



Availability and saccharification of starchy biowastes for bioethanol production in the Kingdom of Saudi Arabia

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Abstract

Bioenergy has received a great interest because of increase in oil price, rapid depletion of fossil fuels, global climate change, and environmental pollution. However, cheap, and fermentable sugar-rich substrates represent a challenge that face production of biofuel on commercial scale. Therefore, this study offers a reliable solution for sustainability of biofuel production by recycling cheap resource (starchy biowaste) that is abundant in Kingdom of Saudi Arabia (KSA). To achieve the goal of this study, we applied different hydrolysis protocols to obtain a high quantity of fermentable sugars from starchy biowaste collected from restaurants as meal leftover. The results approved that starchy biowastes are abundant in the KSA; with the size of the residue per meal ranging from 149 to 5218 g, and starchy materials, mostly waste rice (WR), representing 72.5%. The saccharification of thermochemically pre-treated WR, carried out using an α -amylase and glucoamylase mixture for 4 h, was the most effective technique amongst all the pre-treatment methods, and produced the highest glucose concentration, i.e. 430.6 g/kg WR. Among five yeast isolates that were tested for their ability to produce ethanol from pre-treated WR via fermentation, *Kluyveromyces marxianus* KKU-RDI-11 and *Pichia kudriavzevii* KKU-RDI-18 produced the highest bioethanol concentrations, i.e. 15.44 g/L and 15.62 g/L, respectively. This study recommends application of our technique and the fermentative yeasts on the industrial and commercial scale in KSA, for production of biofuel and recycling of starchy waste materials from restaurants.

Keywords α -Amylase · Biofuel · Biowaste recycling · Fermentation · Glucoamylase · Starch hydrolysis

Abbreviations

KSA	Kingdom of Saudi Arabia
WR	Waste rice
US	United States
DNS	3,5-Dinitrosalicylic acid
YMA	Yeast extract–malt extract agar
GC/MS	Gas chromatography/mass spectrometry
ANOVA	One-way analysis of variance
LSD	Least significant difference

1 Introduction

Nowadays, bioenergy has received a lot of interest owing to the rise in fast fuel prices, rapid depletion of fossil fuels, global climate change, and environmental pollution with fossil fuels [1]. Therefore, new technologies and research results have currently driven interest in the production of liquid biofuels including bioethanol [2, 3]. Production of bioethanol in some countries such as US and Brazil, was established and applied commercially using corn and sugar cane. However, using these crops in biofuel production instead of food could comprise a great challenge facing the developing countries that are lacking to food sources and some of them suffer from famine. So, several substrates have been utilized to produce ethanol, from first to fourth generations such as sugars (sugar cane, sugar beets, molasses, and fruits), starch (grains and root crops), lignocellulose (agricultural and forestry residues, energy crops and biowaste streams) and algal biomass [4]. Utilization of such cheap substrates in production of bioethanol will lower the capital cost of production and makes this type of fuel is

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a competitive to the classical one. Almost of biomass still need pretreatments before fermentation including saccharification using chemical and/or biological processes [3]. Starch is among the most important and readily available solid bio-waste that could emerge as a promising source for bioethanol production. Starch is a complex, branched homopolymer of glucose. Depending on its natural source, it consists of two main components: amylose (16–30%) and amylopectin (65–85%). It has a wide range of molecular weights and sizes [5]. Most ethanol-fermenting microorganisms cannot directly consume starch molecules, so before starchy materials can be considered ready for bioethanol production, they must be hydrolyzed into more simple fermentable sugars [6]. Simple physicochemical pre-treatment of starch is sufficient to provide easier access to enzymatic hydrolysis [7]. Sulphuric and hydrochloric acids are used in pretreatment of starch, and then hydrolysing enzymes such as α -amylase and glucoamylase are applied for saccharification [8, 9].

Food waste is the most readily available, and abundant biomass that could be use in the production of bioethanol [10]. Food waste comprises comparable waste from food processing facilities as well as kitchen waste from homes, workplaces, restaurants, caterers, wholesalers, canteens [11, 12]. In Saudi Arabia, food waste makes up the majority of municipal solid waste; it makes up approximately 50.57% of the solid waste that is collected in municipal areas [13].

