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A REFEREED PAPER

EFFECT OF SEASONAL VARIATION ON ENZYMATIC HYDROLYSIS OF VALENCIA ORANGE PEEL WASTE

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Abstract. **Approximately 10 million tons of oranges are processed in Florida each year, producing approximately 5 million tons of waste consisting of peel, seeds and segment membranes. Most of this peel is currently dried and pelletized to produce citrus pulp pellets, a low value cattle feed. Several researchers have converted orange peel waste into valuable sugars using enzymatic hydrolysis. After hydrolysis, many of these sugars can be utilized to produce ethanol and other chemicals. This study focuses on the effect of harvest time on sugar yields from enzymatic hydrolyses of Valencia orange peel. Valencia oranges were obtained from the same tree at four times during the 2005 harvest season. A commercial juice extractor was used to extract juice and the processing waste collected for hydrolysis. Cellulose, hemicellulose and pectin were hydrolyzed using pectinase, cellulase and beta-glucosidase enzymes to produce sugars. Arabinose and galacturonic acid yields were affected by the harvest season. Dry matter content of the peel increased over the harvest season. Potential ethanol yields also increased over the harvest season as a result of increased peel dry matter content.**

Over 4.5 million Mg (5 million tons) of orange peel waste (peel, segment membranes, and seeds) were produced by orange juice processors in 2003-04 from 9 million Mg (10 million tons) of oranges (U.S. Dept. Agr., 2004). Most of this peel waste was dried, pelletized, and sold as low-value cattle feed called citrus pulp pellets. The production of citrus pulp pellets requires a large capital investment by the processor with little if any return on investment. Due to high capital costs, many small citrus juice processors are unable to install feed mills to produce citrus pulp pellets, leaving them with tons of peel waste for disposal that has little if any value.

Several studies have been published investigating the potential of using enzymes to breakdown cellulose and pectin in Valencia orange peel waste to produce sugars that could be fermented by microorganisms to produce ethanol (Grohmann et al., 1994; Grohmann et al., 1995a). Valencia oranges are the major variety used to produce orange juice in Florida. Grohmann et al. (1994) produced ethanol from Valencia orange peel waste by hydrolyzing cellulose and pectin in the peel with pectinase, cellulase and β-glucosidase enzymes to produce sugars, and then fermenting some of the sugars using *Saccharomyces cerevisiae* yeast to yield ethanol. Prior to hydrolysis, the peel waste contained 28.5% dry weight fermentable sugars (glucose, sucrose, fructose and galactose). After hydrolysis, the hydrolysate contained 44.4% dry weight fermentable sugars, an increase of 56%. Fermentation of enzymatic peel hydrolysate achieved yields of greater than 4% (w/v) ethanol. Ethanol has also been produced from Valencia orange peel waste using recombinant *E. coli* K011, a strain that can ferment arabinose, xylose, galacturonic acid and six carbon sugars into ethanol (Grohmann et al., 1995a). Galacturonic acid is a product of pectin hydrolysis that makes up 17-20% dry weight of enzymatic peel hydrolysate and cannot be fermented by yeast (Grohmann and Baldwin, 1992; Grohmann et al., 1994). *Escherichia coli* K011 also produces acetic acid during galacturonic acid fermentation, a valuable coproduct.

The influence of fruit maturity on sugar yields from enzymatic hydrolysis is not known. Researchers have measured Valencia orange peel composition over the course of the growing season (Hendrickson and Kesterson, 1954; Ting and Deszyck, 1961). Hendrickson and Kesterson (1954) observed that the dry weight of peel and segment membranes of Valencia oranges increases as the fruit matures, while Ting and Descyck (1961) observed the opposite. Ting and Deszyck (1961) observed that total sugars and cellulose increased in Valencia orange peel waste while pectin decreased during the growing season. Changes in peel waste composition, particularly during the harvest season for Valencia oranges from March to May, may affect the amount of sugars that can be produced from the peel, and thus, the amount of ethanol and other fermentation products that can be produced. The objective of this study is to measure the effect of fruit maturation on the yields of sugars produced from enzymatic hydrolysis of Valencia orange peel waste.

