Effect of chemical and enzymatic pretreatment on bioethanol production from starchy food wastes via novel yeast strains


¹Institute of Chemical Engineering Sciences, 11 Stadiou st., Plataní, Patras, GR 26504, Greece.
²School of Chemical Engineering, National Technical University of Athens, GR 15780 Athens, Greece.
³Department of Chemical Engineering, University of Patras, 1 Karatheodori st., GR 25400, Greece
⁴King Khalid University, College of Science, Department of Biology, Abha 61413, Saudi Arabia.
⁵Assiut University, Faculty of Science, Botany and Microbiology Department, Assiut, 71516, Egypt.

Abstract

In the present study the exploitation of a starchy food waste containing mainly wasted rice (WR) was investigated for bioethanol production via novel yeast strains. The saccharification efficiency of different pretreatment schemes was initially evaluated aiming to the maximum liberation of free sugars, and their subsequent fermentation towards enhanced ethanol yields. Three approaches of pretreatment were investigated i.e. enzymatic pretreatment via commercial amylolytic enzymes, thermochemical pretreatment via HCl and combined chemical-enzymatic pretreatment with initial solids loading of WR 10 - 30% TS WR. Fermentation tests of the pretreated WR were performed via different newly isolated yeast and the fermentation efficiency was comparatively assessed. It was shown that the combined pretreatment led to the maximum saccharification of the waste, whereas ethanol yields were in in all cases quite high, exceeding 85% of the theoretical maximum.

Introduction

Due to the steadily increasing population growth and industrial development, the conventional energy sources can hardly meet the energy demands. As such, exploring alternative energy sources become a great strategic interest worldwide. Bioethanol is a clean and renewable energy carrier, being a strong candidate to fill the gap of energy demand. Nevertheless, the main challenge for its sustainable production is the cost of the raw material and its maximum bioconversion using cost efficient and eco-friendly methodologies. The current study aspires to offer a new biotechnological solution for this problem through the bioconversion of an abundant type of biowaste worldwide i.e. starchy food wastes into biofuel bioethanol using novel yeast strains via which maximum exploitation of substrates, ethanol yields and productivities can be achieved. Towards this direction the effect of the pretreatment of the biowaste, thermochemically and enzymatically on the saccharification and fermentation efficiency, was assessed.

Materials and Methods

Feedstock used

The starchy biowastes containing mainly wasted rice (WR) was collected from various restaurants of Asir region, Saudi Arabia. Immediately upon collection the WR was transferred to the laboratory and dried at 50-55°C for 24h and subsequently was air dried, grinded at particle size of ≤2.5mm, homogenized and packed in polypropylene bags in batches of 2 kg. Prior to pretreatment and fermentation experiments, WR was further grinded using a laboratory stainless steel mill to a particle size of ≤0.5mm.

Pretreatment conditions

The pre-treatment of the biowastes was considered essential in order to hydrolyse the complex carbohydrates contained in the wastes to sugars than can be fermented by the yeast strains and as such enhance the fermentation efficiency of the process in terms of ethanol. WR was subjected to pre-treatment. Three approaches were investigated i.e. the enzymatic pre-treatment via commercial amylolytic enzymes, the thermochemical pre-treatment via HCl and the combined chemical-enzymatic pre-treatment. In all cases three solid loadings of the waste were tested i.e. 10% TS WR/v, 20% TS WR/v and 30% TS WR/v. Enzymatic pre-treatment of WR was performed in duplicates at pH 4.8 and 50 °C using a fungal α-amylase, FA (α-amylase from Aspergillus oryzae, Sigma-Aldrich) and amylglucosidase, A, (amylglucosidase from Aspergillus niger, Sigma-Aldrich) at enzymatic loadings 50 FA U/g starch and 25 A U g starch, respectively. For the pH adjustment, 0.1 M sodium acetate buffer was used. To avoid bacterial contamination, 2% sodium azide was added to the suspension. Thermochemical pre-treatment was conducted using 1g HCl/g Ts (w/w) at 121 °C for 20 min. After cooling, the pH was adjusted to 4.8 using 6 N NaOH. The combined pre-treatment included two sequential steps, i.e. thermochemical pretreatment as described above and then enzymatic hydrolysis of the remaining starch with of
50 FA U/g starch and 25 A U g starch. The efficiency of each pre-treatment approach was assessed by estimating the saccharification efficiency, SE, using Equation (1).

