On the exploitation of kitchen biowastes for ethanol production via co-cultures of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*

Menıs N.[1,2], Antonopoulou G.[1,2], Ntaıkou I.[1,2*] And Lyberatos G.[1,2]

[1]Institute of Chemical Engineering Sciences, Foundation for Research and Technology, GR 26504, Patra, Greece

[2] School of Chemical Engineering, National Technical University of Athens, Zografou Campus, GR 15780, Athens, Greece

*corresponding author: Ioanna Ntaikou

e-mail: ntaikou@iceht.forth.gr

Abstract

In the present study, the biotransformation of the pre-dried and shredded organic fraction of kitchen wastes (OFKW) to ethanol was investigated, using mono- and co-cultures of the yeasts *Saccharomyces cerevisiae* and *Pichia stipites*. OFKW represents one of the main type of food wastes that are generated in huge quantities annually and consists of up to 60% of simple and complex carbohydrates. Preliminary experiments with glucose as the sole carbon source were performed, in order to investigate the effect of different operational parameters on the ethanol production efficiency of the co-culture. Subsequently, the ethanol production efficiency from the OFKW was assessed via simultaneous saccharification and fermentation experiments. It was shown that the pH control and the addition of nitrogen were among the key factors for the optimization of the process, and also that the co-cultures led to higher ethanol yields and substrate consumption.

Keywords: bioethanol, kitchen wastes, co-cultures

1. Introduction

It is estimated that almost 90 million tons of food are wasted annually in the EU, when at the same time one billion people become hungry every year, and another billion are undernourished (FAO, 2011). Food wastes (FW) are generated at various points of the food supply chain (FSC) and different strategies should be explored in order either to prevent their production or to identify ways for recovering nutrients, energy of high added value products. In contrast to other commodity flows, FW is biological material subject to degradation, and different types of food stuff have different nutritional values. Thus, FW are exploitable constituting an abundant source of renewable biomass containing large amounts of protein, carbohydrates, lipids and other nutrients that could be actually recovered and/or converted to other high added value products. Different strategies have so far been proposed for the valorization of different types of food wastes, depending on their composition and source. Domestic food wastes i.e. FW occurring at the end of the FSC, are considered an ideal substrate for the production of various biofuels via microbiological processes, due to their high content in readily fermentable carbohydrates, such as sugars and starch and the necessary nutrients that can support efficient growth of different types of microorganisms. The production of bioethanol has attracted great interest during the previous decades, aiming to the replacement of fossil fuels. In the automobile sector, bioethanol may be used as a) octane enhancer in unleaded gasoline in place of the methyl tertio butyl ether (MTBE), b) oxygenated compound for clean combustion of gasoline, c) alternative fuel for reducing CO_2 emissions and d) renewable energy carrier to partly substitute oil and to increase security of supply (Gnansounou and Dauriat, 2005). Different carbohydrate-based feedstocks have been proposed as substrates, including energy "crops", ligniocellulosic biomass, organic residues and wastes and, more recently, algal biomass resulting to the so called first-second- and third-generation bioethanol, respectively (Jambo *et al*., 2016). Different yeast and bacterial strains have so far been proposed as biocatalysts for ethanol production. Using co-cultures during alcoholic fermentation can be quite advantageous in terms of both substrate exploitation and ethanol yields. In principle, when selecting combined microbial species for a co-culture bioethanol generating system, the first step is to choose a glucose-fermenting microorganism and a xylose-fermenting microorganism, then test their compatibility and finally study their co-fermentation performance. According to the literature. the yeast genus *Saccharomyces* is preferably used as the glucose-fermenting strain whereas *Pichia* is often proposed as the xylose-fermenting strain. Co-cultures of various strains of *S. cerevisiae* and *P. stipitis* have also been assessed mainly due to the fact that the pHs and temperatures at which *S. cerevisiae* ferments glucose to ethanol are compatible with those of *P. Stipitis* (Chen, 2011) The aim of the present study, was to investigate the efficiency of second generation bioethanol production from the organic fraction of kitchen wastes (OFKW) generated and collected at municipality level, using co-cultures of the yeasts *Saccharomyces cerevisiae* and *Pichia stipites*. The effect of key factors such as pH, nitrogen efficiency and substrate concentration on the final ethanol yields was assessed