Materials and Methods

Twenty Valencia oranges (*Citrus sinensis* cv. Valencia) were hand picked from a tree at the University of Florida Citrus Research and Education Center (Lake Alfred, Fla.) in mid-March, early-April, mid-April, and early-May. Fruit was picked evenly from all sides of the tree during each harvest time. Juice was extracted from the fruit using a single fruit juice extractor (Fresh n' Squeeze, FMC FoodTech, Chicago, Ill.) and peel waste was collected. Peel waste was ground to particles less than 7 mm (0.25 inches) in diameter using a food processor (Pro Custom 11, Cuisinart, East Windsor, N.J.). Total dry

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matter content of ground peel waste was determined by drying at 70°C (158°F) for 20 h, followed by drying in a vacuum oven at 70°C (158°F) at 700 mm Hg (27.5 inches) vacuum for 1 h. Peel waste was diluted in 250 mL (15.3 inches^3) glass bottles to 1% (w/v) peel dry matter with 50 mM sodium acetate buffer (pH 4.7), which is within the optimal pH range (pH = 4.5-5.0) for the enzymes used, to produce 100 mL (6.1 inches^3) of peel waste/water slurry (Grohmann et al., 1994). Chloramphenicol and cyclohexamide were added at the level of 30 $\mu g \cdot mL^{-1}$ to protect hydrolyses from microbial contamination (Grohmann and Baldwin, 1992).

Hydrolyses were carried out with pectinase, cellulase, and beta-glucosidase enzymes for 24 h at 45°C (113°F) in bottles rotated at 10-12 rpm in an incubator. Pectinase (Pectinex Ultra SP), cellulase (Celluclast 1.5L) and beta-glucosidase (Novozym 188) were obtained from Novozymes A/S (Bagsvaerd, Denmark). Nitrogen contents of enzymes were measured using a Carbo Erba NA 1500 (Thermo Electron Corp., Waltham, Mass.) and multiplied by 6.25 to obtain protein content (ASTM, 2002). Enzyme loadings were 1.4 mg cellulase protein/g peel dry matter, 1.7 mg pectinase protein/g peel dry matter, and 1.5 mg beta-glucosidase protein/g peel dry matter (Grohmann et al., 1994).

After hydrolysis was complete, samples were heated in an oven at 105°C (221°F) for 15 min to inactivate enzymes, and then stored at 3°C (37°F). Prior to analysis for sugars and dissolved dry matter yields, hydrolysates were homogenized with a high shear mixer for 30 s (Brinkmann homogenizer, Brinkmann Instruments, Westbury, N.Y.) to provide a uniform sample for analyses.

Insoluble dry matter was determined by vacuum filtering approximately 10 g of hydrolysate through a 1.2 µm, 9.0 cm (3.5 inches) diameter glass fiber filter with rinsing using deionized water. Filters with insoluble solids were dried according to the procedure described for peel dry weight determination and weighed to 0.001 g accuracy. The difference between hydrolysate dry matter (1% w/v) and insoluble dry matter on the filter was determined to be the dissolved dry matter. Arabinose, fructose, galacturonic acid, glucose, rhamnose, sucrose, and xylose concentrations after hydrolysis were determined by HPLC using a CarboPac PA1 column (Dionex Corp., Sunnyvale, Calif.) regulated at 30°C. A mobile phase was used consisting of aqueous 16 mM NaOH for 15 min, then a linear gradient to aqueous 100 mM NaOH and 150 mM sodium acetate buffer over 30 min, step gradient to aqueous 200 mM NaOH for 15 min as a column wash, and finally a 15 min re-equilibration step at aqueous 16 mM NaOH (Clarke et al., 1991). The mobile phase flow rate at 1 $mL \cdot min^{-1}$ was maintained by a ThermoFinnigan P4000 pump (Thermo Electron, Corp., Waltham, Mass.). Sugars were detected using an electrochemical detector with pulsed-amperometric detection using a potential of 0.10 V for 480 ms, which was followed by 0.95 V for 120 ms and -0.80 V for 70 ms (ED50, Dionex Corp., Sunnyvale, Calif.). Individual sugars were quantitated by the use of internal and external standards to calibrate the detector response for each sugar (Grohmann et al., 1995b). Four replications were performed for each harvest date using the enzyme loadings described earlier. Also, one control bottle for each harvest date containing peel but no enzymes was prepared and exposed to the same conditions as the hydrolyses.

