ACS Sustainable Chemistry & Engineering

Research Article

pubs.acs.org/journal/ascecg

Kinetic Analysis of a Crude Enzyme Extract Produced via Solid State Fermentation of Bakery Waste

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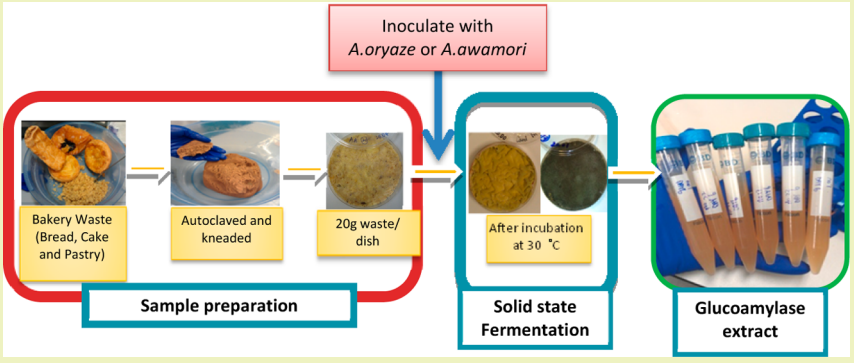

ABSTRACT: In this study, glucoamylase extract was produced from various bakery wastes under solid state fermentation by *Aspergillus awamori* and *Aspergillus oryzae*. The highest glucoamylase activities obtained from bread and cake waste with *A. awamori* were 240.7 U/g of bread (db) and 134.9 U/g of cake (db), respectively, and 83.3 U/g of pastry (db) using *A. oryzae*. Kinetic studies showed that glucoamylase obtained from bakery wastes should have at least two conformations under the two temperature ranges of 30−55 °C and 60−70 °C. Thermodynamics stated that the range 30−55 °C was considered to be the optimal temperature for glucoamylase activity and subsequent food waste hydrolysis. The crude enzyme extracts were then used in the hydrolysis of restaurant food waste to produce a glucose-rich hydrolysate. It was found that using combined crude glucoamylase (produced from *A. awamori* and *A. oryzae*) could achieve higher glucose production (0.515 g of glucose/g of food waste) than using glucoamylase alone (produced from *A. awamori*). Results obtained from this study demonstrate that the proposed bioprocess could be successfully applied for the bioconversion of food waste into value-added products in a sustainable way.

KEYWORDS: *Bakery waste, Food waste, Glucoamylase, Enzymatic hydrolysis, Solid state fermentation*

INTRODUCTION

Food waste is a major problem in developed countries, such as the United Kingdom, United States, Japan, and Korea.[1] This problem is also thriving in Hong Kong because of a rapid depletion of the limited landfill space that is left. Over 3,500 tons of food waste is produced in Hong Kong every day,[2] which accounts for nearly 40% of the total municipal solid waste.[3] One third of the food waste is originated from the commercial and industry (C&I) sector, and the remaining comes from households. In Hong Kong, a significant amount of bakery waste is produced, and ∼1 tonne of bakery waste is received by the Hong Kong Organic Recycling Centre every day.[4] Landfilling, incineration, and food waste recycling are commonly adopted strategies for waste treatment. Unfortunately, direct disposal to landfills and incineration are no longer sustainable because of the limited capacities of the landfills in Hong Kong and dioxin/greenhouse gas generation.[5] However, food waste recycling could be a sustainable way to solve the waste problem. The major conventional recycling methods for food waste have been employed for the production of animal feed and fertilizer. Although the conversion efficiency is very high, the values of animal feed and fertilizer are low and not in great demand in commercial-based cities, such as Hong Kong and Singapore.