2. Materials and Methods

2.1. Feedstock

The OFKW was collected at municipality level 2 times a week from 240 houses of the Municipality of Chalandri, Greece. Upon collection, OFKW was subjected to simultaneous heat-drying at 80^{o}C and shredding, resulting to a homogeneous organic product with the following characteristics: Total solids (TS), 91.28±0.75%, volatile solids (VS), 92.34±0.73%, dissolved sugars, 0.21±0.02 g/g TS, total carbohydrates, 0.43±0.03 g/g TS, total Kjeldahl nitrogen, 1.63±0.17 g/100g TS, proteins, 10.17±1.06% (w/w TS). Drying and shredding were applied in order to prevent biodeterioration of the waste and to ensure its stable and unchanging composition during its storage.

2.2. Microorganism, media and growth conditions

All fermentation tests were performed using the yeasts *S. sereviceae*, CECT 1332 and *P. stipitis*, CECT 1922. Both strains were stored at 4^{0}C in slant solid cultures in the following medium (g.L^{-1}): yeast extract 3; malt extract 3; myco-peptone 5; d-glucose 10; agar 20. For the startup of each experiment, slant cultures were used for the inoculation of 100mL fresh liquid medium of the above composition under sterile conditions. Cultures were incubated at 27^{0}C, under mechanical agitation at 150rpm for 24 h in order to obtain cells at the same growth stage for every experiment. The cells contained in equal volumes of *S. cereviceae and P. stipites* cultures were then harvested via centrifugation and used as inoculum for each experiment. Four sets of experiments were performed with glucose as the sole carbon source in order to study the effect of a) initial substrate concentration (C_{Sin}), b) initial pH, c) constant control of pH using buffer and d) addition of organic nitrogen. In all cases the following medium was used (g.L^{-1}): d-glucose 20-60, KH_2PO_4 1, $MgCl_2.6H_2O$ 1, $(NH_4)_2SO_4$ 1. Additionally, NaOH 3M and HCl 3M were used for pH initial adjustment; KH_2PO_4 with K_2HPO_4 buffer solutions were used for pH control, whereas 1g.L^{-1} yeast extract (y.e.) was added in the case of N-effect experiments. Subsequently two set of experiments with OFKW were performed in order to investigate a) the performance of each microorganism solely and b) the effect of enzymatic loading. The solids loading was 10% (wTS/v) in all cases, whereas 2-30 FPU/g TS of Celluclast and Novozym 188 at a ratio 3:1 were also added under sterile conditions before inoculation.

2.3. Analytical methods

Total solids (TS), volatile solids (VS) and Total Kjeldahl Nitrogen (TKN) in raw and extract-free samples were quantified according to Standard Methods (APHA, 1995). Crude protein content was determined by multiplying TKN by a factor of 6.25 (Monlau *et al*., 2011). Glucose and ethanol were quantified via HPLC-RI (Shodex) with an Aminex HPX–87H column (Biorad) at 60^{0}C and a Cation H micro-guard cartridge (biorad Laboratories), with H_2SO_4 0.006N mobile phase at a flow rate of 0.6mL.min^{-1}.

