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Utilization of Fruit Waste for Bioethanol Production by Co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae*

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Abstract

The purpose of this research was to study the effectiveness of simultaneous fermentation of fruit waste with co-cultures of *Aspergillus niger* TISTR 3063 and *Saccharomyces cerevisiae* TISTR 5606 in production of ethanol. The effect of fermentation temperature on ethanol yield was also observed. Pomelo and banana peels were selected as substrates and prepared by chopping into small rectangular pieces. Fermentation of batches of fruit waste was carried out using a 250 mL Erlenmeyer flask with glucose as a control. Analysis of the composition of the fruit waste included sugar, pH, TS, VS, ash, moisture, COD and TKN. From the results, it was found that maximum yields of 90.71% and 104.90% for pomelo and banana peel, respectively, were achieved at a temperature of 40°C within 24 h. The analysis also showed that fermentation temperature affected ethanol yield. When the fermentation temperature was raised from 30°C to 40°C, maximum ethanol yield from pomelo peel fermentation with 10% of inoculum was increased from 73.86% to 90.71%, significant at p-0.05 Maximum yield from banana peel fermentation showed a similar trend. This study establishes the potential for upgrading fruit wastes such as pomelo and banana peels as high value substrates for ethanol production. Pomelo peel in particular shows high potential as a substrate for ethanol fermentation at 40°C for 24 h, with inoculum of 10% (w/w) of each fungus and yeast.

Keywords: Simultaneous fermentation; Ethanol; Ethanol yield

Introduction

Ethanol has assumed increasing importance as a biofuel as it can be produced through fermentation of various types of biomass. In Thailand, ethanol has been phased in as an alternative fuel by mixing with benzene to create a fuel known as gasohol. The Thailand Alternative Energy Development Plan (2008-2022) [1] aims to see ethanol usage reach 9 million liters per day. Government policies and promotion measures for the “eco-ethanol” industry focus mainly at development of logistic

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systems to reduce cost of research, as well as identification of alternative sources of biomass as feedstock.

Since utilization of wastes via fermentation offers a low-cost alternative for bio-ethanol production, it is important to assess the potential of alternative sources of biomass as substrate. Numerous studies have been conducted on waste products such as empty fruit bunches from oil palm [2-4], fruit wastes [5-10] and food waste [11-12]. Ethanol yields have been reported as dependent on substrate; carob pod extract yielded 24.51 g L^{-1} of ethanol, while Kinnow mandarin yielded 42 g L^{-1} [9, 13]. Various strains of yeasts (*Saccharomyces cerevisiae*) are typically used for the fermentation process.

Factors affecting ethanol yield include pre-treatment of the substrate; this is especially important for substrates containing high amounts of cellulose. Acid or alkaline pre-treatment using either hydrochloric acid or aqueous ammonia have been proposed, under different conditions including steam purging, microwave and ultrasonic wave treatment [7, 14-15]. The use of enzymatic hydrolysis to accelerate release of sugars has also been reported [4, 11]. Nevertheless, fermentation temperature is one of the most important determinants of ethanol yield. Optimal fermentation temperature has been widely reported in the range of 30-38ºC [4, 7, 9-11, 16]. In addition, the use of co-cultures has also been studied as a means of further increasing ethanol yield [17-22]. This provides an alternative fermentation path when biomass high in lignocellulose is used as feedstock [17]. For bio-wastes, co-cultures of yeasts (*Saccharomyces cerevisiae* and *Pichia stipites*) achieved ethanol concentrations up to 45 g L^{-1} [18]. Until recently, co-cultures for simultaneous saccharification and co-fermentation employed combinations of bacteria and yeast [17, 19], yeast and yeast [18, 20] or fungi and yeast [21-22], depending on substrate and fermentation conditions. The use of co-cultures not only increased ethanol yield, but can also reduce overall process costs.

