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OPTIMISATION OF MICROWAVE PRETREATMENT CONDITIONS OF ORANGE AND PLANTAIN PEELS FOR POLYGALACTURONASE PRODUCTION BY *ASPERGILLUS AWAMORI* CICC 2040

Adedeji, O.E.[1,2] and Ezekiel, O.O.[2]

[1]Department of Food Science and Technology, Federal University Wukari, PMB 1020, Wukari, Nigeria,
[2]Department of Food Technology, University of Ibadan, Ibadan, Nigeria.
[*]Corresponding author: jdadedeji@gmail.com

ABSTRACT

This study investigated the optimisation of microwave pretreatment of orange and plantain peels for polygalacturonase (PG) production, by Aspergillus awamori CICC 2040, using response surface methodology. The microwave pretreatment factors interacted were particle size (PS) (<0.4250, 0.4250<PS<0.8025, and 0.8025<PS<1.1800 mm), microwave power (240, 480 and 720 W) and time (2.50, 6.25, and 10.00 min.). These factors were interacted to determine combinations for maximum polygalacturonase activity (MPA). Pretreated orange and plantain peel powders were inoculated with 10^6 spores/mL Aspergillus awamori CICC 2040, incubated at 28^{o}C for 5 days, and crude polygalacturonase was extracted and its activity determined. Same microwave pretreatment combination, 0.8025<PS<1.1800 mm, 720 W and 10.00 min, gave MPA for orange and plantain peels. The MPA from orange and plantain peels was 26.21 and 26.72 U/mL, respectively. F and p values obtained for orange peel powder were 35.42 and 0.00, respectively while those obtained for plantain peel powder were 5.71 and 0.006, respectively. R^2 and R^2 (adjusted) of 96.96 and 94.22%, respectively were obtained for PG activity produced using orange peel powder while 90.71 and 79.04% were recorded for PG activity produced using plantain peel powder. Optimised microwave pretreatment conditions of orange and plantain peels for MPA from Aspergillus awamori CICC 2040 were established.

***Keywords**: Aspergillus awamori, Fruit peel, Microwave, Optimisation, Polygalacturonase, Pretreatment,*

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 used in food, paper and pulp, animal feed, waste management, and pharmaceutical industries (Tapre and Jain, 2014) and represents 10% of estimated commercialised enzymes (Anuradha et al, 2014). Polygalacturonase has been produced via solid-state fermentation and submerged fermentation 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 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 concern of un-utilized wastes stems 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 (Ptichkina et al, 2008; Anuradha et al, 2010; Anuradha et al, 2014) and among these, orange and plantain peels have enjoyed high preference due to their wide availability (Li et al, 2015; Castillo-Isreal et al, 2015).

The utilisation of agricultural residues and fruit processing wastes as substrates for microorganisms for subsequent elaboration of bio-products are limited due to 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 enzyme thereby reducing its degradation efficiency (Ju et al, 2013). Cellulose has been considered a factor that limits accessibility of microorganisms to agricultural residues. The severity of this occurrence is dependent on residue's surface area, crystalline, and amorphous ratio of cellulose as well as its degree of polymerisation (El-shishtawy et al, 2015). Previous studies demonstrated

that the bio-conversion rate of residues is dependent on properties of cellulose (Li et al, 2015; Yang et al, 2017; Lai et al, 2017).

Properties of enzymes e.g. cellulase and xylanase produced from pretreated agricultural by-products are well documented (Rahnama et al, 2013; Salihu et al, 2015). Increased PG activity was reported for alkaline-pretreated highly ligno-cellulosic materials (wheat straw and palm leaves) using *Trichoderma reesei* under SSF (El-Shishtawi et al, 2015). The findings of Li et al (2015) showed that the microwave pretreatment of orange peel before the production of exo-pectinase, by *Aspergillus japonicus* under submerged fermentation, resulted in a significant increase in the activity of the enzyme. However, information on the properties of PG produced from *Aspergillus* species using pretreated pectin-rich agricultural by-products under solid-state fermentation is sparse. Furthermore, there is paucity of information on the optimisation of microwave pretreatment operation conditions of agricultural residues for improved PG production. Hence, the objective of this study was to optimise microwave pretreatment conditions of orange and plantain peels for maximum PG activity from *Aspergillus awamori* CICC 2040 using response surface methodology.