3. Results

3.1. Effect of substrate concentration, pH and nitrogen addition

The effect of initial substrate concentration (C_{Sin}) during alcoholic fermentation of glucose with *S. cereviceae*, and *P. stipitis* co-cultures is illustrated in Fig.1 (a, b, c). It is obvious that glucose uptake was incomplete in all cases. The amount of consumed glucose was 10.53±1.53 g.L^{-1}, 10.04±0.35 g.L^{-1} and 9.5±0.05 g.L^{-1} corresponding to ~53%, ~23% and only ~16 % (table 1) of maximum glucose uptake for C_{Sin} 20 g.L^{-1}, 40 g.L^{-1} and 60 g.L^{-1} respectively. As also shown in Fig.1, the pH drop exhibits the same profile regardless the C_{Sin} and it actually seems that, when the pH reaches 2.5, growth is inhibited. Maximum ethanol concentration in the fermentation broth and also maximum ethanol yield in terms of consumed glucose ($Y_{EtOH/Gl\ cons}$) was observed for C_{Sin} 60 g.L^{-1}. However, since the glucose uptake was minimum for the higher C_{Sin} the estimated ethanol yield in terms of initial substrate concentration ($Y_{EtOH/Gl\ in}$) was actually the lowest (table 1). When the pH was adjusted to higher values, glucose uptake exhibited an increasing tendency, reaching 11.25±0.70 g.L^{-1} and 12.79±0.09 g.L^{-1} for an initial pH of 5.4 and 5.8, respectively. As shown in table 1, the effect on $Y_{EtOH/Gl\ cons}$ and $Y_{EtOH/Gl\ in}$ increase was actually negligible. The limiting factor seems to be again the pH drop, since as illustrated in Fig1.e, pH drop profiles were similar, reaching a lowest value of 2.5. In order to further study the effect of pH on the performance of the co-cultures, the pH was adjusted via the addition of buffer solution which has the ability to prevent dramatic pH drops. This is illustrated in Fig. 2.b, where it is shown that the minimum pH values are 3.5 and 3.7 for initial pH values of 6 and 5.5. respectively, whereas the pH of control cultures (i.e with no buffer addition) drops to 2.5. The positive effect of pH control was even more obvious when estimating the percentage of substrate uptake and $Y_{EtOH/Gl\ in}$ which was actually doubled and tripled when 5.5. and 6 pH buffers were used. In order to investigate the effect of nitrogen source addition on the performance of the co-culture, experiments with different pH adjustment and supplementation with yeast extract were performed. Results are illustrated in Fig.2 (d,e,f). It is apparent that the addition of yeast extract enhanced significantly the uptake of glucose even in the case of no pH control. The substrate uptake reached 36.10±0.49 g.L^{-1} and 37.11±0.09 g.L^{-1} for pH buffers of 5.5 and 6, leading thus to a four-fold $Y_{EtOH/Gl\ in}$, compared to the control in both cases. Previous studies with co-cultures of *S. cereviceae* and *P. stipites* have found similar ethanol yields to those achieved in the present study. Batch co-cultures of *P. stipitis CBS5773* with *S. cerevisiae no. 7* (Taniguchi *et al*., 1997) and *P. Stipitis CCY39501* with *S. cerevisiae V30* (Kordowska-Wiater and Targoński, 2001) using a glucose/xylose mixture under contolled pH at 5 and 5.5. respectively, achieved $Y_{EtOH/Gl\ cons}$ of 0.39g EtOH/g sugars in both cases.

3.2. Ethanol production from OFKW

Kitchen wastes are complex organic substrates that contain different types of sugars and carbohydrates, as well as different nitrogen sources. The OFKW that was used in the present study consisted of ~43% (w/wTS) total carbohydrates ~21% of which correspond to free sugars. In Fig. 3 the profiles of ethanol production from 10% OFKW suspensions (wTS/v) are illustrated, corresponding to an initial concentration of carbohydrates ~40g/L, using both mono- and co- cultures of *S. cereviceae* and *P. stipitis*. Since none of the microorganisms that were used performs hydrolysis of complex carbohydrates, a mixture of cellulose- degrading enzymes was added so as to increase the quantities of free. As shown in Fig.3a and Fig.3c pH in