\[
SE (\%) = \frac{\text{Final concentration of sugars} - \text{Initial concentration of sugars (g/L)}}{\text{Initial concentration of total carbohydrates (g/L)}}
\]  

(1)

**Fermentation experiments**

Fermentation tests with the yeast isolates were performed in duplicate, using as the sole carbon source the pre-treated WR at solids loading 10% initial TS (w/v) and 30% initial TS (w/v). Fermentations were performed at 160 mL serum vials with a working volume 60 mL were used. The experiments were performed in duplicate in batch mode and incubated at 150 g and 30 °C. The vials were sealed with rubber stoppers and equipped with 0.22 µm filters for CO₂ venting and sterilization. In all experiments, cells were harvested from pre-culture of the yeast strains that were newly isolated from WR and spoiled fruits KKU12, KKU13, KKU14 and KKU25 (identification of the strains is in progress) at 10% v/v of the final fermentation volume of each experiment, leading to an initial biomass concentration of 0.2–0.25 g/L. For the inoculation, the estimated volume of pre-culture was centrifuged at 4500 g for 15 min and the yeast pellet was re-suspended in a solution containing KH₂PO₄, MgCl₂, 6 H₂O and (NH₄)₂SO₄ each at final concentrations of 1 g/L culture.

The efficiency of alcoholic fermentation of biowastes was assessed by estimating bioethanol yields in terms of carbohydrate uptake, Yₑ, fermentation efficiency, FE and initial feedstock bioconversion, Yₑ/Waste, using Equations (2), (3) and (4) respectively:

\[
Y_{E/S} (g/g) = \frac{\text{Bioethanol concentration (g/L)}}{\text{Total utilized sugar (g/L)}}
\]  

(2)

\[
FE (\%) = \frac{\text{Obtained yield (g/g)}}{\text{Theoretical yield (g/g)}}
\]  

(3)

\[
Y_{E/Waste} (g/kg) = \frac{\text{Bioethanol concentration (g/L)}}{\text{Waste concentration (kg/L)}}
\]  

(4)

**Analytical methods**

Total solids (TS), total suspended solids (TSS), humidity, volatile solids (VS), volatile suspended solids (VSS), ash (salts and minerals), dissolved and Total chemical oxygen demand (d-COD, t-COD) and Total Kjeldahl Nitrogen (TKN) 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., 2012). Glucose, soluble sugars and total carbohydrates were quantified according to DuBois et al. (1956). Reducing sugars were quantified by the DNS (3,5-dinitrosalicylic acid) method and was expressed as glucose equivalents (Miller, 1959). Starch content will be determined via a total starch assay kit (Megazyme). Lipids and oils content was estimated in a Soxlet apparatus. Ethanol was quantified via HPLC-RI (Shodex) with an Aminex HPX-87H column (Biorad) at 60°C and a Cation H micro-guard cartridge (biorad Laboratories), with H₂SO₄ 0.006N mobile phase at a flow rate of 0.6mL/min. The measurement of the pH was done using a HANNA (pH 211) pH-meter with a HANNA electrode (HI 1230).

**Statistical Analysis**

The statistical analysis of the obtained data was conducted with the use of the SPSS Inc.17 software package. After checking for homogeneity of the variance (Levene’s test of equality of error variances), the significant differences among each treatment were assessed non-parametrically, using the Mann Whitney u test (p < 0.05, ANOVA).

**Results**

**Characterization of raw DPF**

The composition of the WR was: TS (%), 92.59 ± 0.06; VS (% TS), 95.87 ± 0.02; ash (% TS), 2.82 ± 0.03, sugars (%), 7.42 ± 0.32; starch (%), 69.70 ± 0.02; t-COD, (%), 98.89 ± 12.13; TKN, (%), 0.13 ± 0.01; proteins, (%), 0.72 ± 0.03, lipids and oils, 3.74 ± 0.02 (%).

