Optimisation of alkaline pretreatment conditions of orange and plantain peels for polygalacturonase production by *Aspergillus awamori* CICC 2040

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Abstract

This study investigated the optimisation of alkaline pretreatment of orange and plantain peels for polygalacturonase (PG) production by *Aspergillus awamori* CICC 2040 using response surface methodology. The factors evaluated were particle size, PS (< 0.4250, 0.4250 < PS < 0.8025 and 0.8025 < PS < 1.1800 mm), NaOH molarity (0.010, 0.055, and 0.100 M), and time (1.0, 6.5, and 12.0 h). These factors were interacted to determine the most suitable combinations for maximum polygalacturonase activity (MPA). The pretreated orange and plantain peel powders were inoculated with 10⁶ spores/mL *Aspergillus awamori* CICC 2040 was incubated at 28 °C for 5 days, and crude PG was extracted and its activity determined. The alkaline pretreatment combinations that gave MPA were <0.4250 mm, 0.100 M, and 1.0 h, and 0.8025 < PS < 1.1800 mm, 0.010 M, and 1.0 h for orange and plantain peel powders, respectively. The MPA obtained from the pretreated orange and plantain peel powders were 38.46 and 38.82 U/mL, respectively. Optimised alkaline pretreatment conditions of the orange and plantain peels for MPA, produced by *Aspergillus awamori* CICC 2040, were established.

Keywords: *Aspergillus awamori* CICC 2040, Peel, Optimisation, Polygalacturonase, Pretreatment  
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Introduction

Polygalacturonase (PG) (E.C. 3.2.1.15) is a pectinase involved in the degradation of polygalacturonan in plant’s cell walls through the hydrolytic breakdown of glycosidic bonds that bind galacturonic acid moieties (Heerd et al., 2012). Polygalacturonase is employed in food, paper and pulp, animal feed, waste management, and pharmaceutical industries (Tapre and Jain, 2014) and represents 10% of the estimated commercialised enzymes (Anuradha et. al., 2014). Polygalacturonase has been produced via solid-state fermentation (SSF) and submerged fermentation (SF) processes (Khatri et. al., 2015).

In recent times, there has been considerable interest in the use of food wastes and agricultural residues as substrates for the production of bio-products, both from the economic and environmental viewpoints. The utilisation of agricultural residues is increasing due to the high cost of traditional feedstocks (Wadhwa et. al., 2015). The environmental concerns of un-utilised wastes stem from the generation of hazardous materials that are released to nature as a result of their degradation. This results in environmental pollution, which has both short and long term effects (Obi et. al., 2016). Different pectin-rich agricultural by-products have been used as substrates for PG production (Ptitchkina et. al., 2008; Anuradha et. al., 2010; Anuradha et. al.,...
2014) and among these, orange and plantain peels have enjoyed high preferences due to their availability (Li et. al., 2015; Castillo-Isreal et. al., 2015).

The utilisation of agricultural residues and fruit processing wastes as substrates for microorganisms for the subsequent elaboration of bio-products are limited due to the high concentration of lignin, cellulose, and hemicelluloses, which are physical barriers that limit microbial and enzymatic hydrolysis of biomasses (Yu et. al., 2015). Specifically, lignin is known to adsorb enzymes, thereby reducing its degradation efficiency (Ju et. al., 2013). Cellulose has been considered a factor that limits the accessibility of microorganisms to agricultural residues. The severity of this occurrence is dependent on the residue’s surface area, crystalline and amorphous ratio of cellulose as well as its degree of polymerisation (El-shishtawy et. al., 2015). Several studies have demonstrated that the bio-conversion rate of residues is dependent on the properties of cellulose (Li et. al., 2015; Yang et. al., 2017; Lai et. al., 2017).