MATERIALS AND METHODS

Materials

Peels of orange (*Citrus sinensis* L. Osbeck) and plantain (*Musa paradisiaca* Linn.) were obtained from small-scale food processing factories in Ibadan, Nigeria. 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 ^{o}C for 3 min), rinsed, and dried in a hot air oven (NL9023A, Genlab Ltd, Cheshire, England) at 60 ^{o}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 ^{o}C for subsequent analyses (Adedeji and Ezekiel, 2019).

Microwave pretreatment of orange and plantain peel powders

The microwave pre-treatment was carried out based on the procedure reported by Inan et al (2016). Substrate flour (5% w/v) was added to distilled water in a container and the mixture was treated in a laboratory microwave oven (NX-802, Nexus, Beijing, China, with 25 L capacity, 800 W power output and frequency of 2450MHz) at varying power level for different pre-treatment time. Thereafter, the residue was oven-dried (NL9023A, Genlab Ltd, Cheshire, England) at 60 ^{o}C to a final moisture content of 10 %.

Experimental design for microwave pretreatment of orange and plantain peels

Face centered central composite design under the response surface methodology (RSM) was used for the evaluation of three independent variables. Factors interacted were particle size, PS (<0.4250, 0.4250<PS<0.8025, 0.8025<PS<1.1800 mm), microwave power (240, 480, and 720 W) and pretreatment time (2.50, 6.25, and 10.00 min). The factors were interacted to determine combination for maximum polygalacturonase activity.

Culturing of microorganism

The fungal strain was maintained on malt extract agar (MEA) at 28^{o}C for 6 days. Inoculums for the experiments were prepared from heavily sporulated MEA slants.

Solid state production of polygalacturonase

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-dox medium (2.5 g/L $NaNO_3$, 1 g/L KH_2PO_4, 0.5 g/L KCl and 0.5 g/L $MgSO_4.2H_2O$) at pH 4.0 in ratio 1:2 (w/v) in a 250 mL Erlenmeyer flask and autoclaved (121^{o}C, 15 psi) for 15 min. Subsequently, the substrate was inoculated with 10^6 spores/mL of the culture and incubated in an incubator (CLN115, Pol Eko Aparatura, Poland) at 28^{o}C for 5 days. After this, 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, Poland) at 30 ^{o}C for 1 h and centrifuged (K24IR, Centurion Scientific Ltd, UK) at 2200 × g for 10 min. The supernatant was separated using Whatman No. 1 filter and PG assay conducted. Enzyme was stored at -20^{o}C until required.

ANALYSES

Determination of polygalacturonase activity

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 (NL42OS, Genlab Ltd, Cheshire, England) at 50 ^{o}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 ^{o}C for 15 min. The mixture was rapidly cooled and absorbance read at 575 nm with the aid of 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 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

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

$$Y = \alpha_0 + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n} \alpha_{ii} {X_i}^2 + \sum_{i=1}^{n-1} \sum_{i=1}^{n} \alpha_{ij} X_i X_j \quad (1)$$

Where Y represents predicted response, PG activity (U/mL), X_1, X_2, X_3…….Xn are independent variables, α_0 is a constant, and αi, αii and αij are linear, squared and interaction effects, respectively. Multiple regression model was evaluated with the aid of analysis of variance and quality of fit was tested by determining the coefficient of determination (R^2). These were achieved using the Minitab software, version 16.2.1 (Stat-Ease Inc., USA).

RESULTS AND DISCUSSION

Optimisation of microwave pretreatment condition of orange and plantain peels for polygalacturonase production

Table 1 shows the PG activity produced from *Aspergillus awamori* CICC 2040 using microwave (MW) pretreated orange and plantain peels. Polygalacturonase activity ranged from 6.97 (Run 3) to 26.21 U/mL (Run 8) and 10.22 (Run 13) to 26.72 U/mL (Run 8) on OPP and PPP, respectively. Results obtained showed that MW pretreatment variables: substrate particle size, MW power and pretreatment time had significant ($p < 0.05$) effect on PG activity. For OPP and PPP, maximum PG activity of 26.21 and 26.72 U/mL, respectively were obtained at Run 8, which corresponded to a substrate particle size of 0.8025<PS<1.18 mm, MW power of 720 W and pretreatment time of 10 min. The similarity observed may be due to the high efficiency of microwave pretreatment, which probably resulted in substrates with similar properties. Microwave treatment has been described as a technology with a high degree of heating efficiency and uniformity (Nomanbhay et al, 2013). Predicted PG activity of 24.70 and 26.03 U/mL for OPP and PPP, respectively showed that both the experimental and predicted values were highly correlated. The lowest PG activity of 6.97 U/mL was recorded for OPP with <0.425 mm particle size pretreated at 720 W for 2.5 min (Run 3). However, Run 13 which corresponded to PG activity produced from PPP with particle size of 0.8025<PS<1.18 mm, 480W MW power and pretreatment time of 2.5 min gave the lowest value of 10.22 U/mL.

