Citrus Peels: A Potential Feedstock for Bioethanol Production

Diomi Mamma • Paul Christakopoulos*  

ABSTRACT  
Orange peels (OPs) and water unextractable orange peels (WUOPs) were evaluated as feedstocks for bioethanol production, applying simultaneous saccharification and co-fermentation (SSCF). The fungi, Fusarium oxysporum F3 and Neurospora crassa DSM 1129, were grown aerobically under solid state cultivation (SSC) in order to produce the necessary enzymes for hydrolyzing the polysaccharides present in OPs. Following aerated growth and production of hydrolytic enzymes, OPs and WUOPs were fermented to bioethanol. Factors affecting bioethanol production such as, OP and WUOP concentration and the use of single fungal or mixed culture with S. cerevisiae, were investigated. Both microorganisms were capable of producing bioethanol in single or mixed cultures with S. cerevisiae. F. oxysporum F3 was a better ethanol producer than N. crassa in single or mixed cultures. Yields as high as 23 g of ethanol/100 g of added OPs and 19.98 g of ethanol/100 g of added WUOPs corresponding to 65% and 74%, respectively, of the theoretical yield based on total carbohydrate content of OPs or WUOPs, were achieved with F. oxysporum F3.

Keywords: Fusarium oxysporum, Neurospora crassa, orange peels, simultaneous saccharification and co-fermentation, solid state cultivation

INTRODUCTION
Citrus fruits constitute an important group of fruit crops produced all over the world. The family of citrus fruits consists of oranges, kinnow, khatta, lime, lemon, grapefruit, malt, sweet orange, among others (Dhillon et al. 2004). World citrus production has increased significantly since the 1980s. For example, orange production is projected at 66.4 million in 2010, which represents a 14% increase in the fruit produced between 1997 and 1999. About 30.1 million MT of the orange production will be processed to yield juice, essential oils and other by-products (FAO 2003). Citrus peels are the principal solid by-product of the citrus processing industry and constitute about 50% of fresh fruit weight (Garzón and Hours 1992). The disposal of fresh peels is becoming a major problem for many factories. Usually, citrus juice industries dry the residue, which is either sold as raw material for pectin extraction or pelletized for use as animal feed, though none of these processes is particularly profitable (Garzón and Hours 1992). In Greece, approximately 35,000 tons (dry weight) of citrus peels are produced each year, and only a small fraction is used as cattle feed (Ververis et al. 2007). Various microbial transformations have been proposed for the utilization of food processing waste for producing valuable products like biogas, ethanol, citric acid, chemicals, various enzymes, volatile flavouring compounds, fatty acids and microbial biomass (Dhillon et al. 2004).

With the inevitable depletion of the world’s petroleum supply (Kerr 1998) and the increased price for oil, there has been an increasing worldwide interest in alternative, non-petroleum-based sources of energy. Ethanol is one of the most important renewable fuels that contribute to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels (Cardona and Sánchez 2007). The primary difficulty for commercialization of ethanol produced by fermentation is the high cost of production relative to the local cost of gasoline. The most important parameters affecting economic outcomes include the feedstock cost, and the plant capacity, which influences the capital cost (Gable et al. 2007).

Mature technologies for ethanol production are crop-based, and utilize substrates such as, sugar cane juice and cornstarch. Since the cost of raw materials can be as high as 40% of the bioethanol cost, recent efforts have concentrated on using lignocellulose (Zaldivar et al. 2001). The fact that many lignocellulosic materials are by-products of agricultural activities, industrial residues or domestic wastes offers huge possibilities for the large scale production of fuel ethanol as a renewable fuel.

Four biological events occur during conversion of lignocellulose to ethanol via processes featuring enzymatic hydrolysis: production of hydrolytic enzyme (cellulases and hemicellulases), hydrolysis of the polysaccharides present in pretreated biomass, fermentation of hexose sugars (glucose, mannose and galactose), and fermentation of pentose sugars (xylose and arabinose). The hydrolysis and fermentation steps have been combined in simultaneous saccharification and fermentation (SSF) of hexoses and simultaneous saccharification and co-fermentation (SSCF) of both hexoses and pentoses schemes. The ultimate objective would be a one-step “consolidated” bioprocessing (CBP) of lignocellulose to bioethanol, where all four of these steps occur in one reactor and are mediated by a single microorganism or microbial consortium able to ferment pretreated biomass without added saccharolytic enzymes (van Zyl et al 2007).

