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Two Stage Pretreatment Of Date's Seeds For Fermentable Sugar Production

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ABSTRACT

Date's seeds contain hemicellulose and cellulose that can be hydrolyzed into sugars to be fermented for bioethanol production. Dilute sulphuric acid is popularly used to hydrolyze hemicelluloses into xylose, but it leaves the cellulose largely intact. This work uses a two-stage pre-treatment process consisting of dilute acid treatment followed by enzymatic hydrolysis using a cellulase enzyme to convert cellulose into glucose. The sugar content obtained using effect of rotational speed (rpm) on dilute sulphuric acid hydrolysis is 29.81 g/L, and is significantly increased to 61.21 g/L using enzymatic hydrolysis at optimum conditions 10h reaction time, 40˚C temperature, and 90 FPU/g enzyme activity. This study demonstrates the two stage pre-treatment process as an excellent method to obtain a high yield of sugars, and also as feasible and economical for future scaled-up production of bio-ethanol.

KEYWORDS: date seeds, biomass, acid hydrolysis, enzymatic hydrolysis, fermentable sugar.

INTRODUCTION

At the present time, due to the depletion of fossil fuels and global warming, the world resorted to renewable energy production from biomass; there are various methods for the production of biofuels from renewable biomass that are eco-friendly [1]. Bioethanol is an important resource at the present time. Ethanol produced from different materials such as corn that contains a high amount of starch [2]. Also bioethanol have been produced from potatoes, sugar cane, wood fibre, wheat, apple pomace [3] and Palm dates. However, there still remains large amount of biomass that is very appropriate for exploitation. Biomass resources comprise those which are received from cultivation and forestry as well as from agro- and wood industries. It also includes waste sources from construction and demolition as well as municipal wastes. According to the European Environment Agency (EEA) [4], the use of biomass for clean energy generation in the European Union could be significantly increased in the next decades without harming biodiversity, soil and water resources. The potential biomass available in Europe seems to be sufficient to support the ambitious renewable energy targets in an environmentally responsible way. Extracted from agriculture, forestry and organic waste, biomass can provide heat, power and transport fuels in an environmentally friendly way [4].

Palm trees are also a remarkable glucose source. Palm trees has tolerant high salinity, drought and high temperatures so, it is considered an important symbol of life in the desert [5]. As the Palm trees is one of the oldest trees that has been cultivated since ancient times that can take advantage from date fruit, branches, roots and seeds [5]. Cellulose percentage in palm fronds 58% and 22% hemicellulose, these are important sources to ethanol production [6]. Date seeds are produced by palm trees. In 2010 the total global production of dates has exceeded 7 million tonnes, meaning that around 1 million ton of seeds were produced in that year [7]. This

Advances in Environmental Biology, 10(7) July 2016, Pages: 294-300

research proposes the use of date seeds as raw material for ethanol production. There are more than 20 kinds of dates all over the world [7].

The Arab countries are the major manufacturer and exporter of dates in the world. Date palm trees are grown in more than 40 countries, with annual countries manufacture of about 7.4 million tons and the Arab countries produce around 5.4 million tons [8]. The Arab world has more than 84 million date palm trees [8].

Dates are a suitable resource for bioethanol production. They contain considerable amounts of inverted sugars (glucose and fructose), [9]. The flesh of dates contains about 73% to 83% sugars. A second-grade (or low-grade) dates showed the same sugar content as dates of high quality. Fermentation of sugars is an anaerobic biological process in which sugars are converted to alcohol by the action of microorganisms, usually yeast [6].

