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Bioethanol Production from Acid Hydrolysates of Date Palm Fronds Using a Co-culture of Saccharomyces cerevisiae and Pichia stipitis

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Abstract: A two-stage acid hydrolysis was carried out with different concentrations of perchloric acid using date palm frond. In the first stage, most of hemicellulose and crystalline cellulose were hydrolyzed, as well as lignin, which was partially decomposed.In the second stage, the hydrolysis of crystalline cellulose and some additional decomposition of lignin were observed. The combined hydrolysates from the two-stage acid hydrolysis were concentrated by vacuum evaporation to increase the concentration of fermentable sugars and detoxified by over liming and activated charcoal adsorption to decrease concentration of fermentation inhibitors. A higher ethanol yield was obtained with the co-culture of Saccharomyces cerevisiae and Pichiastipitis, than the ethanol produced with a monoculture of Saccharomyces cerevisiae. The ethanol concentration reached 10.32 g/l in 66 h of incubation, with a yield, productivity and fermentation efficiency of 0.45 g/g, 0.16 g/l.h and 88.6%, respectively by using a co-culture of Saccharomyces cerevisiae and Pichiastipitis.

Keywords: Date palm frond, two-stage acid hydrolysis, bioethanol, co-culture, fermentation.

1. Introduction

The date palm (Phoenix dactylifera L.) belongs to the Palmae (Arecaceae) family and it is considered a symbol of life in the desert, because it tolerates high temperatures, drought and salinity more than many other fruit crops [1]. As one of the oldest known fruit crops, the date palm has been cultivated in North Africa and the Middle East for at least 5000 years [2]. Today the date palm is found in both the Old World (Near East, North Africa, Spain) and the New World (Australia and American continent) where dates are grown commercially in large quantities [3].An adult date palm has approximately 80 to 120 green leaves with an annual formation of 12 to 15 new leaves and consequently the same amount can be expected to be cut as part of the maintenance of the palm. A date palm tree can produce 13.5-20 kg of dry fronds annually [4], that 1.15 million ha were planted with palm trees in 2009 [5] and assuming an average density of 100-125 trees/ha, it can be estimated that 1.9-2.4 million tons of dry fronds are available each year [6].

Compared to other types of feedstock for bio-ethanol, DPF is essentially free, being an agricultural waste, low cost in terms of cropping practice, collection and storage. In terms of economic and environmental considerations, the conversion of DPF to bio-ethanol would be a strategic and synergistic move for the date palm industry. DPF is a lignocellulosic material, a renewable resource that can be processed either chemically or biologically to biofuel such as bioethanol. Lignocellulosic DPF consist of three main components; cellulose, hemicellulose and lignin, and an average material contains them in the range of 40–50, 20–35 and 15–35%, respectively [7].

Generally, hydrolysis of lignocellulosic material into its corresponding monomer i.e. sugar was performed by acid, enzymatic and microbial hydrolysis [8]. Enzymatic hydrolysis is an interesting way to produce sugars from cellulosic wastes because of its mild operating conditions and the absence of by-products. However, in most cases, the cellulosic hydrolysis cannot be done directly because of the microcrystalline structure of cellulose and the lignin barrier [9]. Therefore, pretreatment of these materials is necessary to increase the rate of hydrolysis of cellulose to fermentable sugars [10]. Chemical hydrolysis involves exposure of lignocellulosic materials to a chemical for a period of time at a specific temperature, and results in sugar monomers from cellulose and hemicellulose polymers. Acids are predominantly applied in chemical hydrolyses. Sulfuric acid is the most investigated acid, although other acids such as HCl have also been used [11]. Acid hydrolysis is becoming more popular due to its lower cost and greater effectiveness than enzymatic hydrolysis [12]. However, several by-products may be formed or released in this step. If highly toxic hydrolysates are formed, a detoxification stage is necessary prior to fermentation. On the other hand, in enzymatic hydrolysis, the sugars released inhibit the hydrolysis reaction [13]. Acid hydrolysis can be divided into two groups: (a) concentrated-acid hydrolysis and (b) dilute-acid hydrolysis [11]. Dilute acid hydrolysis is a method that can be used

either as a pretreatment preceding enzymatic hydrolysis, or as the actual method of hydrolysing lignocellulose to the sugars [14]. Concentrated-acid processes are generally reported to give a higher sugar yield (e.g. 90% of the theoretical glucose yield), and consequently a higher ethanol yield, than dilute-acid processes [11]. In addition, concentrated-acid processes can operate at a low/medium temperature and pressure leading to formation of low amounts of degradation products, which is a clear advantage over dilute-acid processes [15]. In order to avoid degradation of monosaccharaides at a high concentration of acid and formation of inhibitors, acid hydrolysis is carried out in two stages [12]. In the first stage, which should be carried out under relatively dilute concentration of acid, hemicellulose is converted to sugar monomers. In the second stage, the residual solid is hydrolyzed under more concentrated acid concentration, allowing cellulose to be hydrolyzed [12, 16]. The "two-stage" acid hydrolysis is usually preferred to a one-stage acid hydrolysis because the separate stages for hydrolysis of the hemicellulose and cellulose should result in a higher sugar yield. In addition, the energy consumption should be minimized with less sugar degradation from the hydrolyzed materials in the first stage is leading to a higher overall yield of sugars, and with fewer fermentation-inhibiting components are being formed during the two-stage hydrolysis [13].

