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Optimization of the process parameters for the utilization of orange peel to produce polygalacturonase by solid-state fermentation from an *Aspergillus sojae* mutant strain

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Abstract: The effect of orange peel concentration, HCl concentration, incubation time and temperature, and inoculum size on the spore count and activity of polygalacturonase (PG) enzyme produced from *Aspergillus sojae* M3 by solid-state fermentation was screened using 2^k factorial design. Orange peel and HCl concentrations and incubation time were significant factors affecting the responses. Optimum conditions favoring both PG and spore production from *Aspergillus sojae* M3 were determined as 2% orange peel and 50 mM HCl concentrations at 22 °C and 4.3 days of incubation. An overlay plot was constructed for use as a practical chart for production of high enzyme activity (>35.0 U/g substrate) and spore count (9.0×10^8 to 2.0×10^9 spore/mL) by superimposing the contours of PG activity and spore count responses. The accuracy and reliability of the constructed models on the responses was validated with the maximum calculated error rate between the predicted and actual activities at 14.1% and 22.4%, respectively.

Key words: *Aspergillus sojae*, polygalacturonase, spore production, solid-state fermentation, orange peel, response surface methodology

Introduction

Enzymes are a key component in the textile, ethanol, and pharmaceutical industries as well as in the manufacture of food and beverages. Among food enzymes, pectinases are the complex and diverse group of enzymes that degrade pectic substances (1). Pectin and other pectic substances are complex polysaccharides that play an important role in the firmness of plant tissues. Pectinases, which hold a 25% share in global food enzyme sales, are produced from microbiological sources. They have a variety of applications in the food industry such as clarification of fruit juices, extraction of vegetable oils, curing of coffee and cocoa, refinement of vegetable fibers, and manufacture of pectin-free starch (2-4). Therefore, it is important to discover new pectinase-producing microbial strains and optimize their enzyme production conditions in order to meet increasing demand.

Solid-state fermentation (SSF) is considered an attractive alternative method to submerged fermentation (SmF) for the production of industrially demanded enzymes that employ microorganisms (5). SSF was defined by Pandey (6) as a fermentation process involving solids in the absence (or near absence) of free water; among microorganisms that

are capable of growing on solid substrates, only filamentous fungi can grow to a significant extent in the absence of free water (7). SSF has some advantages over SmF such as nonaseptic conditions, use of raw materials as substrates, use of a wide variety of matrices, low capital cost, low energy expenditure, less expensive downstream processing, lower water usage, and lower wastewater output. Higher volumetric productivity, higher concentrations of the products, high reproducibility, less fermentation space, easier control of contamination, absence of foam formation, and generally simpler fermentation media are other benefits of SSF (8,9).

Polygalacturonases (PGs; EC 3.2.1.67) which are involved in the hydrolysis of polygalacturonic acid chains with the addition of water, are the most abundant pectinolytic enzymes (3). Commercial preparations of PGs used in the food industry are usually derived from fungal sources, especially *Aspergillus* and *Penicillium* species (10,11). Many researchers have focused on producing PGs from *Aspergillus* species by using low-cost agroindustrial residues such as wheat and soy bran (12); lemon peel, sorghum stem, and sunflower head (13); wheat (14); orange peels and pulps (15); apple pomace (16); and sugarcane bagasse (17). Moreover, orange peel was utilized as a SSF medium by Ismail (18) on a local strain, *Aspergillus niger* A-20, and it favored both fungal growth and pectinase production. According to statistics from the United States Department of Agriculture (USDA), a total of 22,916,000 t of oranges were produced for processing in 2009 and 2010 by the major orange producer countries of the world (19). Since about 50% (in weight) of orange fruits are discarded as waste peels, membranes, juice vesicles, and seeds in the orange juice production process (20), orange peel is a sustainable by-product for the microbial production of industrially important enzymes by SSF. Moreover, utilization of this by-product to produce a value-added enzyme will also help to solve the pollution problems encountered by orange juice manufacturers.

Ramachandran et al. (21) stated that one of the major applications of SSF is the production of spores. SSF was described as the model technology, dominating over SmF, as it yields a high quantity of quality spores. It also assures spore production with higher productivity, end concentration, and product stability. The production of spores, which are widely used as inoculum in many industrial fermentation processes, is highly affected by substrate selection and many environmental factors (21). Therefore, in addition to enzyme production with high activity, the spore-production potential of a strain in SSF is an important point to consider.