Orange peels (OPs), are rich in pectin, cellulose, and hemicellulose (Mamma et al 2008). Cellulose can be efficiently hydrolysed into monomers by treatment with dilute mineral acids, but given the severity of conditions required, the chemical hydrolysis of cellulose is not attractive. Enzymatic hydrolysis offers the potential for higher glucose yields and milder processing conditions (Mielenz 2001). However, one of the major research challenges is to improve the hydrolysis of carbohydrates through more efficient and less expensive pretreatment methods and by enhanced enzymatic hydrolysis with superior enzymes at a reduced enzyme production cost. The latter is one of the most
uncertain costs in most economic analyses (Gable et al. 2007). An alternative approach includes a direct process in which one or more microorganisms carry out simultaneous production of cellulases, hydrolysis and fermentation in the same bioreactor (Lynd et al. 2002). A few microbial species such as, Neurospora, Fusarium, Paecilomyces and Monilia, have been reported to hold the ability of fermenting cellulose directly to ethanol (Singh et al. 1992). Should F. oxysporum F3 and N. crassa be used as fermentation organisms, it will not be necessary to perform a separate enzymatic hydrolysis of the lignocellulosic raw material, as these microorganisms are capable of producing the necessary enzymes (Mamma et al. 2008).

Among the processes used for enzyme production, solid state cultivation (SSC), which can be defined as “the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water” (Pérez-Guerra et al. 2003), is attractive because it presents higher productivity per reactor volume, lower capital and operating costs, lower space requirements, simpler equipment and easier downstream processing compared to that of submerged fermentation (SmF) (Pandey et al. 2000a). Several authors have reviewed various applications of SSC (Rainbault 1998; Pandey et al. 2000b; Pérez-Guerra et al. 2003; Hölker and Lenz 2004). There is also evidence that some enzymes are less affected by catabolic repression, than those obtained by SmF (Martins et al. 2002).

Among the processes used for bioethanol production from lignocellulosic biomass, simultaneous saccharification and co-fermentation (SSCF) is a promising integration alternative that includes pentose fermentation in the simultaneous saccharification and fermentation (SSF) process (Cardona and Sánchez 2007). It should be noted that due to the high amounts of citrus wastes available in the USA, researchers of the US Department of Agriculture worked with commercial enzymes to economically hydrolyze pectin, cellulose and hemicellulose 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). Florida Power & Light Energy LLC (FPL Energy LCC) 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 converting citrus peel waste to ethanol (Ames 2008).

In previous work, it was possible to control the simultaneous production of pectinolytic, cellulolytic and xylanolytic enzymes using fungal strains of the genera, Fusarium and Neurospora. Multienzyme activities were generated using a simple growth medium consisting of a solid by-product of the citrus processing industry and a mineral medium, under SSC (Mamma et al. 2008). The present study was undertaken to investigate the feasibility of using orange peels as feedstock for bioethanol production with the fungi, F. oxysporum F3 and N. crassa DSM 1129, applying SSCF. Factors affecting bioethanol production such as, carbon source concentration and the use of single fungal or mixed cultures with S. cerevisiae were investigated.

**MATERIALS AND METHODS**

**Materials**

Dry OPs of the greek Navel variety (Citrus sinensis) were provided by a local orange processing industry. Dry material was subjected to extraction, as described elsewhere (Mamma et al. 2008), in order to remove all water soluble compounds. The dried material (water unextractable orange peels, WUOPs) was also used in the present study.