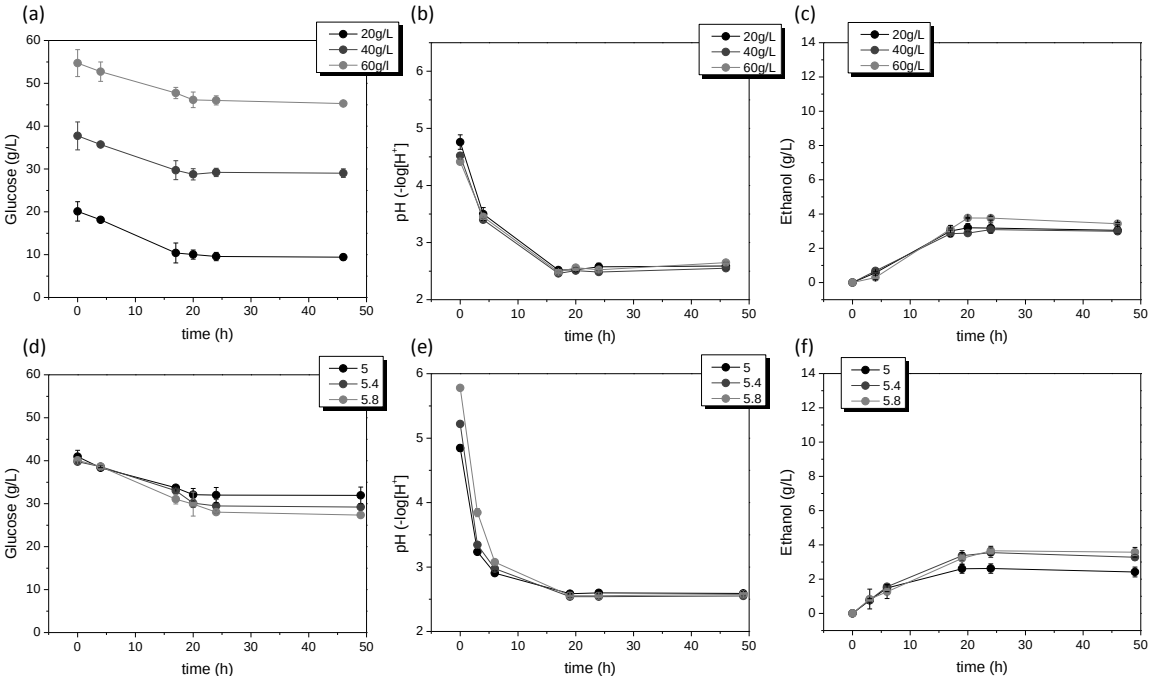

Figure 1. Effect of initial substrate concentration and pH on glucose consumption (a,d), pH drop (b,e) and ethanol production (c, f) during batch co-cultures of *S. cereviceae* and *P. stipitis* with glucose as the sole carbon source.

