activities (Benavente-Garcia *et al*. 1997; Carrol *et al.* 1999; Moure *et al.* 2001). It is believed that they act as scavengers of free radicals, as well as modulate the activities of metabolic enzymes involved in the initiation of low-density lipoprotein oxidation (e.g. xanthine oxidase, glutathione reductase, lipoxygenase, and NADPH-oxidase) and inhibit cellular proliferation (Saleh *et al*. 1998; Duthie and Crozier 2000). In addition, they have been implicated in the defense of plants against invading pathogens, including bacteria, fungi, and viruses (Moure *et al.* 2001).

Moreover, while flavonoids are abundant elsewhere in the plant kingdom, there are several compounds (e.g. flavanones, flavanone glycosides and polymethoxylated flavones) unique to citrus, which are relatively rare in other plants (Moyer *et al*. 2002; Manthey and Grohmann 2001).

Enzymatic extraction of flavonoids

Traditionally the extraction process of citrus flavonoids based on organic solvents. The method worked well and a high percentage extraction was possible. An alternative process, enzyme-assisted extraction was used with similar results using food-grade enzymes, containing glucanase and pectinase activities such as Cellulase® MX, Cellulase® CL, Kleerase® AFP (Li *et al.* 2006) and Pectinase 690L, Pectinase 62L, Cellulase C013P (Mandalari *et al*. 2006). One of the main advantages of the enzymatic extraction of phenolics is that during this process 90% of the flavonoid glycosides present were cleaved to their aglycones (Mandalari *et al.* 2006). Many of the ingested dietary flavonoids (e.g., rutin, rutinosides, neohesperosides, and complex acylated flavonoid glycosides) reach the colon without degradation. In the colon, they are substrates for the complex indigenous microflora which are known to contain species and genera able to exert varying effects on the health of the host (Schneider *et al.* 2000; Aura *et al.* 2002, 2005). While the majority of dietary phytochemicals are hydrolyzed by the colonic microflora, small percentages are taken up during transit through the small intestine, and in some cases, the presence of a glucose moiety may enhance absorption (Hollman *et al.* 1999; Hollman and Arts 2000). It has also been demonstrated that the bioavailability of some xenobiotics is dependent mainly on small intestinal uptake (Hollman *et al*. 1996; Morand *et al.* 2000). Polyphenol glycosides, however, are relatively hydrophilic and do not diffuse passively across biological membranes. Simple flavonoid glucosides can be taken up into cells via SGLT1 (sodium-dependent glucose transporter 1) and other hexose transporters, and the aglycones are readily absorbed by passive diffusion. Rutinosides and some other glycosides are not absorbed in the small intestine. Thus, adeglycosylation step is critical for the absorption of dietary flavonoids (Nemeth *et al.* 2003). The low solubility of the released flavonoid aglycones could be increased by addition of food/pharmaceutical grade cyclodextrins as has been recently demons-trated for naringenin and hesperetin (Tommasini *et al*. 2004a, 2004b).

Enzymatic esterification of flavonoids

The use of flavonoids in several domains is limited by their low stability and solubility in the fatty and aqueous phases (Miyake *et al.* 1991; Kitao *et al.* 1993; Sakai *et al*. 1994; Tommasini *et al.* 2004a). To improve their properties, several authors have studied the modification of their structure by chemical, enzymatic or chemo-enzymatic reactions. Two reactions (glycosylation and acylation) have received particular attention. The first allowed flavonoids to reinforce their hydrophilic character by adding sugars, whereas the second reaction makes them more hydrophobic by fatty acid linkage. The chemical acylation of flavonoids by various fatty acids has been patented (Perrier *et al.* 1998; Bok *et al.* 2001), but this process is not regioselective and leads to an unwanted functionalisation of phenolic hydroxyl groups which are responsible for the antioxidant activity of flavonoids (Rice-Evans *et al.* 1996). However, the enzymatic acylation of flavonoids by lipases with phenolic acids is more regioselective than chemical acylation and may enhance not only their solubility in various media, but also their stability (Fossen *et al*. 1998; Ishihara and Nakajima 2003) and their antioxidant activity (Tamura and Yamagami 1994). Enzymatic modification of flavonoids by lipases is described in quite a lot of studies. Flavonoid glycosides were acylated by butanoic acid in the presence of subtilisin (Danieli *et al.* 1990) or by vinyl esters of fatty acids as well as phenolic acids using *Candida antarctica* as biocatalyst (Sakai *et al.* 1994; Tommasini *et al.* 2004a). More specifically, several groups have already performed enzymatic acylation of rutin and naringin (Fossen *et al.* 1998; Perrier *et al.* 1998; Bok *et al*. 2001).

