Glucoamylase production from food waste by solid state fermentation and its evaluation in the hydrolysis of domestic food waste

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Abstract

In this study, food wastes such as waste bread, savory, waste cakes, cafeteria waste, fruits, vegetables and potatoes were used as sole substrate for glucoamylase production by solid state fermentation. Response surface methodology was employed to optimize the fermentation conditions for improving the production of high activity enzyme. It was found that waste cake was the best substrate for glucoamylase production. Among all the parameters studied, glucoamylase activity was significantly affected by the initial pH and incubation time. The highest glucoamylase activity of 108.47 U/gds was achieved at initial pH of 7.9, moisture content of 69.6% (by weight), inoculum loading of $5.2*10^5$ cells/gram substrate (gs) and incubation time of 6 days. The enzyme preparation could effectively digest 50% (w/v) domestic food waste in 24 h with about complete saccharification using an enzyme dose of only 2U/g food waste at 60°C.

Keywords:

Glucoamylase; *Aspergillus awamori;* Food waste; Saccharification; Solid state fermentation; Response surface methodology.

1 Introduction

Food waste (FW) is a kind of organic waste discharged from households, cafeterias and restaurants. According to (FAO 2012), one third of food produced for human consumption (nearly 1.3 billion tones) is lost or wasted globally throughout the food supply chain and it is increasing dramatically while almost 1 billion people worldwide are classified as starving. Besides, every tone of FW means 4.5 ton of CO_2 emissions (Smith et al. 2001). Currently, the majority of FW in Singapore is incinerated with other combustible municipal wastes for heat or energy production, while residual ash is then disposed of in landfills. However, incineration is an expensive waste conversion technique and can cause severe air pollution (El-Fadel et al. 1997). From an environmental viewpoint, there is an urgent need for appropriate management of FW. Due to its chemical complexity, high moisture content, easy degradation and nutrient rich composition, FW should be treated as a useful resource for higher value products, such as fuels and chemicals through fermentation. Recently, there is a growing interests on the biochemicals production from FW (Han and Shin 2004, Ohkouchi and Inoue 2007, Sakai and Ezaki 2006, Wang et al. 2005, Yang et al. 2006, Zhang et al. 2013, Zhang et al. 2010, Koike et al. 2009). Starch is an important biopolymer in foods, as such, it is a significant part of kitchen waste (Arooj et al. 2008). Hence, the saccharification of FW is a key step for its bioconversion into value-added products. For this, commercial enzymes, particularly glucoamylases, were often used to promote the bioconversion of polymers to bioproducts. To produce lactic acid from FW, (Sakai et al. 2004) used glucoamylase to saccharify the production medium. In other studies commercial glucoamylase, alpha-amylase and cellulase solutions were used to saccharify the kitchen wastes for ethanol production (Kim et al. 2008, Uncu and Cekmecelioglu 2011, Yan et al. 2012). If the enzymes could be produced in-situ without downstream treatments and

integrated with the biochemicals production, the cost of the process will be decreased (Merino and Cherry 2007, Wang et al. 2010). Moreover, the transportation cost and enzyme inactivation during storage could be avoided. If the crude enzyme activity is high, it would be feasible and economical for it to be used directly without any recovery process. Such strategy has been explored by several researchers (Leung et al. [61] + Meligloku) who produced succinic acid from waste bread. *Aspergillus awamori* and *Aspergillus oryzae* produced an enzyme cocktail rich in amylolytic and proteolytic enzymes to hydrolyze waste bread in SSF. The resulting fermented solids were added directly to a bread suspension to generate a hydrolysate rich in glucose and free amino nitrogen. The bread hydrolyzate was then used as the sole feedstock for *A. succinogenes* fermentation.

The microorganisms reported to be active producers of amylolytic enzymes are *Aspergillus awamori, Aspergillus foetidus, Aspergillus niger, Aspergillus oryzae, Aspergillus terreus, Mucor rouxians, Mucor javanicus, Neurospora crassa, Rhizopus delmar, Rhizopus oryzae and Thermomucor indicae-seudaticae* (Norouzian et al. 2006). Although glucoamylases have been produced by submerged fermentation traditionally, the solid state fermentation (SSF) processes have been increasingly applied for the production of this enzyme in recent years (Ellaiah et al. 2002). SSF has advantages over submerged fermentation in that it is simpler, requires less capital, has superior productivity, lower energy requirement, requires simpler fermentation media, does not require rigorous control of fermentation parameters, uses less water, produces less waste water, allows for the easy control of bacterial contamination, and has a lower downstream processing cost (Ellaiah et al. 2002, Anto et al. 2006, Melikoglu et al. 2013a).