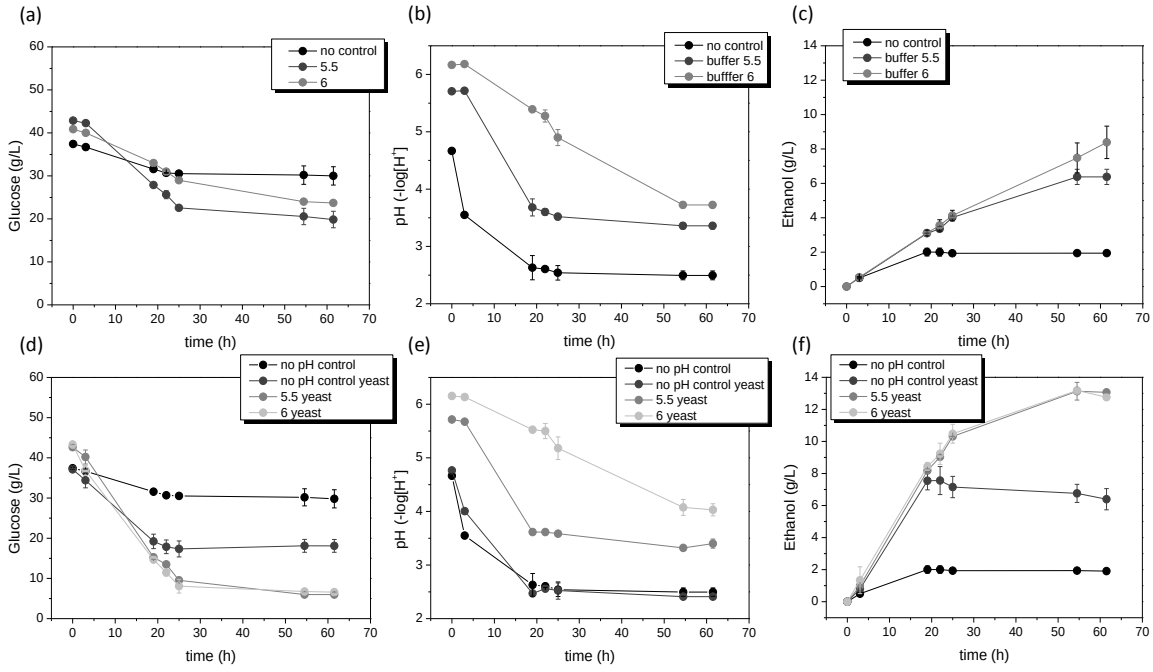

Figure 2. Effect of pH control and supplementation with yeast extract on glucose consumption (a,d), pH variation (b,e) and ethanol production (c, f) during batch co-cultures of *S. cereviceae* and *P. stipitis* with glucose as the sole carbon source.

Table 1. Effect of initial substrate concentration, pH and addition of yeast extract on the consumption of glucose and the yields of produced ethanol during batch co-cultures of *S. cereviceae* and *P. stipitis* with glucose as the sole carbon source (*no buffer, no added N).

Parameter tested	range	Substrate uptake (%)	$Y_{EtOH/Gl\ cons}$ (g EtOH/g consumed glu.)	$Y_{EtOH/Gl\ in}$ (gEtOH/g initial glu.)
$C_{s.init,}$ (no pH control, no N addition).	20g/L	52.55 ± 0.03	0.31 ± 0.03	0.16 ± 0.02
	40g/L	22.91 ± 0.03	0.35 ± 0.10	0.08 ± 0.02
	60g/L	15.94 ± 0.03	0.43 ± 0.06	0.07 ± 0.01
pH_{init} ($C_{s.init.}$:40g/L, no N addition).	5	22.35 ± 1.9	0.32 ± 0.07	0.08 ± 0.02
	5.4	28.98 ± 0.78	0.36 ± 0.09	0.09 ± 0.03
	5.8	31.80 ± 0.99	0.36 ± 0.01	0.09 ± 0.02
pH control ($C_{s.init.}$:40g/L, no N addition).	no buffer	21.88 ± 3.03	0.32± 0.03	0.08 ± 0.00
	buffer, pH 5.5	41.20 ± 1.37	0.38 ± 0.01	0.16 ± 0.00
	buffer, pH 6	71.93 ± 4.89	0.39 ± 0.03	0.21 ± 0.03
N source ($C_{s.init.}$:40g/L).	Control*	20.88± 3.03	0.31± 0.03	0.07± 0.00
	no buffer, + y. e.	54.09± 4.02	0.39 ± 0.01	0.20 ± 0.02
	pH 5.5, + y. e.	83.81± 0.97	0.37 ± 0.01	0.31 ± 0.02
	pH 6, + y. e.	84.42 ± 0.67	0.37± 0.00	0.30± 0.00