The global market for industrial enzymes was worth nearly 4.5 billion U.S. dollars in 2012 and $4.8 billion in 2013. This market is expected to reach around U.S. $7.1 billion by 2018, a

Received: April 17, 2015
Revised: June 22, 2015
Published: July 20, 2015

DOI: 10.1021/acssuschemeng.5b00323
ACS Sustainable Chem. Eng. 2015, 3, 2043−2048

compound annual growth rate of 8.2% from 2013 to 2018.[6] Li et al.[7] reported that almost 75% of industrial enzymes are hydrolytic. Carbohydrases, proteases, and lipases dominate the enzyme market, accounting for over 70% of the enzyme market. These enzymes are typically used as bulk enzymes in applications such as detergents, textile, pulp and paper industries, organic synthesis, and the biofuel industry. Glucoamylase is one of the most industrially important enzymes that is used to produce crystalline glucose or glucose syrup from starch for biofuel, chemical, and wine production. Recently, the feasibility of glucose production through fermentation from various municipal food wastes or agriculture residues, such as bakery waste, restaurant food waste, wheat byproducts, and cowpea waste have been investigated.[8]

Glucose is the principle carbon source in many biotechnological processes, and it is of great importance for the production of fermentative chemicals. Commercial glucoamylase is usually used to recover glucose from food waste; however, this inevitably increase the operation costs.[9] Utilization of crude fungal enzymes from the *Aspergillus* genus, a known secretor of glucoamylase, could however be a low-cost alternative.[10]

Considering these problems, conversion of food waste to high-value products, such as biofuel and platform chemicals,[11,12] could be an alternative, environmentally friendly option. Previously, we had reported that pastry waste could be used as a promising substrate for glucoamylase production with a yield of 253.7 ± 20.4 U/g of pastry (db) through solid state fermentation by *Aspergillus awamori*.[13] As a follow up, in this study, our primary interest was to conduct an in-depth study to characterize the kinetic properties of the crude glucoamylase produced from pastry waste and other bakery wastes (bread and cake) in order to determine the most suitable substrate for glucoamylase production. Therefore, the aim of this study can be summarized as (i) produce crude glucoamylase extracts from cake and bread wastes via solid state fermentation; (ii) characterize and compare the kinetic properties of glucoamylase in these enzyme extracts, and (iii) demonstrate the application of crude glucoamylases in glucose production from restaurant food waste.

MATERIALS AND METHODS

Microorganisms. *Aspergillus awamori* (ATCC 14331) and *Aspergillus oryzae*, isolated from a soy sauce starter provided by the Amoy Food Ltd., Hong Kong,[2,13] were used in solid state fermentations to produce glucoamylase. Detailed information on the storage, sporulation, and inoculum preparation of *Aspergillus awamori* and *Aspergillus oryzae* were provided in a previous publication.[14]

Raw Materials. Bakery wastes (cake and bread) were collected from Starbucks in Hong Kong and separately blended in the laboratory using a blender. All portions of the cake and bread waste were pooled in a mixing bowl and further mixed by a spatula, kneaded into a homogeneous dough by hand, and stored at −20 °C in a freezer. The pretreated bakery wastes were autoclaved at 120 °C for 30 min before being subjected to solid state fermentation (SSF). The restaurant food waste was obtained from a local canteen and lyophilized before being used in the food waste digestion experiment.

Solid State Fermentation (SSF). The initial moisture contents of the bakery wastes were adjusted to 60−65% wet basis through the addition of sterilized distilled water to bakery waste prior to SSF. The optimum initial moisture content for fungal solid state fermentation was suggested by Melikoglu.[16] Sterilized bakery wastes of 20 g were placed in a Petri dish (10 cm diameter) and inoculated with either *A. awamori* or *A. oryzae* spore suspensions with a constant inoculum size of 5×10^5 spores/g of bakery waste. Batch fermentations were carried at 30 °C in a static incubator.

Enzyme Extraction. As soon as the fermentations were terminated, whole plate contents of the fermented solids were homogenized using a blender containing 20 mL of demineralized water. The mixture was transferred to a 500 mL bottle and swirled with a stirrer bar at 30 °C for 30 min in a water bath. The suspension was centrifuged at 22,000*g* for 10 min at 4 °C and then collected by performing suction filtration using Whitman no. 1 filter paper. The filtrates were crude glucoamylase extracts and were stored at −20 °C refrigerator until being used for enzymatic analysis.