Date seeds are traditionally used as animals feed, Also use alternative coffee after that toasted and crushed to produce caffeine-free coffee In Arabic countries [10]. Date seeds oil has been utilized to replace a part of another vegetable oils in shampoos, body creams, and shaving soap formulations. In general, the quality of these products are enhanced [11]. In Table 1, the six varieties of date seeds are altaboni, albekrari, alkhdraia, albudi, aldikala, saidi and Halima. Observed in this table that date seeds contain 20% starch and 2.46 reducing sugar, In addition, some other substances exist such as non- reducing sugars 1.98%, fat 9.20%, protein 6.43%, calcium 0.038%, phosphorus 0.112%, potassium 0.244%, sodium 0.082%, chlorine 0.161%, manganese 15.7 ppm, Iron 30.4 ppm and copper 8.1 ppm. Starch found in seeds can be converted into simple sugars for the production of bioethanol by pre-treatment methods, date seeds have the potential to be used as the raw material in ethanol fermentation, due to its carbohydrates content (62.51%) [12]. The chemical content for Iraqi dates was estimated as follows: Moisture 3.1 – 7.1%, Protein 2.3 – 6.4%, Fat 5.0 – 13.1%, Ash .9 - 1.8% and dietary fiber 22.5 – 80.2% [13].

Table 1: The average contents of six varieties of Libyan date seed [12].

Material	Content (Dry weight)
Starch	20.64%
Reducing sugars	2.46%
Non- reducing sugars	1.98%
Fat	9.20%
Proteins	6.43%
Calcium	0.038%
Phosphorus	0.112%
Potassium	0.244%
Sodium	0.082%
Chlorine	0.161%
Manganese	15.71 ppm
Iron	30.4 ppm
Copper	8.1 ppm

Date seeds have a solid body; it has rectangular shape, tapered at both ends and occupies the center of the fruit. The weight ranges between 0.5–4 g and length between 12-20 mm, width from 6 to 15 mm, and usually the length of the seed is equal to three times the width and it occupies from 10-20% full weight of the fruit. Parts of date seeds: Seed coat, it is a thick wall of solid surrounded by embryo and endosperm. Embryo is the small body, fluffy white, elliptical length of 2 mm and thickness of 1 mm. Endosperm, represent the bulk of the seed and created from solid half-transparent (hemicelluloses). Single cotyledon is consist of three types of cells, pranchyma, procambial, protodermal.

Date seeds as raw material was used for bioethanol production. The high dietary value of date seed is based on their nutritional fiber content. Insoluble nutritional fiber (hemicellulose, cellulose, and lignin) is considered to be the major constituent of seed fiber [11] that can be broken into simple sugars. These sugars can be used for ethanol production. The advantages of using date seeds are that, they are cheap raw materials and don't compete with food supply.

In this research date seeds were used as a raw material to fermentable sugar production for many reasons and advantages: date seeds contain 62.5% of carbohydrates. Date seeds don't affect the global food supply. Hence we develop ways of converting lignocelluloses from date seeds rather than crops to produce renewable bio-fuels due to large quantities of date seeds available in the Arab countries.

Research gap for my project is not getting enough studies about production of bioethanol from date seeds till now.

2. Literature Review:

There are many studies about bioethanol production from different biomass. Meinita *et al*. [14] used 10% of sulphuric acid at 130 ˚C for hydrolysis of *Kappaphycus alvarezii*. They obtained 38.45 g/L reducing sugar and 23.87 g/L glucose after 15 min. Bujang *et al*. [15] studied effect of 1% of dilute sulphuric acid hydrolysis of coconut dregs at 130˚C. Higher glucose concentration (0.38 g/l) was reached at a residence time of 60 min.

Advances in Environmental Biology, 10(7) July 2016, Pages: 294-300

Sulieman *et al*. [6] used a wild strain of *S. cerevisiae* to bioethanol production from low-quality dates by anaerobic fermentation of sugars extracted from at 33ºC operating temperature. Ethanol yields of 91.3%, 68.7% and 54.8% for the 10, 15 and 20% initial sugar concentrations, respectively were obtained. Abd-Rahim *et al*. [16] used 8% sulphuric acid for hydrolysis of *Kappaphycus alvarezii* at 110˚C. The reducing sugar was 34.28 g/L after 90 min. Tyagil *et al*. [17] used commercial enzymes Cellulase I & Cellulase II of bagasse pith for bio-ethanol production. Result of the fermentation study of enzymatic hydrolyzate bagasse pith by S.cerevisiae showed that ethanol production efficiency is 7.73 % (v/v). Noparat *et al*. [18] used 3% of sulfuric acid to pretreat old oil palm trunk at 170 ˚C, the result of glucose was 92.9% after 20 min.