In light of these points, this study investigated both the PG and spore-producing potential of a randomly mutated type of *A. sojae*, coded M3, by SSF, taking the effects of orange peel concentration, HCl concentration, incubation temperature, incubation time, and inoculum size as the main factor variables. Interaction among the above factors is expected. Hence, experimental design and statistical tools were employed to investigate the effects of these factors and their interactions with each other. This study not only serves as a basis for the combination of all the aforementioned parameters, but also introduces a new strain. To the best of our knowledge, this strain has not been previously considered for this purpose in biotechnology.

Materials and methods

Microorganism and propagation

A. sojae ATCC 20235 was purchased in lyophilized form from Procochem Inc., an international distributor of the American Type Culture Collection (ATCC) in Europe. This wild-type culture was randomly mutated using ultraviolet light exposure at Jacobs University gGmbH, Bremen, Germany, and coded as *Aspergillus sojae* M3. According to the modified procedure of De Nicolás-Santiago et al. (22), mutagenesis was performed at an exposure time during which 90% of spores were inactivated (LD_{90}). All experiments in this study were conducted using this strain. The culture was propagated on yeast malt extract (YME) agar plate medium containing malt extract (10 g/L), yeast extract (4 g/L), glucose (4 g/L), and agar (20 g/L) and incubated at 30 °C until sporulation (1 week). Stock cultures of these strains were prepared with 20% glycerol water and stored at −80 °C.

Preparation of inoculum

After the propagation step performed on YME agar using the stock cultures, spore suspensions used as inoculum were obtained on molasses agar slants, optimized by Gögus et al. (23), containing glycerol (45 g/L), peptone (18 g/L), molasses (45 g/L), NaCl (5 g/L), $FeSO_4 \cdot 7H_2O$ (15 mg/L), KH_2PO_4 (60 mg/L), $MgSO_4$ (50 mg/L), $CuSO_4 \cdot 5H_2O$ (12 mg/L), $MnSO_4 \cdot H_2O$ (15 mg/L), and agar (20 g/L). Slant cultures were incubated at 30 °C for 1 week. Spores were harvested from the slants using 5 mL of sterile Tween 80 water (0.02%). The spore suspension was collected in a sterile falcon tube and stored at 4 °C until the inoculation step. The initial spore counts and viability counts were recorded.

SSF fermentation conditions

SSF was conducted in 250-mL Erlenmeyer flasks containing 10 g of sterilized (121 °C/15 min) solid media composed of ground (dried) bitter orange peel (concentrations given in Table 1) and wheat bran. Orange peel and wheat bran were purchased from a local market in Bremen, Germany. The prepared solid media were treated with 7 mL of HCl solution at different concentrations (Table 1). The inoculation was carried out using prepared spore suspension in the range of 10^4 to 2×10^7 total spores at the screening step and 10^4 total spores at the optimization step. The total liquid amount in each flask was 12 mL; 7 mL of distilled water was used for HCl addition and 5 mL of distilled water was used for inoculation to maintain the homogenous spread of spores onto the solid-state media. The inoculated flasks were incubated at various temperatures, and durations are given in Table 1.

Enzyme extraction and spore count

At the end of fermentation, each flask was filled with 100 mL of sterile Tween 80 water (0.02%) and shaken at 150 rpm and 30 °C for 30 min. The flask contents were then filtered through sterile cheese cloth and centrifuged at 4 °C and 6000 rpm for 15 min. The supernatant was separated and immediately used for the enzyme assay.

Total spore counts were obtained by taking into account the extracted volume for each run, which varied between 89 and 99 mL, and were expressed as spore/mL. Spores were counted manually using a Thoma bright-line hemocytometer (Marienfield, Germany). Spores/g substrate was estimated by dividing the total spores obtained into the amount of substrate used in SSF.

Enzyme assay

PG activity was assayed according to the procedure given by Panda et al. (24) using 2.4 g/L of polygalacturonic acid as the substrate at pH 4.0 and 26 °C. The amount of substrate and enzymes used were 0.4 and 0.086 mL, respectively. The amount of enzyme that catalyzes the release of 1 micromole of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions was defined as 1 unit of enzyme activity. Galacturonic acid (Sigma, St Louis, MO, USA) was used as a standard for the calibration curve of PG activity. Enzyme activity was expressed as U/g solid substrate:

Activity (U/g solid substrate) = microgram of galacturonic acid/212.12 × 1/20 × 1/0.086 × 1/10.