The properties of enzymes, such as cellulase and xylanase, produced from pretreated agricultural by-products are well documented (Rahnama et. al., 2013; Salihu et. al., 2015). An increase in PG activity was reported for alkaline-pretreated highly lignocellulosic materials, such as wheat straw and palm leaves, by Trichoderma reesei under SSF (El-Shishtawi et. al., 2015). The findings of Li et. al., (2015) also showed an increase in exopectinase activity of microwave-pretreated orange peels using Aspergillus japonicus under SF. However, information on the properties of PG produced by Aspergillus species using pretreated pectin-rich agricultural by-products under SSF is sparse. Furthermore, there is a paucity of information on the optimisation of pretreatment operation conditions of agricultural residues for improved PG production. Therefore, the objective of this study is to optimise the alkaline (NaOH) pretreatment conditions of orange and plantain peels for maximum activity of PG, produced by Aspergillus awamori/CICC 2040, using response surface methodology.

**Materials and Methods**

**Materials**

Peels of orange (Citrus sinensis L.) and plantain (Musa paradisiaca Linn.) were obtained from fruit and vegetable vendors at the premises of the University of Ibadan, Ibadan. The fungal strain, Aspergillus awamori CICC (China Centre of Industrial Culture Collection) 2040, was obtained from China National Research Institute of Food and Fermentation, Beijing, China. All reagents used were of analytical grade.

**Methods**

**Production of orange and plantain peel powders**

The orange and plantain peels were blanched (80 °C for 3 min), rinsed, and dried in a hot air oven (NL9023A, Genlab Ltd, Cheshire, England) at 60 °C for 48 h. The dried peels were milled into powders and sieved into 3 different particle sizes with the aid of 0.4250, 0.8025, and 1.1800 mm sieves (United States Pharmacopoeia Standard Sieves). The powders were packaged in polyethylene containers (ZipLock, China) and stored at -20 °C for subsequent analyses (Adedeji and Ezekiel, 2019).

**Alkaline pretreatment of orange and plantain peels**

The alkaline pretreatment method outlined by Salihu et. al. (2015) was adopted with little modification in the molarity of NaOH. Substrate flours (5% w/v) were treated with NaOH (Loba Chemie, India) at varying pretreatment conditions. Pretreated samples were rinsed with distilled water to a pH value of 7, dried in a hot air oven (NL9023A, Genlab Ltd, Cheshire, England) at 60 °C for 24 h, and stored at -20 ± 2 °C for subsequent analyses.

**Experimental design for alkaline pretreatment variables of orange and plantain peels**

Face centered central composite design under the response surface methodology was used for the evaluation of three independent variables for alkaline pretreatment. The variables were particle size, PS (< 0.4250, 0.4250 < PS < 0.8025, and 0.8025 < PS < 1.1800 mm), NaOH molarity (0.010, 0.055, and 0.100 M) and pretreatment time (1.0, 6.5, and 12.0 h). The factors were interacted to determine the most
suitable combinations for maximum polygalacturonase activity.

Production of polygalacturonase

Culturing of microorganism

The fungal strain was maintained on malt extract agar (MEA) at 28 °C for 6 days. Inoculums for the experiments were prepared from heavily sporulated MEA slants (Adedeji and Ezekiel, 2019).

Solid-state production of polygalacturonase

The solid-state fermentation procedure described by Dey et al., (2014) was adopted. Orange peel powder (OPP) and plantain peel powder (PPP) were mixed with Czapek-doxy medium (2.5 g/L NaNO₃, 1 g/L KH₂PO₄, 0.5 g/L KCl and 0.5 g/L MgSO₄·7H₂O) at pH 4.0 in ratio 1:2 (w/v) in a 250 mL Erlenmeyer flask and autoclaved (121 °C, 15 psi) for 15 min. Subsequently, the substrate was inoculated with 10⁶ spores/mL of the culture and incubated in an incubator (CLN115, Pol Eko Aparatura, Słaski, Poland) at 28 °C for 5 days. After this, the fermented mass was suspended in distilled water to form a 50 g/L suspension. The suspension was placed in an incubator (CLN115, Pol Eko Aparatura, Słaski, Poland) at 30 °C for 1 h and centrifuged (K24IR, Centurion Scientific Ltd., Chichester, UK) at 2200 × g for 10 min. The supernatant was separated using Whatman No. 1 filter and PG assay conducted. The enzyme obtained was stored at -20 °C until required.