**Microorganisms and aerated growth conditions**

The microorganisms used throughout the present study were: (a) the wild-type strain F3 of Fusarium oxysporum, isolated from cu-

min (Christakopoulos et al. 1989), (b) Neurospora crassa DSM 1129, which was obtained from DSMZ (Deutsche Sammlung von Microorganismen und Zellkultur GmbH), Germany and (c) Saccharomyces cerevisiae 2541, which was kindly provided by Pro-
fessor Li Ze-Lin, Shichuan Academy of Food and Fermentation Industries, Chengdu, P.R. of China. Stock cultures were maintained on potato dextrose agar (PDA) slants at 4°C. F. oxysporum F3 and N. crassa DSM 1129 were grown aerobically under SSC. WUOPs were used as solid substrate. WUOPs (2.5 g) were placed in 100 ml Erlenmeyer flasks and moistened with Toyama’s mineral medium (Toyama and Ogawa 1978). Initial culture pHs for F. oxysporum F3 and N. crassa DSM 1129 were 6.0 and 5.0, respectively, while initial moisture content was adjusted at 90% and 80% (w/v), respectively. Following heat steriliza-
tion (121°C, 20 min), the flasks were inoculated with 1 ml of spore suspension (5 × 10^7 conidia/ml) and incubated at 30°C for 6 days.

Aerated growth of S. cerevisiae 2541 was performed as described elsewhere (Mamma et al. 1995).

**Ethanol fermentation**

Following aerated growth of the two fungal strains (as described above), various amounts of OPs or WUOPs and Toyama’s mineral medium were added in order to achieve a final OPs or WUOPs concentration 2, 4, 5 and 6% (w/v). Furthermore, mixed fungi cul-
tures with the yeast S. cerevisiae 2541 were applied. A part of Toyama’s mineral solution was substituted with certain volume of S. cerevisiae culture (Mamma et al. 1995). Flasks were sealed with rubber stoppers carrying a one-way valve to allow release of car-on dioxide produced during fermentation and ensure anaerobic conditions. The flasks were inoculated on a rotary shaker (100 rpm) at 30°C. Samples were taken every 24 hours for a period of 5 days and analysed for ethanol, reducing sugars and glucose, as described herein.

**Enzyme extraction**

Ten volumes of distilled water were added to each flask. Extrac-
tion of the enzymes was carried out on a rotary shaker (250 rpm) at 28 ± 2°C for 1 h. The slurry was squeezed through cheese cloth. The extract was clarified by centrifugation at 12,000 × g (4°C) for 15 min. The clear supernatant was used in enzyme activity mea-
surements.

**Enzyme assays**

Endoglucanase, xylanase, polygalacturonase, pectate lyase and invertase activities were assayed on carboxymethyl cellulose (Sigma Chemical Co., St Louis, MO), birchwood xylan (Sigma), polygal-
acturonic acid (Sigma), pectic acid (Sigma) and sucrose (Sigma), respectively (Cheilas et al. 2000; Jayani et al. 2005). The release of reducing sugars was determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). A total of 1 U of enzyme activi-
ty was defined as the amount of enzyme liberating 1 µmol of reducing sugars per min.

β-Glucosidase and β-xyllosidase activities were determined by a photometric assay using the respective p-nitrophenyl glycosides (Sigma) as substrates (Mamma et al. 1995, 1996). A total of 1 U of enzyme activity was defined as the amount of the enzyme liberating 1 µmol p-nitrophenol per min.

All assays were carried out at 40°C, while the pH of the sub-
strate depended on the microorganism used. Enzyme activities from F. oxysporum F3 were assayed at pH 6.0, while those from N. crassa DSM 1129 were assayed at pH 5.0. For all enzyme assays, blank samples consisting of inactivated enzyme (after boiling for 15 min at 100°C) were used as a reference. Enzyme production was expressed in units per gram of initial dry WUOPs (U/g WUOP).

**Analyses**

Ethanol was determined by a Perkin-Elmer, model 8500, gas chro-
matography apparatus equipped with a DB-WAX column (15 m ×
0.54 mm) and a flame ionisation detector as described elsewhere.
Glucose was estimated by a glucose oxidase/peroxidase assay kit (Sigma). Reducing sugars were determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959).