Experimental design and statistical analysis

Experimental design and statistical analysis were carried out in 2 steps: screening and optimization of the process variables.

Table 1. The investigated factors and their levels in the experimental designs of screening and optimization processes.

		Actual factor levels				
		Screening		Optimization		
		−1	**+1**	**−1**	**0**	**+1**
Orange peel concentration (%)	X_1	5	30	2	8.5	15
HCl concentration (mM)	X_2	20	200	50	150	250
Incubation temperature (°C)	X_3	30		22	29.5	37
Incubation time (days)	X_4	4	8	3	4.5	6
Inoculum size (total spores)	X_5	10^4	2×10^7		10^4	

In the screening part of this study, the effects of orange peel concentration (X_1), HCl concentration (X_2), incubation time (X_3), and inoculum size (X_4) on spore count and the PG enzyme produced from *Aspergillus sojae* M3 by SSF were investigated. A double replicate 2^k factorial design with 32 experimental runs was employed. The blocking number was 2 since 2 incubators were used.

In the optimization, inoculum size was kept at 10^4 total spores based on the screening results, and a face-centered composite design (FCCD) with 30 runs was employed using the above factor variables in addition to temperature. The blocking number was 3 because 3 incubators were used. A second-order polynomial regression equation was fitted to the response data:

$$Y = \beta_0 + \sum_{i=1}^{\kappa} \beta_i X_i + \sum_{i=1}^{\kappa} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j + \varepsilon$$

where Y is the predicted response, k the number of factor variables, β_0 the model constant, β_i the linear coefficient, X_i the factor variable in its coded form, β_{ii} the quadratic coefficient, β_{ij} the interaction coefficient, and ε the error factor.

Analysis of data and response surface graphics generation was performed with Design Expert 7.0.0 software in both screening and optimization stages. The range and levels of the variables investigated in both screening and optimization are given in Table 1. A natural logarithm transformation was applied to the spore count response values. The following equation was used for coding the actual values of the factors between (−1) and (+1):

x = [actual − (low level + high level)/2)] / [(high level – low level)/2].

Results and discussion

Screening of the factors affecting PG activity and spore count

According to Montgomery (25), the 2^k design is especially useful in factor screening experiments when there are many factors to be investigated since it provides the smallest number of runs with *k* factors that can be studied in a complete factorial design. Therefore, in the screening step of this study, a 2^4 factorial experimental design with double replicate was employed in order to investigate the effects of orange peel concentration, HCl concentration, incubation time, and inoculum size and their interactions on PG activity and spore count. According to the results, maximum PG activity was 42.3 U/g substrate at 5% orange peel and 200 mM HCl concentrations, 4 days of incubation, and 10^4 total spore inoculum levels.

ANOVA results indicated that the constructed model is significant with a P-value of 0.0004, and the most important factors affecting PG activity were the orange peel concentration (X_1), HCl concentration (X_2), and incubation time (X_3) main factors and the orange peel concentration-incubation time (X_1X_3) interaction. As a result of these findings, we decided to study these factors in order to optimize conditions and maximize PG activity even further.

The results showed that neither inoculum size nor its interactions have a significant effect on PG activity. Based on this information, in the optimization segment of this study, the inoculum size was fixed at 10^4 total spores, where the maximum PG activity was obtained.

ANOVA results for spore count response indicated that orange peel concentration (X_1) and incubation time (X_3), incubation time-inoculum size (X_3X_4), orange peel concentration-HCl concentration-incubation time ($X_1X_2X_3$), orange peel concentration-HCl concentration-inoculum size ($X_1X_2X_4$), HCl concentration-incubation time-inoculum size ($X_2X_3X_4$), and the interaction of all 4 factors ($X_1X_2X_3X_4$) have significant effects on total spore count. The maximum spore count was 1.2×10^8 spores/mL at 30% orange peel concentration, 200 mM HCl concentration, and 8 days of incubation using 10^4 total spore inoculum levels.