However, the scale up of the SSF is a great challenge due to hardship of mixing, difficulty of heat removal and restricted water content which cause rapid change of moisture.

In order to attain higher enzyme activities, a number of factors need to be optimized. The statistical methods for optimization are gaining growing interest and application as they have proved to be cost and time saving. Recently, several statistical experimental design methods have been employed for optimizing enzyme production (Soni et al. 2012). Among the optimization methods used, central composite design using response surface methodology (RSM) is a method suitable for identifying the effects of individual variables and seeking the optimal conditions for a multivariable system efficiently. This approach reduces the number of experiments, improves statistical interpretation possibilities and reveals possible interactions among parameters. To develop a viable process it is important to determine the most appropriate substrate and to optimize the fermentation conditions.

Although there are some reports explaining the production of various enzymes from agro-industrial biomass, the effect of different FW constituents on glucoamylase production was not compared up to date and the produced enzymes were not evaluated for their suitability to hydrolyze starch in FW and produce fermentable sugars. In this study, different FWs were evaluated to produce glucoamylase using solid state fermentation. The overall strategy was to find out the best substrate for glucoamylase production, and optimize the yield in order to hydrolyze raw mixed food waste. This novel strategy can help to produce fermentable sugars for the production of biofuels or chemicals. The fermentation conditions such as particle size, initial moisture content, inoculum loading, pH and incubation time for high activity glucoamylase

production were optimized statistically. Finally, as part of an integrated solution the effect of the produced enzyme solution on the hydrolysis of domestic FW was evaluated.

2 Material and methods

2.1 Materials

Aspergillus awamori was used to produce glucoamylase (GA) in solid state fermentation for FW hydrolysis. It is originally obtained from ABM Chemicals Ltd (Cheshire, England). The enzyme was stored and prepared according to the procedures explained by Wang et al. (2007). The waste cakes used in this study was collected from local catering. The waste cakes were ground, sieved and then stored at -20^{o}C pending further experiments. The mixed FW (MFW) and domestic FW used in this study were collected from a cafeteria at Nanyang Technological University and a local food court, respectively. Potatoes, fruits and vegetables were obtained from a local supermarket. These were discarded from the packaging line due to a lower quality. The FWs were homogenized in a blender and directly stored in zipped plastic bags at -20˚C pending use in experiments.

2.2 Methods

2.2.1 Effect of particle size on SSF

To determine the effect of particle size the substrate was sieved through mesh number 5, 10, 16 and 230 corresponding to size cut-off of 0.6 mm, 1.18 mm, 2 mm and 4 mm, respectively (Endecotts Ltd., UK). After sieving the moisture content was adjusted to 70% (wb) and the SSF was carried out with an inoculum loading of 10^6 spores/g substrate at neutral initial pH and 30^{o}C

for 4 days as these conditions were reported to be the optimum for GA production from *A.awamori* by SSF by (Melikoglu et al. 2013a).

2.2.2 Experimental Design for Enzyme Production

A 2^4 full factorial design was used in the optimization of GA production from cake waste. Initial pH (X_1), moisture content (X_2, %, w/w), inoculum loading (X_3, inoculum/g substrate) and time (X_4, day) were chosen as independent input variables as they are the most important parameters for enzyme production during SSF (Hashemi et al. 2010, Kumar and Satyanarayana 2004, Garg et al. 2011). The GA activity (Units/gram dry solid or U/gds) was used as dependent output variables. A total of 30 experiments that included 16 cube points (runs 1-16), 8 star points (runs 17-24), and 6 replicas of the central point (runs 25-30) were performed to fit a second order polynomial model. The experimental range and the levels of the variables are defined and presented in Table 1. The ranges of variables used in this work were selected based on literature (Ellaiah et al. 2002, Melikoglu et al. 2013a, Wang et al. 2009, Pandey 1991).