Analysis of variance (Table 2) showed the adequacy of the models in the characterisation of the independent variables. F and p values obtained for OPP were 35.42 and 0.00, respectively while those obtained for PPP were 5.71 and 0.006, respectively. These values implied that the models were significant ($p<0.05$). R^2 and R^2 (adjusted) of 96.96 and 94.22%, respectively were obtained for PG activity produced from OPP while 90.71 and 79.04% were respectively recorded for PG activity produced form PPP. This suggested that 96.96% and 90.71% of the variation in the predicted and experimental data of PG obtained from pretreated OPP and PPP, respectively were covered by the models. For PG activity produced from OPP, X_2 (MW power), X_3 (pretreatment time), X_3^2 (pretreatment time × pretreatment time), and X_2X_3 (MW power × pretreatment time) were significant ($p < 0.05$) in the model. The quadratic equation after deleting the terms that were not significant ($p>0.05$) is presented in Equation 2.

$$Y = 25.89 - 0.069X_2 - 3.83X_3 + 0.17X_3^2 + 0.004X_2X_3 \quad (2)$$

The following terms were significant ($p < 0.05$) in the regression model for PG activity produced from MW-pretreated PPP: X_1 (particle size), X_2 (MW power), X_1^2 (particle size × particle size), X_1X_2 (particle size × MW power) and X_1X_3 (particle size × pretreatment time). The residual terms in the model are shown in Equation 3.

$$Y = 5.71 - 75.93X_1 - 0.08X_2 + 28.60X_1^2 + 0.03X_1X_2 + 1.85X_1X_3 \quad (3)$$

Effect of microwave pretreatment conditions of orange and plantain peels on polygalacturonase activity

The activity of PG as influenced by the synergistic effect of substrate particle size and MW power on OPP and PPP are presented in Figure 1a and b, respectively. The PG activity obtained from both OPP and PPP increased with increasing MW power and substrate particle size. For both peels, maximum PG activity was obtained at the highest boundary of MW power and substrate particle size. Li et al (2015) reported 11.8% increase in exo-pectinase activity from *Aspergillus japonicus* using OPP pre-treated at high MW power of 630 W and substrate particle size of 0.850 mm. According to Woldesenbet et al (2012), microwave radiation at high power level results in accelerated rupturing of substrates, due to high thermal energy dissipation, which makes polysaccharides to be more susceptible to microbial proliferation.

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Table 1. **Activity of polygalacturonase produced by *Aspergillus awamori* on microwave-pretreated orange and plantain Peels**

				PG activity (U/mL)			
	Independent variables			From orange peel		From plantain peel	
Run	Particle size (mm)	Power level (W)	Time (min)	Experimental	Predicted	Experimental	Predicted
1	<0.425	240	2.5	14.04	15.42	22.84	23.68
2	0.8025<x<1.18	240	2.5	25.43	24.27	11.29	10.52
3	<0.425	720	2.5	6.97	6.05	19.69	17.32
4	0.8025<x<1.18	720	2.5	17.12	18.15	13.27	16.19
5	<0.425	240	10	10.13	8.97	22.22	19.44
6	0.8025<x<1.18	240	10	15.04	15.83	14.27	16.79
7	<0.425	720	10	13.55	14.59	15.74	16.66
8	0.8025<x<1.18	720	10	26.21	24.70	26.72	26.03
9	<0.425	480	6.25	7.13	6.79	15.09	18.48
10	0.8025<x<1.18	480	6.25	15.43	16.27	20.56	16.58
11	0.425<x<0.8025	240	6.25	14.99	15.13	15.80	15.99
12	0.425<x<0.8025	720	6.25	14.53	14.88	18.22	17.44
13	0.425<x<0.8025	480	2.5	15.62	15.29	10.22	9.59
14	0.425<x<0.8025	480	10	14.51	15.34	12.36	12.39
15	0.425<x<0.8025	480	6.25	13.06	12.93	13.33	13.45
16	0.425<x<0.8025	480	6.25	13.11	12.93	13.06	13.45
17	0.425<x<0.8025	480	6.25	13.01	12.93	13.25	13.45
18	0.425<x<0.8025	480	6.25	13.16	12.93	13.12	13.45
19	0.425<x<0.8025	480	6.25	13.00	12.93	13.42	13.45
20	0.425<x<0.8025	480	6.25	13.21	12.93	13.35	13.45