Enzymatic halogenation of flavonoids

The effects of flavonoids, on the central nervous system have been considered. They process anxiolytic activity and low sedative or myorelaxant effects (Medina *et al*. 1997). Among the most active compounds, a number of halogenated flavones have been reported; in particular, 6-bromoflavone and 6-bromo-3-nitroflavone showed activities close to or higher than that of diazepam, a benzodiazepine derivative which is a classical anxiolytic, anticonvulsant, sedative and skeletal muscle relaxant drug. In order to show these activities, the presence of electro-donating or withdrawing substituents on the aromatic ring of the flavonoids seems to be essential. In the literature, several methods for halogenating aromatic compounds are reported. Direct bromination, e.g. with elemental bromine, is a highly polluting method which, in addition, involves serious difficulties con-

nected with the handling of a highly corrosive agent. Other methods, including NBS-amberlyst, (Goldberg and Alper 1994) metal-oxo-catalysed KBr–H2O2 (Clagueand Butler 1995) and KBr–$NaBO_3$ (Roche *et al.* 2000) suffer from harsh conditions or require complex or laborious work-up.

The use of microbial enzymes for the transformation of organic compounds has been employed as a powerful method in metabolism studies and in modern synthetic organic chemistry for decades (Smith and Rosazza 1975). One of the enzymatic reactions that has been widely studied is a chloroperoxidase-catalyzed halogenation (Franssen *et al.* 1987). Chloroperoxidase from *Caldariomyces fumago* (CPO; EC 1.11.1.10) is a well-known enzyme, capable of halogenating a great variety of organic compounds such as *β*-ketoacids (Shaw and Hager 1959), cyclic *β*-diketones (Hager *et al.* 1966), steroids (Levine *et al.* 1968), alkenes (Yamada *et al.* 1985), activated aromatic compounds (Wannstedt *et al.* 1990) and heterocylcic compounds (Franssen 1994). The reaction mechanism of CPO involves the formation of a halogenium ion (X+) or hypohalous acid (HOX) as an intermediate which can effect electrophilic substitution with electron-rich substrates (Yamada *et al.* 1985; Libby *et al.* 1992).

The whole cells and the chloroperoxidase enzyme of *Caldariomyces fumago* were capable of halogenating the flavanones, naringenin and hesperetin, at C-6 and C-8 in the presence of either Cl^- or Br^- (Yaipakdeea and Robertsonb 2001). The biohalogenated products of naringenin and hesperetin were isolated and found to be identical to those obtained from chemical reactions using molecular halogen and hypohalous acid.

CITRUS BY-PRODUCTS AS SOURCES FOR DIETARY FIBRES

Dietary fibre consists of a variety of non starch polysaccharides which include cellulose, hemicellulose, pectin, β-glucans, gums, and lignin (Lamghari *et al.* 2000; Gallaher and Schneeman 2001). Dietary fibre is composed mainly by remnants of edible plant cells; parenchymatous tissues are known to be the most important source of vegetable fibre (de Vries and Faubion 1999; Eastwood 1992). Cell walls of fruits, vegetables, pulses and cereals make up most of the dietary fibre intake (Jiménez *et al*. 2000).

Dietary fibre plays an important role in human health (Anderson *et al.* 1994). High dietary fibre diets are associated with the prevention, reduction and treatment of some diseases, such as diverticular and coronary heart diseases (Anderson *et al.* 1994; Gorinstein *et al.* 2001; Villanueva-Suarez *et al.* 2003). The physiological effects are related to the physicochemical and functional properties of dietary fibre. It is widely known that dietary fibres obtained by different methods and from different sources, behave differently during their transit through the gastrointestinal tract, depending on their chemical composition and physicochemical characteristicsand on the processing that food undergo (Jiménez *et al.* 2000; Chau and Huang 2003). Fiber is often classified as soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) (Gorinstein *et al.* 2001) the SDF/IDF ratio is important for both, dietary and functional properties. It is generally accepted that those fibre sources suitable for use as food ingredient should have an SDF/IDF ratio close to 1:2 (Schneeman 1987; Jaime *et al*. 2002)

Fibre derived from fruits and vegetables have a considerably higher proportion of soluble dietary fibre, whereas cereal fibres contain more insoluble cellulose and hemicellulose. Plant fibres show some functional properties, such us water-holding capacity, swelling capacity, viscosity or gel formation, bile acid binding capacity, and cation-exchange capacity which have been more useful for understanding the physiological effect of dietary fibre, than the chemical composition alone (Femenia *et al.* 1997; Gallaher and Schneeman 2001). These properties are related to the porous matrix structure formed by polysaccharide chains which can hold large amounts of water through hydrogen bonds (Kethireddipalli *et al.* 2002). Functional properties of plant fibre depend on the IDF/SDF ratio, particle size, extraction condition and, vegetable source (Jaime *et al.* 2002).

Currently, there is a great variety of raw materials, mainly processing by-products, from which dietary fibre powders are obtained (Femenia *et al.* 1997). According to Larrauri (1999), the ''ideal dietary fibre'' should meet, among others, the following requirements; have no nutritionally objectionable components, be as concentrated as possible, be bland in taste, colour and odour; have a balanced composition and adequate amount of associated bioactive compounds; have a good shelf life; be compatible with food processing; have the expected physiological effects.

Residues from orange juice extraction are potentially an excellent source of DF because this material is rich in pectin and may be available in large quantities (Grigelmo-Miguel and Martín-Belloso 1998). Citrus fibres have better quality than other dietary fibres due to the presence of associated bioactive compounds, such as flavonoids, polyphenols and carotenes (Fernández-Ginés *et al.* 2003; Figuerola *et al.* 2005).