**Effect of pretreatment on the saccharification of WR**

The pre-treatment of WR aimed to the saccharification of the waste in order to enhance its subsequent fermentation efficiency towards ethanol. More specifically, what was targeted was the enzymatic or chemical hydrolysis of the starch content of the waste to glucose, maltose or oligosaccharides. As such the pre-treatment methods that were assessed were a) the enzymatic pre-treatment via commercial amylolytic enzymes, b) the thermochemical pre-treatment via HCl and c) the two stepped chemical-enzymatic pre-treatment, using in all cases three solid loadings WR i.e. 10% TS WR/v, 20% TS WR/v and 30% TS WR/v.
In Fig. 1, the liberation of sugars and sugars is illustrated for the different handlings, expressed as contestation of soluble sugars and reducing sugars. It is obvious that the higher solids loading resulting to higher concentrations of both soluble and reducing sugars in all cases. It can also be noted that both enzymatic and combined chemical/ enzymatic pre-treatment led to much enhanced saccharification compared to the chemical saccharification. It can be assumed thus that the chemical pre-treatment solely cannot be a proposed method for the efficient exploitation of the waste towards ethanol, since a considerable amount carbohydrates are not hydrolysed and thus will remain un-exploitable by the yeasts. Chemical pre-treatment on the other hand seems to facilitate to some extent the enzymatic hydrolysis, since as it can be observed in Fig. 1a, the combined pre-treatment leads to higher liberation of sugars for loading 20% and 30%.

As it regards the effect of different pre-treatments on the liberation of reducing sugars, the comparison of Fig 1a and 1b, reveals that in the case of enzymatic and combined pre-treatment the soluble sugars are all reducing, since the values do not have any significant statistically difference, actually. This is not the case however for the chemical pre-treatment too, for which the concentration soluble sugars seem to be considerable higher than the concentration of reducing sugars. For the direct comparison of the values the ratio of reducing to soluble sugars was estimated for all cases, and the values are presented in Table 1.

![Figure 1](image_url)

**Figure 1.** Effect of enzymatic, chemical and combined pre-treatment of 10%, 20% and 30% aquatic suspensions of WR TS on the liberation of soluble sugars (a) and reducing sugars (b). Initial, concentration of sugars before pre-treatment; Enz24, after 24 h of enzymatic hydrolysis with 50 U AG/g TS; Enz48, after 48 h of enzymatic hydrolysis with 50 U AG/g TS; Chem, after hydrolysis with 1g HCl/g TS, at 121°C for 20 min; Cmb24, after chemical hydrolysis followed by 24 h of enzymatic hydrolysis with 50 U A/g TS; Cmb48, after chemical hydrolysis followed by 48 h of enzymatic hydrolysis with 50 U A/g TS. Experimental points represent the mean value of a duplicate culture ±SD (N≥4).

The values that were estimated for the ratios of reducing to soluble sugars proved indeed, that the above assumption was correct, i.e. the whole amount of measured the soluble sugars are actually reducing ones in all cases of enzymatic and combined pre-treatment with no significant statistically differences being observed among values.

On the contrary, all those values are statistically much higher than the ones estimated from the concentrations of sugars after solely chemical pre-treatment. This finding could be attributed to the different effect of enzymatic and chemical pre-treatment. Indeed, enzymatic pre-treatment that was performed by the synergetic action of α-amylase and amyloglucosidase and as such is expected to lead to the complete hydrolysis of all carbohydrates to glucose (reducing). This is due to the mechanism of those enzymes that involve the hydrolysis of the 1,4-α-glucosidic bonds of starch to oligosaccharides of three or more molecules of glucose by α-amylase whereas amyloglucosidase hydrolyses further oligosaccharides to glucose monomers. On the contrary the effect of thermo-acid hydrolysis of starch via hydrochloric acid leads to random breakdown of the 1,4-α-glucosidic bonds that could result to a mixture of monosaccharides and oligosaccharides that might not be reducing.

**Table 1.** Ratio of concentrations of reducing to soluble sugars that were liberated after the enzymatic, chemical and combined pre-treatment of 10%, 20% and 30% aquatic suspensions of WR TS. Experimental points represent the mean value of a duplicate culture ±SD (N≥4).
For the further better evaluation of the efficiency of each process in terms of the saccharification of the waste due to its hydrolysis, the parameter of saccharification efficiency $SE$, was estimated in each case using the Equation (1). The results are presented graphically in Fig. 2. As expected, the $SE$ is in all cases much higher for enzymatic and combined pre-treatment than for chemical pre-treatment reaching in the case of combined pre-treatment almost complete saccharification of the waste. Interestingly enough, it seems that the effectiveness of enzymatic pre-treatments seems to be depended highly by the solid loading. Indeed, the enzymatic treatment seems to be more effective for the lower loading i.e. 10% than for the higher ones. This can probably be attributed to the difficulty in the mixing of the slurries for higher loadings which was much thicker than for the 10% loading. On the contrary, in the case that chemical pre-treatment precedes the enzymatic one this effect is not only eliminated but is actually inverted, leading thus to enhanced $SE$s compared to the application of enzymatic pre-treatment only even after 24h of treatment.