Thermal Deactivation of Crude Glucoamylase Extract. Thermal deactivation of glucoamylase was studied by incubating the crude enzyme extract at particular temperatures in the absence of the substrate. Standard amounts of enzyme extracts were placed into test tubes and in water baths (i) at temperatures between 30 and 55 °C with +5 °C increments for 120 h and (ii) at temperatures between 60 and 70 °C with +5 °C increments for 120 min. The experimental details have been previously described elsewhere.[17]

Food Waste Hydrolysis with Crude Glucoamylase Extracts. Food waste hydrolysis was started by adding 0.4 mL of crude enzyme extract (10.5 U/mL) to test tubes containing 50 mg of lyophilized food waste in 1.8 mL of 0.2 M sodium acetate (pH 5.5). The reaction mixture was incubated at 55 °C and mixed by pipetting every 30 min. Aliquots of samples were withdrawn regularly from the reaction mixture and mixed with 10% (w/v) trichloroacetic acid prior to glucose determination.

Analytical Methods. Glucoamylase activities were measured according to Pleissner's study.[5] One unit (U) of glucoamylase activity was defined as the amount of enzyme required for the release of 1 μmol of glucose in 1 min under the reaction conditions. Glucose concentration was measured by an Analox GL6 analyzer, Analox Instruments Ltd., UK. Dry weight of the samples was determined by weight loss after lyophilization.

RESULTS AND DISCUSSION

Production of Glucoamylase from Bakery Wastes. As shown in Table 1, the bakery wastes are rich in carbohydrates and protein, which could be used for glucoamylase production by *Aspergillus awamori* through solid state fermentation. To evaluate the most suitable bakery waste for glucoamylase production, we used two kinds of commonly available bakery wastes (cake and bread) as substrates in this study. Glucoamylase activities from different bakery wastes were recorded during the course of fermentations and shown in Figure 1.

Table 1. Compositions of Various Food Wastes Used in This Study[a]

composition	bread	pastry	cake	restaurant food waste
moisture (g)	22.3	34.5	45.0	N/A
carbohydrate	46.8	33.5	62.0	47.0−73.8
starch	59.8	44.6	12.9	36.2−61.2
lipids	0.9	35.2	19.0	7.4−37.4
sucrose	3.0	4.5	22.7	N/A
fructose	N/A	2.3	11.9	N/A
protein (TN × 5.7)	8.9	7.1	17.0	5.8−9.9
total phosphorus	trace	1.7	1.5	N/A
ash	N/A	2.5	1.6	N/A

[a]Per 100 g, dry basis.

It was observed that the glucoamylase activity increased significantly from day 4 to day 8 during bread waste fermentation. The trend of glucoamylase production is similar to that of Melikoglu's study[15] using waste bread (white loaf)

DOI: 10.1021/acssuschemeng.5b00323
ACS Sustainable Chem. Eng. 2015, 3, 2043−2048

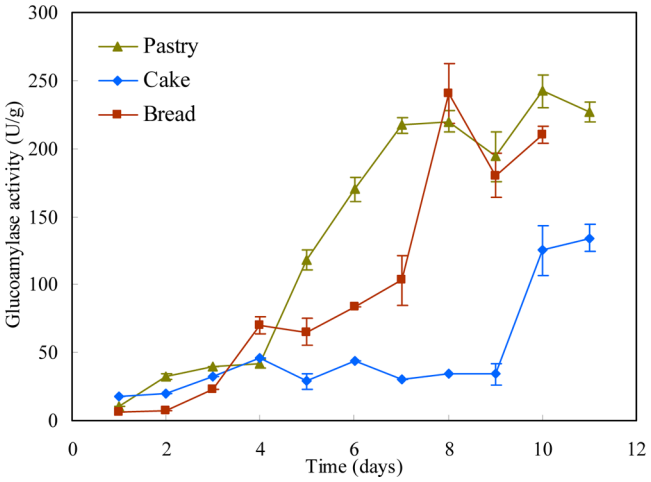

Figure 1. Glucoamylase production by *A. awamori* cultivated on bread or cake and compared to pastry waste under solid state fermentation conditions.