Optimization of the factors affecting PG activity and spore count

The results of the screening step of this study show that orange peel concentration, HCl concentration, and incubation time were the most important factors affecting PG activity. In addition to these variables, another factor variable (incubation temperature), which was excluded in the screening step due to its known significant effect, was included in the optimization step in order to determine the optimum temperature range and its possible interactive effect

on the other variables. In fact, Krishna (8) stated that the most important of all the physical variables affecting SSF performance is temperature, because growth and production of enzymes or metabolites are usually sensitive to temperature. Furthermore, many researchers have observed that incubation temperature is a major parameter affecting the production of pectinases in SSF (8,13,26). Patil and Dayanand (13) explained this by pointing out that temperature is known to influence the metabolic rate of the organism involved in the process, and this determines the amount of the end product. It is well known that fungi can grow from 20 to 55 °C; nevertheless, the optimum growth temperature may be different from the optimum for product formation (27). Hence, all of these variables were taken into consideration in the optimization step, and their effects on PG activity and spore count were investigated using FCCD (Table 2).

Table 2. FCCD design and experimental results of PG enzyme activity and spore count responses.

Run	Block (incubator number)	X_1 (%)	X_2 (mM)	X_3 (°C)	X_4 (days)	PG activity (U/g substrate)	Spore count (spores/mL)
1	1	2.0	250	37.0	6.0	32.4	1.1×10^8
2	1	15.0	50	22.0	3.0	32.2	2.2×10^8
3	1	8.5	150	29.5	4.5	26.8	9.1×10^8
4	1	15.0	250	22.0	6.0	28.9	1×10^9
5	1	8.5	150	29.5	4.5	30.3	1.1×10^9
6	1	2.0	50	37.0	3.0	30.9	7.3×10^7
7	1	15	250	37.0	3.0	30.8	5×10^7
8	1	2.0	250	22.0	3.0	38.4	2.7×10^8
9	1	2.0	50	22.0	6.0	27.6	8.8×10^8
10	1	15.0	50	37.0	6.0	31.6	7.9×10^7
11	1	15.0	250	22.0	3.0	34.5	2.7×10^8
12	2	2.0	250	37.0	3.0	31.0	7×10^7
13	2	15.0	250	37.0	6.0	42.5	8.9×10^7
14	2	8.5	150	29.5	4.5	40.1	1×10^9
15	2	15.0	50	37.0	3.0	31.7	1×10^8
16	2	2.0	250	22.0	6.0	34.0	8.6×10^8
17	2	2.0	50	22.0	3.0	40.7	2.2×10^8
18	2	2.0	50	37.0	6.0	29.1	7.3×10^7
19	2	8.5	150	29.5	4.5	31.6	1.1×10^9
20	2	15.0	50	22.0	6.0	32.7	8.4×10^8
21	3	8.5	150	29.5	4.5	32.2	7.9×10^8
22	3	15.0	150	29.5	4.5	34.6	6.2×10^8
23	3	8.5	150	29.5	4.5	36.8	1.9×10^9
24	3	8.5	50	29.5	4.5	38.0	1.1×10^9
25	3	8.5	150	29.5	3.0	34.7	3.9×10^8
26	3	8.5	150	37.0	4.5	37.4	6×10^7
27	3	8.5	150	29.5	6.0	24.6	5.5×10^8
28	3	2.0	150	29.5	4.5	38.8	9.9×10^8
29	3	8.5	250	29.5	4.5	33.2	8.3×10^8
30	3	8.5	150	22.0	4.5	41.7	5.5×10^8

Levels of the factors chosen for the optimization process were redetermined according to results obtained at the screening step. It was observed in the screening step that orange peel concentration and incubation time at their low levels and HCl concentration at its high level led to higher PG activity. Based on this information, orange peel concentration, incubation time, and HCl concentration were investigated at 2%-15%, 3-6 days, and 50-250 mM ranges, respectively, in the optimization step. Additionally, the range of the incubation temperature investigated was 22-37 °C. These levels were also in accordance with the optimization of the factors affecting the spore count response.