The fermentation of the lignocellulosichydrolysates is more difficult than the well-established processes of ethanol production from e.g. sugar-cane juice or grains. The hydrolysates contain a broader range of inhibitory compounds, whose composition and concentration depend on the type of lignocellulosic materials and on the chemistry and nature of the pretreatment and hydrolysis processes [11]. Secondly, almost one-third of the reducing sugars obtained from hydrolyzed lignocellulosic materials are pentoses, composed primarily of D-xylose [17]. Therefore, the fermenting microorganism should be able to produce ethanol from the hydrolysates with a high yield and productivity, withstand potential inhibitors, and produce ethanol from pentoses. Baker's yeast (S. cerevisiae) is the most commercial used microorganism for ethanol production, but it cannot ferment xylose [11]. Yeasts that have the ability to convert xylose to ethanol have been reported, and one of the earliest identified with this unique capability was P. stipitis[18]. For efficient conversion of all sugars to ethanol, a co-culture of (OVB 11) S. cerevisiae and P. stipitis(NCIM 348) can be used to co-ferment hexoses and pentoses to ethanol [12, 17].

A study on the optimization of acid (HCl) hydrolysis of date palm leaf to produce bioethanol was reported by [19]. The production of acetone-butanol-ethanol from spoilage date palm fruits by a mixed culture of Clostridium acetobutylicum and Bacillus subtilis was investigated [20]. The synergistic effect of pretreatment and hydrolysis enzymes on the production of fermentable sugars from date palm lignocellulosic waste was studied [21]. This work has been proposed to replace the conventional pretreatment and hydrolysis steps with biological processes, using enzymes. Several studies were done on the production of ethanol from biomass produced in the oil palm industry waste and these included: palm pressed fiber [22], empty fruit bunches [23, 24], fronds [25-27], and trunks [28, 29]. In this work, DPF was treated with different concentrations of perchloric acid in a two-stage hydrolysis to fermentable sugars. In addition, vacuum evaporation and overliming of the hydrolysate were investigated [30]. The fermentability of the hydrolysate was evaluated using a monoculture of S. cerevisiaeand a co-culture of S. cerevisiaeand P. stipitis. The ethanol that was produced was 10.32 g/l in 66 h of incubation, with a yield of 0.45 g/g, productivity of 0.16 g/l.h, and fermentation efficiency of 88.6% by using a co-culture of S. cerevisiae and P. stipites. Perchloric acid was used because of its double function as an oxidizing agent and a hydrolysing agent, and because it can be recycled from its byproduct, $KClO_4$ salt.

2. Material and Methods

2.1. Materials

2.1.1. Date palm frond

Sun dried date palm fronds, cultivated in June 2011, were obtained from local harvest, Tuzkhwrmatow, Iraq. It was further air dried in an oven at 80°C for 12 h then reduced to small pieces in order to facilitate grinding into a meal. These were put in a prototype hammer mill and screened. DPF meals passing through an 80-mesh screen and retained on a 100-mesh screen were collected for further use in experiments.

2.1.2. Microorganisms and Culture Media

2.1.2.1. Hexose yeast

Dry baker's yeast, S. cerevisiae, commonly used in bakery and brewery industries (Mauri-Pan, Instant yeast, AB Mauri Malaysia Sdn. Bhd.) was used for ethanol fermentation of glucose. The yeast (10 g/l) was inoculated in YM broth (pH 6.0), which consisted of glucose (10 g/l), peptone (5 g/l), yeast extract (3 g/l), and malt extract (3 g/l) in distilled water (up to 1 L). The culture was incubated at 30°C 100 rpm for 48 h.

2.1.2.2. Pentose yeast

P. stipitis CBS 5773, was purchased from CBS culture collection center, Netherlands, and used for ethanol fermentation of xylose. It was maintained on a GPYA (ATCC 144) agar plate on glucose (40 g/l), yeast extract(5 g/l), peptone (5 g/l), agar(15 g/l)) medium. It was grown in GPYA medium at 30 C, 100 rpm for 48 h, after which xylose (40 g/l) was substituted for glucose in the medium, and the yeast was incubated at 30 °C, 100 rpm for 72 h. The pre-cultured yeast cells were collected by centrifugation at 6000 x g for 10 min. The cells were washed twice with distilled water prior to inoculation.

2.2. Methods

2.2.1. Characterization of date palm frond

Determination of the extractives in DPF was carried out according to the laboratory analytical procedure for the determination of extractives in biomass, LAP 010 [31]. Determination of lignin, α-cellulose and ash in DPF was carried out according to the applicable Tappi standards for the different components, lignin (Tappi T-222), α-cellulose (Tappi T-203 0S-61), and ash (Tappi T-211). Holocellulose was determined with the method of Wise [32]. Hemicellulose content was determined by subtracting the α-cellulose content from the holocellulose content [33].