pieces as substrate. However, the maximum glucoamylase yield obtained from this study at day 8 was 240 U/g of bread (db), which is two times higher than the reported yield of 114.0 U/g of bread (db), probably due to differences in the physical properties and compositions of the two substrates. In that study, waste bread (white loaf) pieces were used as substrate in solid state fermentation. However, the bread waste used in this study was a mixture of croissants, sandwiches, and baguettes containing ham, bacon, vegetables, eggs, and cheese, which was blended and kneaded into a homogeneous form. It was found that the glucoamylase yield from wheat pieces under solid state fermentation conditions using the same fungus was lower than glucoamylase yield from bread, which is basically produced from wheat. Therefore, it is highly plausible that glucoamylase activity could be related to the physical properties of particle size and distribution of the substrate together with its biochemical properties. This is an important finding in terms of fermentation engineering.

The glucoamylase production trend and yield during bread waste fermentation is comparable to those of pastry waste fermentation in our previously reported data[13] in which a maximum glucoamylase yield of 253.7 ± 20.4 U/g of pastry (db) was observed at day 10. In contrast, glucoamylase production from cake waste fermentation was significantly lower. The maximum glucoamylase yield was 134.9 U/g of cake (db) at day 11. The higher glucoamylase yields observed from bread and pastry waste are likely due to their higher starch contents (Table 1), as starch is reported to be an inducer of glucoamylase synthesis by some *Aspergillus* producers.[1,11]

Kinetic Studies on Crude Glucoamylase by *A. awamori* from Different Bakery Wastes. Variation in carbon source, nitrogen source, or their ratios could have a crucial influence on different types of enzyme production[18] and kinetic properties of the enzyme extracts because *A. awamori* is capable of secreting an array of hydrolytic enzymes.[19] Consequently, it is important to understand the kinetic behavior (such as thermal deactivation) of the crude glucoamylase extracts produced from different bakery wastes before scaling up the bioprocess.

Thermal deactivation is a major problem for enzymatic hydrolysis reactions, which should be investigated before

