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# Citrus Peels: A Potential Feedstock for Bioethanol Production

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## 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, malta, 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 food (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

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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* and *N. crassa* be used as the 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 (Raimbault 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 concerting 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 mateial (water unexractable 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 cumin (Christakopoulos *et al.* 1989), (b) *Neurospora crassa* DSM 1129, which was obtained from DSMZ (Deutsche Sammlung von Microorganismen und Zellkulture GmbH), Germany and (c) *Saccharomyces cerevisiae* 2541, which was kindly provided by Professor 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/w), respectively. Following heat sterilization (121°C, 20 min), the flasks were inoculated with 1 ml of spore suspension ($5.8 \times 10^7$ 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 cultures 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 carbon dioxide produced during fermentation and ensure anaerobic conditions. The flasks were incubated 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. Extraction 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 12000 × g (4°C) for 15 min. The clear supernatant was used in enzyme activity measurements.

### Enzyme assays

Endoglucanase, xylanase, polygalacturonase, pectate lyase and invertase activities were assayed on carboxymethyl cellulose (Sigma Chemical Co., St Louis, MO), birchwood xylan (Sigma), polygalacturonic 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 activity was defined as the amount of enzyme liberating 1 μmole of reducing sugars per min.

*β*-Glucosidase and *β*-xylosidase 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 μmole *p*-nitrophenol per min.

All assays were carried out at 40°C, while the pH of the substrate 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 ($U/g_{WUOPs}$).

### Analyses

Ethanol was determined by a Perkin-Elmer, model 8500, gas chromatography apparatus equipped with a DB-WAX column (15 m × 0.54 mm) and a flame ionisation detector as described elsewhere

(Mamma *et al*. 1995).

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 asses 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.

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

| Component | % (w/w dry basis) (± standard error) |
|---|---|
| Crude fat | 3.9 ± 0.1 |
| Water soluble materials [(2)] | 41.1 ± 1.2 |
| Pectin | 14.4 ± 0.3 |
| Protein | 7.9 ± 0.1 |
| Cellulose | 16.2 ± 0.5 |
| Hemicellulose | 13.8 ± 0.3 |
| Ash | 1.7 ± 0.1 |
| Lignin | 1.0 ± 0.02 |

[(1)] Moisture content 4.96% (w/w)
[(2)] Glucose 14.6 ± 0.4% (w/w, dry basis), fructose 15.5 ± 0.5% (w/w, dry basis) and sucrose 10.9 ± 0.3% (w/w, dry basis)
Values are the mean of three determinations.

### 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 under 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 Woodanms 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/$g_{WUOPs}$, respectively) between the 4$^{th}$ and 5$^{th}$ day of incubation and remained constant over time. Endoglucanase and *β*-glucosidase activities increased continuously over time and exhibited maximum values at the 9$^{th}$ and 10$^{th}$ day of cultivation, respectively (138.52 and 7.91 U/$g_{WUOPs}$, respectively) (**Fig. 1A**). *β*-xylosidase 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/$g_{WUOPs}$) was registered on the 6$^{th}$ 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/$g_{WUOPs}$, respectively) at the 8$^{th}$ day and remained practically constant during the last 2 days of cultivation. On the other hand, *β*-glucosidase exhibited its maximum activity at the 3$^{rd}$ day (0.92 U/$g_{WUOPs}$) and decreased sharply thereafter. Finally, *β*-xylosidase activity was maximum (0.092 U/$g_{WUOPs}$) at the 2$^{nd}$ day and remained practically constant until the 8$^{th}$ 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.