Based on a recent statistical analysis, Saudi Arabia ranks first globally in terms of monthly food expenditure per family and food waste that could be around 33%, worth SR40 billion (\$10.77 billion) annually [14]. It was reported that KSA has the highest rate of food waste per capita in the world, wasting about 1.3 kg of food every day, or 470 kg annually. This amounts to food waste of \$US 35 million per day, or \$US 13 billion annually [15]. About half of this waste is generated at the home level, and the other half is generated in restaurants. The most starch-based food that is wasted in KSA households and restaurants is rice [16]. The average annual food waste per person at the restaurant and household levels is approximately 250 kg; of this, 35% is made up of baked goods and 30% is rice. This enormous volume of waste confirms that starchy waste is available for use in the Kingdom of Saudi Arabia's large-scale commercial production of biofuel.

Therefore, the aim of this work was to apply different pretreatments protocols to hydrolyse the wasted rice into fermentable sugars to make the production of biofuel from biowastes a competitive resource for energy supplies in the future. Aso, novel yeast isolates were tested for their potential of fermentation of the hydrolysed WR into bioethanol.

2 Materials and methods

2.1 Food waste

The food waste used was collected from local restaurants in Abha and Khamis Mushayt cities, KSA. Samples were collected in plastic baskets and stored at 4 °C until used. The characteristics of the food waste, i.e., carbohydrate (rice, bread, pasta), protein (fish, beef, chicken), and vegetable/fruit contents were analysed. Starchy waste, primarily wasted rice (WR), was air-dried and ground into a uniform slurry with a particle size of less than 0.5 mm using a laboratory blender after being dried at 50–55 °C for 24 h.

2.2 Characterization of WR

The WR composition was characterized using either the raw or dried WR according to the requirements of each protocol. All analyses were performed on at least 3 different samples. Estimates were made of the physical and chemical characteristics of WR, such as pH, moisture content, total solids, sugars, protein, lipids, fibers, ash, and metals [17]. In order to calculate the glucose concentration, the 3,5-dinitrosalicylic acid (DNS) reagent was used, as directed by Miller [18].

2.3 Pretreatment of WR

2.3.1 Physical pretreatment

Physical pre-treatment of WR was conducted using two protocols. In the first protocol, samples (10 g) were added to 1-L Erlenmeyer flasks, combined with 100 mL of distilled water, and heated on a hotplate for 15, 30, 45, 60, 75, 90, and 100 min. In the second protocol, 4, 6, 8, and 10 g of WR were each poured into Erlenmeyer flasks (1 L), mixed with 100 mL distilled water, and then boiled for 90 min. The pre-treated WR was cooled at room temperature. The free glucose was estimated spectrophotometry (570 nm) using 3,5-dinitrosalicylic acid (DNS) reagent [18]. The experiment was repeated twice, and three replicates were used for each treatment.

2.3.2 Thermo-acidic pretreatment

Thermo-acidic pretreatment of WR was carried out by mixing 10 g samples with 100 mL distilled water in 1L-Erlenmeyer flasks. A serial concentration of HCl (0.5%, 1%, 1.5%, 2%, 2.5%, 3%) was used. The solution was boiled for 90 min and then cooled at room temperature. The pretreated WR was neutralized to a pH of 4.8. In separate experiments, two sets of samples of 10 g of WR were poured into Erlenmeyer

flasks (1 L) and mixed with 100 mL distilled water, and 3% HCl. The first set of samples was boiled for 90 min on a hotplate whilst the second set was autoclaved at 121 °C for 90 min. The pretreated WR was cooled at room temperature and neutralized to pH 4.8. Glucose resulting from hydrolysis was estimated spectrophotometry as mentioned before. The experiment was repeated twice, and three replicates were used for each treatment.

2.3.3 Thermochemical-enzymatic pre-treatment of WR

The enzymes: α -amylase from *Aspergillus oryzae* (working temperature is 50 °C) and glucoamylase from *Aspergillus niger* (working temperature is 30 °C) were purchased from Sigma company and used in this experiment. A combined thermochemical-enzymatic pretreatment of WR was carried out using two protocols. In the first protocol, the sample consisted of 10 g of WR, 100 mL of distilled water, and 3% HCl (v:v). The mixture was autoclaved for 90 min at 121 °C, then was cooled at room temperature, and neutralized to a pH of 4.8. Subsequently, 50 u/g of WR of α -amylase was added, and it was incubated at 30 °C for one to four h.