3. Methodology:

In this research, experiments were carried out in Erlenmeyer flasks using dates seeds as the raw material. Thus two stages pretreatment is started with acid hydrolysis followed by enzymatic hydrolysis. The concentration of reducing sugar after both steps was measured using DNS method. The objectives were to analyze the effect of varied pretreatment. Dilute acid concentration, temperature, shaking speed and time of reaction on acid hydrolysis and optimize it use OFAT. While for the enzymatic hydrolysis using cellulase enzyme (Celluclast, Novozymes, Denmark).

3.1 Preparation of raw material:

AL-Hashedi date seeds collected from Yemen were dried and crushed. The seeds went through a size-reduction step to make the pre-treatment process more efficient. In this step, the surface area of the seeds increases. Firstly, the seeds are washed and then grinded in a grinding machine until the consistency of a very fine powder is obtained.

3.2 Pre-treatment:

The pre-treatment of the date seeds have two-stages, the first stage is by diluting sulphuric acid with the seeds and the second stage is by adding commercial cellulase enzyme to the hydrolyzed date seeds.

3.2.1 Effect of rotational speed on Diluted acid hydrolysis:

Rotational speed of 50 - 250 rpm was applied. 10 g of powdered date seeds were added to 50 ml of 1% sulphuric acid in 250 mL flasks. This is following 1:5 the solid/ liquid ratio. The mixtures were agitated using an orbital shaker (Sartorius, Malaysia) for 2 hours under 70˚C. The acid hydrolysed date seeds were then centrifuge (Sartorius, Germany) at 10,000 rpm for 15 min at 4˚C. The supernatant is then analyzed for its reducing sugar content.

3.2.2 Enzymatic hydrolysis:

The acid hydrolysed solid obtained after the acid hydrolysis was washed to remove sulphuric acid residue and by- products such as acetic acid, formic acid and furfural [19] and added with the cellulase enzyme (Celluclast, Novozymes, Denmark) at pH 6 [20]. For each experiment, 10 g of date seeds powder was loaded in 250 ml flasks with 50 ml of phosphate buffer (pH 6). Cellulase enzyme in the range of 10 – 90 FPU/g was used for the enzymatic hydrolysis, at 50˚C and 200 rpm for 2 hours, Samples of 1 ml were collected after 2 hours and directly submerged in an ice bath, to inactivate the enzyme. The liquid was separated after centrifugation and then stored at 4˚C for sugar analysis.

4. Reducing sugar analysis:

10 g of 3, 5-dinitrosalicylic acid, 2 g Phenol, 0.5 g Sodium sulphate (Na_2SO_4) and 10 g of NaOH were dissolved in 1 L of distilled water. The reagent was stored in the refrigerator at 4 ˚C in a black colored bottle [21].

Reducing sugar was estimated by miller method. Reaction mixture 1 ml of sample in test tube then add 3 ml of DNS reagent to same sample, heated the mixture at 95 ˚C for 5-10 minutes to change the red-brown colour. After that has been added 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution to prove the colour. Then the mixture was cooled at room temperature. Finally, 5 ml of distilled water was added to 10 ml. Absorbance was read with a spectrophotometer (Genesys, Canada) at 575 nm.

RESULTS AND DISCUSSION

To gain high concentrations of sugars from dates seeds, the pre-treatment and hydrolysis of the date seeds are most crucial. The activity of hydrolysis is affected by the characteristics of the biomass and catalyst and its reaction [22]. In this study, sulphuric acid-catalysed pre-treatment was used, followed by enzymatic hydrolysis to obtain sugars from date seeds.