An analysis of variance (ANOVA) ($P < 0.05$) was calculated using SAS, Version 9.1, GLM (SAS Institute, Cary, N.C.) with harvest date as the independent variable and arabinose, fructose, galactose, galacturonic acid, glucose, rhamnose, sucrose, xylose, total fermentable sugars (FS) by *S. cerevisiae* (fructose + galactose + glucose + sucrose) and dissolved dry matter as dependent variables. Data from the control samples were not included in the ANOVA. Means of dependent variables that were significant ($P < 0.05$) were separated by Duncan's multiple range test, 5% level.

Results and Discussion

Hydrolyzed peel. The ANOVA indicated that harvest time had an effect on yields (% peel solids) of arabinose and galacturonic acid ($P < 0.05$) (Table 1). Harvest time had no effect on yields (% peel solids) of fructose, galactose, glucose, rhamnose, sucrose, xylose, FS and dissolved dry matter ($P > 0.05$). Arabinose peaked in early-April peel and decreased during the last two harvest times. Galacturonic acid yields were similar for all harvest times except in mid-April peel, which was greater than the other harvest times. Arabinose and galacturonic acid are products of pectin hydrolysis. Arabinose is found in pectin side chains, while polymers of galacturonic acid form the backbone of pectin molecules (Carpita and Gibeault, 1993).

Gross and Sams (1984) observed that arabinose content in cell walls declined as fruit ripened in 14 of the 17 species they tested, although they did not test any citrus varieties. The loss of arabinose has been associated with pectin degradation during ripening of pears and peaches (Ahmed and Labavitch, 1977; Brummell et al., 2004). A similar loss of arabinose as the oranges ripened was also observed in this study.

Brummell et al. (2004) observed in peaches that the amount of galacturonic acid equivalents in pectins bound loosely to the cell wall through ionic bonds increased slightly from early ripe fruit to mid ripe fruit, peaked in fully ripe fruit, and then declined in overripe fruit below the levels seen in early ripe fruit. In this study, the amount of galacturonic acid produced by enzymatic hydrolysis also peaked in the middle of the harvest season. Loosely bound pectins bonded to the cell wall by ionic bonds may be more susceptible to enzymatic attack than are more tightly bound pectins bonded to

Table 1. Sugar yields[z] for enzymatic hydrolysates of Valencia orange peel waste harvested at different times.[y]

	Ara[x]	Fruc[x]	Gal[x]	GA[x]	Glucose	Rham[x]	Sucr[x]	Xylose	FS[x]
Mid-March	3.23 b	13.15 a	3.39 a	13.45 a	22.39 a	0.82 a	0.50 a	0.83 a	39.43 a
Early-April	3.71 a	13.52 a	3.57 a	13.52 a	22.83 a	0.89 a	0.79 a	0.90 a	40.70 a
Mid-April	2.71 c	12.49 a	3.48 a	15.10 b	22.93 a	0.80 a	0.59 a	0.91 a	39.49 a
Early-May	2.59 c	13.66 a	3.24 a	13.52 a	23.11 a	0.83 a	0.56 a	0.78 a	40.56 a