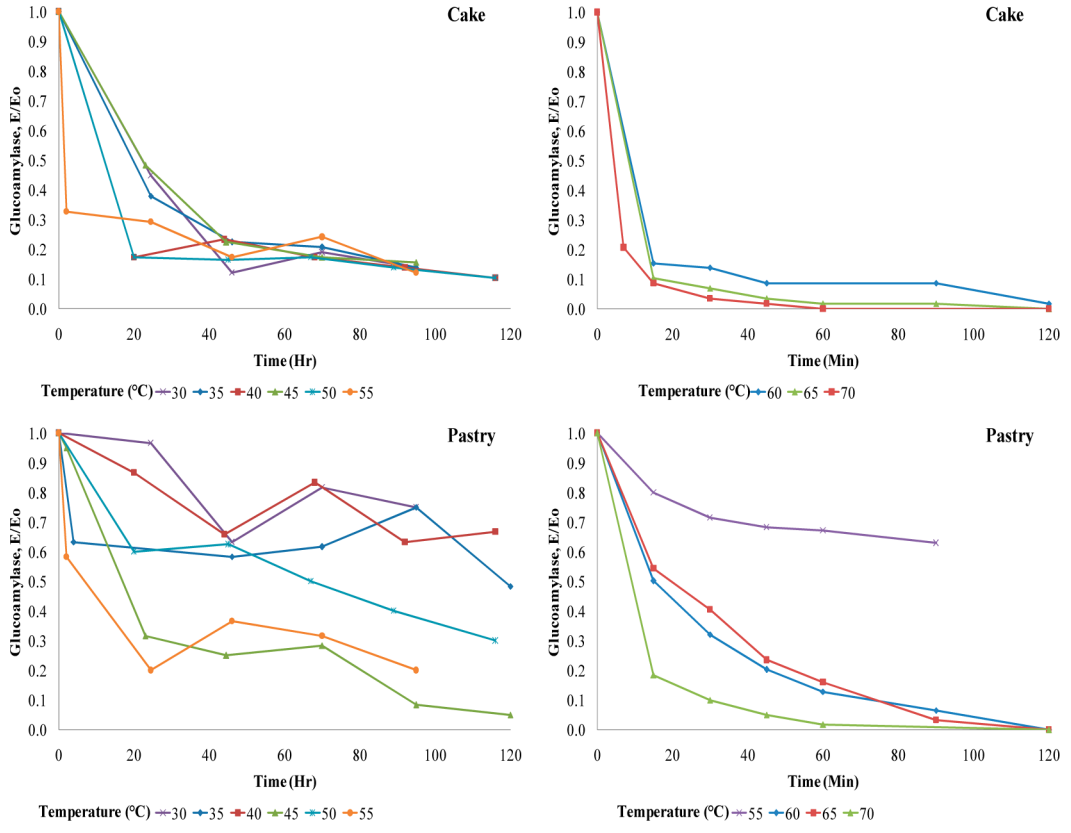

Figure 2. Thermal deactivation of glucoamylase from *A. awamori* produced by solid state fermentation of cake and pastry at 30−70 °C with +5 °C increments.

DOI: 10.1021/acssuschemeng.5b00323
ACS Sustainable Chem. Eng. 2015, 3, 2043−2048

commercial utilization of the crude enzyme extract produced. At the beginning of the kinetic studies, the initial glucoamylase activities of cake and pastry wastes were measured as 134.9 U/g of cake (db) and 253.7 ± 20.4 U/g of pastry (db), respectively. Deactivation of the enzymes are expressed as the ratio of the measured activity E at time t of heat treatment to the initial enzyme activity E_0. Thermal deactivation of glucoamylase produced from cake and pastry waste by *A. awamori* at temperatures between 30 and 70 °C, with +5 °C increments, are shown in Figure 2. It was observed that the thermal deactivation at moderate temperatures, between 30 and 55 °C, was much slower than the thermal deactivation at higher temperatures between 60 and 70 °C. These results are similar to those of Norouzian's study[20] in that the optimal reaction temperatures for glucoamylse activity by *Aspergillus* were in the range of 50−60 °C, whereas the enzyme activity decreased with the temperature further increasing, probably due to enzyme denaturation at high temperature.

The thermal deactivation of enzymes could be expressed as an exponential decay (eq 1),[21] where E_0 is the initial enzyme activity, E is the enzyme activity at time t, and k_d is the deactivation rate constant. The k_d values for glucoamylase at each incubation temperature could be calculated using eq 1, and the experimental data together with the values reported by Melikoglu et al.[17] using waste bread are given in Table 2. It was found that the k_d values for glucoamylase in the range of 30−55 °C from cake and pastry wastes were significantly lower than the values obtained from in temperatures ranging from 60 to 70 °C. Therefore, the crude glucoamylase activity with temperatures of 30−55 °C were more thermo-stable. The results awere similar to the reported values obtained by Lam et al.[13] using waste bread.

Table 2. Deactivation Rate Constants of Glucoamylase Extract Produced from Cake and Pastry Waste by *Aspergillus awamori* from Solid State Fermentation at 30−70°C with +5°C Increments

	k_d (hour^{-1})		
temperature (°C)	cake	pastry	bread[a]
30	0.021	0.003	0.0012
35	0.019	0.006	0.0024
40	0.019	0.005	0.0032
45	0.020	0.024	0.0035
50	0.015	0.009	0.0050
55	0.017	0.014	0.0109
60	1.144	1.804	0.6615
65	2.487	2.191	1.2341
70	4.981	3.837	4.2351

[a]Values reported by Melikoglu et al. (2013) in ref 17.