2.2.3 Solid State Fermentation and Enzyme Extraction

Substrates were moistened with the calculated amount of 0.1 M phosphate and citrate buffer solutions in 500 mL Erlenmeyer flasks depending on the targeted initial pHs. After sterilization by autoclaving (120^{o}C for 20 min), the flasks were cooled down, inoculated with inoculum to obtain a certain spore concentration and the contents were mixed thoroughly with a sterile spatula. Then, 10 g of the content was distributed into each Petri dish and incubated at 30°C under stationary conditions. Petri dishes, in duplicate, were withdrawn at regular time intervals

and the content was extracted with 60 mL of distilled sterile water. This was then centrifuged at 6,000 rpm for 10 min and cell free supernatant was used for assaying the GA activity at pH 7.

2.2.4 GA assay

The activity of GA was determined at 55°C at neutral pH using 2% (w/v) soluble starch (Sigma) as substrate in a Na acetate buffer (100mM) at pH 5. The glucose concentration was determined with Optium Xceed blood glucose monitor (Abbott Diabetes Care, Oxon, UK) (Bahcegul 2011). One unit (1 U) of GA activity was defined as the amount of enzyme releasing 1 micromole glucose equivalent per minute under the assay conditions.

2.2.5 Statistical analysis

The data obtained from the central composite design experiments were analyzed using Design Expert (Stat-Ease Inc., Minneapolis, USA) (Version 8.0.7.1) software, and response surface curves, corresponding contour plots, regression coefficients and *F* values were obtained. Analysis of variance (ANOVA) was applied for the response function. The effects of the variables were estimated by the following second-order quadratic equation:

$$Y = b_0 + \sum bi\mathrm{Xi} + \sum bij\mathrm{Xij} + \sum bi^2\mathrm{Xi}^2 + \text{error} \quad (1)$$

where Y is the predicted response for GA activity (U/gds); b_0 is the intercept; bi is the coefficient for linear direct effect; bij is the coefficient for interaction effect; bi^2 is the coefficients for quadratic effect (a positive or negative significant value implies possible interaction between the medium constituents); X_i and X_{ij} are the independent variables. The quality of fit to the second

177 order equation was expressed by the coefficient of determination (R^2) and its statistical
178 significance was determined by the F-test. Variables with probability below 95% ($P > 0.05$)
179 were regarded as not significant for the final model. Three dimensional surface plots were drawn
180 to illustrate the main and interactive effects of the independent variables on the dependent
181 variables. The influence of experimental error on the central composite design was assessed with
182 six replications at the central point of the experimental domain. Experiments were carried out in
183 triplicates. Results were presented as the average of three independent trials. To maximize the
184 enzyme activity, numerical optimization was used for determination of the optimal levels of the
185 four variables.
186
187 **2.2.6 Model validation**
188 One set of experiment was performed to validate the model. Solid state fermentation was
189 conducted using an initial pH of 7.9, moisture content of 69.6%, inoculum loading of $5.2*10^5$/gs
190 and incubation time of 6 days to obtain the highest GA activity: All experiments were performed
191 in triplicate, and the mean and standard deviations of the triplicates were reported.
192
193 **2.2.7 Hydrolysis of domestic FW**
194 Twenty five mL of 10% suspension of domestic FWs from local food court in 0.1 M phosphate
195 buffer, pH 7.0 taken in 100 mL Erlenmeyer flask was mixed with GA produced in-situ from
196 *Aspergillus awamori* with enzyme to substrate ratio of 2U/g FW. The mixture was incubated at
197 60°C in a water bath for 24 h. The extent of saccharification was calculated by estimating
198 glucose concentrations, after centrifugation at 5000 rpm for 5 min. The degree of
199 saccharification was determined in terms of the ratio of glucose formed and the theoretical

obtainable glucose from starch actually degraded (in percentages). Theoretical glucose yield was calculated based on the equation: 1 g starch = 1.11 g glucose. The whole process is described in Figure 1.