[z]Yields expressed as % dry weight and are the means of four replicates.
[y]Mean separation in columns by Duncan's multiple range test, 5% level.
[x]Ara-arabinose, Fruc-fructose, Gal-galactose, GA-galaturonic acid, rham-rhamose, sucr-sucrose, FS-fermentable sugars by *Saccharomyces cerevisiae.*

the cell wall by covalent bonds, resulting in an increase in galacturonic acid yield in fully ripe fruit. This view is supported by the accumulation of low molecular weight molecules in loosely bound pectins in peaches as ripening progressed and activities of native polygalacturonase increased, while the molecular weight distribution in covalently bound pectins remained unchanged during ripening, despite the increase in polygalacturonase activity (Brummell et al., 2004). Polygalacturonases hydrolyze α-(1-4) linkages in galacturonan polymers (Fischer and Bennett, 1991). A change in arabinose and galacturonic acid yields affect theoretical ethanol yields from *E. coli* K011 fermentations. *E. coli* K011 can ferment arabinose and galacturonic acid, as well as fructose, glucose, sucrose, and xylose, to ethanol (Grohmann et al., 1995a).

Unhydrolyzed peel. Total dry matter, galactose, glucose, and total sugars in unhydrolyzed peel waste increased and sucrose decreased as the harvest season progressed (Fig. 1). Fructose decreased between mid-March and early-April, then increased for the rest of the season. Arabinose, galacturonic acid, rhamnose, and xylose were not detected in unhydrolyzed peel waste. Decreases in sucrose with corresponding increase in fructose and glucose are due to the hydrolysis of sucrose into fructose and glucose. Despite increases in sugars in unhydrolyzed peel waste over the course of the season, the yields of sugars from enzymatic hydrolysis were not affected by harvest time.

As noted earlier, total dry matter in unhydrolyzed peel waste was observed to increase as the season progressed. Greater dry matter contents in unhydrolyzed peel waste are beneficial for the production of ethanol or other fermentation products because there is decreased dilution of sugars and theoretical product yields are greater. For example, FS mean yields expressed as % peel solids for early-April and early-May peel waste were similar (40.70% and 40.56%, respectively) (Table 1), but because of early-May peel waste's greater dry matter content, wet weight FS yields for early-April and early-May peel waste were 9.92% weight and 10.68%, respectively (Fig. 2). These wet weight FS yields correspond to a theoretical ethanol yield of 5.07% and 5.46%, respectively. Similarly, the fermentable sugar yield for *E. coli* K011 (total sugars minus rhamnose) decreased from 59.03% in early April peel waste to 57.43% in early May peel waste, but the wet weight yields increased 14.40% to 15.16% due to increase in dry matter, resulting in an increase in theoretical ethanol yield from 6.43% to 6.76% (Fig. 2). Theoretical ethanol yields from *S. cerevisiae* (Eqn. 1) and *E. coli* K011 fermentation (Eqn. 2) are calculated by the following equations (Grohmann et al., 1995a; McMillan, 1996; Russell, 2003):

$$EtOH\ (\%\ weight.) = 0.511 * FS * \%\ dm * 0.01 \quad (1)$$

$$EtOH\ (\%\ weight.) = [0.511*(FS+ara+xyl)+0.237*(GA)]* \%dm*0.01$$

where *EtOH* is theoretical ethanol expressed as % weight, FS, ara, GA, and xyl are fermentable sugars, arabinose, galacturonic acid, and xylose expressed in % peel solids, respectively, and dm is dry matter. Increased ethanol concentration not only results in more ethanol produced per ton of peel, but also reduced energy costs (Madson, 2004). An increase in ethanol concentra-