Based on the above it could be assumed that for the selection of the appropriate methodology for the most efficient hydrolysis should be made taking into account the factors of time, energy and materials required in each process in relation to the relative increase in soluble carbohydrates.

**Figure 2.** Saccharification efficiency of enzymatic, chemical and combined chemical-enzymatic pre-treatment on 10%, 20% and 30% aquatic suspensions of WR. Enz24, after 24 h of enzymatic hydrolysis with 50 U AG/g TS; Enz48, after 48 h of enzymatic hydrolysis with 50 U AG/g TS; Chem, after hydrolysis with 1g HCl/g TS, at 121°C for 20 min; Cmb24, after chemical hydrolysis followed by 24 h of enzymatic hydrolysis with 50 U A/g TS; Cmb48, after chemical hydrolysis followed by 48 h of enzymatic hydrolysis with 50 U A/g TS. Experimental points represent the mean value of a duplicate culture ±SD (N≥4).

*Ethanol production from pretreated WR*

The fermentation tests of the pretreated WR showed that the enzymatic and combined pretreatment led to considerably higher ethanol yields and fermentation efficiencies ($FE$) compared to the chemically pretreated waste. Indeed the chemical pretreatments, although leading to the liberation of considerable amount of free sugars was proven to produce an insufficient substrate for further fermentation. It can be assumed that the selective conditions of chemical pretreatment and are not severe enough to warranty the efficient saccharification of the complex carbohydrates of the dried WR to monosaccharides and disaccharides, such as glucose and maltose, but to oligosaccharides which are not fermentable by the selected yeast strains. Indeed, in previous studies in which a similar waste was assessed as substrate for ethanol production the ethanol yields were quite high (Hashem et al., 2021). In that study the biowaste was being subjected to acid pretreatment with HCl in more...
severe conditions and without being priory dried, which has probably affected positively its composition in free sugars and its subsequent bioconversion to ethanol.

With regard to the enzymatic and combined pretreatment the isolates KKU12, KKU13 and KKU14, exhibited similar FE, which exceeded in all cases 90% of the theoretical maximum. The performance of the strain KKU25 on the other hand was not as high with its FE ranging from 78% to 89% of the theoretical maximum. In general, the FE did not seem to be affected by the solids loading.

On the contrary, solids loading affected the $Y_{\text{ENWASTE}}$, which was in all cases considerably higher for the lowest solids loading i.e. 10% TS waste, as shown in Table 2, and could possibly be attributed either to nitrogen limitation which could lead to the cease of the metabolic activity of the strains, or product inhibition due to the high accumulation of ethanol in the fermentation broth. Both hypotheses will be further investigated experimentally.

It has to be mentioned though that the solids loading of 10% TS waste is hardly efficient for providing the ethanol concentrations that required for successful distillation of from the fermentation broth, which is reported to be 40 g/L (Wingren et al., 2004). Indeed, the ethanol titter that was noticed for 10% TS ranged from 35-47 g/L, whereas the highest ethanol titter was 103 g/L and it was achieved from the isolate KKU13 from combined pretreated waste with 30% initial TS.

![Figure 3. Ethanol concentrations (a) and estimated ethanol yields during the alcoholic fermentation of enzymatically, chemically and combined chemically-enzymatically pre-treated WR at initial solids loadings 10% and 30%. Experimental points represent the mean value of a duplicate culture ±SD (N≥4).](image)

![Table 2. Maximum consumption of sugars the alcoholic fermentation of enzymatically, chemically and combined chemically-enzymatically pre-treated WR at initial solids loadings 10% and 30%. Experimental points represent the mean value of a duplicate culture ±SD (N≥4).](image)

<table>
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<th>10% ENZ</th>
<th>30% ENZ</th>
<th>10% CMB</th>
<th>30% CMB</th>
<th>10% CHEM</th>
<th>30% CHEM</th>
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<td>10% ENZ</td>
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References