Table 2. **Analysis of variance of fitted models of polygalacturonase from microwave-pretreated orange and plantain peels**

		Orange peel					Plantain peel			
Source	DF	Sum of square	Mean square	F-value	P-value	DF	Sum of square	Mean square	F-value	P-value
Model	9	396.79	44.09	35.42	0.00	9	305.55	33.95	5.71	0.006
X_1	1	224.71	12.98	10.43	0.09	1	8.97	109.25	18.37	0.002
X_2	1	0.16	39.52	31.75	0.00	1	5.21	59.74	10.05	0.01
X_3	1	0.01	39.67	31.87	0.00	1	19.60	0.96	0.16	0.70
X_1^2	1	8.26	5.37	4.31	0.07	1	103.79	45.68	7.68	0.02
X_2^2	1	28.38	11.93	9.58	0.01	1	17.49	29.34	4.92	0.051
X_3^2	1	15.68	15.68	12.59	0.005	1	16.64	16.36	2.80	0.125
X_1X_2	1	5.30	5.30	4.26	0.07	1	72.36	72.36	12.17	0.006
X_1X_3	1	1.97	1.97	1.58	0.24	1	55.13	55.13	9.27	0.012
X_2X_3	1	112.28	112.28	90.19	0.00	1	6.37	6.37	1.07	0.325
Residual error	10	12.45	12.45			10	59.47	5.95		
Lack of fit	5	12.41	2.48	353.84	0.227	5	59.37	11.86	604.92	0.183
Pure error	5	0.04	0.01			5	0.10			
Total	19	409.24				19	365.03			
R^2	96.96%					90.71%				
R^2 (adj)	94.22%					79.04%				

X_1- particle size; X_2- Microwave power; X_3- pretreatment time; DF- degree of freedom