The most important factors affecting PG activity were selected ($P < 0.1000$) according to the ANOVA results of the responses and a model was developed. This analysis indicated that incubation time-incubation temperature (X_3X_4), orange peel concentration-incubation temperature (X_1X_4), and orange peel concentration-incubation time (X_1X_3) interactions and the quadratic forms of incubation time (X_3^2) and temperature (X_4^2) were the most important factors. Orange peel concentration (X_1), incubation time (X_3), and incubation temperature (X_4) main factors were found to be insignificant terms, but they were included in the model because of their strong interactions with incubation time and temperature (i.e. X_3X_4, X_1X_3, and X_1X_4). HCl concentration (X_2) was not included in the model due to its insignificant effect on PG activity.

The P-value of the model, according to ANOVA, was 0.0138. This indicated that the constructed model was significant and that the terms included in this model had an important effect on PG activity. Moreover, the lack-of-fit value of the model (0.8343) proved the validity of the factors included in the model. The model equation that expressed PG activity in terms of coded factors was as follows:

$$\text{PG activity} = 33.42 - 0.19X_1 - 1.19X_3 - 0.73X_4 + 1.52X_1X_3 + 1.59X_1X_4 + 2.12X_3X_4 - 4.73X_3^2 + 5.14X_4^2.$$

The response surface plots obtained according to the above model can be seen in Figure 1. Figure 1A shows that high PG activity was obtained at a middle level of time and a low level of orange peel concentration. Nevertheless, a low level of orange peel concentration and both high and low temperature levels resulted in high PG activity (Figure 1B). According to Figure 1C, at the middle level of time and both high and low levels of temperature, PG activity was also high.

The improvement in PG activity with the optimization process relative to the screening process can be clearly observed by comparing histograms of PG activity values obtained at the screening (Figure 2A) and optimization (Figure 2B) steps. The PG activity values of the screening step were obtained in the range of 0-40 U/g substrate and increased to 25-42.5 U/g substrate at the optimization step. This improvement was also pronounced in the mean activity values (14.9 U/g substrate for screening and 33.7 U/g substrate for optimization step) presented in Figures 2A and 2B.

It was concluded from ANOVA results from the other response, spore count, of the FCCD that incubation temperature (X_3) and time (X_4) main factors, their interaction with each other (X_3X_4), and the quadratic forms of incubation time (X_3^2) and temperature (X_4^2) were the most important factors affecting this response. Therefore, the mathematical model developed for spore count in terms of coded factors is as follows:

$$\ln(\text{Spore count}) = 20.75 - 0.92\,X_3 + 0.35X_4 - 0.28X_3X_4 - 1.37\,X_3^2 - 0.43X_4^2.$$

Compared to our previous SSF study using wild type *Aspergillus sojae* (28) where maximum PG activity was 31.7 U/g solid, the investigated factors and their levels in this study improved PG activity by at least 25%, which provides a rough estimate as to the PG-producing potential of *Aspergillus sojae* M3. Similarly, this optimization process increased spore count production by approximately 56 times relative to our previous SSF study (28). These improvements may be attributed to the mutation and screening process, the optimization routine, and the change in cultivation system.

In order to provide the end user with certain alternatives depending on their interest, the following criteria, summarized in Table 3, were used for numerical optimization of the 2 responses. The aim of Criterion 1 was to reach maximum PG activity in PG production processes with no limitation on spore production. In Criterion 2,