In the second protocol, a mixture of 10 g of WR, 100 mL of distilled water, and 3% HCl (v:v) was autoclaving 90 min at 121 °C. The mixture was cooled at room temperature before being neutralized to a pH of 4.8. Then, α -amylase (50 u/g of WR) and glucoamylase (25 u/g of WR) were added and the samples were incubated at 30 °C for different times (1–4 h). After each period, the mixture was centrifuged at 5000 rpm for 10 min. The concentration of glucose was estimated in the supernatant spectrophotometry at 570 nm using a 3,5-dinitro salicylic acid (DNS) reagent as mentioned before. The experiment was repeated twice, and three replicates were used for each treatment.

2.4 Bioethanol production from the pretreated WR

2.4.1 Isolation and identification of fermentative yeasts

The fermentative yeasts were isolated from natural sources including wasted rice, spoiled dates, and rotten fruits by mixing 25 g from each sample with 100 mL distilled water in 250-mL Erlenmeyer flasks, sealed with a fit cotton plug, and agitated at 25 °C in a rotatory shaker incubator until spontaneous fermentation took place. Then, 0.1 mL was spread on yeast extract–malt extract agar (YMA) medium using the dilution plate method at 25 °C for 48–72 h [19, 20]. For each sample, five dishes were utilized. During the incubation period, the growing yeast colonies were isolated and purified by streaking them onto the same agar medium. The purified isolates were then stored in slants with the same medium at 4 °C.

The D1/D2 domain of the 26S rDNA regions was sequenced to identify the isolated yeasts using the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTT CAAGACGG-3') from Macrogen, Korea. The acquired yeast 26S rDNA sequences were compared to similar sequences in the GenBank database, and percentage homology scores were produced to determine the identity of the yeasts.

2.4.2 Preparation of the WR for fermentation

Based on the effective pre-treatment methods, a mixture of 10% WR, 3% HCl, and 90% distilled water (w:v:v) was autoclaved at 121 °C for 90 min. After cooling, the mixture was neutralized to pH 4.8. Then α -amylase (500 u) and glucoamylase (250 u) were added and incubated at 30 °C for 4 h. The pretreated produced medium was utilized for the fermentation process to produce bioethanol. In 160 mL serum vials, 50 mL of the fermentation medium was inoculated with 5% (v:v) of the desired yeast isolate. The vials were then sealed with fit rubber stoppers and incubated for 48 h at 35 °C, and 150 rpm agitation. Five mL of the fermented medium were transferred to a glass tube and centrifuged for 15 min at 10,000 rpm. The ethanol concentration was measured using 1 mL of the supernatant. The experiment was repeated twice, and three replicates were used for each treatment.

2.5 Quantitative estimation of ethanol

Ethanol concentration was estimated using a BR-SWax separation column (FS 30 m, 0.25 mm ID, 0.25 μ m df) in gas chromatography/mass spectrometry (Agilent 6890 N/5975 B Germany). Utilizing helium at a rate of 2 mL/min, the carrier gas. High-purity hydrogen flow rates were set at 30 mL/min and zero air at 300 mL/min, respectively. The injection temperatures were 225 °C and 285 °C. The oven was pre-heated to 45 °C for 2 min, and then it was gradually raised to 240 °C in increments of 45 °C per min. The injection volume was 1 μ L.

2.6 Statistical analysis

All treatments were repeated twice, and 3 replicates were used for each treatment. The recorded data was statistically analyzed. Standard variation (\pm) was calculated in case of physicochemical composition analysis of WR. One-way analysis of variance (ANOVA) was used to identify statistically significant differences among the means of the data obtained from the experiments of effect of boiling time, concentration of biowaste, HCl, α -amylase and glucoamylase on hydrolysis and glucose production from the biowaste, as well as ethanol production by yeast isolates. The least significant difference (LSD) test was used at $p < 0.05$ to identify the

significant differences between the means among the treatments. Before carrying out the analysis, the homogeneity of variance and normality of distribution of the data were assessed. Means of effect of heating time on WR hydrolysis were compared using *T*-test ($p < 0.001$).