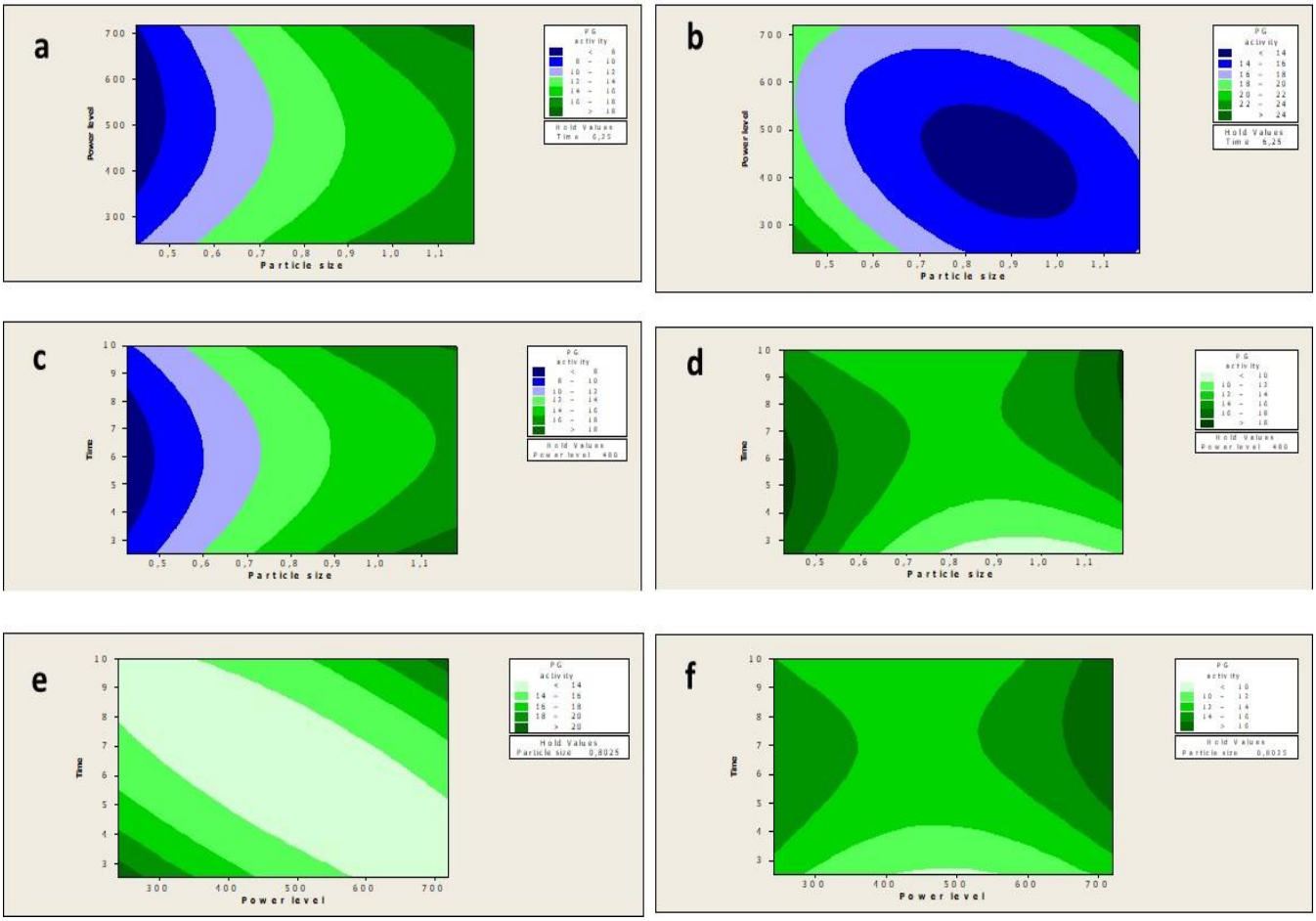

Figure 1. Effect of microwave pretreatment condition on PG activity (a) effect of particle size and NaOH molarity on PG activity produced using pretreated orange peel, (b) effect of particle size and NaOH molarity on PG activity produced using pretreated plantain peel, (c) effect of particle size and time on PG activity produced using pretreated orange peel, (d) effect of particle size and time on PG activity produced using pretreated plantain peel, (e) effect of NaOH molarity and time on PG activity produced using pretreated orange peel, (f) effect of NaOH molarity and time on PG activity produced using pretreated plantain peel.

The iinteractive effect between substrate particle size and pre-treatment time on PG activity produced from OPP and PPP is shown in Figures 1c and d, respectively. The activity of PG produced using OPP increased with increasing particle size irrespective of the pre-treatment time. The findings of Inan et al (2016) also showed an increase in sugar concentration with increasing particle size of barley straw (x < 1.0 mm) irrespective of pre-treatment time (2.5 – 10 min). Low PG activity recorded in OPP with small particle size could be due to the disintegration of pectin molecules, the main inducer substrate for PG production, as a result of intensive size reduction operation. For PG produced using PPP, maximum activity was produced at substrate particle size of >1.0 mm and pre-treatment time of 6 – 10 min.

Figures 1e and f are contour plots showing the interaction between MW power and pretreatment time on the activity of PG. The activity of PG from OPP increased between MW power of 480 and 720 W, and pretreatment time 7 to 10 min. In a similar pattern, PG activity from PPP increased with increasing MW power and pretreatment time. Differences in substrate composition may be responsible for the variation. This study is in agreement with the work of Inan et al (2016) who observed a decrease in total sugar from barley straw pretreated at a microwave power level of 300 W and pretreatment time of 2.5 min. This result did not agree with the report of Tiwari et al (2017) who reported maximum enzymatic hydrolysis of mango peel obtained at MW power of 450 W and exposure time of 4 min.

Model validation for microwave pre-treatment of orange and plantain peels

The percentage deviation for experimental and predicted data of OPP and PPP was 4.81 and 2.58%, respectively. Suitability of the model in fitting the experimental data was thus validated since these values were less than 5.0% (Ezekiel and Aworh, 2018).

CONCLUSIONS

This study established microwave pretreatment conditions of orange and plantain peels for maximum PG activity. The same microwave pretreatment conditions, i.e. 720 W microwave power, 0.8025<PS<1.1800 mm particle size and pretreatment time of 10 min, gave maximum PG activity. Besides, the maximum PG activity from orange (26.21 U/mL) and plantain (26.72 U/mL) peels was very similar. For the PG activity produced from OPP, the terms including X_2 (MW power), X_3 (pretreatment time), X_3^2 (pretreatment time × pretreatment time) and X_2X_3 (MW power × pretreatment time) were significant ($p<0.05$) in the model, however, X_1 (particle size), X_2 (MW power), X_1^2 (particle size × particle size), X_1X_2 (particle size × MW power) and X_1X_3 (particle size × pretreatment time) were significant for the PG activity produced from PPP.

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