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Article

Production of Bioethanol from Carrot Pomace Using the Thermotolerant Yeast *Kluyveromyces marxianus*

Chi-Yang Yu, Bo-Hong Jiang and Kow-Jen Duan *

Department of Bioengineering, Tatung University, 40 Chungshan N. Rd. Sec. 3, Taipei 10452, Taiwan; E-Mails: chrisyu@ttu.edu.tw (C.-Y.Y.); hk12737goddlesss@yahoo.com (B.-H.J.)

* Author to whom correspondence should be addressed; E-Mail: duan@ttu.edu.tw; Tel.: +886-2-2182-2928 (ext. 6318); Fax: +886-2-2585-4735.

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Abstract: Carrot pomace, a major agricultural waste from the juice industry, was used as a feedstock for bioethanol production by fermentation with the thermotolerant yeast *Kluyveromyces marxianus*. Treatment of the carrot pomace with Accellerase™ 1000 and pectinase at 50 °C for 84 h, resulted in conversion of 42% of its mass to fermentable sugars, mainly glucose, fructose, and sucrose. Simultaneous saccharification and fermentation (SSF) at 42 °C was performed on 10% (*w*/*v*) carrot pomace; the concentration of ethanol reached 18 g/L and the yield of ethanol from carrot pomace was 0.18 g/g. The highest ethanol concentration of 37 g/L was observed with an additional charge of 10% supplemented to the original 10% of carrot pomace after 12 h; the corresponding yield was 0.185 g/g. Our results clearly demonstrated the potential of combining a SSF process with thermotolerant yeast for the production of bioethanol using carrot pomace as a feedstock.

Keywords: bioethanol; carrot pomace; *Kluyveromyces marxianus*; simultaneous saccharification and fermentation

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1. Introduction

In recent years, bioethanol has drawn an immense amount of attention as a clean, safe, and renewable alternative energy source because of the exhaustion of fossil fuels and ever-increasing air pollution. It is by far the most widely used biofuel for transportation worldwide [1]. Production of

bioethanol from renewable biomass will reduce the environmental pollution and postpone the depletion of crude oil.

Sugar-containing, starch-containing, and lignocellulosic materials are often used as feedstocks for the production of bioethanol. Common feedstocks include sugarcane, sugar beet, corn, and wheat. Compared to other feedstock materials, lignocellulosic materials offer several unique and desirable features such as a secure source of supply, limited conflict with land use for food and feed production, and low fossil fuel inputs [1]. Common sources of lignocellulosic materials include crop residues, forest residues, and municipal solid waste [2].

Carrot pomace is a lignocellulosic material produced in large quantities during the process of juice extraction in the industry. Although this agricultural residue may be used as an animal feed, it is usually discarded as waste [3]. Juice processing companies produce about six thousand tons of carrot pomace annually in Taiwan (personal communication). Carrot pomace is composed of 28% cellulose, 2.1% pectin, 6.7% hemicellulose, and 17.5% lignin on dry weight basis [4]. Bioethanol production with carrot pomace as a feedstock using a separate hydrolysis and fermentation (SHF) process has been reported in other studies [5].

We explored the potential of bioethanol production from carrot pomace using a simultaneous saccharification and fermentation (SSF) process in combination with a thermotolerant yeast. The major advantage of SSF over SHF is the higher rate of ethanol production because the cellulase-inhibiting glucose, product from the saccharification of biomass, is rapidly converted to ethanol by the yeast [6]. Additionally, only one bioreactor is required for SSF which leads to lower cost. However, a major drawback of the SSF process is that the operating conditions are often compromises between saccharification and fermentation. For instance, the enzymatic hydrolysis has an optimum temperature around 50 °C but most fermenting microorganisms have an optimum temperature between 30 °C and 37 °C. We used a thermotolerant yeast, *Kluyveromyces marxianus*, which grows rapidly even at temperatures above 40 °C, to improve the yield of ethanol with the SSF process [7]. Additional advantages of using a thermotolerant yeast include higher saccharification yields, decreased risk of contamination, and the possibility of continuous ethanol removal [7]. In this study, the compositions of fermentable sugars of the hydrolysate from carrot pomace were determined, and then different fermentation strategies were applied to improve the concentration and yield of ethanol with the SSF process.