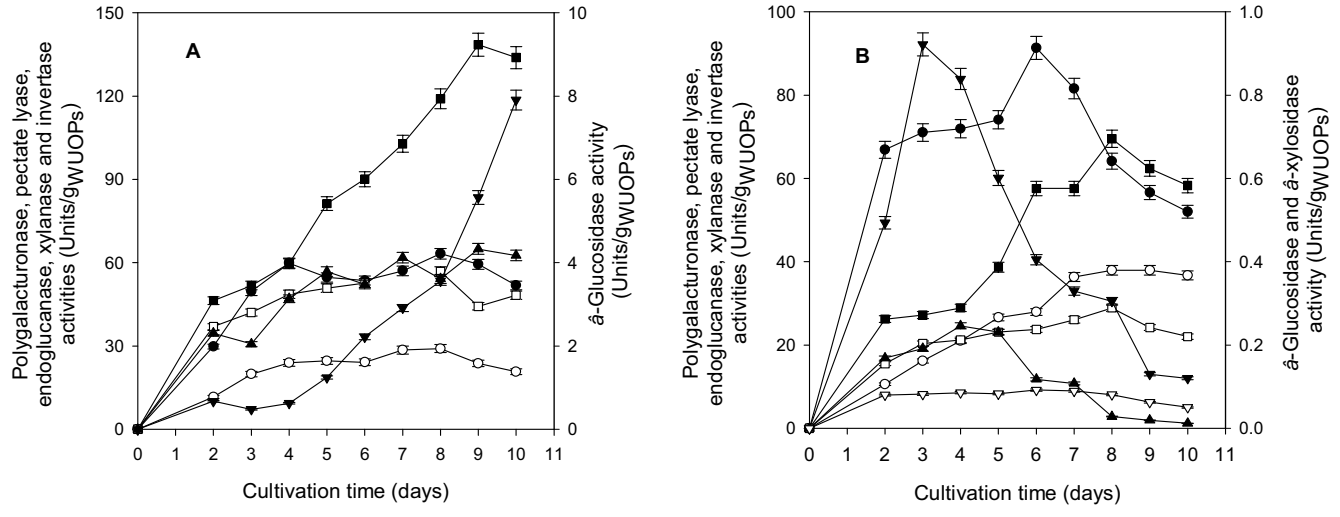


**Fig. 1** Polygalacturonase (●), pectate lyase (○), endoglucanase (■), xylanase (□), invertase (▲), *β*-glucosidase (▼) and *β*-xylosidase (▽) activities produced by **A** *N. crassa* DSM 1129 (initial moisture content: 80% (w/w) and initial culture pH 5.0) and **B** *F. oxysporum* F3 (initial moisture 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 Baldwin 1992; Grohmann *et al.* 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* KO11 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; Panagiotou *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 4rth 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 compared to single fungal cultures, could probably be attributed to the inhibitory effect of OP oil and/or D-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 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).

**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. cerevisiase* 2541 (values are the means of three determinations and the standard deviation was below 5% in all cases).

| OP concentration (% w/v) | Ethanol yield (g/100 g of OPs) | |
|---|---|---|
| | *N. crassa* DSM 1129 | *F. oxysporum* F3 |
| 2 | 17.30 | 23.55 |
| 4 | 14.75 | 15.78 |
| 5 | 13.98 | 16.60 |
| 6 | 15.52 | 12.75 |
| | *N. crassa* DSM 1129 + *S. cerevisiae* 2541 | *F. oxysporum* F3 + *S. cerevisiae* 2541 |
| 2 | 17.40 | 23.25 |
| 4 | 15.30 | 16.43 |
| 5 | 16.24 | 15.00 |
| 6 | 15.17 | 15.52 |