3 Results and discussion

3.1 Biowaste sampling analysis

Examining of 150 meal leftover's samples showed that meal waste comprises large amount, with the weight of 149 g and 5218 g per leftover foodstuff. Rice represented about 72.5% of meal leftovers, meat constituted 25%, and vegetables comprised about 1.5% of the residue (Fig. 1). Based on the review of literature, food waste accounts for about 50.57% of the solid waste collected in municipal areas in Saudi Arabia, making it the predominant type of municipal solid waste [13]. So, food waste is one of the main problems endangering Saudi Arabia's food security. According to a recent statistic, Saudi Arabia is the world's top country in terms of family monthly food expenditure and food waste. Food waste reached around 33%, estimated at SR40 billion (\$10.77 billion) per year in Saudi Arabia [14]. Furthermore, with approximately 1.3 kg wasted per day, or 470 kg annually, Saudi Arabia is the country with the highest rate of food waste per capita in the world. At the household and restaurant levels, this equates to \$US 35 million in wasted food every day, or \$US 13 billion annually [15]. The large quantity of starch meal waste (149–5218 g) that is composed mainly of rice approves availability and sustainability of this waste to produce biofuel on commercial scale in KSA.

Rice has been determined to be a vital component of the KSA strategy's food security. Also, rice represents the most starch-based wasted food in KSA at the household and restaurant level. Approximately 557 thousand tons of WR are deposited in landfills annually [16]. On average, each person's annual food wastage at a restaurant and household level

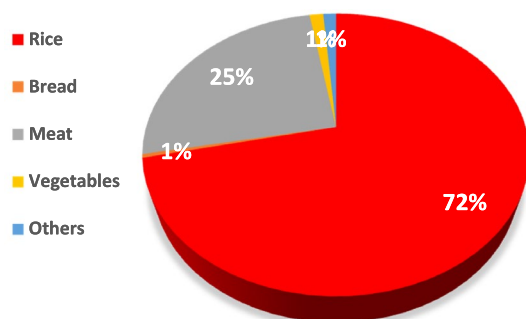


Fig. 1 Percentage of different constituents of the leftover of meals

amounts to around 250 kg; 35% consists of baked goods and 30% is rice. The abundance of biodegradable starchy materials supports the main study assumption, i.e. that the availability of high quantities of starchy waste in KSA means that synthesizing biofuels from starchy wastes is potentially a continuous, economic, and feasible process.

The biowaste "WR" was characterized in terms of its physicochemical composition according to the protocols described in the materials and methods section. The main characteristics of WR are presented in Table 1. Soluble carbohydrates, total carbohydrates, and starch formed 7.99%, 80.68%, and 75.28%, respectively, when measured on a dry basis. The physicochemical composition of WR is complex, as it is rich in many components including soluble carbohydrates and starch. The composition of WR therefore makes it an excellent feedstock to produce biofuels through microbial conversion. In agreement with the current findings, Tu et al. [21] observed that two rice varieties have total starch content ranging from 70 to 80%. Nath et al. [22] reported that the carbohydrate content of ten rice types was in the range of 77–80%. Guadamuz-Mayorga [23] recorded the carbohydrate content of five rice types to be within the range, of 78.8–81.3%.

3.2 Pre-treatment and saccharification of WR

Physical, chemical, and biological pre-treatment methods of the WR starch content were targeted toward waste saccharification to generate fermentable sugars and to enhance their subsequent fermentation efficiency to ethanol.

Table 1 Physicochemical composition of WR based on a dry basis

Analysis	Wasted rice (WR) content
TS (%)	92.59 ± 0.06
Humidity (%)	7.41 ± 0.08
VS (%TS)	95.87 ± 0.02
Ash (% TS)	2.82 ± 0.03
pH (10% aqueous solution)	4.77 ± 0.02
Soluble carbohydrates (%)	7.99 ± 0.43
Total carbohydrates (%)	80.68 ± 2.05
Starch (%)	75.28 ± 0.84
Soluble COD (%)	11.05 ± 1.23
Total COD (%)	106.81 ± 13.10
TKN (NH ₃ -N, %)	0.14 ± 0.02
Proteins (%)	0.78 ± 0.04
Cu (ppm)	1.204
Pb (ppm)	0.353
Ni (ppm)	0.397
Cr (ppm)	0.280
Zn (ppm)	2.218
Cd (ppm)	0.037