$$E = E_0 \times e^{-k_d t} \quad (1)$$

The half-life ($t_{1/2}$) is the time when the enzyme activity decreases to half of its original value. This is a very important parameter for the economic feasibility of bakery waste reutilization because a longer half-life at a specific operating temperature represents higher thermostability. Mathematically, the half-life of an enzyme can be calculated by replacing E with $E_0/2$ in eq 1, as shown in eq 2. Therefore, the half-life of glucoamylase from different bakery wastes could be calculated, and the results are shown in Table 3. The optimal half-life from cake (40.07−45.9) was obtained in the range of 50−55 °C, which should be regarded as the optimal temperature for glucoamylase activity and subsequent food waste hydrolysis. The largest half-life of glucoamylase in pastries was at 30 °C. This is consistent with the findings reported by Mehmet,[17] in which the largest half-life of glucoamylase in bread was also at 30 °C, and the half-lives decreased with higher temperature.

Table 3. Half-Life of Glucoamylase Extract Produced from Cake and Pastry Waste by *Aspergillus awamori* from Solid State Fermentation at 30−70°C with +5°C Increments

	$t_{1/2}$ (hours)		
temperature (°C)	cake	pastry	bread[a]
30	33.81	216.61	560.4
35	35.73	111.80	294.7
40	36.67	138.63	216.6
45	35.01	28.64	200.6
50	45.90	76.17	138.7
55	40.07	49.51	63.6
60	0.61	0.38	1.0
65	0.28	0.32	0.6
70	0.14	0.18	0.2

[a]Values reported by Melikoglu et al. (2013) in ref 17.

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (2)$$

Using the Arrhenius equation given in eq 3 and the deactivation rate constants given in Table 2, activation energies for denaturation of glucoamylase could be calculated. In eq 3, A is the pre-exponential factor, E_{ad} is the activation energy of denaturation, R is the universal gas constant (8.314 J mol^{-1} K^{-1}), and T is the absolute temperature. When the temperature was in the range of 30−55 °C, the activation energies for the cake and pastry were 7.331 and 48.13 kJ/mol, respectively. However, both the activation energies for cake and pastry increased to 139.8 and 71.47 kJ/mol when the temperature increased to 60−70 °C. This is consistent with the findings reported by our earlier study,[13] which suggested that glucoamylase obtained from each bakery waste has at least two conformations under the two temperature ranges of 30−55 and 60−70 °C.

$$k_d = Ae^{-E_{ad}/RT} \quad (3)$$

The Eyring absolute rate equation (eq 4) could be used to estimate the thermodynamic data for glucoamylase. In eq 4, h is the Planck constant of 6.63 × 10^{-34} J s, k_B is the Boltzmann constant of 1.38 × 10^{-23} J/K, T is the absolute temperature, ΔH is the enthalpy of activation in kJ/mol, and ΔS is the entropy of activation in J mol^{-1} K^{-1}. The enthalpy of activation (ΔH) given in eq 4 was calculated using the activation energies for denaturation as shown in eq 5. Similarly, the free energy of activation (ΔG) could be calculated using eq 6. Finally, entropy of activation (ΔS), represented in eq 4, could be calculated using the enthalpy and free energies of activation as shown in eq 7.

$$k_d = (k_B T/h)e^{(-\Delta H/RT)}e^{(\Delta S/R)} \quad (4)$$

$$\Delta H = E_{ad} - RT \quad (5)$$

DOI: 10.1021/acssuschemeng.5b00323
ACS Sustainable Chem. Eng. 2015, 3, 2043−2048

Table 4. Thermodynamic Parameters for Thermal Deactivation of Crude Glucoamylase Extract from *Aspergillus awamori* Produced by Solid State Fermentation with Cake and Pastry Wastes