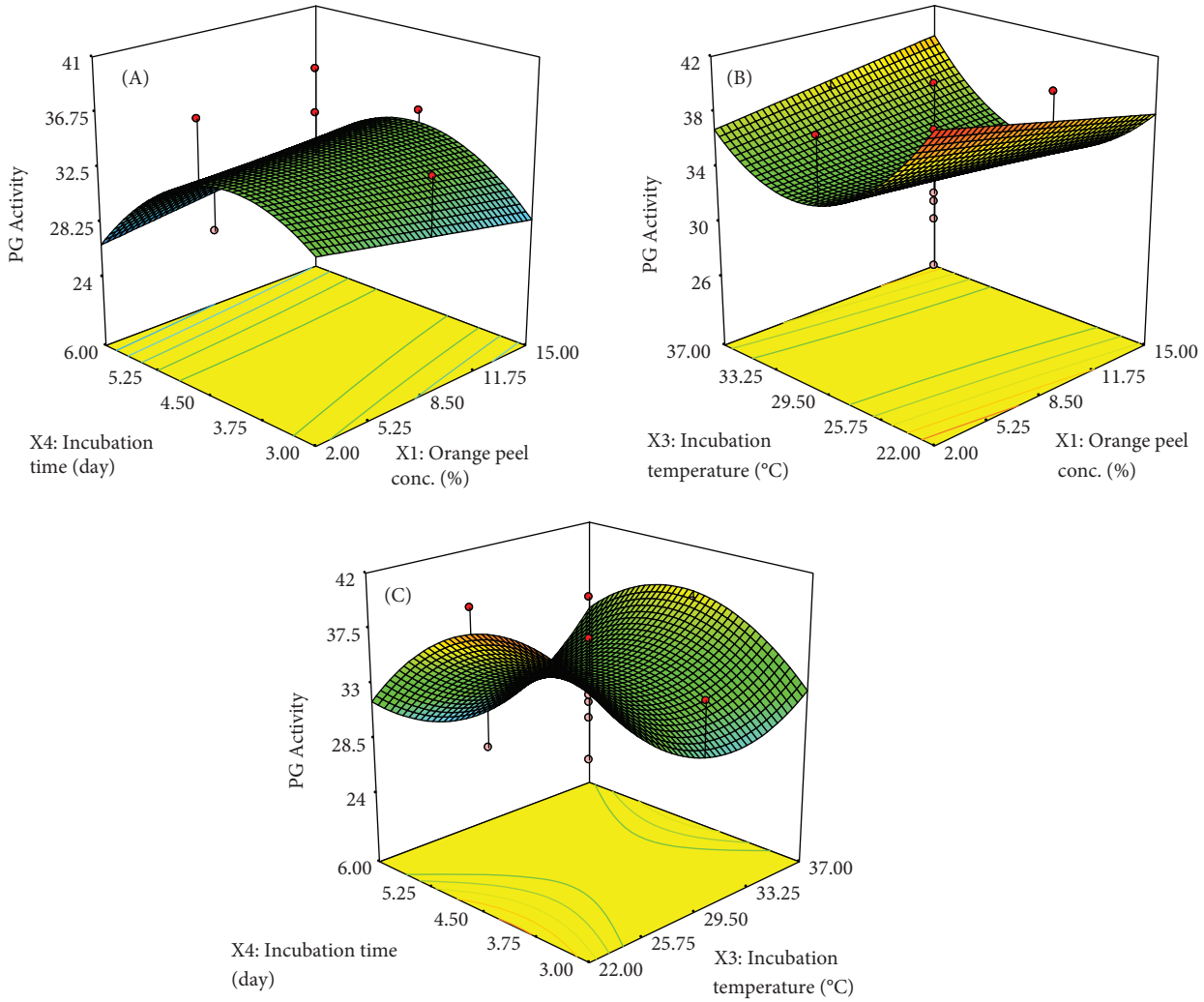

Figure 1. A) Response surface plot showing the interaction between orange peel concentration and incubation time (constant values; HCl concentration: 150 mM, incubation temperature: 29.5 °C).
B) Response surface plot showing the interaction between orange peel concentration and incubation temperature (constant values; HCl concentration: 150 mM, incubation time: 4.5 days).
C) Response surface plot showing the interaction between incubation time and incubation temperature (constant values; orange peel concentration: 8.5%, HCl concentration: 150 mM).

providing optimum conditions for the processes in which spore production is the main purpose. Criterion 3 is designed for processes where both high PG activity and spore production are desired. From the solution of obtained models and based on Criterion 1, the optimum PG activity and spore production conditions are 2% orange peel concentration, 50 mM HCl concentration, 22 °C incubation temperature, and 3.8 days of incubation time. This result is in accordance with the conditions obtained in the response surface plots of the PG activity response (Figures 1A-1C). The optimization results for Criterion 2, given in Table 3, show that, when compared with the optimum conditions of maximum PG activity (Criterion 1), nearly 1.5 more incubation days and slightly higher temperatures were required in order to obtain maximum spore count. Furthermore, the optimum conditions will be very close to those obtained with Criterion 1 when both maximum PG activity and spore count are targeted.