Despite the numerous research attention on this subject, there remains considerable need to study the utility of diverse sources of bio-wastes under various process conditions. The upgrading of wastes from fruit processing as a high-value substrate for bio-ethanol production is of particular interest in this regard, as a practical industrial-scale solution. Therefore, the objective of this study was to study ethanol production obtained from simultaneous saccharification and co-fermentation (SSCF) using a co-culture of fungus and yeast. The effect of fermentation temperature on ethanol production was also studied.

Materials and methods

1) Preparation of substrate and inoculums

1.1) Preparation of the substrates

Two types of fruit wastes were used as substrates for experimental batch fermentation using SSCF. These were fruit peel from pomelo (*Citrus maxima* (*Burm*.) *Merr*.) and fruit peel from banana (*Musa sapientum Linn*.) taken from a fresh market located in the Min Buri area of Bangkok. The materials were chopped for homogenization and to increase the substrate surface area, and stored at 4°C prior to use (Figure 1). The substrate was then analyzed [23] to determine chemical composition.

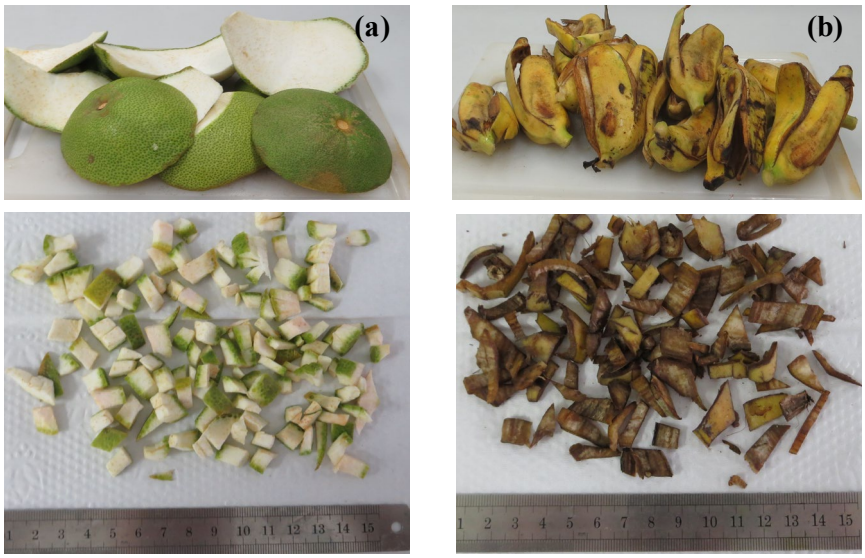

Figure 1 Physical image of the substrates ((a) pomelo and (b) banana peels) before and after preparation by chopping.

1.2) Preparation of *Aspergillus niger*

A freeze-dried culture of *Aspergillus niger* TISTR 3063 was provided by the Culture Collection Center of the Thailand Institute of Scientific and Technological Research. The culture was initially activated using potato dextrose agar (PDA) under 30ºC for 48 h. After that, it was transferred to potato dextrose broth (PDB) under 30ºC for 48 h.

1.3) Preparation of *Saccharomyces cerevisiae*

The culture (TISTR 5606) was also obtained from TISTR Collection Center in freeze-dried form. The culture was activated and cultivated using yeast mold agar (YMA) under 30ºC for 48 h and consequently transferred to be cultivated using yeast mold broth media (YMB) under 30ºC for 48 h.