CITRUS BY-PRODUCTS AS BIOSORBENS FOR HEAVY METAL REMOVAL

Heavy metals have been excessively released into the environment due to rapid industrialization and have created a major global concern. Cadmium, zinc, copper, nickel, lead, mercury and chromium are often detected in industrial wastewaters, which originate from metal plating, mining activities, smelting, battery manufacture, tanneries, petroleum refining, paint manufacture, pesticides, pigment manufacture, printing and photographic industries, etc. (Williams *et al*. 1998; Kadirvelu *et al.* 2001). Unlike organic wastes, heavy metals are non-biodegradable and they can be accumulated in living tissues, causing various diseases and disorders; therefore they must be removed before discharge. Research interest into the production of cheaper adsorbents to replace costly wastewater treatment methods such as chemical precipitation, ion-exchange, electroflotation, membrane separation, reverse osmosis, electrodialysis, solvent extraction, etc. (Namasivayam and Ranganathan 1995) are attracting attention of scientists. Adsorption is one the physico-chemical treatment processes found to be effective in removing heavy metals from aqueous solutions.

The emerging process of 'biosorption' uses nonviable or viable biological materials to bind contaminants *via* physico-chemical mechanisms, whereby factors like pH, size of biosorbent, ionic strength and temperature influence metal biosorption (Volesky and Schiewer 1999). Plant wastes are inexpensive as they have no or very low economic value and thereby most of the adsorption studies have been focused on untreated plant wastes. Ngah and Hanafiah (2008) reviewed the potential application of plant wastes for the removal of heavy metals.

Adsorption of divalent heavy metal ions particularly Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} onto acid (HNO_3) and alkali (NaOH) treated banana and orange peels was performed by Annadurai *et al*. (2002). In general, the adsorption capacity decreases in the order of $Pb^{2+} > Ni^{2+} > Zn^{2+} > Cu^{2+} > Co^{2+}$ for both adsorbents. The reported maximum adsorption capacities using orange peels were 7.75 (Pb^{2+}), 6.01 (Ni^{2+}), 5.25 (Zn^{2+}), 3.65 (Cu^{2+}) and 1.82 mg/g (Co^{2+}) using orange peel. Acid treated peels showed better adsorption capacities followed by alkali and water treated peels. Based on regeneration studies, it was reported that the peels could be used for two regenerations for removal and recovery of heavy metal ions.

Dhakal *et al*. (2005) studied the removal of six heavy metal ions particularly Fe^{3+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Mn^{2+} using orange waste treated with $Ca(OH)_2$ in order to form two types of saponified gels (SOW) (Ca^{2+}-form and H^+-form). The authors suggested that cation exchange was the main mechanism for the removal of heavy metal ions as

the pH of solutions decreased after adsorption. The order of removal for Ca^{2+}-form SOW gel was $Pb^{2+} > Fe^{3+} > Cu^{2+} > Cd^{2+} > Zn^{2+} > Mn^{2+}$. In the case of H^{+}-form SOW gel, the order of removal was $Pb^{2+} > Fe^{3+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Mn^{2+}$.

Li *et al.* (2006) investigated orange peels as an adsorbent for cadmium (Cd^{2+}) adsorption and the effect of different citric acid concentrations on the adsorbent characters was studied. It was also reported that cadmium adsorption occurred via ion-exchange mechanism as the pH of the solution decreases after adsorption, which indicates the presence of more protons in the effluents. Desorption experiment revealed that Cd^{2+} ions could be removed when the concentration of hydrochloric acid was increased and maximum percentage recovery of cadmium was 94% with 0.15 M HCl solution. The reported value of maximum adsorption capacity was 101.16 mg/g. The untreated orange waste however could only adsorb 0.43 mmol/g or 48.33 mg/g Cd (Pérez-Marín *et al*. 2007).

Ajmal *et al*. (2000) reported that Ni^{2+} had a higher affinity to orange peels than Cu^{2+}, Pb^{2+}, Zn^{2+} and Cr^{2+} and described kinetics of divalent cation adsorption by orange peels with a first-order model with respect to the binding sites. Data for binding of Hg^{2+}, Pb^{2+} and Zn^{2+} by *Citrus sinensis* skin (grapefruit) and coffee husk were in good agreement with the Freundlich isotherm model (Jumle *et al.* 2002).

Schiewer and Patil (2008) studied the removal of Cd^{2+} by fruit wastes (derived from several citrus fruits, apples and grapes). Citrus peels were identified as the most promising biosorbent due to high metal uptake in conjunction with physical stability. The metal uptake increased with pH, with uptake capacities ranging between 0.5 and 0.9 meq/g of dry peel.

FINAL REMARKS

Citrus by-products represent a great potential for use as substrates in biotechnological processes. Several studies have been described regarding the employment of this residue for the production of value-added compounds, such as enzymes, biofuels, biopolymers, SCP, organic acids, prebiotic compounds and natural antioxidants among others. Biotechnological applications of the citrus by-products are interesting not only from the point of view of low-cost substrate, but also in solving problems related to their disposal.

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