Determination of polygalacturonase activity

The activity of PG was determined based on the procedure outlined by Dey et. al. (2014). A 0.5 mL each of PG and 0.5% polygalacturonic acid was prepared in acetate buffer (pH 5.0) and the mixture incubated in a water bath (NL420S, Genlab Ltd., Cheshire, England) at 50 °C for 10 min. Thereafter, a 3 mL of freshly prepared 3, 5-di-nitro salicylic acid solution was added and the mixture heated at 90 °C for 15 min. The mixture was rapidly cooled and absorbance read at 575 nm with the aid of a UV/VIS spectrophotometer (Jenway 6850, Cole-Parmer, Staffordshire, UK). One unit of PG activity was calculated as the amount of enzyme required to release 1 µmol of D-galacturonic acid per minute of reaction (µmol/min). A blank was prepared by mixing a buffer, DNS, and distilled water, and subjected to similar treatment as the enzyme solution. Polygalacturonase activity was expressed in unit of activity per mL (U/mL).

Statistical analyses

The experiments were conducted in triplicates and means of the measured variables were used to generate the response (PG activity). A linear equation was fitted to the data by a multiple regression procedure (Equation 1).

\[ Y = a_0 + \sum_{i=1}^{n} a_i X_i + \sum_{i=1}^{n} \alpha_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \alpha_{ij} X_i X_j \]  

(1)

Where Y represents predicted response, PG activity (U/mL), X₁, X₂, X₃, X₄ are independent variables, ai is a constant, and a₀, a₁, a₂, and a₃ are linear, squared and interaction effects, respectively. The multiple regression model was evaluated with the aid of analysis of variance (ANOVA) and the quality of fit was tested by determining the coefficient of correlation (R²). These were achieved using the Minitab software, version 16.2.1 (Stat-Ease Inc., Minneapolis, USA).

Results and Discussion

Optimisation of alkaline pretreatment conditions of orange and plantain peels for maximum polygalacturonase activity

The activity of the PG obtained at different combinations of alkaline pretreatment variables for OPP and PPP is presented in Table 1. Polygalacturonase activity ranged from 11.05 (Run 2) to 38.46 U/mL (Run 3), and 12.59 (Run 7) to 38.82 U/mL (Run 2) on OPP and PPP, respectively. For OPP, a maximum PG activity of 38.46 U/mL was obtained at Run 3 i.e. < 0.425 mm, 0.1 M NaOH and 1 h. However, a maximum PG activity of 38.82 U/mL was obtained from Run 2 i.e. 0.8025 < PS < 1.1800 mm, 0.01 M and 1 h for PPP. The difference in the chemical composition of the orange and plantain peels might have been responsible for the variation. According to Li et. al., (2015), chemical constituents, such as pectin and sugars, partly determine enzymatic activity. Earlier studies have also shown the differences in the activity of PG obtained from different substrates (Anuradha et. al., 2010; Padma et. al., 2012; Heerd et. al., 2014). The activity of...
PG varied significantly (p<0.05) depending on the particle size of the peel powders, the molarity of NaOH, and pretreatment time. The variation validated the need for optimisation of substrate pretreatment before enzyme production. The low PG activity recorded for Runs 2, 6, and 10 for OPP and 7 and 9 for PPP could be due to the low proliferation of *Aspergillus awamori* CICC 2040 under the prevailing conditions. The slow microbial growth that results from the unfavourable nutrient and non-nutrient based factors reduces the rate of metabolism and hence, the yield of the bioproducts (Akinpelu et al., 2016). The experimental and predicted PG activity obtained at each pretreatment combination was significantly close. For example, the experimental and predicted PG activity obtained from OPP at Run 8 were 38.46 and 38.10 U/mL, respectively. This is an indication of a high degree of positive correlation. This implied suitability of the response surface methodology in the analysis of pretreatment variables for improved PG activity.