2) Batch set-up and monitoring

SSCF by *A. niger* and *S. cerevisiae* was conducted in batch experiments using 250 mL Erlenmeyer flasks. The treatments were duplicated at fermentation temperatures of 30ºC and 40ºC. Each flask contained 10 g (w/w) of substrate, with the working volume adjusted using distilled water. In case of 5% and 10% inoculums, 90 and 80 mL of distilled water was added, respectively. The flasks were then plugged with cotton wool and sterilized by autoclave at 121ºC for 15 min (Figure 2). Liquid inoculums of *A. niger* and *S. cerevisiae* in 5% and 10% (w/w) were introduced to all flasks containing the sterile substrates, and the flasks incubated at the desired fermentation temperatures of 30ºC or 40ºC. A control flask used glucose at 50 g L^{-1} with yeast extract of 2.5 g L^{-1} combined with nutrients (NH_4Cl, KNO_3, Na_2HPO_4 and $CaCl_2$) at rates of 0.62, 1.17, 3.0 and 1.0 g L^{-1}, respectively.

Daily samples of 5-10 mL were collected, and pH value was measure throughout the sampling period. To preserve the samples, 34% (w/w) phosphoric acid was added before storage of the samples in the freezer. Sample preparation for analysis of sugar and ethanol content was performed by separation of liquid phase by centrifuge at 13,400 rpm for 7 min. The liquid phase was then transferred into vials prior to measuring ethanol concentrations via gas chromatography (GC: FID).

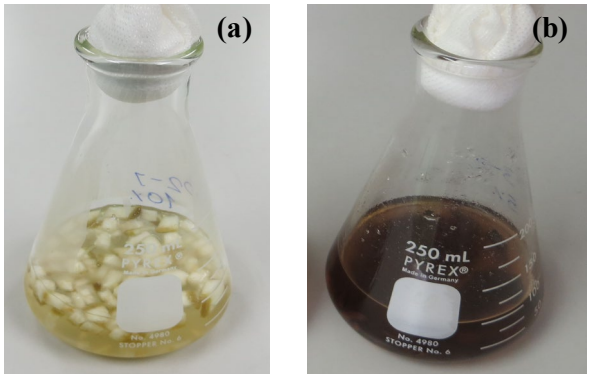

Figure 2 Experimental batches for ethanol fermentation from (a) pomelo and (b) banana peels.

3) Analytical methods

Determination of substrate composition including total solids (TS), volatile solid (VS), ash, moisture, pH, chemical oxygen demand (COD), and the total Kjeldahl nitrogen (TKN) was carried out according to the Standard Methods for the Examination of Water and Wastewater [23]. Analysis of initial sugar (in glucose form) contained in the substrates was achieved via HPLC (Agilent 1100: UV/VIS detector). Reduction of sugar (glucose) over the duration of the experiment was then calculated based on production of ethanol. Ethanol production was quantified with a gas chromatograph (Shimadzu GC-14A) equipped with a thermal FID detector.

4) Evaluation of data

Experimental data covered three aspects: composition of substrates, ethanol production from batch tests, and the effect of fermentation temperature on ethanol production. Evaluation of substrate composition and ethanol production data were conducted using descriptive statistics. Additionally, a statistical program package was used to conduct paired t-tests for hypothesis testing.

Result and discussion

1) Ethanol yield

Using pomelo peel as substrate, simultaneous saccharification and co-fermentation with *A. niger* and *S. cerevisiae* at rates of 5% or 10% (w/w) at a fermentation temperature of 30°C for 24 h, resulted in ethanol yields of 75.70% and 73.86%. At 40°C ethanol yield increased to 81.17% and 90.71% at 24 h, further increasing to 92.91% after 48 h. of fermentation (Figure 3). Under the same fermentation conditions at 30°C, the use of banana peel substrate resulted in lower ethanol yields of 32.88% and 45.29%. However, yield increased to 83.43% and 104.90% at 40°C (Figure 3). These high yields were achieved from the co-cultures due to less carbon consumption for biomass production and synergistic metabolic interactions between them; this is consistent with the conclusions of a previous study by Abouzied and Reddy [24].