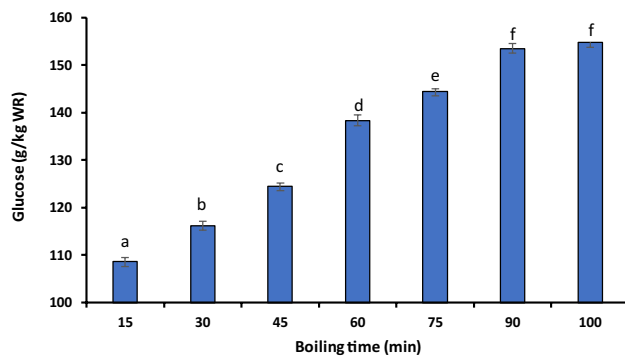


Fig. 2 Effect of boiling time on WR hydrolysis. Columns followed by the same letter are not significantly different at $p < 0.05$

3.2.1 Physical pre-treatment of WR

Results of physical pre-treatment showed that there was a direct positive correlation between boiling time and concentration of the produced glucose with the highest glucose concentration up to 90 min that yielded 153.5 g/kg WR. However, there was no significant difference between 90 and 100 min of boiling time (Fig. 2). This could be because starch's properties that may change as a result of the rearrangement of the amylose and amylopectin chains caused by heat moisture treatment [24]. Moreover, when heated in the presence of water, starchy foods' granules absorb moisture, swell, and gelatinize, whereas amylose breaks down and leaves the solution [25]. Longer cooking times also cause a higher degree of gelatinization, which may account for the rise in produced sugar content and moisture content in boiled rice [26]. Generally, an increase in time during hydrothermal treatment caused an increase in solubility and digestibility of the starch until a specific point, beyond this period, the solubilization of the starch gradually decreased and thus the generation of sugar reaches a constant state [26]. In agreement with the present finding, Thuengtung et al. [7] found a constant starch digestibility rate when raising the heating time from 12 to 18 min. Thuy et al. [25] observed that increasing boiling time from 3 to 5 min resulted in increased starch degradation. Martín-Lara et al. [27] reported that increasing boiling time from 10 to 40 min had a positive effect on sugar production which resulted in an increased sugar recovery, from 20.3 to 61.0%.

Studying the effect of substrate concentration on saccharification rate, approved that 10% (w/v) of WR was the best concentration that could liberate glucose (153.5 g/kg) as shown in Fig. 3. Concentration higher than 10% was not used to avoid the formation of a viscous slurry of the substrate, based on preliminary investigation (data not shown). The disruption of starch during the pretreatment process is stimulated by variables like the starch's concentration and source [28]. The rate of sugar generation may

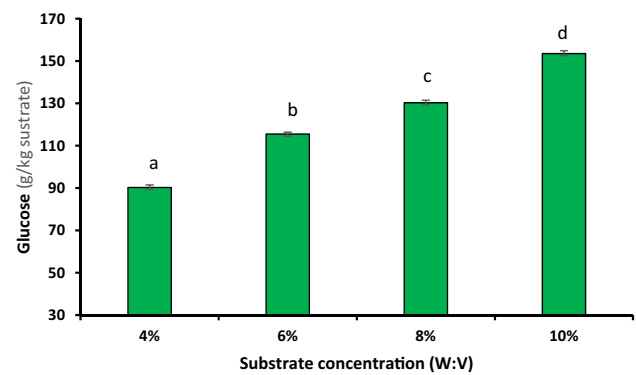


Fig. 3 Effect of WR concentration on hydrolysis and glucose production. Columns followed by the same letter are not significantly different at $p < 0.05$

be significantly accelerated by increasing the starch concentration [29]. This is because during the boiling process, more substrate molecules will collide with water molecules, resulting in the formation of more products. But beyond a certain point, any additional starch content could produce a glutinous solution and slow down the pretreatment process because the substrate concentration would no longer be the limiting factor [29]. This phenomenon is known as starch gelatinization, which depends on its dissolved water content; so, if starch concentration was increased behind this specific point the mixture would be viscous and fail to demonstrate high glucose productivity [29]. Our results could be supported by the findings of Aguilar et al. [30], who found that sugar concentration increased gradually with substrate content rising from 15 g/L to 30 g/L, which enhanced sugar generation from 9 to 13 g/L. Also, Olosunde et al. [31] recorded an increase in sugar production from 0.17 to 0.253 g/L with increasing substrate concentration from 0.5 to 3%. In the same context, Zhang et al. [32] reported that substrate concentration of 10 g, generated the highest sugar concentration of 1.5 g/L.