The remaining sugars, at the end of ethanol fermentation of OPs and WUOPs were determined by high performance anion exchange chromatography (HPAEC). The system consisted of a Jasco quaternary gradient pump (Jasco PU-1580I, Jasco Ltd, UK), a Rheodyne injector and Borwin software. The column was a CarboPack PA1, 4 × 250 mm column (Dionex Corp., USA) with a CarboPack PA1 guard column (Dionex). The separation of sugars was monitored with a pulse amperometric detector (HPAEC-PAD) (Dionex). Fucose (50 μM) was added in each sample as an internal standard. The column was eluted isocratically with 17.5 mM NaOH at a flow rate of 1 ml/min. The injection volume was 20 μl. All analyses were performed in triplicate.

**Statistical analysis**

Analysis of variance (ANOVA) was used to assess differences between factors using Microsoft Excel 2003 (Microsoft Corp.). The significance of main effects and interactions between factors was assessed by P values. During the reporting of the results in this paper the term “significant” refers to a 95% confidence interval.

**RESULTS AND DISCUSSION**

**Composition of OPs**

Dry OPs contained a high amount of water soluble compounds (41.1% w/w, dry basis), which were identified as glucose (14.6% w/w, dry basis), fructose (15.5% w/w, dry basis) and sucrose (10.9% w/w, dry basis), by HPAEC-PAD analysis. OPs were also rich in pectin, cellulose and hemicellulose (Table 1). WUOPs were obtained following extraction of OPs.

**Production of pectinolytic, cellulolytic and xylanolytic enzymes under solid state fermentation by F. oxysporum F3 and N. crassa DSM 1129**

Polysaccharides in OPs had to be hydrolyzed to ethanol fermentable sugars and this role was accomplished by the two fungi under investigation. Both F. oxysporum F3 and N. crassa DSM 1129 were grown aerobically on SSC using WUOPs as the sole carbon source and produced a multienzyme complex consisting of pectinases, cellulases and xylanases. It is generally agreed that pectinase, cellulase and hemicellulase production is inducible and also is affected by the nature of the substrate used in fermentation (Hang and Woodmanns 1994; Solis-Pereira et al. 1994; Naidu and Panda 1998; Kang et al. 2004).

Polygalacturonase, pectate lyase and xylanase activities, produced by N. crassa DSM 1129, reached their maximum values (63.25, 29.09 and 56.84 U/gWUOPs, respectively) between the 4th and 5th day of incubation and remained constant over time. Endoglanucanase and β-glucosidase activities increased continuously over time and exhibited maximum values at the 9th and 10th day of cultivation, respectively (138.52 and 7.91 U/gWUOPs, respectively) (Fig. 1A). β-xylanolysidase activity was not obtained in N. crassa cultures.

Time course of enzymes production by F. oxysporum F3 is presented in Fig. 1B. Maximum polygalacturonase activity (91.36 U/gWUOPs) was registered on the 6th day of cultivation and decreased the last 4 days. Pectate lyase, endoglucanase and xylanase activities reached the maximum values (37.95, 69.49 and 28.92 U/gWUOPs, respectively) at the 8th day and remained practically constant during the last 2 days of cultivation. On the other hand, β-glucosidase exhibited its maximum activity at the 3rd day (0.92 U/gWUOPs) and decreased sharply thereafter. Finally, β-xylanolysidase activity was maximum (0.092 U/gWUOPs) at the 2nd day and remained practically constant until the 8th day of cultivation (Fig. 1B).

Owing to the high cost of pure enzymes, the use of a simple two-stage process which includes the SSC growth of the fungi on orange peels followed by the hydrolysis of the added amounts of it by the in situ produced multienzyme systems and the conversion of polymeric carbohydrates to monomeric sugars for further conversion to ethanol, is a very promising process.

**Table 1** Composition of dry orange peels (compiled from Mamma et al. 2008).

<table>
<thead>
<tr>
<th>Component</th>
<th>% (w/w dry basis) ± standard error</th>
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<tr>
<td>Crude fat</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>Water soluble materials</td>
<td>41.1 ± 1.2</td>
</tr>
<tr>
<td>Pectin</td>
<td>14.4 ± 0.3</td>
</tr>
<tr>
<td>Protein</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>Cellulose</td>
<td>16.2 ± 0.5</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>13.8 ± 0.3</td>
</tr>
<tr>
<td>Ash</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Lignin</td>
<td>1.0 ± 0.02</td>
</tr>
</tbody>
</table>