2.2.8 Optimization of FW hydrolysis

The hydrolysis of FW was optimized with respect to the main influencing parameters, i.e., the temperature, enzyme dose and FW concentration. All the experiments were performed at an enzyme to substrate ratio of 2U/g FW in the reaction mixtures made with 0.1 M phosphate buffer, pH 7.0 containing 10% of FW at 60˚C for 24 h unless otherwise stated. The temperature levels of 50, 60, 70, 80, and 90˚C, enzyme dosage levels of 2, 5, and 10 U/g FW, and FW concentration levels of 10, 20, 30, 40 and 50% w/v FW were used in the optimization of hydrolysis process.

2.2.9 Analytical methods

Moisture and ash contents were determined according to analytical gravimetric methods (AOAC 2001). Crude protein content was determined using HR Test'n tube TN kit (HACH, US) and calculated according to the Kjeldahl method with a conversion factor of 6.25. Starch content was determined using Megazyme's TN kit (Bray, Ireland). The lipid content was determined by hexane/isopropanol (3:2) method (Hara and Radin 1978). The glucose concentration was determined with Optium Xceed blood glucose monitor (Abbott Diabetes Care, Oxon, UK) (Bahcegul 2011). Reducing sugars were quantified to monitor the saccharification of FW according to the dinitrosalicylic acid (DNSA) method using glucose as standard (Miller 1959).

3 Results and discussion

In order to understand the effects of different substrates, the wastes were characterized (Table 2). As can be seen in the table, the food wastes composed of different constituents. Bread has the highest starch content (71.6%) followed by potato (47.6%), cake (45.8%) and savory (45.7%). The reducing sugar content of cake (16.8%), fruit (11.7%) and potato (1.2%) were higher than that of bread (1.5%).

The influence of different FW such as bread, cake, savory, vegetable, fruit, potato and mixed type FW (MFW) from a cafeteria on GA production by *Aspergillus awamori* was investigated for 10 days (Figure 2). The incubation time is governed by characteristics of the culture, its growth rate and enzyme production. Maximum GA production normally occurs after 2-5 days of incubation as reported by other researchers working with solid state cultures involving bacteria and fungi (Melikoglu et al. 2013a, Soni et al. 2003). The fungus used in the present study colonized well the waste materials, and exhibited a good growth on the surface after 24 hours. The high reducing sugars in cake, fruit and potato wastes may have triggered the GA production, so it was higher than savory and mixed type FW on day 1. The growth and enzyme yields improved gradually, and the maximum activity of GA was obtained using waste cakes on the 4^{th} day of fermentation (Figure 2). It was followed by bread, potato and fruit wastes. The protein content of cake waste (14.1%) was also higher than that of bread (8.6%) which may have resulted in a better fungal growth and higher GA activity. To our knowledge this is the first study demonstrating that cake waste is a better substrate for GA production. The optimization of GA production from cake waste which resulted in the highest enzyme activity was afterward investigated.

The utilization of the substrate during solid state fermentations by the fungi was not only influenced by its nutritional quality but also by the particle size of the solid substrate (Schmidt and Furlong, 2012). Experimental studies shown in Figure 3 validated that particle size has a direct effect on GA production during solid state fermentation. The highest GA activity, 63.06 U/gds was measured with a particle size of $0.6 \leq X \leq 1.18$ mm. In solid state fermentations, smaller particle size provided a larger contact area. However, reduction in particle size increased the packing density, which causes a reduction in the void space between the particles, which results in reduction in microbial growth and enzyme production (Ruiz et al. 2012). Therefore, there must be an optimum for particle size. As the highest GA activity was obtained using $0.6 \leq X \leq 1.18$ particle size, it was adjusted to that particle range in the following set of experiments.

To determine the optimum pH, moisture content, inoculum loading and time that maximize GA activity, thirty experiments were designed using a Central Composite Design. The experimental conditions and the responses were presented in Table 3. A quadratic model was chosen from several models and fitted to the results. The regression equation obtained after the analysis of variance (ANOVA) represented the level of enzyme activity as a function of initial pH, moisture content, inoculum loading and time.