The significance (p < 0.05) of each variable as assessed by ANOVA is presented in Table 2. High F-value of 71.42 and 21.88 for PG activity produced using OPP and PPP, respectively, showed the significance of the model. This is corroborated by a very low p-value of 0.00 and non-significant (p > 0.05) lack of fit. \( R^2 \) and \( R^2 \) (adjusted) values of 98.47% and 97.09%, respectively, for PG activity from pretreated OPP validated that the model was in good agreement with the experimental data. Also, \( R^2 \) and \( R^2 \) (adjusted) values of 95.17% and 90.82%, respectively, showed good agreement of the model with PG activity obtained from pretreated PPP. The findings suggest the models covered 98.47% and 95.17% of the variations in the predicted and experimental data of the PG obtained from the pretreated OPP and PPP, respectively.

![Image](image-url)

For the activity of PG produced from pretreated OPP, one term each of linear (\( X_1 \) - particle size), quadratic (\( X_1^2 \) - particle size × particle size) and two cross-product combinations, \( X_1X_2 \) (particle size × NaOH molarity), and \( X_2X_3 \) (NaOH molarity × time) were found to be significant. Therefore, the remaining non-significant (p > 0.05) terms were deleted from the regression equation (Equation 2).

\[
Y = 43.47 - 1.34X_3 - 20.47X_1^2 + 81.83X_1X_2 + 1.30X_1X_3
\]  

(2)

The significant (p<0.05) terms for PG activity obtained from the pretreated PPP were \( X_3 \), \( X_1^2 \), \( X_1X_2 \), and \( X_2X_3 \) (Equation 3).

\[
Y = 15.31 - 0.80X_3 - 0.09X_3^2 + 87.42X_1X_2 + 7.38X_2X_3
\]  

(3)

**Effect of alkaline pretreatment of orange and plantain peels on polygalacturonase activity**

The alkaline pretreatment variables, substrate particle size, NaOH molarity, and pretreatment time, had a significant (p<0.05) effect on PG activity. Figure 1a and b show the contour plots for the interactive effects of substrate particle size and molarity of NaOH on the PG activity obtained from OPP and PPP, respectively. The maximum PG activity was obtained at the lowest OPP’s particle size (< 0.4250 mm) and the highest NaOH molarity (0.10 M) and vice versa. Maeda et al., (2011) also reported an increase in the enzymatic hydrolysis of sugarcane bagasse pretreated with 1-4% NaOH and a reduction at NaOH concentration of 0.5%. The reduction in particle size increases substrates’ surface area, which in turn increases the bioavailability of nutrients for microbial proliferation, hence, improved yield of bio-products (Salihu et al., 2015). Low PG activity was obtained at low NaOH molarity probably due to the non-inhibition of chemical barriers at low NaOH concentration. For example, OPP is a rich source of limonene and essential oils, which are inhibitory to enzymatic hydrolysis (Li et al., 2015; Wu et al., 2017). However, the maximum PG activity was produced from PPP, with 0.8025<PS<1.18 mm particle size, pretreated with 0.01 M NaOH. This is advantageous because of the reduction in the NaOH consumption and the energy required for size reduction. Low et al., (2015) also reported a reduction in the glucanase activity produced using banana pseudostem pre-treated with highly concentrated NaOH. The low PG activity produced from PPP pretreated at the high molar concentration of NaOH could be due to the destruction of carbohydrates that might have resulted in the production of inhibitory compounds (Pandey and Negi, 2015).
Table 1: The activity of polygalacturonase from *Aspergillus awamori* CICC 2040 on alkaline pretreated orange and plantain peels