**Fig. 1** Polygalacturonase (○), pectate lyase (□), endoglucanase (■), xylanase (□), invertase (▲), β-glucosidase (▼) and β-xylanolysidase (▼) activities produced by A N. crassa DSM 1129 (initial moisture content: 80% (w/w) and initial culture pH 5.0) and B F. oxysporum F3 (initial moisture content: 90% (w/w) and initial culture pH 6.0) grown on WUOPS, under solid state cultivation.
Ethanol production from OPs and WUOPs by single fungal cultures and mixed fungal cultures with S. cerevisiae

Following aerated growth of N. crassa DSM 1129 and F. oxysporum F3 under SSC using WUOPs as a carbon source for the production of hydrolytic enzymes, bioethanol production was carried out by adding various amounts of OPs or WUOPs. Factors affecting bioethanol production such as, the concentrations of OPs and WUOPs and the use of single fungal or mixed cultures with S. cerevisiae 2541 were investigated.

Previous researchers have successfully hydrolyzed both orange and grapefruit peel waste to glucose, galactose, fructose, arabinose, xylose, rhamnose, and galacturonic acid using cellulase and pectinase enzymes (Grohmann and Bas 1994a; Grohmann et al. 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 S. 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). Escherichia coli K011 is a recombinant bacterial strain developed to ferment arabinose and xylose as well as hexoses to ethanol (Beall et al. 1991).

One of the major advantages of the present study’s approach is that it is not necessary to perform a separate hydrolysis of the raw material since both F. oxysporum F3 and N. crassa DSM 1129 can produce the necessary enzymes. Furthermore, both fungal strains have the ability of fermenting the hexoses as well as the pentoses present in the hydrolyzate to bioethanol (Deshpande et al. 1986; Panaigiotou et al. 2005a, 2005b). Further, the use of mixed microbial cultures is beneficial to the process due to the fact that enzymes hydrolyze polysaccharides into sugars, which are immediately consumed by S. cerevisiae 2541 to produce ethanol and consequently hydrolysis rates are increased by reducing product inhibition of enzymes. Mixed microbial cultures were successfully applied in our previous studies on bioethanol production from sweet sorghum, an energy crop containing a high amount of sugars (Mamma et al. 1995, 1996).

Ethanol production from OPs

The results of bioethanol production using OPs as feedstock by single or mixed microbial cultures are presented in Table 2. In order to determine the statistical differences in the results presented in Table 2, a two factor Analysis of Variance (ANOVA) was performed. Practically, no significant differences were observed between single N. crassa DSM 1129 and mixed N. crassa DSM 1129 with S. cerevisiae 2541, cultures as judged by the ANOVA results (P-value = 0.346), as well as between the different OP concentrations (P-value = 0.154). Varying OPs concentration in cultures of F. oxysporum F3 resulted in significant statistical differences (P-value = 0.017), while no differences were observed between single F. oxysporum F3 and mixed F. oxysporum F3 with S. cerevisiae 2541 (P-value = 0.708).

Maximum ethanol yields were registered between the 3rd and 4th day of fermentation. Yields as high as 23.5 g/100 g of OPs were achieved by F. oxysporum F3 (Table 2). The fact that the mixed microbial cultures did not increase ethanol yield could suggest that the fungal cultures could not be attributed to the inhibitory effect of OP oil and/or limonene on yeast growth. According to Grohmann et al. (1994a), in order for the yeast to ferment hexose sugars, OP oil concentration in the hydrolysate must be reduced prior to fermentation. The inhibitory effect on yeast growth due to orange peel oil and/or d-limonene, a monoterpenone 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).

At the end of ethanol fermentation, the remaining reducing sugars were determined by the DNS method as well as by HPAEC-PAD analysis. The results of the DNS method are presented in Fig. 2A-2B. Practically no statistical differences were observed between single or mixed microbial cultures for both fungal strains (Fvalue = 0.728 and PFusarium-value = 0.488). In all cases, increasing OPs concentration resulted in increased amounts of total reducing sugars (Pvalue = 0.0033 and PFusarium-value = 0.0014) (Fig. 2A-2B). HPAEC-PAD analysis revealed that

Table 2 Ethanol production from OPs applying SSCF, in single cultures of N. crassa DSM 1129 and F. oxysporum F3 or mixed fungal cultures with the yeast, S. cerevisiae 2541 (values are the means of three determinations and the standard deviation was below 5% in all cases).