2.2.2. Acid hydrolysis

In this study, the rotary evaporator set (Wagtech RE200B, Made in the UK by BIBBY STERILIN LTD, STONE STAFFORDSHIRE ENGLAND ST15 OSA) was used to a heating process during the hydrolysis of the DPF but without running vacuum pump, removing the collection flask, and locking its connection tube as shown in the Fig. 1. This was used for preventing the loss of water vapor during the hydrolysis process and to guarantee a good mixing process. This set is called the hydrolysis set after the above modification. The powdered DPF (10.0 g) was taken in the flask of the hydrolysis set for biphasic perchloric acid hydrolysis at a solid to liquid ratio of 1:4. Initially, it was carried out with 15% acid at 100 °C for 30 min. Then it was cooled in ice bath and filtrated by a pressure filter set. The DPF residue from the filtration was kept for the second stage of hydrolysis. The pH of the liquid phase was adjusted to pH 7 by adding 10 M of KOH, and the precipitated $KClO_4$ was separated by a pressure filter set. The liquid phase is called the hemicellulose hydrolysate. In the second phase the DPF residue from the filtration of the first stage was hydrolysed with 35% acid at 100 °C for 60 min. It was cooled in an ice bath and filtrated by a pressure filter set .The pH of the liquid phase was adjusted to pH 7 by adding 10 M of KOH, and the generated $KClO_4$was separated by a pressure filter set. The liquid phase is called the cellulose hydrolysate. Sugars,acetic acid, phenolics and furans from the hemicellulose hydrolysate and the cellulose hydrolysate were identified and estimated.

The hydrolysis yield of cellulose (HY_{ce}) and hemicellulose (HY_{he}) were calculated using the following formula [34].

$$HY_{ce} = \{(m_g \text{ after hydrolysis} \times 0.9)/(m_{ce} \text{ in raw material})\} \times 100 \quad \cdots (1)$$

Where m_g is mass of glucose, m_{ce} is mass of cellulose in raw material (DPF) and m_{ce} is equal to:

$$m_{ce} = \text{mass of the raw material} \times \text{cellulose\% in raw material} \quad \cdots (2)$$

$$HY_{he} = \{(m_{hes} \text{ after hydrolysis} \times 0.88)/(m_{he} \text{ in raw material})\} \times 100 \quad \cdots (3)$$

Where m_{hes} is mass of hemecellulose sugars (xylose, galactose, mannose and arabinose), m_{he} is mass of hemicellulose in raw material (DPF) and m_{he} is equal to:

$$m_{ce} = \text{mass of the raw material} \times \text{hemicellulose\% in raw material} \quad \cdots (4)$$

2.2.3. Concentrating the sugars

The concentration of DPF hydrolysate to increase sugar concentration was carried out by vacuum distillation (Buchi Vacuum or Rotary evaporator, Rotavapor R-215 V SJ 24 23122V100). During the vacuum distillation, the boiling temperature of the liquid was maintained at 80°C under vacuum. The volume of the dilute acid hydrolysate used for the concentration process was 500 ml. Sugars, phenolics and furans were checked before and after the concentration process. The procedure was triplicated and average values were calculated.

2.2.4. Detoxification

After vacuum distillation to the hydrolysate, calcium oxide was added while stirring, until the pH of the hydrolysate reached 10. Then it was incubated for half an hour followed by centrifugation (3000 g, 20 min) and filtration. Later the pH of the hydrolysate was brought back to pH 6 by $HClO_4$ (10 M). After over liming, 3.5% of activated charcoal was added to the hydrolysates and stirred for 1 h. The mixture was again centrifuged (3000 g, 20 min) and vacuum filtered [35]. Sugars, phenolics and furans were estimated before and after detoxification process. The treated hydrolysate was then used for the fermentation studies.The procedure was triplicated and average values were calculated.

2.2.5. Ethanol fermentation

2.2.5.1. Ethanol fermentation by mono-culture (S. cereviae)

The detoxified hydrolysates (100 ml) were taken along with supplementation of 0.1% (w/v) yeast extract, peptone, NH_4Cl, KH_2PO_4 and 0.05% of $MgSO_4.7H_2O$, $MnSO_4$, $CaCl_2.2H_2O$, $FeCl_3.2H_2O$ and $ZnSO_4.7H_2O$ in a 250 ml conical flask adjusting the pH to 5.5 and autoclaved at 115 °C for 15 min [35]. After cooling the media to room temperature it was transferred to a 500 ml jar (Laboratory Fermentor, B.E. MARUBISHI (THAILAND) CO., LTD) under sterile conditions. The yeast starter culture (10 ml) was inoculated, and incubated anaerobically at 30°C, 150 rpm for 48 h. Samples from the medium were withdrawn periodically at various intervals from the fermentor flasks and centrifuged at 6000 g for 10 min at 10°C and analyzed for ethanol. These procedures were triplicated and average values were calculated.