Advances in Environmental Biology, 10(7) July 2016, Pages: 294-300

5.1 Effect of shaking speed on acid hydrolysis:

To increase the rate of reaction during hydrolysis, shaking speed is important. The effect of shaking speed on acid hydrolysis is as shown in Fig.1. The other parameters kept constant were 1% sulfuric acid, 70˚C and 2h reaction time. It can be observed that there is an increasing trend of total sugar production due to the increasing speed from 50 to 200 rpm.

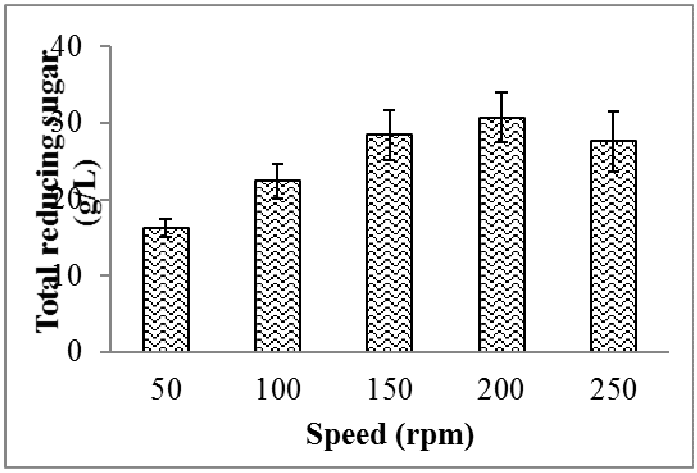

Fig. 1: Effect of shaking speed (rpm) on acid hydrolysis, under the conditions of 1% sulphuric acid, 70˚C and 2h reaction time.

A maximum total sugar concentration of 29.81 g/L was obtained at 200 rpm. However, when the rotational speed was further increased to 250 rpm, the total concentration of sugar decreased to 27.58 g/L. This may be due to the reaction between the acid and powder of date seeds increased. As a result, the effectiveness of hydrolysis also increased. The use of agitation speed higher than 200 rpm causes the biomass particles to be destroyed due to the excessive collisions, producing undesirable material such as furfural. At this value, the particles collision becomes accelerated and reaction can happen at a higher rate to produce the desired sugar yield.

4.2 Enzymatic pretreatment:

4.2.1 Effect of enzyme concentration on enzymatic hydrolysis:

This work uses concentration of enzyme between 10 – 90 FPU/g at a starting temperature of 50°C and 200 rpm for 2h. The temperature of 50°C was selected based on previous studies for ethanol production.

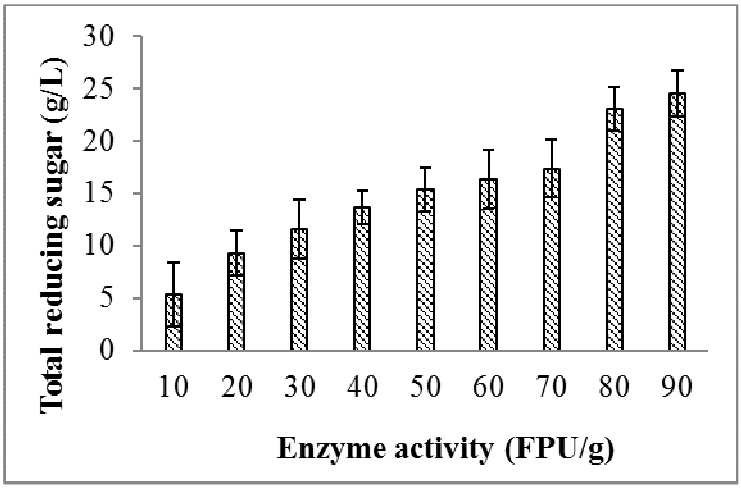

Fig. 2: Effect of enzyme activity on enzymatic hydrolysis at 50˚C and 200 rpm for 2h.