3.2.2 Thermo acidic pre-treatment of WR

There was a direct correlation between HCl concentration and liberated glucose amount. The highest glucose concentration was obtained using 3% HCl, which produced 247.4 g/kg WR as indicated in Fig. 4. The efficiency of acid in hydrolysis of starchy materials could be because hydroxonium ion interacts with the glycosidic bond's oxygen atom during acid hydrolysis, causing the bond to hydrolyze. An acid acts on the starch granules' surface first, then penetrates into the interior of the starch. Without changing the grain morphology, acidic modifications significantly change the structural and functional characteristics of starch [33]. Increased hydrogen ions released into the suspension due to

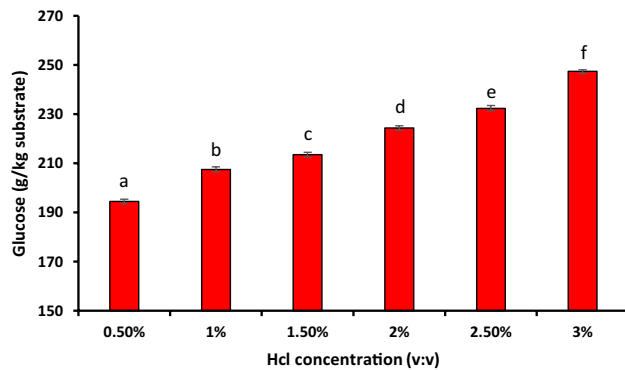


Fig. 4 Effect of HCl concentration on WR hydrolysis. Columns followed by the same letter are not significantly different at $p < 0.05$

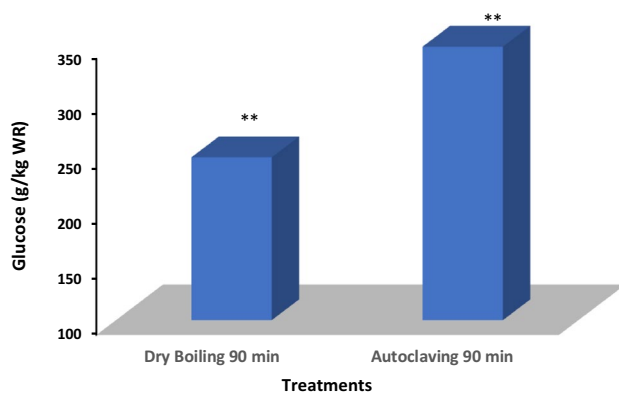


Fig. 5 Effect of heating time on WR hydrolysis. ** means high significant difference between the treatments using *T*-test ($p < 0.001$)

a higher acid concentration cause more cleavage of glycosidic bonds in the crystalline and amorphous portions of the starch [28]. Additionally, starch's solubility was enhanced, and its viscosity was decreased by acid hydrolysis. It has been reported that during acid hydrolysis of starch, the starch viscosity decreases while its solubility and sugar content increase depending on the increase in acid concentration [34]. In agreement with the present results, Liu et al. [8] revealed that the solubility of starch increased significantly with increasing HCl concentration. Additionally, González-Mendoza et al. [34] reported that increasing HCl concentration from 2 to 4% enhanced sugar content from 0.5 to 2.5 g/L. Also, Nassar et al. [35] recorded an enhancement in sugar yield from 13.9 to 19.2 mg/mL when increasing HCl concentration from 10 to 30%.

Autoclaving of 10% of the WR substrate in presence of 3% HCl gave rise to the highest glucose concentration, 347.5 g/kg WR compared with dry boiling of the same substrate concentration in the presence of 3% HCl that produced only 247.4 g/kg WR glucose (Fig. 5). It was clear

that autoclaving is an effective method for the hydrolysis of starch pre-treated with HCl. This could be because dry boiling is carried out above the glass transition temperature and below the starch gelation temperature [9]. However, when the wet boiling method is used, steam is released at a specific pressure, raising the temperature of the starch above its saturation point [9]. Therefore, it has greater productivity than methods carried out at boiling temperatures and normal pressure. Moreover, the autoclaving method is superior to conventional hydrothermal modification methods in different ways such as, it is safer than other hydrothermal methods [9]. This is because the method's high heat transfer coefficient increases the mobility of starch molecules, which facilitates the modification process [9]. Furthermore, it is often necessary to steam food wastes prior to fermentation to improve the product's purity and yield [36]. In similar studies, Zhang et al. [37] found a significant increase in the total soluble sugar content in steamed than boiled sweet potato samples. Thuy and Van [25] reported that wet boiling enhanced starch hydrolysis more than the dry boiling technique. Gruduls et al. [38] confirmed that the autoclaving method had the greatest effect on substrate conversion when biowaste was pre-treated using autoclaving and boiling methods.