The fermentation efficiency was calculated using the following formula [35].

$$FE\% = \text{Practical yield}/\text{Theoretical yield} \times 100 \quad \cdots (5)$$

Practical yield is the ethanol produced and the theoretical yield is:

$$\text{Theoretical yield} = \text{total produced sugars} \times 0.511 \quad \cdots (6)$$

2.2.5.2. Ethanol fermentation by co-culture (S. cereviae and P. stipitis)

The detoxified hydrolysates (100 ml) were taken along with supplementation of 0.1% (w/v) yeast extract, peptone, NH_4Cl, KH_2PO_4 and 0.05% of $MgSO_4.7H_2O$, $MnSO_4$, $CaCl_2.2H_2O$, $FeCl_3.2H_2O$ and $ZnSO_4.7H_2O$ in a 250 mL conical flask adjusting the pH to 5.5 and autoclaved at 115°C for 15 min [35]. After cooling the media to room temperature it was transferred to a 500 ml flask (Laboratory Fermentor, B.E. MARUBISHI (THAILAND) CO., LTD) under sterile conditions. After transferring, the yeast starter culture (10 ml) was inoculated and incubated anaerobically at 30°C, 150 rpm for 30h. Then, P. stipitis inoculum was added at a rate of (10 g/l) and fermentation was allowed to continue at 30°C, 300 rpm for 84h. Samples from the medium were withdrawn periodically the fermentor flasks and centrifuged at 6000 g for 10 min at 10°C and analyzed for ethanol. These procedures were triplicated and average values were calculated.

2.2.6. Analytical methods

The concentrations sugars, ethanol, acetic acid, furfural, HMF, ethyl vanillin and syrigaldehyde were determined using Waters HPLC system with a refractive index as a detector (Waters 600E Multisolvent Delivery System, Waters 717plus Autosampler, Waters TCM column heater- item 2989 HPLC, Waters 2414 Refractive Index Detector). Separation of sugars was carried out using a Pinnacle II Amino 3 µm column (150 x4.6 mm, Restek, 9217365-700) at 80°C. The mobile phase was (75:25) acetonitrile:waterat a flow rate of 0.6 ml/min and the injection volume was 10 µl. Separation of ethanol, acetic acid, furfural, HMF, ethyl vanillin and syrigaldehyde was carried out using a Rezex column (ROR-Organic Acid 00F-0138-K0, 8% H, 150 x 7.8 mm). The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 ml/min and the injection volume was 10 µl. All measurements were performed in triplicate and values were averaged. Results are given as means and standard deviation established using Microsoft Excel software.

3. Result and Discussion

3.1. Characterization of date palm frond

The pulverized DPF was found to contain (36.7% ± 2.8) α-cellulose, (60.5% ± 4.9) holocellulose, (23.8% ± 1.7) hemicellulose, (14.5% ± 1.1) lignin,(3.4% ± 0.3) water extractable,(4.3% ± 0.3) ethanol extractable,(8.4% ± 0.5) ash and (8.9% ± 0.6) moisture content. In agreement to our studies, Mirmehdi et al. (2010) also reported that the chemical composition of DPF is around of an average in the range of 40–50% α-cellulose, 20–35% hemicellulose and 15–35% lignin.

3.2. Acid hydrolysis

The use of perchloric acid was due to its double function as an oxidizing and hydrolysing agent. In addition to the acid hydrolysing effect of $HClO_4$, its oxidizing function will help in the delignification and reduce time and energy required when compared to other acids pretreatments used [36]. Also the neutralization of the excess $HClO_4$ with KOH leads to the precipitation of the insoluble $KClO_4$ and this could be recycled to $HClO_4$.

3.2.1. First stage of acid hydrolysis

The effects of four different temperatures (50, 75, 85, 100 ^{0}C) were investigated on the hydrolysis of DPF. The effects of different heating times from 10 min to 60 min were also investigated at each of the above temperatures. In this study, the ratio of powdered DPF to the volume of perchloric acid was 1:4, and the concentration of perchloric acid was 15%. Reducing sugars from the hydrolysis increased as the temperature and the heating time increased as shown in Figs. 2 and 3. However, at 100^{0}C, the concentrations of xylose and arabinose slightly increased after 30 min of the hydrolysis and approximately stabilized after 50 min of the hydrolysis. This indicated that the hemicellulose hydrolysis of DPF was nearly completed when it was hydrolyzed at 100^{0}C for 50 min. The by-products concentrations such as furfural, HMF, ethyl vanillin, syringaldehyde and acetic acid were increased by increasing the temperature and time as shown in Figs. 3 and 4. This indicates the reason why there were no observed degradation products at 50^{0}C. However at 100°C the concentrations of the by-products increased after 30 min of the hydrolysis. Based on the results and for reconciliation between larger produced sugars concentrations and smaller by-products concentrations the optimum conditions for the first stage of the hydrolysis were 100^{0}C for 30 min.The hydrolysis yields of cellulose and hemicellulose after the first stage of acidic hydrolysis were 17.0% and 79.1%, respectively, for DPF at optimum conditions.