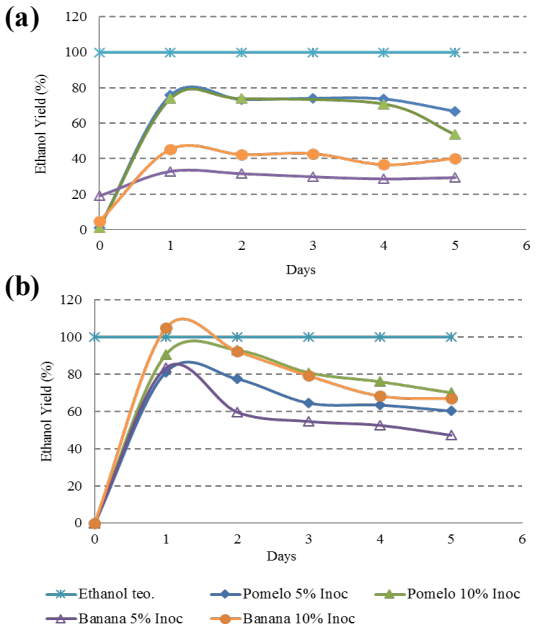

Figure 3 Ethanol yield under fermentation temperatures of (a) 30°C and (b) 40°C.

Ethanol yield per gram of sugar from the co-cultures in this experiment reached 0.46 for pomelo and 0.54 for banana peel, using 10% w/w of inoculum at 40°C fermentation temperature (Figure 4). This is consistent with the findings of a recent study by Izmirlioglu and Demirci, who reported yields of 0.41 g/g) [22]. The effect of the co-cultures in reducing consumption of

carbon source for biomass production was observed under this experiment as mentioned by Abouzied and Reddy [24]. In addition, the control batch showed fermentation process reached completion within 48 h. using 50 g L^{-1} glucose. This suggests that the use of the co-cultures (*A. niger* and *S. cerevisiae*) can provide high ethanol yield even without chemical pre-treatment. *A. niger* could play a synergistic role in the hydrolysis process as it can boost the release of initial sugar, which is subsequently fermented to ethanol by *S. cerevisiae*.

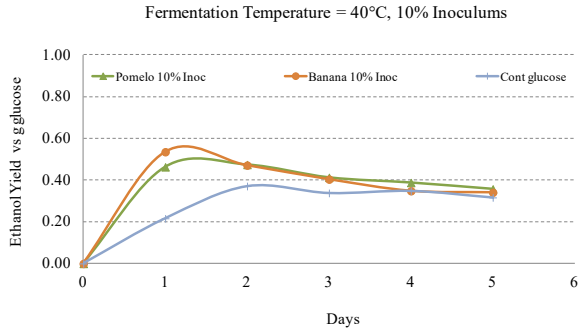

Figure 4 Ethanol yield per gram sugar from the experiment.

Untreated pomelo and banana peels were submitted to the SSCF process for ethanol production and resulted in ethanol yields of 474.6 and 535.8 g kg^{-1} DW, respectively (Figure 5). Despite significant differences in substrate composition, the abundance in fermentative carbohydrates of 53.37% w/w DW and 54.80% w/w DW fell within a similar range, although the moisture content differed considerably (76.29% for pomelo peel and 87.95% for banana peel).

Using pomelo and banana peel as substrate, the mass balance based on the fermentation equation (Eq. 1) indicated theoretical ethanol yields of 6.15 g L^{-1} and 2.82 g L^{-1}, respectively, while the maximum obtained values from the pomelo batch experiment were 2.02 and 2.79 g L^{-1} under 30°C and 40°C fermentation temperatures, using 10% of each culture. Lower ethanol yields of 0.32 and 1.49 g L^{-1} were achieved with banana peel as substrate with the same amount of co-cultures. During the fermentation process, the co-cultures consumed nutrient (glucose) for their internal metabolism, at rates of 0.3, 0.3, and 0.27 g/g substrate for the control, pomelo, and banana peel treatments, respectively. The calculation highlights the observation that ethanol yield without internal utilization using pomelo peel as substrate was higher than that obtained with banana peel (32.84% and 11.34% with 10% of each culture). However, the resembled values of 45.35% and 52.79% were obtained under 40°C (Table 1). In the control treatment, 92.36% of glucose utilization was observed, with 13.6 g of glucose utilized under the same fermentation conditions in this study. Higher glucose utilization by the co-cultures was observed at 30°C, which resulted in reduced production efficiency.