Fig.2 shows that the sugar yield using enzymatic hydrolysis increased from 5.3 g/L to 24.6 g/L as the enzyme concentration was increased from 10 FPU/g to 90 FPU/g. The poor enzymatic digestibility of cellulose at 10 FPU/g may be due to the mild pretreatment being insufficiently adequate to decompose hemicelluloses

Advances in Environmental Biology, 10(7) July 2016, Pages: 294-300

[18]. From previous studies, Saliu & Sani [23], pretreated corn cob using enzymatic hydrolysis with 0.046 FPU/g of cellulase and reported the maximum sugar 7.63 g/L was obtained. de Cassia Pereira et al. (2016) reported that 4.99 g/L glucose was obtained of sugarcane bagasse hydrolysis at 60˚C for 8h, using 10 FPU/g of enzyme.

4.2.2 Effect of temperature on enzymatic hydrolysis:

The structure of enzymes is very susceptible to heat damage at high temperatures and for that reason, most studies used temperature in the range of 30°C to 60°C to utilize the enzyme efficiently. However, the efficiency of the enzyme at very low temperatures such as 0°C is equal to zero. In this study, temperatures between 30 to 70°C were applied to observe its effect on total sugar production. The experiments were carried out using cellulase enzyme at 90 FPU/g activity, 200 rpm for 2h and the results is shown in Fig.3.

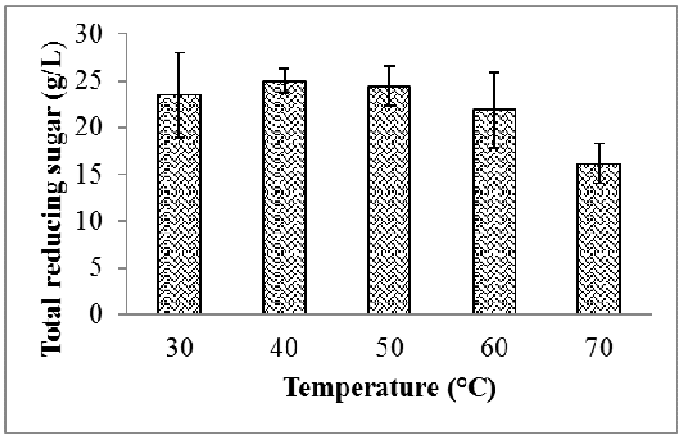

Fig. 3: Effect of temperature on enzymatic hydrolysis. Condition: 90 FPU/g cellulase, 200 rpm for 2h.

The total sugar concentration increased when the hydrolysis temperature was increased from 30°C to 40°C. The result was due to the increase in lignin removal [24]. The highest total sugar yield obtained was 25 g/L, which was achieved at 40°C after 2 h of hydrolysis. The total sugar concentration decreases at temperatures higher than 50°C. High temperatures enhances the denaturation of the conformational enzyme structure, resulting in the lowering of its action and consequently, lower hydrolysis rate. The activity of enzyme decreased due to the fact that the heat supplies enough energy to break some of the intermolecular attractions between polar molecules such as hydrogen bonding, dipole-dipole attractions, ionic interactions and hydrophobic forces between non-polar groups within the enzyme structure. In this study, the optimal temperatures for the activity of cellulase were within the range of 40°C – 50°C for date seeds pretreatment using enzymatic hydrolysis. The enzyme should have effective cellulase absorption at temperatures below 65°C and it was analyzed that the maximum absorption of cellulase occur at 40°C.

4.2.3 Effect of reaction time on enzymatic hydrolysis:

Fig. 4 shows the effect of enzyme loading and pretreatment time length on the obtained total sugar concentration from powdered date seeds.