The effects of perchloric acid concentration on the hydrolysis were investigated. Perchloric acid concentration ranged from 15% to 40% (w/w). Glucose concentration from the hydrolysis increased significantly as perchloric concentration increased until it reached 35% and after which slightly increased as shown in Table 1. The xylose and arabinose concentration slightly increased to 20% after which they declined as shown in Table 1. These results showed that the hemicellulose hydrolysis of DPF was approximately completed when it was hydrolyzed by 15% perchloric acid at 100^{0}C for 30 min, but the cellulose of DPF still remained and continued for further hydrolysis. However, the concentration of furfural, ethyl vanillin and syringaldehyde increased at the beginning of increasing concentration of perchloric acid, but later decreased. This is due to their oxidation to formic acid, isovanilic acid and syringic acid respectively by $HClO_4$[36]. The concentration of HMF continuously increased with the increase in concentration of the acid, which was probably due to the conversion rate of furfural which it is about four times faster than that of HMF [16].Based on the results, the optimum concentration of perchloric acid for hydrolysis of cellulose was conducted by using 35% $HClO_4$, which is guaranteed to produce a high yield of glucose. In order to prevent degradation of xylose and arabinose and to minimize the amount of by-products, it was decided to separate the hydrolysis of the hemicellulose and cellulose into two separate stages.

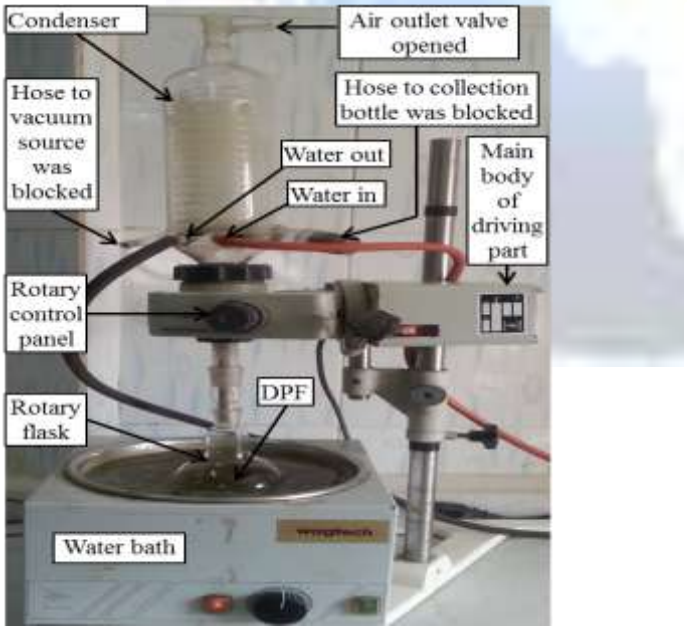

Figure1. A photographic diagram of the rotary evaporator set which was subjected to a heating process during the hydrolysis of the DPF.

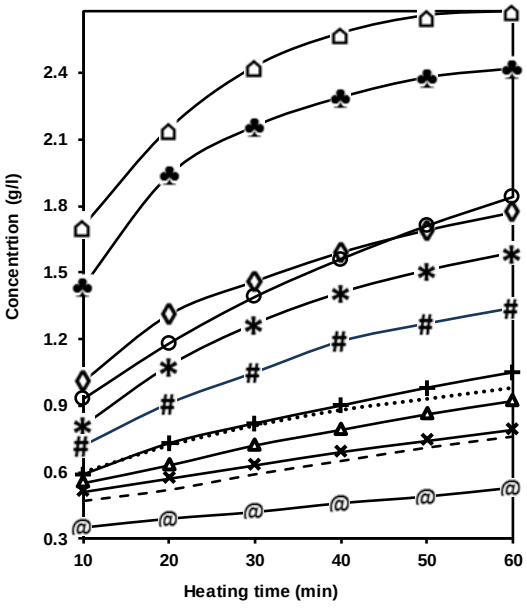

Figure 2. Effects of the temperature and the heating time on the hydrolysis of DPF (the first stage), (X) xylose; (G) glucose; (AA) acetic acid; (⌂) X at100 °C; (♣) X at 85 °C; (◊) X at 75 °C; (○) G at 100 °C; (*) G at 85 °C; (#) AA at 100 °C; (+) G at 75 °C; (….) AA at 85 °C; (Δ) X at 50 °C; (×) AA at 75 °C; (----) G at 50 °C; (@) AA at 50 °C.

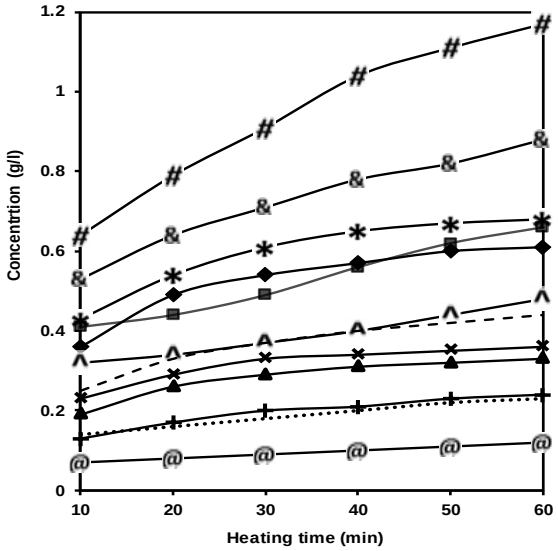

Figure 3. Effects of the temperature and the heating time on the hydrolysis of DPF (the first stage), (Ar) arabinose; (M) mannose; (Ga) galactose; (#) Ar at 100 °C; (&) Ar at 85 °C; (*) M at 100 °C; (♦) M at 85 °C; (■) Ar at 75 °C; (^) Ar at 50 °C; (----) M at 75 °C; (×) Ga at 100 °C; (▲) Ga at 85 °C; (+) G at 75 °C; (….) M at 50 °C; (@) Ga at 50 °C.