<table>
<thead>
<tr>
<th>OP concentration (%) w/v</th>
<th>Ethanol yield (g/100 g of OPs)</th>
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<tbody>
<tr>
<td></td>
<td>N. crassa DSM 1129</td>
</tr>
<tr>
<td>2</td>
<td>17.30</td>
</tr>
<tr>
<td>4</td>
<td>14.75</td>
</tr>
<tr>
<td>5</td>
<td>13.98</td>
</tr>
<tr>
<td>6</td>
<td>15.52</td>
</tr>
<tr>
<td>N. crassa DSM 1129 + F. oxysporum F3 + S. cerevisiae 2541</td>
<td>17.40</td>
</tr>
<tr>
<td>2</td>
<td>15.30</td>
</tr>
<tr>
<td>4</td>
<td>16.24</td>
</tr>
<tr>
<td>6</td>
<td>15.17</td>
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</tbody>
</table>

Fig. 2 Total reducing sugars remaining after OPs fermentation to ethanol applying: A: single culture of N. crassa DSM 1119 (■) or F. oxysporum F3 (□), B: mixed microbial of N. crassa DSM 1119 with S. cerevisiae 2541 (■) or F. oxysporum F3 with S. cerevisiae 2541 (□). Total reducing sugars after WUOPs fermentation to ethanol applying: C: single culture of N. crassa DSM 1119 (■) or F. oxysporum F3 (□), D: mixed microbial of N. crassa DSM 1119 with S. cerevisiae 2541 (■) or F. oxysporum F3 with S. cerevisiae 2541 (□).
only glucose and xylose were present in the culture broth at the end of ethanol fermentation. It should be noted that in all cases traces of glucose (less than 0.5 g/l) were detected. Furthermore, xylose was detected and, in all cases, its concentration was in the range of 0.7-1.5 g/l. The difference between total reducing sugars concentration measured by DNS method and the results obtained by HPAEC-PAD analysis could probably be attributed to uronic acids produced during hydrolysis of the pectin content of the substrate. In this study, uronic acids were not be fermented by the microorganisms to bioethanol.

The percentage of the theoretical yield based on the carbohydrate content (cellulose and hemicellulose) of WUOPs is presented in Table 2. Maximum ethanol yields were registered between the 3rd and 4th day of fermentation. Statistical differences were observed between single N. crassa DSM 1129 and mixed N. crassa DSM 1129 with S. cerevisiae 2541 cultures as judged by the ANOVA results (P-value = 0.019), as well as between the different WUOPs concentrations (P-value = 0.002). WUOPs concentration seem to affect more ethanol production by the different F. oxysporum F3 cultures (P-value = 0.0006). Mixed F. oxysporum F3 cultures slightly improved fermentation of WUOPs to ethanol compared to single fungal cultures (P-value = 0.032). Yields as high as 19.98 g/100 g of WUOPs were achieved by F. oxysporum F3 (Table 3).

At the end of ethanol fermentation, the remaining reducing sugars were determined by the DNS method as well as by HPAEC-PAD analysis. The results of the DNS method are presented in Fig. 3C, 2D. Practically no statistical differences were observed between single or mixed microbial cultures for both fungal strains (P-value = 0.489 and P-value = 0.250). Glucose and xylose were the only sugars detected by HPAEC-PAD analysis. Once more, traces of glucose (less than 0.3 g/l) were detected, while the xylose concentration ranged from 1.0-2.5 g/l.

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study are quite promising. Further investigations concerning pretreatment of WUOPs or the use of fed-batch process in order to increase ethanol yields are warranted.

CONCLUSIONS

The use of the simple process proposed, which includes the growth under SSC of *F. oxysporum* F3 and *N. pseudocerasus* DSM 1129 on WUOPs followed by the SSSF of OPs or WUOPs to ethanol, resulted in promising yields. Further investigations of the process are warranted.

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