2. Results and Discussion

2.1. Composition of the Hydrolysate from Carrot Pomace

Three major sugars, glucose, fructose, and sucrose, were identified in the hydrolysate and their concentrations were 12, 3, and 6 g/L, respectively, after treatment of 5% (*w*/*v*) carrot pomance with Accellerase™ 1000 and pectinase. The amount of these fermentable sugars accounted for 42% (*w*/*w*) of the carrot pomace. The concentrations of the fermentable sugars showed little change after increasing the doses of Accellerase™ 1000 and pectinase, which indicated that almost all the fermentable sugars were released from the carrot pomace.

2.2. SSF of Carrot Pomace Using Different Fermentation Strategies

The results of the SSF process with 10% (*w*/*v*) carrot pomace as a feedstock are shown in Figure 1. After 24 h, the ethanol concentration reached a plateau of 18 g/L and almost all the glucose was consumed. The yield of ethanol from the carrot pomace was 0.18 g/g (maximum mass of ethanol produced/mass of substrate added). When an additional 5% (*w*/*v*) of the carrot pomace was supplemented to the initial charge of 10% (*w*/*v*) at 12 h, the concentration of ethanol increased to 30 g/L at 36 h, and a slight increase in glucose was observed after 48 h (Figure 2).

Figure 1. Time course of ethanol (□), glucose (●), fructose (○), and sucrose (▼) during the SSF process with 10% (*w*/*v*) carrot pomace as the feedstock.

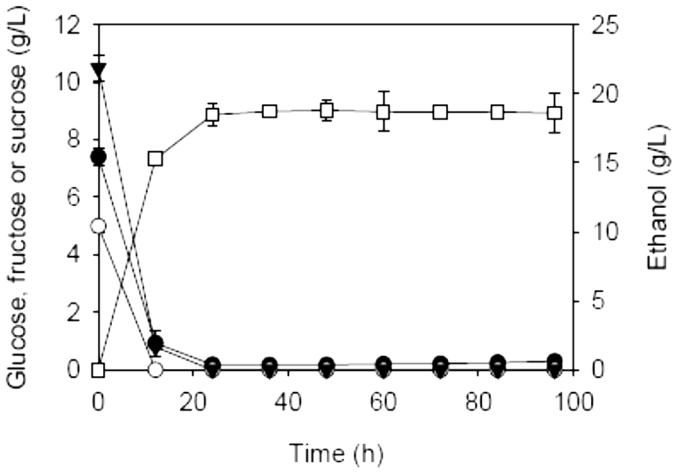
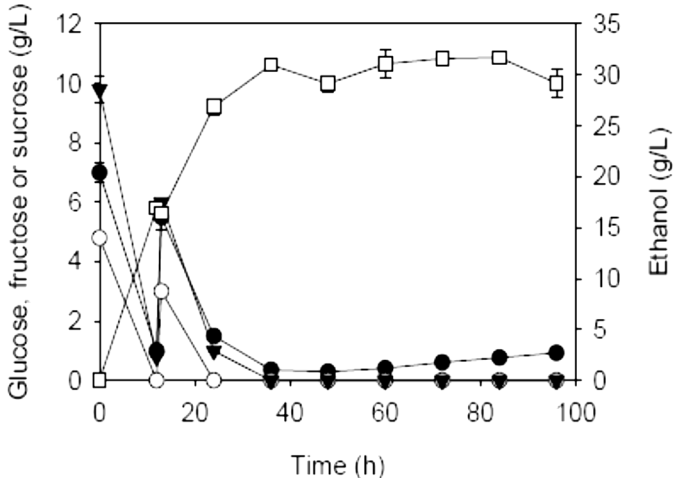


Figure 2. Time course of ethanol (□), glucose (●), fructose (○), and sucrose (▼) during the SSF process with 15% (*w*/*v*) carrot pomace as the feedstock. The initial carrot pomace charge was 10% (*w*/*v*) and an additional 5% (*w*/*v*) was added at 12 h.