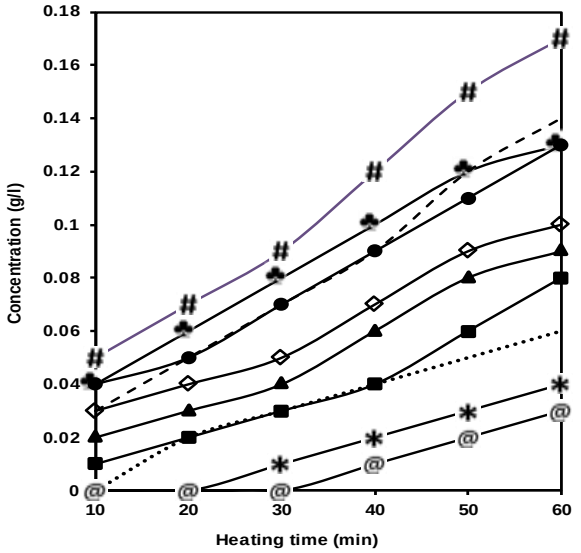

Figure 4. Effects of the temperature and the heating time on the hydrolysis of DPF (the first stage), (E) ethyl vanillin; (S) syringaldehyde; (F) furfural; (HMF) hydroxymethylfurfural; (#) E at 100 °C; (♣) E at 85 °C; (---) S at 100 °C; (●) F at 100 °C; (◊) S at 85 °C; (▲) F at 85 °C; (■) HMF at 100 °C; (….) E at 75 °C; (*) S at 75 °C; (@) F at 75 °C.

Table 1. Effects of HClO4 concentration on the hydrolysis of DPF at 100 °C and for 30 minutes

$HClO_4$ (%)	G (g/l)	X (g/l)	Ar (g/l)	Ga (g/l)	M (g/l)	AA (g/l)	HMF (g/l)	F (g/l)	E (g/l)	S (g/l)
15	1.39 ± 0.05	2.43 ± 0.07	0.91 ± 0.04	0.32 ± 0.02	0.61 ± 0.03	1.05 ± 0.04	0.03 ± 0.00	0.07 ± 0.01	0.09 ± 0.02	0.06 ± 0.01
20	2.91 ± 0.09	2.68 ± 0.07	1.15 ± 0.06	0.36 ± 0.02	0.67 ± 0.02	1.09 ± 0.05	0.07 ± 0.01	0.09 ± 0.01	0.12 ± 0.02	0.10 ± 0.01
25	4.23 ± 0.10	2.67 ± 0.06	1.15 ± 0.04	0.35 ± 0.01	0.67 ± 0.03	1.23 ± 0.05	0.10 ± 0.02	0.11 ± 0.03	0.13 ± 0.02	0.13 ± 0.03
30	5.19 ± 0.11	2.65 ± 0.07	1.11 ± 0.04	0.35 ± 0.01	0.66 ± 0.03	1.35 ± 0.04	0.12 ± 0.02	0.12 ± 0.01	0.13 ± 0.02	0.14 ± 0.02
35	5.83 ± 0.12	2.58 ± 0.06	1.02 ± 0.05	0.34 ± 0.01	0.65 ± 0.02	1.40 ± 0.03	0.13 ± 0.03	0.13 ± 0.02	0.12 ± 0.02	0.14 ± 0.02
40	6.02 ± 0.12	2.50 ± 0.07	0.93 ± 0.03	0.33 ± 0.01	0.63 ± 0.03	1.42 ± 0.05	0.15 ± 0.04	0.13 ± 0.03	0.11 ± 0.02	0.14 ± 0.03

Data are means of triplicates ± SD. (G, glucose; X, xylose; Ga, galactose; M, mannose; Ar, arabinose; AA, acetic acid; F, furfural; E, ethyl vanillin; S, sryngaldehyde)

3.2.2. Second stage of acid hydrolysis

The effects of various heating time from 10 min to 90 min on the hydrolysis of the DPF residual from the first stage were investigated. In this study, 40 ml of 35% $HClO_4$ and the DPF residue were added to the flask of hydrolysis set and the hydrolysis was done at 100°C. The glucose concentration from the hydrolysis increased significantly as the time of heating increased to 60 min and it declined slightly after that, as shown in Table 2. However, the amount produced of xylose, arabinose, galactose and mannose were small and by increasing the heating time until 60°C was reached as shown in Table 2. This is yet another evidence to that the hemicellulose hydrolysis of DPF was approximately completed in the first stage of the hydrolysis, but the cellulose of DPF still remained and may be continued for further hydrolysis. The concentration of acetic acid, furfural, ethyl vanillin and syringaldehyde increased with the increase in heating time until 60-70°C was reached, and then stabilized as shown in Table 2.

The concentration of HMF continuously increased with the increase in heating time. Based on the result, 60 min was selected as the optimum heating time for the second stage of the hydrolysis. The total hydrolysis yields of cellulose and hemicellulose after two stages of acidic hydrolysis were 88.4% and 90.4%, respectively, for the DPF at the optimum conditions.