| Run | Particle size (mm) | Molarity (M) | Time (h) | Orange peel powder | Plantain peel powder |
|-----|--------------------|--------------|----------|--------------------|---------------------|
|     |                    |              |          | Experimental       | Predicted            |
|     |                    |              |          |                     |                     |
| 1   | <0.4250            | 0.010        | 1.0      | 35.23              | 36.82               |
| 2   | 0.8025<PS<1.1800   | 0.010        | 1.0      | 11.05              | 10.68               |
| 3   | <0.4250            | 0.100        | 1.0      | 38.46              | 38.10               |
| 4   | 0.8025<PS<1.1800   | 0.100        | 1.0      | 16.73              | 17.52               |
| 5   | <0.4250            | 0.010        | 12.0     | 30.05              | 29.36               |
| 6   | 0.8025<PS<1.1800   | 0.010        | 12.0     | 13.59              | 14.05               |
| 7   | <0.4250            | 0.100        | 12.0     | 33.53              | 34.00               |
| 8   | 0.8025<PS<1.1800   | 0.100        | 12.0     | 25.74              | 24.24               |
| 9   | <0.4250            | 0.055        | 6.50     | 34.09              | 33.07               |
| 10  | 0.8025<PS<1.1800   | 0.055        | 6.50     | 14.50              | 15.12               |
| 11  | 0.4250<PS<0.8025   | 0.010        | 6.50     | 26.47              | 25.48               |
| 12  | 0.4250<PS<0.8025   | 0.100        | 6.50     | 30.62              | 31.22               |
| 13  | 0.4250<PS<0.8025   | 0.055        | 1.00     | 29.01              | 27.36               |
| 14  | 0.4250<PS<0.8025   | 0.055        | 12.00    | 25.74              | 27.00               |
| 15  | 0.4250<PS<0.8025   | 0.055        | 6.50     | 26.35              | 27.02               |
| 16  | 0.4250<PS<0.8025   | 0.055        | 6.50     | 26.11              | 27.02               |
| 17  | 0.4250<PS<0.8025   | 0.055        | 6.50     | 26.78              | 27.02               |
| 18  | 0.4250<PS<0.8025   | 0.055        | 6.50     | 27.01              | 27.02               |
| 19  | 0.4250<PS<0.8025   | 0.055        | 6.50     | 27.57              | 27.02               |
| 20  | 0.4250<PS<0.8025   | 0.055        | 6.50     | 27.50              | 27.02               |
Table 2: Analysis of variance of fitted models of polygalacturonase from alkaline pretreated orange and plantain peels

| Source        | DF  | Sum of square | Mean square | F-value | P-value | DF  | Sum of square | Mean square | F-value | P-value |
|---------------|-----|---------------|-------------|---------|---------|-----|---------------|-------------|---------|---------|
| Orange peel powder |     |               |             |         |         |     |               |             |         |         |
| Model         | 9   | 994.58        | 110.51      | 71.42   | 0.00    | 9   | 880.10        | 97.79       | 21.88   | 0.00    |
| $X_1$         | 1   | 805.51        | 0.31        | 0.20    | 0.67    | 1   | 689.40        | 13.63       | 3.05    | 0.11    |
| $X_2$         | 1   | 82.31         | 6.25        | 4.04    | 0.072   | 1   | 85.32         | 14.19       | 3.17    | 0.11    |
| $X_3$         | 1   | 0.33          | 18.74       | 12.11   | 0.006   | 1   | 2.83          | 6.63        | 8.48    | 0.03    |
| $X_1^2$       | 1   | 20.68         | 23.40       | 15.12   | 0.003   | 1   | 13.89         | 1.47        | 0.33    | 0.58    |
| $X_2^2$       | 1   | 6.22          | 4.89        | 3.16    | 0.106   | 1   | 15.90         | 3.76        | 0.84    | 0.38    |
| $X_3^2$       | 1   | 0.07          | 0.07        | 0.05    | 0.832   | 1   | 21.94         | 21.94       | 5.91    | 0.04    |
| $X_1X_2$      | 1   | 15.46         | 15.46       | 9.99    | 0.01    | 1   | 17.64         | 17.64       | 4.95    | 0.04    |
| $X_1X_3$      | 1   | 58.64         | 58.64       | 37.90   | 0.00    | 1   | 0.22          | 0.22        | 0.05    | 0.827   |
| $X_2X_3$      | 1   | 5.64          | 5.65        | 3.65    | 0.085   | 1   | 32.97         | 32.97       | 7.38    | 0.022   |
| Residual error| 10  | 15.47         | 1.55        |         |         | 10  | 44.70         | 4.47        |         |         |
| Lack of fit   | 5   | 13.71         | 2.74        | 7.79    | 0.121   | 5   | 44.42         | 8.88        | 2.65    | 0.193   |
| Pure error    | 5   | 1.76          | 0.35        |         |         | 5   | 0.28          | 0.06        |         |         |
| Total         | 19  | 1010.65       |             |         |         | 19  | 9224.80       |             |         |         |