The increase in glucose could be explained by the low viability of the thermotolerant yeast because of high ethanol concentration; few viable yeast cells were found as determined with a methylene-blue based viability assay. The growth inhibition of *Kluyveromyces* sp. by ethanol has been reported [8]; devices such as an air stripper has been employed to remove ethanol from the fermenter to alleviate its

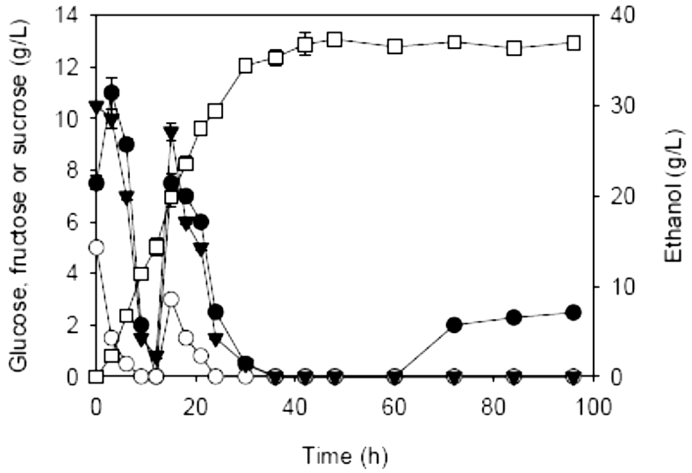
toxic effects [9]. When two additions of 5% (*w*/*v*) carrot pomace were supplemented at 12 and 18 h to the initial charge of 10% (*w*/*v*); however, such a strategy had little effect on the concentration of ethanol, which reached 32 g/L at 36 h (Table 1). Addition of 10% (*w*/*v*) of the carrot pomace to the initial charge at 12 h resulted in the highest ethanol concentration of 37 g/L after 42 h (Figure 3), which was significantly higher than 28 g/L reported by Patle and Lal using a SHF process [5]. The accumulation of glucose after 60 h was also observed.

Table 1. Maximum ethanol (EtOH) concentrations, yields ($Y_{E/S}$), and productivities with carrot pomace as a feedstock using different fermentation strategies.

Initial charge of carrot pomace (%, *w*/*v*)	Addition (%, *w*/*v*)		Prehydrolysis dosages of Accellerase™ 1000 (FPU/g dry matter) & pectinase (U/g dry matter)	Max. EtOH (g/L)	Theoretical max. EtOH (g/L) [a]	$Y_{E/S}$ (g/g) [b]	Q_E (g/L·h)[c]
	t = 12 h	*t* = 18 h					
10			-	18	21.2	0.18	0.75 (24 h)
10	5		-	30	32.2	0.2	0.83 (36 h)
10	5	5	-	32	42.8	0.16	0.89 (36 h)
10	10		-	37	42.8	0.185	0.88 (42 h)
10			15 & 52.3	18	21.4	0.18	0.75 (24 h)
10	10		7.5 & 26.2	30	42.8	0.15	0.83 (36 h)
20			15 & 52.3	15	42.8	0.075	0.21 (72 h)

[a] Theoretical max. EtOH (g/L) = substrate concentration (g/L) × 0.42 (weight percentage of fermentable sugars) × 0.51; [b] $Y_{E/S}$ (g/g) = mass of EtOH (g)/mass of substrate (g); [c] Q_E (g/L·h) (EtOH productivity) = Max EtOH (g/L)/time to reach max. EtOH (in parentheses).