3.2.3 Thermo chemical-enzymatic pre-treatment of WR

Results indicated that when using α -amylase only or combined with glucoamylase, the glucose concentration increases significantly with longer incubation times until 3 h. It then increased at a semi-constant level, reaching the highest concentration, 425.4 and 430.6 g/kg WR, at an incubation time of 4 h, as indicated in Fig. 6. The physicochemical characteristics and creation of the starch-based final product are significantly affected by the reaction time and the used enzymes [9]. Over time, all proteins, including enzymes, experience saturation and subsequently lose their catalytic activity [39]. Therefore, in any enzymatic reaction, the hydrolysis must be done within a range of incubation time that would accommodate with the nature of the enzyme and ensure optimal enzymatic activity. Additionally, reactions catalyzed by enzymes can be reversed [39]. The reaction would only proceed forward at first because there would be little to no product present. But as the reaction goes on, there will be a noticeable build-up of product and a noticeable rate of reverse reaction. As the incubation period increase, the rate of product formation slows down, and an incubation period lowers the enzyme's activity [39]. α -amylase and glucoamylase are frequently combined in the hydrolysis of starch [40]. The primary function of glucoamylase is to hydrolyze the soluble oligomers that are created by the action of α -amylase, thereby releasing glucose or maltose units. One-stage assays using these two enzymes can be combined, or two-stage assays using them one after

Fig. 6 Effect of α -amylase and glucoamylase at different periods on WR hydrolysis. Columns followed by the same letter are not significantly different at $p < 0.05$

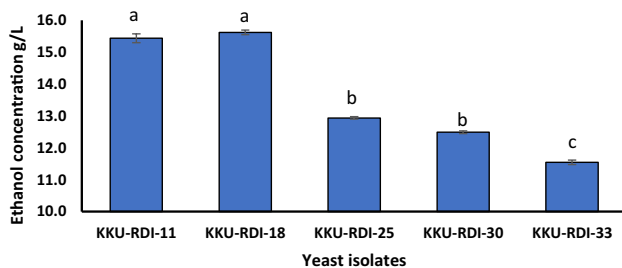
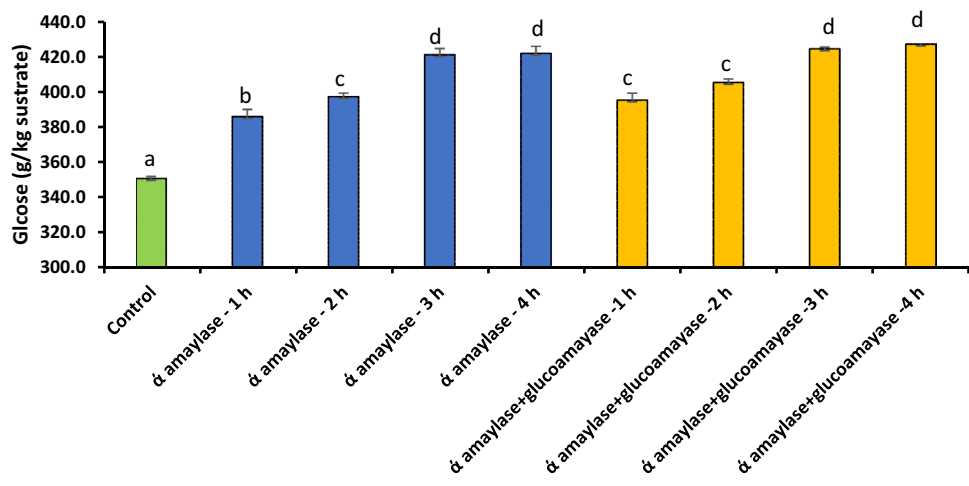


Fig. 7 Ethanol production by yeast isolates using pre-treated WR. Columns followed by the same letter are not significantly different at $p < 0.05$

the other [40]. When utilized in tandem, they exhibit synergistic activity on native starch; α -amylase breaks down the large molecules, supplying glucoamylase with materials, while glucoamylase amplifies the activity of α -amylase by breaking down inhibitory oligosaccharides into glucose and untangling double helical structures [40]. In a similar study, Olosunde et al. [31] recorded the maximum glucose concentration (0.253 g/L) at a reaction time of 40 min, after this duration there was constant sugar production. Liu et al. [41] documented that there was a reduction in enzymatic saccharification rate beyond 25 min. Acosta-Pavas et al. [42] observed that sugar production was enhanced significantly to 150 g/L by prolonging the time from 1 to 3 h.