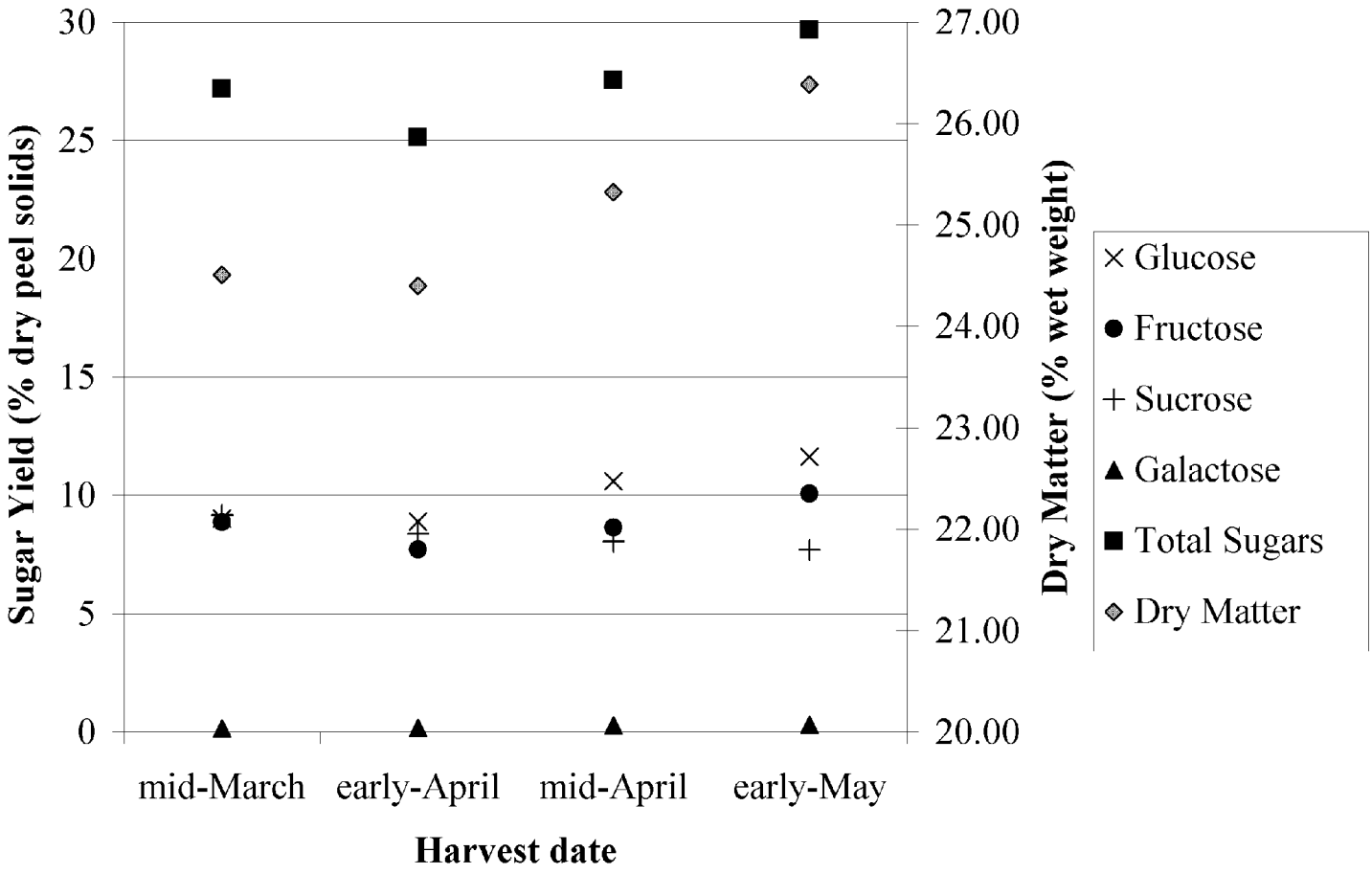

Fig. 1. Sugar yields and dry matter content of Valencia orange peel harvested at different times treated with no enzymes.