Table 2. Effects of heating time on the hydrolysis of DPF residual from the first stage filtration, using 35% of $HClO_4$ at 100 °C.

Time (min)	G (g/l)	X (g/l)	Ga (g/l)	M (g/l)	Ar (g/l)	AA (g/l)	HMF (g/l)	F (g/l)	E (g/l)	S (g/l)
10	1.60 ± 0.04	0.06 ± 0.01	0.00	0.01 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
20	3.21 ± 0.06	0.1 ± 0.02	0.01 ± 0.00	0.03 ± 0.00	0.07 ± 0.02	0.04 ± 0.00	0.06 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
30	4.44 ± 0.07	0.15 ± 0.04	0.02 ± 0.01	0.04 ± 0.01	0.11 ± 0.04	0.06 ± 0.02	0.09 ± 0.02	0.05 ± 0.01	0.03 ± 0.00	0.06 ± 0.01
40	5.13 ± 0.07	0.21 ± 0.05	0.03 ± 0.00	0.05 ± 0.01	0.16 ± 0.04	0.08 ± 0.02	0.13 ± 0.02	0.07 ± 0.01	0.04 ± 0.01	0.08 ± 0.01
50	5.55 ± 0.07	0.24 ± 0.05	0.04 ± 0.01	0.06 ± 0.01	0.2 ± 0.03	0.09 ± 0.02	0.15 ± 0.03	0.08 ± 0.01	0.05 ± 0.01	0.10 ± 0.02
60	5.82 ± 0.09	0.25 ± 0.07	0.05 ± 0.02	0.07 ± 0.02	0.23 ± 0.04	0.1 ± 0.03	0.17 ± 0.04	0.09 ± 0.02	0.06 ± 0.01	0.11 ± 0.02
70	5.81 ± 0.08	0.25 ± 0.04	0.05 ± 0.01	0.07 ± 0.02	0.23 ± 0.03	0.1 ± 0.02	0.19 ± 0.04	0.09 ± 0.02	0.06 ± 0.02	0.12 ± 0.03
80	5.78 ± 0.08	0.25± 0.05	0.05 ± 0.01	0.07 ± 0.02	0.23 ± 0.04	0.10 ± 0.02	0.20 ± 0.03	0.09 ± 0.02	0.06 ± 0.01	0.12 ± 0.02
90	5.68 ± 0.06	0.25 ± 0.06	0.05 ± 0.01	0.07 ± 0.01	0.23 ± 0.03	0.10 ± 0.03	0.21 ± 0.04	0.09 ± 0.01	0.06 ± 0.01	0.12 ± 0.02

Data are means of triplicates ± SD. (G, glucose; X, xylose; Ga, galactose; M, mannose; Ar, arabinose; AA, acetic acid; F, furfural; E, ethyl vanillin; S, sryngaldehyde)

3.3. Concentrating of the sugars

The sugars were concentrated by about two folds from their initial concentrations as shown in Fig. 5. The doubling of the concentration of sugars in the hydrolysate indicates that vacuum distillation did not decompose the carbohydrates [35, 37]. Furfural, HMF, ethyl vanillin, syringaldehyde and acetic acid concentrations reached 0.20, 0.31, 0.28, 0.32 and 2.29 g/l respectively from initial concentrations of 0.16, 0.20, 0.15, 0.18 and 1.15 g/l respectively. The by-products were concentrated at different rates. This may be due to decomposition such as furfural to acetic acid and formic acid [37].

3.4. Detoxification

Partial neutralization, over liming and activated charcoal treatments were used to minimize the effect of microbial inhibitors (the by-products) caused by the acid hydrolysis and improve the formation of ethanol during the fermentation process. This process led to the reduction by 90.0%, 87.1%, 75.5%, 89.3% and 84.4% of furfural, HMF, acetic acid, ethyl vanillin, and syringaldehyde respectively, while lesser amounts of glucose, xylose, galactose, mannose and arabinose, 2.92%, 2.87%, 2.86%, 3.05% and 3.15% respectively were absorbed as shown in Fig. 5. Overliming has caused the decrease of the inhibitors which are present in acidic hydrolysate. While activated charcoal, being hydrophobic in nature removes the hydrophobic inhibitory compounds i.e., furan and phenolics more effectively [35].

3.5. Fermentation

The fermentability of the concentrated and detoxified hydrolysate was evaluated using the baker's yeast starter culture. The highest concentration of ethanol reached was 6.68 g/l in 30 h of incubations shown in Fig. 6. The resulting yield of ethanol was equivalent to 0.29 g/g with volumetric productivity and fermentation efficiency of 0.22 g/l.h and 57.4% respectively, based on the total fermentable sugars 22.79 g/l of the hydrolysate. The ethanol efficiency declined after 30 h of incubation. The reason could be due to the presence of xylose, concentrated by evaporation during incubation, which remained unfermented by baker's yeast as the hydrolysate contains pentoses and hexoses.