On the basis of their P-value, R^2, SD and predicted sum of square values, the adequacy of the quadratic regression model was found to be significant for GA production. The statistical significance of the ratio of mean square variation due to regression and mean square residual error was investigated using the ANOVA. The associated P-value was used to estimate whether

F is large enough to indicate statistical significance. If P-value is lower than 0.05, it indicates that the model is statistically significant. The ANOVA result for the GA production system showed the model F-value of 21.96 indicating that the model is significant (Table 4). There is only a 0.01% chance that a "Model F-Value'' this large could occur due to noise. Considering the P-values of parameters, the effect of terms of X_1, X_4, X_{14}, X_{23}, X_{11}, X_{22}, X_{33} and X_{44} were significant, whereas that of X_2, X_3, X_{12}, X_{13}, X_{24} and X_{34} were negligible. The coefficient of determination (R^2) for the enzyme activity was calculated as 0.9565, showing that the fitted model could explain 95.65% of variability in the response. An adequate precision of 12.74 for the enzyme activity was recorded. A value greater than 4 is desirable in support of the fitness of the model (Muthukumar et al. 2003). The adjusted R^2 corrects the R^2 value for the sample size and the number of terms used in the selected model. If there are many terms in the model and the sample size is not large enough, the adjusted R^2 may be clearly smaller than R^2. The Coefficient of Variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the CV value, the lower is the reliability of experiment. In this study, a CV value of 22.81 indicates a great reliability of the experiments performed. The table also shows a term for residual error, which measures the amount of variation in the response data left unexplained by the model. The analysis showed that the form of the model chosen to explain the relationship between the factors and the response is correct.

The equation (2) in terms of actual factors (confidence level above 95%) as determined by Design of expert software is given below:

291 GA Activity (U/gds) = $-1366.16 + 184.69*X_1 + 17.38 * X_2 + 8.02*10^{-6} *X_3 + 39.82*X_4 - 0.21*$

292 $X_1*X_2 - 6.79*10^{-6}*X_1*X_3 + 11.19*X_1*X_4 + 1.73*10^{-6}* X_2*X_3 - 0.06*X_2*X_4 + 7.32*10^{-6}*X_3*X_4 -$

293 $14.7* X_1^2 - 0.12*X_2^2 - 1.11*10^{-10}*X_3^2 - 10.21*X_4^2$

294 (2)

295

296 where X_1, X_2, X_3 and X_4 are independent variables representing the pH, moisture content,

297 inoculum loading and time, respectively. The negative coefficients for X_{12}, X_{13}, X_{24}, X_{11}, X_{22},

298 X_{33} and X_{44} demonstrate the existence of quadratic and linear interaction effects that decrease the

299 response quantity, while the positive coefficients for X_{14}, X_{23} and X_{34} expose the existence of

300 quadratic interaction effects that enhance the activity of GA. Figure 4 shows the correlation

301 between the experimental and predicted values of the response. The points close to the line

302 indicate a good fit between the experimental and predicted data.

303

304 The optima of the variables for which the responses are maximized are represented by the

305 contour plots (Figure 5). The contour plot of the moisture content and pH effect on the activity

306 of GA illustrates that the neutral pHs led to higher enzyme activity using an initial moisture

307 content of around 66-74% (wb) (Figure 5A). The maximum activity of 92.92 U/gds was

308 determined at pH 7.5 using initial moisture content of 69.6%. Lower initial moisture content

309 provides lower solubility of the nutrients while higher moisture contents cause decreased

310 porosity and decrease in gas exchange. The moisture content range is consistent with the levels

311 reported in the literature for solid state fermentation of waste bread and wheat flour by *A.*

312 *awamori* (Melikoglu et al. 2013a, Wang et al. 2009). Generally, the initial pH for GA production

313 by *A. awamori* using SSF is adjusted to neutral pHs as the fungus grows well at such pHs. Since

the maximum activity of 92.92 U/gds was determined at pH 7.5 using initial moisture content of 69.6%, these conditions were kept constant in the subsequent studies to find the optimum inoculum loading and incubation time.