	ΔH (kJ/mol)		ΔG (kJ/mol)		ΔS (J mol^{-1} K^{-1})	
temperature (°c)	cake	pastry	cake	pastry	cake	pastry
30	4.812	45.611	83.978	88.9	−261.3	−142.8
35	4.770	45.569	85.662	88.6	−262.6	−139.8
40	4.729	45.528	87.094	90.6	−263.1	−143.9
45	4.687	45.486	88.392	87.9	−263.2	−133.4
50	4.646	45.445	90.596	92.0	−266.1	−144.0
55	4.604	45.403	91.699	92.2	−265.5	−142.8
60	137.031	68.701	81.486	80.2	166.8	−34.6
65	136.990	68.660	80.569	80.9	166.9	−36.3
70	136.948	68.618	79.822	80.6	166.5	−34.8

$$\Delta G = -RT\ln\left[\frac{k_d h}{k_B T}\right] \quad (6)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (7)$$

The thermodynamic parameters of crude glucoamylase could be calculated, and the results are tabulated in Table 4. Thermodynamics governs that when ΔH is positive and ΔS is negative, a process is not spontaneous at any temperature, but the reverse process is spontaneous. Therefore, deactivation of glucoamylase in the enzyme extract from cake waste could be reversible between 30 and 55 °C. However, both ΔS and ΔH are positive above this temperature range for cake waste, and this means that the deactivation of enzymes is spontaneous at high temperatures of 60−70 °C. More importantly for pastry, between 30 and 70 °C, ΔH is positive and ΔS is negative, which indicates inactivation of glucoamylase could be reversible at all operating temperatures, and the glucoamylase is more thermally stable than the one produced from cake waste. This is also validated by the higher half-life of glucoamylase from pastry and bread as reported in Table 3.

Enzymatic Hydrolysis of Food Waste. The typical composition of restaurant food waste obtained from a canteen is shown in Table 1. The starch content is high, containing up to 61.2%, making it a suitable substrate for glucose production. The crude enzyme extract from *Aspergillus* usually contains a variety of hydrolytic enzymes[22,23] that can act synergistically to digest the starch portion of food waste with glucose as the end product. Therefore, it is interesting to investigate whether combined crude glucoamylase extract from other *Aspergillus* producers would lead to faster glucose production from food waste hydrolysis.

As shown in Figure 1 and Table 4, it was observed that pastry waste is a better substrate for glucoamylase production with higher glucoamylase yield and thermal stability compared to those of cake and bread waste. Therefore, the crude glucoamylase extract obtained from pastry waste fermentation was used for glucose production from restaurant food waste. Meanwhile, glucoamylase extract produced by *Aspergillus oryzae* from pastry waste under the same conditions was also used to combine with the crude glucoamylase extract from *A. awamori* for glucose production. Figure 3 shows the glucoamylase production by *A. oryzae* from pastry waste. The maximum glucoamylase yield was 83.3 U/g of pastry (db) at day 7. The crude glucoamylase extract was then mixed with the crude glucoamylase extract from *A. awamori* to obtain combined glucoamylase extracts. The combined glucoamylase extracts (0.4 mL) with a glucoamylase activity of 10.5 U/mL was used

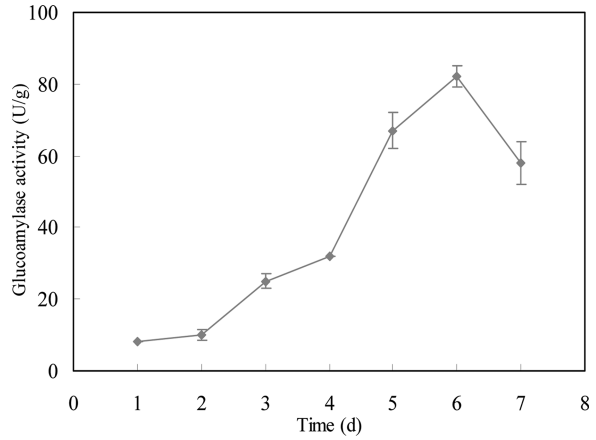

Figure 3. Glucoamylase production by *A. oryzae* cultivated on pastry waste under solid state fermentation conditions.