A co-culture ethanol fermentation using baker's yeast and P. stipitiswas performed; thefermentability of the concentrated and detoxified hydrolysate of DPF was evaluated using the co-culture of baker's yeast and P. stipitis. The highest concentration of ethanol reached was10.32 g/l in 66 h, which is the optimum incubation period for maximum fermentation efficiency of 88.6% as shown in Fig. 6. The resulting yield of ethanol was equivalent to 0.45 g/g with volumetric productivity of 0.16 g/l.h based on the total fermentable sugars 22.79 g/l of the hydrolysate. However, the ethanol productivity declined after 66 h of incubation. The higher ethanol yield is attributed to the fermentation of both hexoses and pentoses and all the consumed sugars in the hydrolysate.

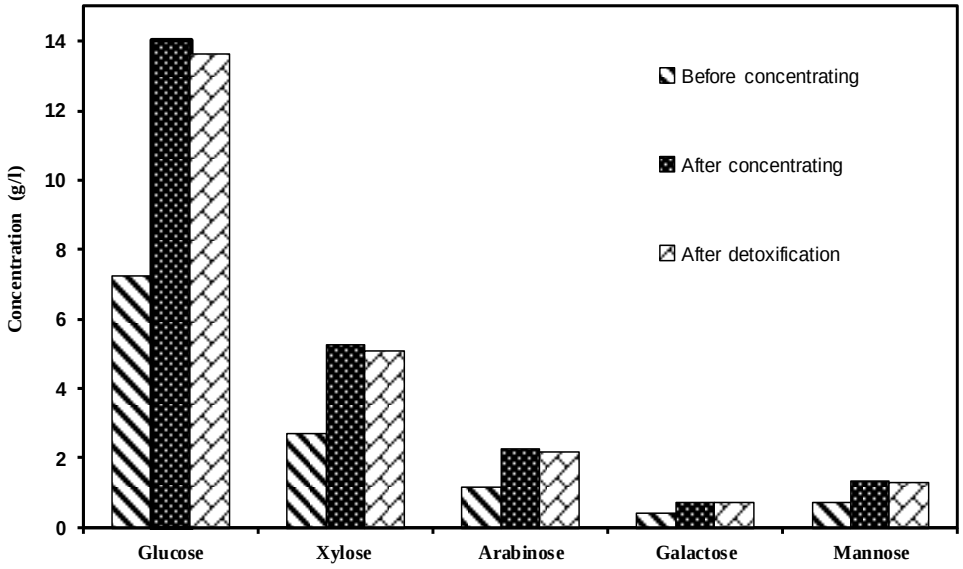

Figure 5: Effects of the hydrolysate concentrating and detoxification on the amounts of sugars

4. Conclusion

Bioethanol was produced from DPF using two different concentrations of perchloric acid hydrolysis in two separate stages. The two-stage hydrolysis was preferred to the one stage hydrolysis due to less sugar decomposition from the hydrolysed materials in the first stage. Fewer fermentation inhibitors formed and less time of heating was required during the two-stage hydrolysis.

The use of a co-culture of S. Cerevisiae and P. Ctipitis for fermentation of the concentrated and detoxified hydrolysate led to the bioconversion of both hexose and pentose sugars with higher ethanol yields than the ethanol fermentation by mono-culture of S. cerevisiae.

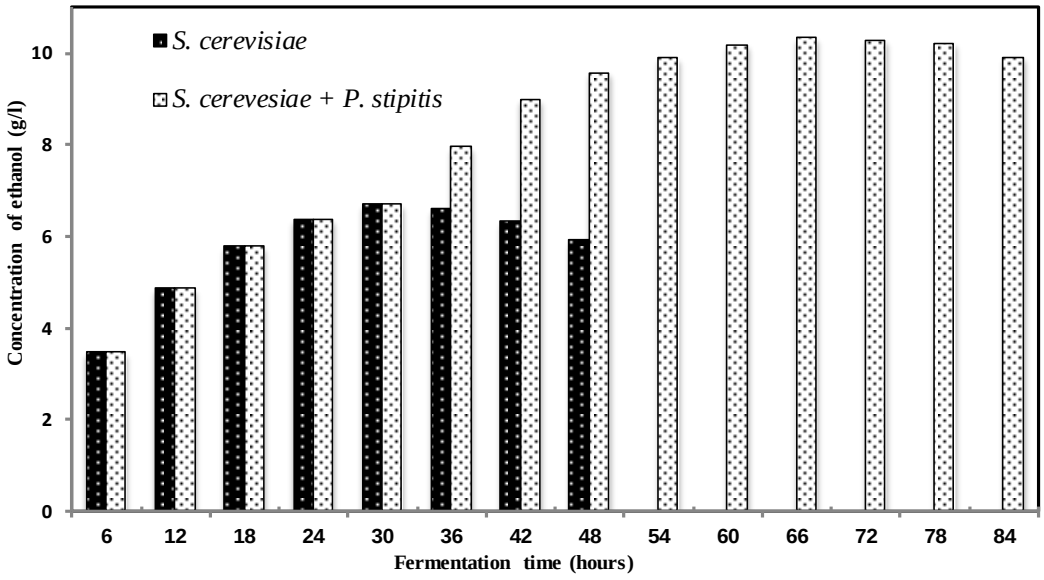

Figure 6:. Effect of incubation time on the ethanol production using mono-culture (S. cerevisiae) and co-culture (S. cerevisiae and P. stipitis)

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