$R^2$  98.47%  95.17%

$R^2$ (adj) 97.09%  90.82%

$X_1$- particle size; $X_2$- NaOH molarity; $X_3$- pretreatment time; DF- degree of freedom
Figures 1c and d show the interactive effect of substrate particle size and pretreatment time on PG activity from pretreated OPP and PPP, respectively. The results show that the variables had a significant (p < 0.05) effect on PG activity. This corroborated the findings of Han et al. (2012) who reported improved enzymatic hydrolysis of wheat straw following the synergistic effect of particle size and pretreatment time. Improved enzymatic activity is related to an increase in the adsorption of microorganisms on carbohydrate fibers, which is a consequence of surface area and time. The maximum PG activity was obtained from OPP with a particle size of < 0.4250 mm pretreated for 1 h. Besides, PPP pretreated for 1 h gave the maximum PG activity, however, at a substrate particle size of 0.8025 < PS < 1.1800 mm. The activity of PG decreased as the substrate pretreatment time increased. Figure 1e shows the effect of NaOH molarity and pretreatment time on the activity of PG produced on OPP. Pre-treatment time had no significant effect (p > 0.05) on PG activity produced from the OPP pretreated with 0.1 M NaOH. The maximum PG activity (> 32 U/mL) was recorded at 0.1 M NaOH irrespective of pretreatment time. However, at a lower NaOH molarity, high pretreatment time (> 4 h) resulted in a reduction in PG activity. A reduction in enzymatic hydrolysis was also reported for wheat straw due to long (> 1½ h) alkaline pretreatment time. The reduction in PG activity at prolonged pretreatment time could be due to the destruction of inducer substrates, such as pectin and sugars, owing to the increased saponification rate of their ester bonds (Santos et al., 2011). For the PG produced from PPP, the maximum PG activity was obtained at low NaOH molarity and high pretreatment time (Figure 1f). Sahare et al. (2012) also reported maximum enzymatic hydrolysis for corncob subjected to prolonged (> 4 h) alkaline pretreatment.

**Model validation for alkaline pretreatment of orange and plantain peels**

The adequacy of the regression equation was validated by conducting the experiment using the terms of the optimised conditions. The percentage deviation was 0.94 and 3.40% for OPP and PPP, respectively. Since the values were less than 5.0%, it can be concluded, therefore, that the model was suitable for the description of the experiment (Ezekiel and Aworh, 2018).

**Conclusions**

This study established the alkaline pretreatment conditions of orange and plantain peels for maximum activity of PG produced by *Aspergillus awamori* CICC 2040. The alkaline pretreatment combinations that gave the maximum polygalacturonase activity were <0.4250 mm, 0.100 M, and 1.0 h and 0.8025 < PS < 1.1800 mm, 0.010 M, and 1.0 h for orange and plantain peel powders, respectively. The maximum polygalacturonase activity obtained from pretreated orange and plantain peel powders were 38.46 and 38.82 U/mL, respectively.

**Acknowledgement**

Special thanks go to the Tertiary Education Trust Fund of the Federal Republic of Nigeria for the Research Fellowship (FUW/REG/S.39/vol.1/024) granted to Adedeji, O.E.
Figure 1. Effect of alkaline pretreatment condition on PG activity (a) effect of particle size and NaOH molarity on PG activity produced using pretreated orange peel, (b) effect of particle size and NaOH molarity on PG activity produced using pretreated plantain peel, (c) effect of particle size and time on PG activity produced using pretreated orange peel, (d) effect of particle size and time on PG activity produced using plantain peel, (e) effect of NaOH molarity and time on PG activity produced using orange peel, (f) effect of NaOH molarity and time on PG activity produced using plantain peel.

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