Figure 3. Time course of ethanol (□), glucose (●), fructose (○), and sucrose (▼) during the SSF process with 20% (*w*/*v*) carrot pomace as the feedstock. The initial carrot pomace charge was 10% (*w*/*v*) and an additional 10% (*w*/*v*) was added at 12 h.



2.3. Effects of Enzymatic Prehydrolysis

Enzymatic prehydrolysis prior to SSF can decrease the viscosity of the fermentation sludge, and thus improve the mass and heat transfer; making the subsequent SSF shorter and compatible with even higher water insoluble substances [10]. Prehydrolysis was performed for 12 h on 10% (*w*/*v*) carrot

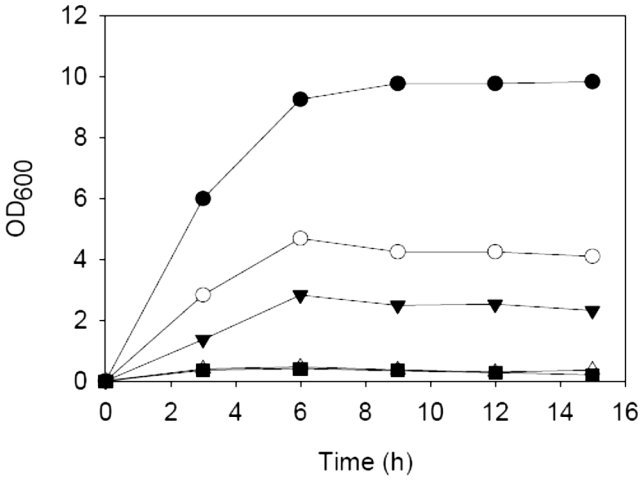
pomace at 50 °C (for the doses of Accellerase™ 1000 and pectinase, see Section 3.4); the time course of ethanol concentration showed little change as compared to that without prehydrolysis. When an additional 10% substrate was supplemented to the initial 10% (*w*/*v*) of carrot pomace prehydrolyzed for 12 h; the maximum ethanol concentration of 30 g/L was reached after 36 h, which was lower than the 37 g/L (at 42 h) obtained after SSF process without prehydrolysis. We further doubled the doses of hydrolytic enzymes to prehydrolyze an initial charge of 20% (*w*/*v*) carrot pomace for 12 h; however, the maximum ethanol concentration was only 15 g/L (at 72 h) and the yield of ethanol from the carrot pomace was 0.075 g/g (Table 1). We suspected that the low ethanol concentration and yield were related to the increased doses of Accellerase™ 1000. We cultured *K. marxianus* K21 under different doses of Accellerase™ 1000 at 42 °C (Figure 4). Our results indicated that the growth of the yeast was inhibited by the presence of Accellerase™ 1000; such inhibition on *K. marxianus* has been reported by others [7]. Maximum ethanol concentrations, yields, and productivities using different fermentation strategies are summarized in Table 1.

3. Experimental Section

3.1. Preparation of Carrot Pomace

The juice of the carrots purchased from a local market was extracted with a juicer (Philips HR1861). The pomace was then collected and dried in an oven at 70 °C for 4 days. Dried pomace was ground, screened (20 mesh), and stored in a dark and dry environment at room temperature.

Figure 4. Effects of different doses of Accellerase™ 1000 on the growth of *Kluyveromyces marxianus* K21 at 42 °C. The doses were 0 (●), 5 (○), 10(▼), 15(Δ) and 20(■) FPU/g dry matter.



3.2. Hydrolysis of Carrot Pomace

Five% (*w*/*v*) suspension of carrot pomace (100 mL) was prepared with 50 mM sodium citrate buffer, pH 5. The hydrolysis was initiated by the addition of Accellerase™ 1000 (Genencor, Rochester, NY, USA) and pectinase (Sigma, St. Louis, MO, USA). Pectinase was used to enhance

sugar conversion from carrot pomace in this study [11]. A pectin-hydrolyzing enzyme, has been used applied to enhance sugar conversion in rice straw treated with ammonia fiber expansion (AFEX) [12].