3.3 Fermentation tests of yeasts on WR

As shown in Fig. 7, the yeast isolates, KKU-RDI-11 and KKU-RDI-18, were most prolific in terms of bioethanol production, reaching 15.44 g/L and 15.62 g/L, respectively. The yeast isolates, KKU-RDI-25 and KKU-RDI-30, demonstrated similar intermediate bioethanol production capacity, yielding 12.94 g/L and 12.49 g/L, respectively. KKU-RDI-33 generated the lowest bioethanol

concentration, 11.55 g/L. Therefore, two yeast isolates were chosen for molecular identification based on their high ethanol production efficiency.

3.4 Identification of yeast isolates

A pair of yeast isolates were chosen for molecular identification due to their high efficiency in producing ethanol. According to the results from the D1/D2 domain 26S rDNA sequencing, 2 identified yeast isolates emerged from 2 different genera and 2 species. Isolate KKU-RDI-11 was identified as *Kluyveromyces marxianus*, and isolate KKU-RDI-18 was determined to be *Pichia kudriavzevii* (Table 2). The similarity percentages of *K. marxianus* KKU-RDI-11 and *P. kudriavzevii* KKU-RDI-18 isolates concerning those in the GenBank were 100% and 99%, respectively. The two sequences were allocated at the GenBank under accession numbers OP990984 for *K. marxianus* KKU-RDI-11, and OP990986 for *P. kudriavzevii* KKU-RDI-18. Various yeast species represented a great performance in the industry of biofuel production over the years. While *Saccharomyces cerevisiae* is the primary organism in this process, other yeasts may also play a significant role in increasing the amount of ethanol produced. Therefore, a key strategy in this field is to look for additional new yeasts to maximize the rate of fermentation. In this regard, Sivarathnakumar et al. [43] reported that *K. marxianus* increased ethanol generation to 18.25 g/L, which is in line with these findings. Audu et al. [44] recorded the highest ethanol concentration (4.91%) during fermentation by *P. kudriavzevii*. Tesfaw et al. [45] reported that when using *K. marxianus*, the highest ethanol production obtained was 12.49 g/L. Akita et al. [46] found that a maximum ethanol concentration of 23.8 g/L was attained with *P. kudriavzevii*.

Table 2 Molecular identification of two yeast isolates

Isolate code	Identity	GenBank accession number	Similarity (%)	Link
KKU-RDI-11	<i>Kluyveromyces marxianus</i>	OP990984	100	https://www.ncbi.nlm.nih.gov/nucleotide/OP990984.1
KKU-RDI-18	<i>Pichia kudriavzevii</i>	OP990986	99	https://www.ncbi.nlm.nih.gov/nucleotide/OP990986.1

4 Conclusion

Our results approved the availability and sustainability of starchy biowastes in KSA as a cheap resource for bioethanol production. New protocol of saccharification of the biowaste, in which α -amylase and glucoamylase mixture was used to enhance the hydrolysis of thermochemically pre-treated WR. This technique involved in production of 430.6 g/kg of glucose from the WR after 4 h of reaction. Our new yeasts: *Kluyveromyces marxianus* KKU-RDI-11 and *Pichia kudriavzevii* KKU-RDI-18 generated high quantity of bioethanol (15.44 g/L and 15.62 g/L, respectively) from the pretreated biowaste. The current study recommends application of our technique and the fermentative yeasts on the industrial and commercial scale in KSA, for production of biofuel and recycling of starchy waste materials from restaurants.

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Author contributions All authors contributed to the study's conception and design. Material preparation was performed by Fadia Al-Malki. Data collection and analysis were performed by Fadia Al-Malki and Mohamed Hashem. The first draft of the manuscript was written by Fadia Al-Malki and Saad Alamri. All authors contributed to writing and editing of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that there is no conflict of interest. We declare that the corresponding author "Mohamed Hashem" is a member of editorial board of the Journal of Umm Al-Qura University for Applied Sciences.

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