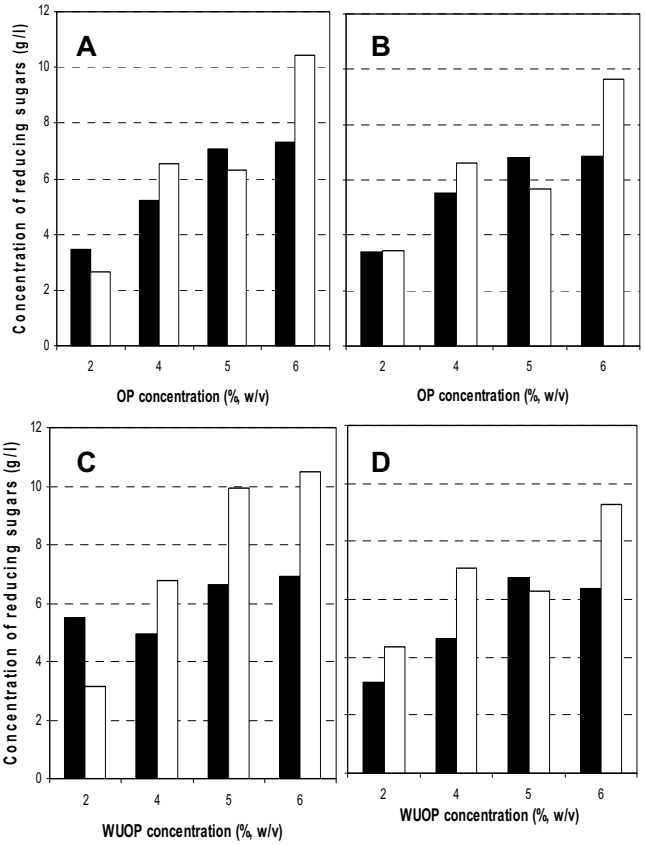


**Fig. 2** Total reducing sugars remaining after OPs fermentation to ethanol applying: **A** single culture of *N. crassa* DSM 1119 (■) or *F. oxysporum F3* (□), **B** mixed microbial of *N. crassa* DSM 1119 with *S. cerevisiae* 2541 (■) or *F. oxysporum F3* with *S. cerevisiae* 2541 (□).Total reducing 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 (□).

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 ($P_{Fusarium}$-*value* = 0.728 and $P_{Neurospora}$-*value* = 0.488). In all cases, increasing OPs concentration resulted in increased amounts of total reducing sugars ($P_{Fusarium}$-*value* = 0.0033 and $P_{Neurospora}$-*value* = 0.0014) (**Fig. 2A-2B**). HPAEC-PAD analysis revealed that