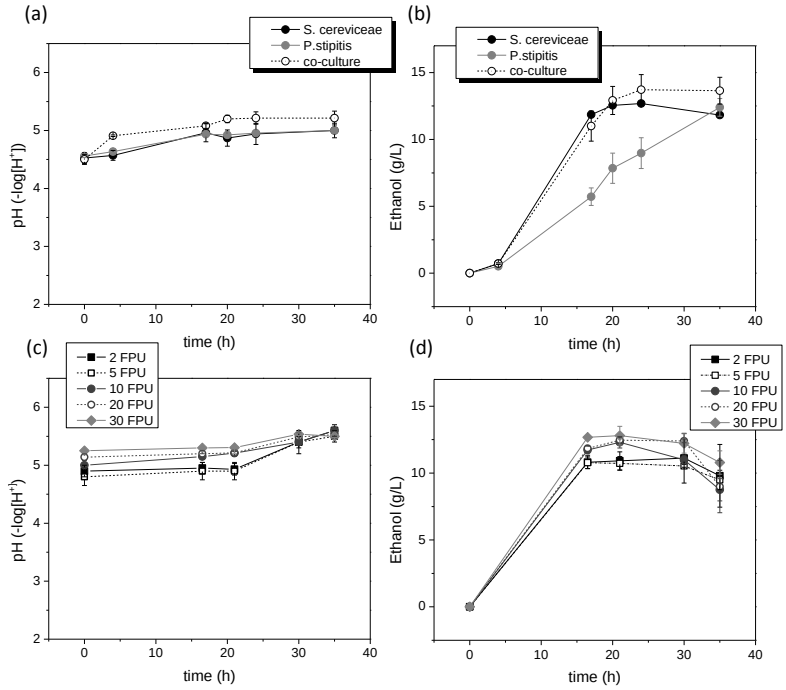

Figure 3. Bioethanol production and pH variation during batch mono-cultures and co-cultures of *S. cereviceae* and *P. stipitis* (a, b) and effect of different enzymatic loading with DFW as the sole carbon and nitrogen source.

Table 2. Effect of the type of microorganism used and the enzymatic loading on the yields of produced ethanol during batch cultures with OFKW as the sole carbon source.

Parameter tested	range	$Y_{EtOH/carb.}$ (g EtOH/g initial carbohydrates)	$Y_{EtOH/OFKW}$ (gEtOH/g initial waste)
strain	*S. cereviceae*	0.29 ± 0.00	0.13 ± 0.00
	P. stipitis	0.31 ± 0.01	0.14 ± 0.01
	Co-culture	0.32 ± 0.01	0.14 ± 0.01
Enzymatic loading	2 FPU	0.26 ± 0.03	0.11 ± 0.02
	5 FPU	0.25 ± 0.00	0.11 ± 0.00
	10 FPU	0.29 ± 0.01	0.12 ± 0.00
	20 FPU	0.29 ± 0.00	0.12 ± 0.00
	30 FPU	0.30 ± 0.02	0.13 ± 0.01

the cultures seems to be self-controlled at ~5, with fermentations. *S. cereviceae* seems to lead to higher ethanol concentrations (C_{EtOH}) compared to *P. stipites*, whereas the co-culture led to the highest C_{EtOH}. The maximum achieved C_{EtOH} was 13.61±1.31 $g.L^{-1}$, which corresponded to a $Y_{EtOH/carb.}$ 0.32g $EtOH.g^{-1}$ initial carbohydrates. The increased amount of enzymes added had a positive effect on the $Y_{EtOH/carb}$ up to the value of 10FPU of Celluclast, with higher amounts of enzymes having a negligible effect on the performance of alcoholic fermentation, as shown by the results summarized in Table 2. Matsakas *et al*. (2014) using household food wastes at 45% solids loading and addition of 10FPU of enzymes have managed to achieve C_{EtOH} of 42.78±0.83 $g.L^{-1}$ and 39.15±0.75 $g.L^{-1}$ via monocultures of common baker's yeast, when performing separate and simultaneous saccharification and fermentation, respectively. It was assumed thus that the saccharification of the waste prior to fermentation contributes to the increase of ethanol yields.

4. Conclusions

S. sereviceae, CECT 1332 and *P. stipitis*, CECT 1922 are compatible during simultaneous fermentation of glucose and complex carbohydrates contained in the OFKW, for ethanol production. Adjustment of pH in the range 5.5-3.5 as well as supplementation with an organic nitrogen source are essential for achieving high substrate uptake and high ethanol yields. Simultaneous saccharification and fermentation for ethanol production is enhanced with an enzymatic loading above 10FPU. In overall it was demonstrated that the OFKW is a promising substrate for ethanol production using co-cultures of the above yeast stains.

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