to hydrolyze restaurant food waste to produce glucose. To compare the glucose production rate and yield, we also used the crude glucoamylase extract produced by *A.awamori* alone with the same volume (0.4 mL) and activity (10.5 U/mL) to hydrolyze the restaurant food waste. The results for glucose yield from food waste hydrolysis are shown in Figure 4. Hydrolysis of restaurant food waste using crude glucoamylase extract from *A. awamori* alone resulted in ∼44.9 g of glucose

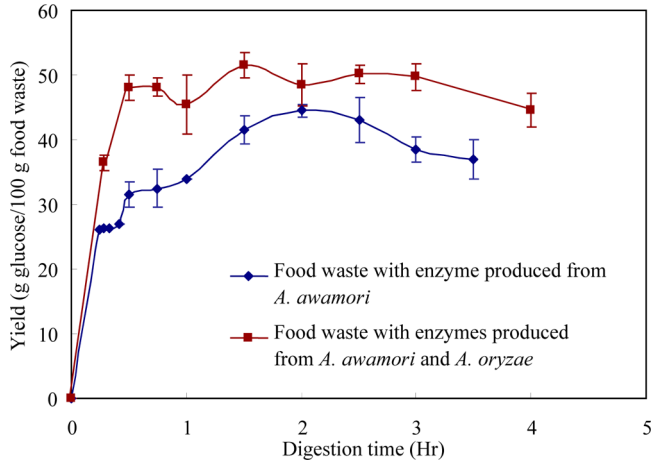

Figure 4. Glucose yield from food waste hydrolysis using crude glucoamylase extract from *A. awamori* and both *A. awamori* and *A. oryzae*.

DOI: 10.1021/acssuschemeng.5b00323
ACS Sustainable Chem. Eng. 2015, 3, 2043−2048

per 100 g of food waste. Although a faster rate of glucose production from food waste was not observed when combined crude glucoamylase extracts were used, interestingly, a higher glucose yield of 51.5 g of glucose per 100 g of food waste was obtained. The possible contribution to higher glucose yield might due to the fact that the enzyme solution from *A. oryzae* contains other glucose-producing enzymes, such as α-amylase and lactase. These enzymes can digest specific components of food waste into glucose. For instance, α-amylase breaks down long-chain carbohydrates into glucose or maltose. Lactase breaks down disaccharide lactose into galactose and glucose monomers. Therefore, a higher resultant glucose yield was obtained using combined enzyme extracts in mixed food waste conversion.

CONCLUSIONS

Various bakery wastes were used as substrates by *Aspergillus awamori* and *Aspergillus oryzae* for the production of glucoamylase and protease via solid state fermentation. Higher starch content in the bakery waste could lead to higher glucoamylase yield. Thermodynamics governs that when the enthalpy of activation (ΔH) is positive and entropy of activation (ΔS) is negative, a process is not spontaneous at any temperature, but the reverse process is spontaneous. Therefore, deactivation of glucoamylase in the enzyme extract from cake waste could be reversible between 30 and 55 °C. However, both ΔS and ΔH are positive above this temperature range for cake waste, which means that deactivation of the enzymes is spontaneous at high temperatures of 60−70 °C. In addition, a higher yield of glucose (0.515 g of glucose/g of food waste) could be achieved from restaurant food waste through enzymatic hydrolysis using combined enzyme extracts from *A. awamori* and *A. oryzae*. This suggested that the use of combined enzymes extract could be a better strategy for glucose recovery from restaurant food waste.

In summary, this study clearly shows that crude enzyme extract produced from bakery waste under solid state fermentation can be applied for valorization of restaurant food waste for the production of a glucose-rich feedstock, which could be further used in the production of fermentative chemicals. This study also highlights the potential of an alternative approach of waste management to reduce the food waste problem.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge the Innovation and Technology Funding from the Innovation and Technology Commission (ITS/323/11) in Hong Kong and a donation from the Coffee Concept (Hong Kong) Ltd. for the "Care for Our Planet" campaign.

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DOI: 10.1021/acssuschemeng.5b00323
ACS Sustainable Chem. Eng. 2015, 3, 2043−2048