The doses of Accellerase™ 1000 and pectinase were 15 filter paper unit (FPU)/g dry matter and 52.3 U/g dry matter, respectively. The hydrolysis was performed at 50 °C for 84 h on an orbital shaker at 150 rpm. The hydrolysate was heated to 95 °C to inactivate the enzymes. The hydrolysate was centrifuged at 14,000 rpm for 10 min, and then the supernatant was filtered with a 0.22 μm filter. The composition of sugars in the hydrolysate was determined using high performance liquid chromatograph (HPLC) by comparing the retention time of the standards.

3.3. Microorganism and Growth Conditions

The thermotolerant yeast *K. marxianus* K21 was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). It was maintained on Yeast-Malt (YM) agar slant containing 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract, and 20 g/L agar. Active culture for inoculation was prepared in a 250 mL Erlenmeyer flask with 100 mL growth medium containing 30 g/L glucose, 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$. The preculture was grown on a rotary shaker at 150 rpm for 12 h at 40 °C.

3.4. SSF of Carrot Pomace

The 450 mL SSF mixture containing 60 g of dried carrot pomace was prepared in a 1 L custom-made jar fermentor and then autoclaved at 121 °C for 30 min. Ninety mL of medium containing 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4 \cdot 7H_2O$ was added to the fermentor. After cooling down, 60 mL of *K. marxianus* K21 inoculum at 1×10^7 CFU/mL was added, followed by the addition of Accellerase™ 1000 and pectinase; the doses were 15 FPU/g dry matter and 52.3 U/g dry matter, respectively. The final concentration of the carrot pomace was 10% (*w*/*v*). The initial pH was adjusted to 5 with 10 N NaOH. SSF experiments were performed at 680 rpm and 42 °C.

3.5. Analytical Methods

The composition of fermentable sugars was determined with a HPLC system equipped with a NH_2-derivatized column (Chromatorex SPS-100-5, Fuji Silysia Chemical LTD, Kasugai, Aichi, Japan) and a refractive index detector (Shodex RI-201H, Showa Denko K.K., Minato-Ku, Tokyo, Japan). The mobile phase was 70% (*v*/*v*) acetonitrile at a flow rate of 0.5 mL/min. The column temperature was fixed at 30 °C. The concentration of glucose was further measured with an YSI M2700 SELECT biochemistry analyzer (Yellow Springs, OH, USA). For the determination of ethanol concentrations, a Transgenomic ICSep ION-300 column (Omaha, NE, USA) was used in the HPLC system; the mobile phase was 0.0085N H_2SO_4 at a flow rate of 0.3 mL/min and the column temperature was fixed at 50 °C. All analyses were performed in duplicates.

4. Conclusions

Addition of 10% (*w*/*v*) carrot pomance at 12 h to an initial charge of 10% (*w*/*v*) of carrot pomace resulted in the highest ethanol concentration of 37 g/L after 42 h with a yield of 0.185 g/g. The

inclusion of a 12-h prehydrolysis step did not show an increase in the ethanol concentration, and increasing the dose of Accellerase™ 1000 had an adverse effect on the growth of the yeast. Nevertheless, cellulases from different sources may alleviate the growth inhibition, leading to an even more efficient fermentation process. To test the possibility of applying such process to other feedstocks, in our preliminary study, ethanol concentration reached 17 g/L after 96 h with 10% (*w*/*v*) orange peel as substrate and the yield was 0.17 g/g. Our results clearly indicate that the bioethanol production from carrot pomace using a SSF process combined with thermotolerant yeast is promising. Because of the fact that no pre-treatment such as steam explosion or AFEX is required, the overall process is much simplified, and thus reduces the capital cost for deriving bioethanol from carrot pomace.

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