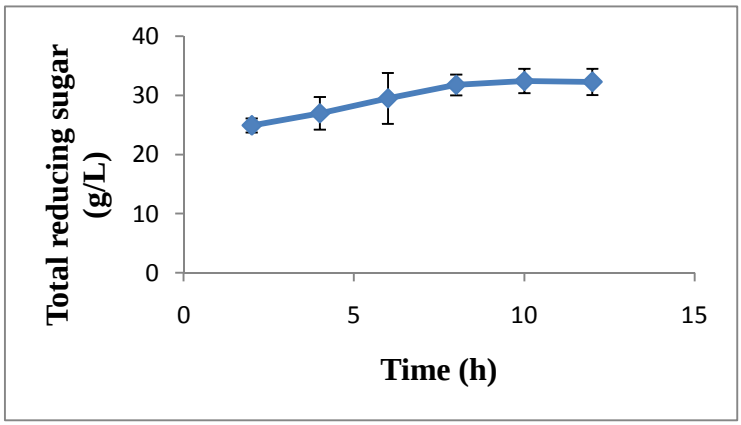

Fig. 4: Effect of reaction time on enzymatic hydrolysis. Condition: 90 FPU/g Cellulase, 200 rpm and 40°C

Advances in Environmental Biology, 10(7) July 2016, Pages: 294-300

Increasing the enzyme loading and time length of hydrolysis significantly increased the total sugar concentration. The reaction time experiment was carried out using 90 FPU/g, 40°C at 200 rpm. The total sugar concentration continues to increase as the duration increase even after 2 hours, indicating that the enzymatic hydrolysis was still incomplete at the 2 hours mark. A constant amount of total sugars was observed after 10 hours of pretreatment. A time length of 10 h for enzymatic hydrolysis and 90 FPU/g enzyme loading were found to be the best for total sugar production, with a total sugar concentration yield of 32.4 g/L. This indicated that the cellulose sites were saturated at an enzyme loading of 90 FPU/g and the cellulose was completely hydrolyzed after 10 h. In a previous study, Gao et al. [25], pretreated sugarcane bagasse using enzyme of 10 FPU/g activity in the range of 6 - 120 h incubation time. The maximum of sugar concentration of 60 % was obtained after 120 h of incubation. The work reported that the hydrolysis time was retarded after 6 h and the hydrolysis velocity was very slow. Fojas & Del Rosario [26], pretreated cogon grass using enzyme of 25 FPU/g activity in the range of 24 - 96 h and reported the maximum reducing sugar (71.29%) was obtained after 96 h. Jin et al. [27], reported the best enzyme hydrolysis time for the reducing sugar produced from catalpa sawdust is 96 h using enzyme of 150 FPU/g activity. Sugiharto et al. [28], reported the best enzymatic hydrolysis time more than 40 hour using enzyme of 70 FPU/g activity for reducing sugar production from empty fruit bunch. The studies show that the total reducing sugar produced is improved with increase in time for enzymatic hydrolysis. It was observed that a long incubation time has a positive effect on total reducing sugar yield, while a short incubation time does not. Thus, to improve the amount of reducing sugar produced, the incubation period should be longer.

Conclusion:

Dilute sulphuric acid pre-treatment successfully removed hemicelluloses from the powdered date seeds, which then enhancedthe following pre-treatment, the enzymatic digestibility of the date seeds. When the enzyme activity is increased, the total sugar concentration also increases. The optimum condition for sugar production requires 10 hours of pre-treatment and 90 FPU/g enzyme activity. A total sugar concentration of 32.26 g/L can be obtained this way. Acidic pre-treatment of 1% sulphuric acid at 70˚C for 2 hours under 200 rpm did not completely hydrolyze the powdered date seeds. The highest amount of total sugars concentration obtained by effective of 200 rpm on dilute sulphuric acid hydrolysis is 32.26 g/L. The optimized enzymatic hydrolysis pre-treatment allows further hydrolysis of the powdered date seeds, bringing the final sugar yield to a maximum of 61.21 g/L.

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