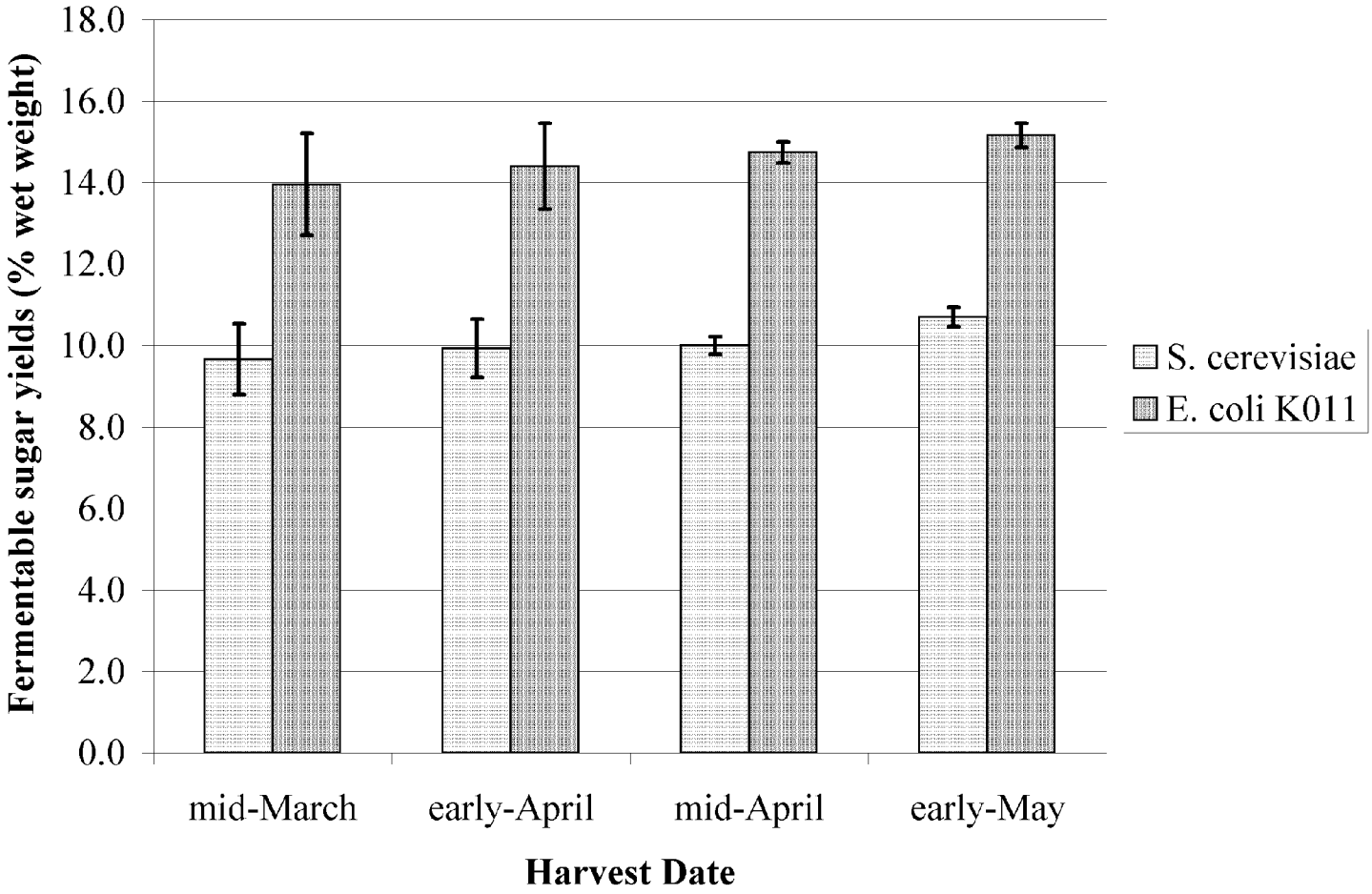

Fig. 2. Sugars fermentable to ethanol by *Saccharomyces cerevisiae* and *Escherichia coli* K011 after enzymatic hydrolysis of Valencia orange peel harvested at different times.

tion from 5.07% weight to 5.46% weight could potentially reduce steam requirements by approximately 3.44 kg steam/L of ethanol produced (2 lb steam/gal) (Madson, 2004).

In conclusion, arabinose yields decreased as the harvest season progressed. Galacturonic acid yields peaked in the mid-April samples, but were constant in the other samples. Dry matter content of the peel increased over the course of the harvest season. Due to the increase in dry matter content, more ethanol and/or other fermentation products could be produced from enzymatic hydrolysis of Valencia orange peel from oranges picked later in the harvest season.

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