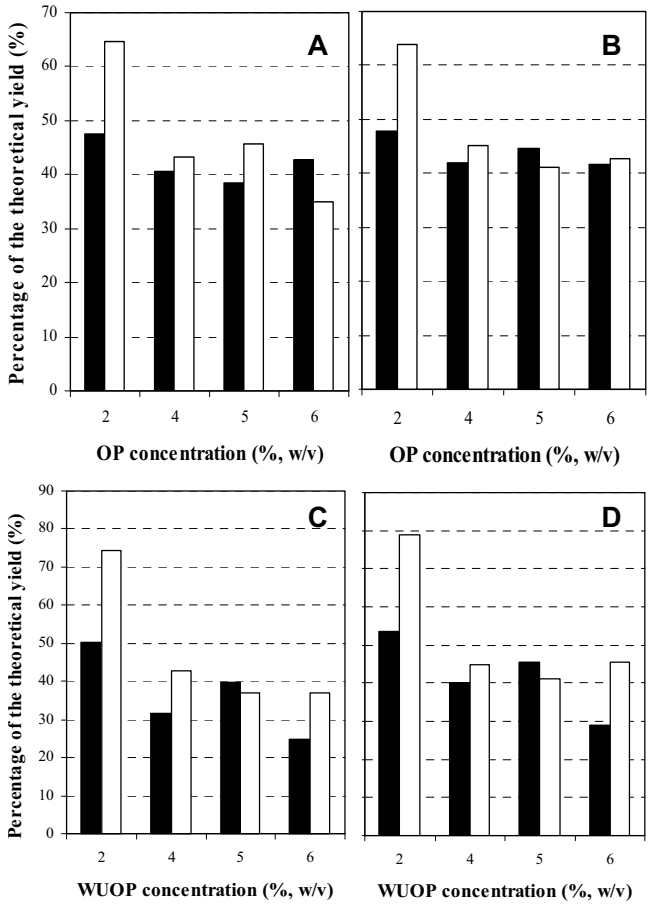


**Fig. 3** Percentage of the theoretical yield during bioethanol production by OPs applying: **A** single culture of *N. crassa* DSM 1119 (■) or *F. oxysporum F3* (□), **B** mixed microbial of *N. crassa* DSM 1119 with *S. cerevisiae* 2541 (■) or *F. oxysporum F3* with *S. cerevisiae* 2541 (□). Percentage of the theoretical yield during bioethanol production by WUOPs 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 (fermentable sugars, cellulose and hemicellulose) of OPs is presented in **Fig. 3A** and **3B** for the single and mixed microbial cultures of the two fungal strains. The percentages of the theoretical yields for the single or mixed cultures of *N. crassa* DSM 1129 were in the range of 45%, which means that only the readily available sugars were fermented to ethanol. On the contrary, single and mixed cultures of *F. oxysporum* F3 exhibited higher percentages of the theoretical yield, reaching 65%. The latter suggests that all sugars as well as part of the polysaccharides (cellulose and hemicellulose) were fermented to ethanol.

### *Ethanol production from WUOPs*

Dry OPs were subjected to aqueous extraction in order to remove all water soluble compounds (sugars), resulting in the WUOPs, a material that contains only polysaccharides in the form of cellulose, hemicellulose and pectin. The results of bioethanol production using WUOPs as feedstock

**Table 3** Ethanol production from WUOPs applying SSCF, in single cultures of *N. crassa* DSM 1129 and *F. oxysporum* F3 or mixed fungal cultures with the yeast *S. cerevisiase* 2541 (values are the means of three determinations and the standard deviation was below 5% in all cases)

| **WUOP concentration (%, w/v)** | **Ethanol yield (g/100 g of WUOPs)** | |
|---|---|---|
| | *N. crassa* DSM 1129 | *F. oxysporum* F3 |
| 2 | 13.55 | 19.98 |
| 4 | 8.48 | 11.48 |
| 5 | 10.70 | 10.00 |
| 6 | 6.70 | 10.00 |
| | *N. crassa* DSM 1129 + *S. cerevisiae* 2541 | *F. oxysporum* F3 + *S. cerevisiae* 2541 |
| 2 | 14.45 | 21.25 |
| 4 | 10.80 | 12.10 |
| 5 | 12.20 | 11.10 |
| 6 | 7.78 | 12.17 |

by single or mixed microbial cultures are presented on **Table 3**. 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. 2C, 2D**. Practically no statistical differences were observed between single or mixed microbial cultures for both fungal strains ($P_{Fusarium}$-*value* = 0.489 and $P_{Neurospora}$-*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.

The percentage of the theoretical yield based on the carbohydrate content (cellulose and hemicellulose) of WUOPs is presented in **Fig. 3C** and **3D** for the single and mixed microbial cultures of the two fungal strains. The percentages of the theoretical yields for the single or mixed culture of *N. crassa* DSM 1129 were in the range of 50%, while single and mixed cultures of *F. oxysporum* F3 exhibited higher percentages of the theoretical yield reaching the 74%.

Wilkins *et al*. (2007b) reported that ethanol produced by *S. cerevisiae* and *K. marxianus* during fermentation of a solution modelling hydrolyzed orange peels waste was 37.1 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 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.

The results of the present study indicated that (a) the enzymatic systems of the fungi tested, namely *F. oxysporum* F3 and *N. crassa* DSM 1129 could efficiently hydrolyse the polysaccharides present in OPs or WUOPs and (b) both fungi ferment the hexoses and pentoses produced during hydrolysis to ethanol. The yields obtained in the present

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. crassa* DSM 1129 on WUOPs followed by the SSCF of OPs or WUOPs to ethanol, resulted in promising yields. Further investigations of the process are warranted.

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