The GA production increased by using an inoculum loading of $2*10^5$ to $9*10^5$/gs for 5 to 7 days and the maximum GA activity of 104.29 U/gds was obtained using $5.2*10^5$/gs inoculum on 6^{th} day of the fermentation (Figure 5B). During the fermentation, medium pH, nutrient concentration, temperature, moisture content, and physical structure of the raw material changes continuously. All these parameters affect microbial growth and enzyme production. According to Melikoglu et al. (2013a), the growth of *A. awamori* on bread pieces increased exponentially between the 3^{rd} and 5^{th} days and GA production reached its maximum level on the 6^{th} day of the fermentation. However, as the medium pH was not controlled, the pH is decreasing during this period (Melikoglu et al. 2013a). They reported that the pH decreased to 3.8 on the 5^{th} day of the fermentation. This may be one of the major causes of deceleration of the growth and enzyme production after 6^{th} day of the fermentation. Therefore, the effect of initial pH was evaluated using the optimized parameters and it was predicted that the GA activity increased from 90.69 U/gds to 107.1 U/gds using initial pH of 7.9 instead of pH 7.0 (Figure 5C). The pH reached 4.5 after the 5^{th} day of the fermentation when the initial pH was 8 and 9. On the other hand, the pH decreased to 3.5 and 4 when the initial pH was adjusted to 6 and 7, respectively. This explains why the microbial growth and GA production was enhanced using an initial pH of 7.9.

To evaluate the accuracy of quadratic polynomial model, a verification experiment was conducted under the predicted optimal conditions and the result was 108.47 U/gds which is

1.37% higher than the predicted value. This is higher than values reported by (Wang et al. 2009) for the same fungus using wheat flour and similar to those reported by (Melikoglu et al. 2013a) on bread pieces. However, higher activities were reported with *A. niger* (695 U/g), but the enzymatic assay was carried out at pH 4.5 and the substrate was wheat bran (Silveira et al. 2006). This high degree of accuracy obtained confirms the validity of the model with minor discrepancy due to the slight variation in experimental conditions. The activity obtained was 1.4 fold higher than the yield obtained by cake wastes on the 6th day of the fermentation without optimization suggesting the important role of RSM for rapid screening of important process variables in the optimization studies.

Many factors affect enzymatic hydrolysis including the temperature, enzyme dose, substrate concentration and the duration. The effect of reaction temperatures on domestic FW (10%, w/v) hydrolysis using in-situ produced GA was evaluated between 50^{o}C and 90^{o}C (Figure 6). During the first 6 hours, the glucose production was the highest at 70^{o}C (6.59 g L^{-1}) and then it slowed down (Figure 6A). After 6 hours, the glucose production at 50°C and 60^{o}C became higher than that at 70^{o}C. This might be because of enzyme denaturation at temperatures higher than 60^{o}C. These findings are similar to the results reported in the literature. Melikoglu et al. (2013b) evaluated the kinetics of the GA using the same microorganism and reported that the maximum enzyme activity (12 U/mL) was obtained at 60^{o}C and started to decrease at higher temperatures which was due to thermal deactivation of the enzyme. The highest glucose concentration of 10.4 g L^{-1} corresponding to a saccharification degree of 97.9% was obtained at 60^{o}C after 24 hours. Hence, the following studies were conducted at 60^{o}C for 24 hours.

The enzyme concentration also affected the enzymatic hydrolysis. FW hydrolysis speeded with an increase in enzyme concentration especially in the first 6 hours of hydrolysis. The glucose concentration obtained using 2 and 5 U/g FW was similar to the concentration obtained using 10U/g FW after 24 hours (Figure 6B). The effect of substrate loading was also evaluated using FW suspensions within the range of 10 and 50% (w/v) (Figure 6C). Glucose production increased with an increase in substrate concentration. Among the various concentrations, 50% (w/v) FW yielded the highest glucose concentration (52.3 g L^{-1} with a saccharification degree of 98.4%) compared to lower FW concentrations showing that there was no substrate inhibition.

4 Conclusions

This study demonstrated the feasibility of effective production of GA with SSF using FWs as sole nutrient source. GA with the highest activity was produced from cake waste using SSF by *A. awamori*. The optimum conditions for GA production from cake waste were determined as initial pH of 7.9, initial moisture content of 69.6%, inoculum loading of $5.2*10^5$/gs and incubation time of 6 days. Under these conditions, GA activity of 108.47 U/gds was obtained. This study showed that waste cakes could be ideal raw materials for production of high-activity enzymes through SSF. The produced enzyme solution can be a potential candidate for the saccharification of FW, so it can significantly reduce the process cost because commercial enzymes are not purchased. The saccharification degree obtained during the hydrolysis may be one of the best reported till date and the glucose concentration obtained is sufficient enough to produce various kinds of biofuels.

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