$$C_6H_{12}O_6 \longrightarrow 2C_2H_6O + 2CO_2 \qquad \text{(Eq. 1)}$$

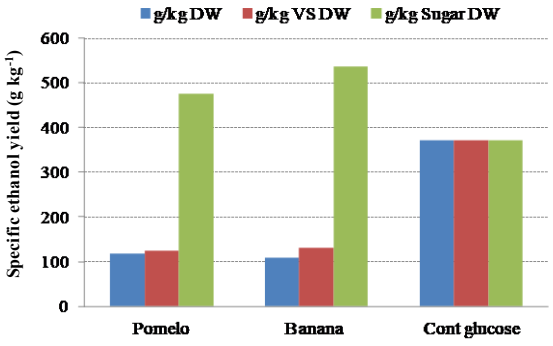

Figure 5 Specific ethanol yields by substrate.

In addition, the mass balance analysis showed that the practical ethanol yield obtained at 30°C using banana peel was lower than the expected theoretical value. However, at 40°C ethanol yield approached the theoretical yield.

Table 1 Ethanol yield at 40°C fermentation temperature

Parameter	Control (glucose)	Banana Peel		Pomelo Peel	
		5% Inoc.	10% Inoc.	5% Inoc.	10% Inoc.
Substrate load (g L^{-1})	50.00	100.00	100.00	100.00	100.00
Volatile solids (%)	100.00	10.07	10.07	22.58	22.58
Specific organic load (g L^{-1})	50.00	10.07	10.07	22.58	22.58
Fermentable sugar (%)	100.00	54.80	54.80	53.27	53.27
Specific fermentable sugar (g L^{-1})	50.00	5.52	5.52	12.03	12.03
Theoretical ethanol yield (g L^{-1})	25.57	2.82	2.82	6.15	6.15
Internal uptake by control factor	13.66	2.72	2.72	6.10	6.10
Control corrected substrate	36.34	7.35	7.35	16.48	16.48
Specific fermentable sugar (g L^{-1})	36.34	2.80	2.80	5.93	5.93
Control corrected ethanol yield (g L^{-1})	18.59	1.43	1.43	3.03	3.03
Practical experimental ethanol yield (g L^{-1})	18.58	1.18	1.49	2.43	2.79
Practical carbon dioxide yield (g L^{-1}).	17.76	1.13	1.42	2.32	2.67
Lost in internal utilization (g $50g^{-1}$, control experiment)	13.66	3.21	2.60	7.28	6.57
Internal utilization (g/g substrate)	0.27	0.27	0.27	0.27	0.27
Production efficiency (%)	99.96	82.42	104.07	80.10	91.97
Production yield without internal utilization (%)	72.66	41.81	52.79	39.50	45.35

2) Substrate composition

Analysis of substrate composition showed that pomelo peel contains lower values for pH, ash, moisture, and TKN than banana peel, with higher values for total solids, volatile solids, COD, and sugar (Table 2). Substrate composition, especially initial sugar concentration, is an important factor influencing ethanol production by the yeast strain [25]. According to the theoretical equation for biological ethanol fermentation, 1 mole of glucose will be converted to 2 moles of ethanol and 2 moles of carbon dioxide, and so higher yields can be expected using pomelo peel as substrate. Moreover, the amount of total solids could be used for pre-assessment of the active sugar and ethanol production from the hydrolysis and the fermentation process. Generally, the concentration of total solids for the hydrolysis process should be in the range of 10-20% (w/w), but can be as high as 40%, depending on fermentation conditions. This study found that pomelo peel contained higher levels of total solids than banana peel (23.70% and 12.05%), which is consistent with the higher ethanol yields obtained using pomelo peel. In other words, 53% of total solids were converted to ethanol by the co-cultures. However, further increasing the substrate fermented area and also homogenizing the substrates are recommended as an effective physical pre-treatment, due to its simplicity, low cost and scalability to full-scale industrial application.

Table 2 Substrate composition

Parameter	Pomelo peel		Banana peel	
	Average	SD	Average	SD
pH	4.28	0.06	6.08	0.00
Total solids (g kg^{-1})	237.07	1.28	120.53	2.79
Volatile solid (g kg^{-1})	225.82	1.15	100.71	2.45
Ash (g kg^{-1})	11.25	0.36	19.81	0.55
Moisture (%)	76.29	0.13	87.95	0.28
COD (g L^{-1})	301.70	8.53	62.64	8.19
TKN (g L^{-1})	12.45	0.40	16.32	0.13
Glucose (g L^{-1})	5.62	0.29	2.09	0.79

3) Effect of fermentation temperature on ethanol yield

The experimental results indicate that fermentation temperature affects the obtained ethanol yield, in agreement with previous studies [e.g. 26]. Theoretically, fermentation temperature affects the rate of growth of microorganisms and their metabolic function in the fermentation process, and thus also the ethanol yield [27]. High fermentation temperature also increases reaction rate for the hydrolysis of starch to available sugar for fermentation. However, this is dependent on the specific function of the microorganism in the fermentation process, and temperature-dependent growth inhibition has also been observed. Moreover, the strain of microorganisms used for the fermentation should be tolerant of higher temperatures. For yeasts (*S. cerevisiae*), 30-32°C is generally an optimal temperature for growth, although many strains will tolerate temperatures up to 45°C [27]. This study observed higher ethanol yield at 40°C (Figure 3). In the same way, a yield of 0.41 g ethanol from 1 g of starch can be achieved by co-cultures of *A. niger* and *S. cerevisiae* under 35°C in SSF, as reported recently [22]. However, the fermentation period also influences ethanol yield. If the period is too short, production efficiency will not reach its potential due to the limitation on rate of growth of the yeast or co-culture. On the other hand, too long a fermentation period may result in inhibition of the microorganism's growth, especially in batch mode of fermentation. Thus, low fermentation temperature requires a longer period and results in lower ethanol yields. Statistical analysis using paired sample T-test indicated that the fermentation temperature has a statistically significantly effect on maximum yield ($p<0.05$). The data also showed that the maximum yield from fermentation of pomelo and banana peel at 30°C for 24 h is significantly lower from the yield obtained at 40°C ($p<0.05$).

Considering the overall cost of industrial scale ethanol production, bio-ethanol fermentation under high fermentation temperature is attractive, due to its increased production efficiency, lower costs of cooling and reduced water usage during the fermentation process. Together these savings result in reduced overall costs of production [28]. If the fermentation was established using a yeast strain, it requires control of fermentation temperature as the fermentation process is exothermic. Therefore, thermo-tolerant yeast strains are necessary under high fermentation temperatures ≥35°C, such as *S. cerevisiae*. In this study, *S. cerevisiae* TISTR 5606 could be activated concurrently with *A. niger* TISTR 3063 at 40°C, resulting in a higher ethanol yield than that obtained at lower temperatures (30°C).

Conclusions

This study confirmed that high ethanol yield from SSCF of pomelo peel under 40°C for 24 h could be achieved using co-cultures of *A. niger* and *S. cerevisiae* in 10% (w/w) of each culture. The results also indicated that fermentation temperature has a significant effect on maximum ethanol yield ($p < 0.05$). Semi-continuous SSCF at lab-scale is needed to further study optimal conditions to enhance productivity. Further investigation on starch-rich wastes streams are also recommended to generate knowledge and optimize substrate mixtures for superior yields and cost-effectiveness at industrial scale.

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