The utilization of optimum conditions for spore production (Criterion 2) will yield 66% more spores than the process targeting maximum PG activity

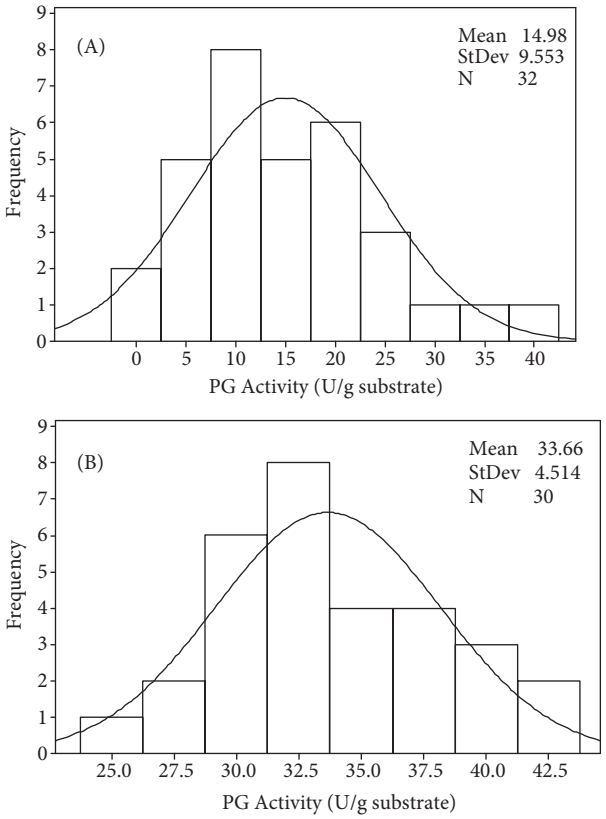

Figure 2. A) Histogram of PG activity response values obtained at the screening step.
B) Histogram of PG activity response values obtained at the optimization step.

(Criterion 1). However, it should be kept in mind by the end user that maximum spore production will cause a reduction of about 24% in PG activity. Similarly, in a process where maximum PG activity is achieved, optimum conditions indicated for Criterion 1 lead to a 66% decrease in spore count. Criterion 3 supplies both maximum PG activity and spore count, which may result in an insignificant decrease in PG activity (1.4%) in addition to a significant increase (24%) in spore count relative to Criterion 1. Criterion 3 may also lead to 1.3 times more PG activity; however, the spore count yield may be 2.3 times lower when compared with Criterion 2.

The graphical optimization of responses built up the overlay plot where the contours of interest for the various response surfaces were superimposed. The overlay plot shown in Figure 3 was constructed with the contours of PG activity greater than 35.0 U/g

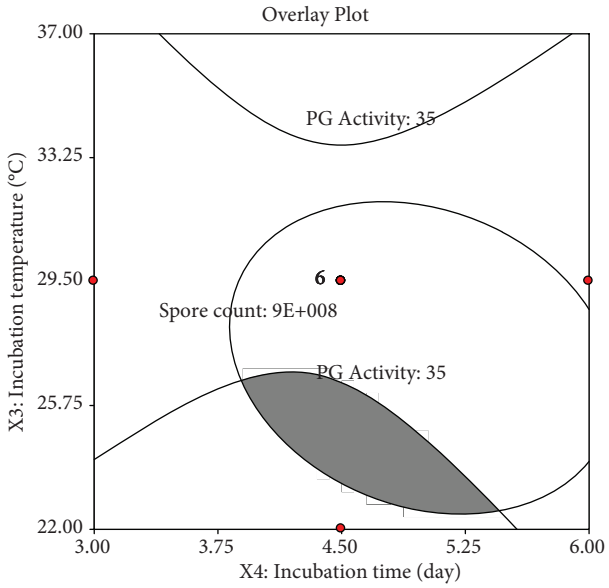

Figure 3. Overlay plot of PG activity (U/g substrate) and spore count (spores/mL) in response to incubation temperature and incubation time (orange peel concentration: 2 g/L, HCl concentration: 50 mM). Optimum area is shaded in grey.

substrate and spore count contours between 9.0×10^8 and 2.0×10^9 spores/mL. This overlay plot (Figure 3) demonstrated an optimum region (shaded in gray) in which each point would represent a combination of fermentation conditions that would provide the maximum PG activity and spore count. In Figure 3, the optimum region for maximum PG activity and spore count is an incubation period of 3.8 to 5.5 days and 22.4 to 27.5 °C incubation temperatures. This overlay plot was prepared for visual presentation of optimum conditions where conditions meet the proposed criteria and can be used practically to select conditions at the preparation step of the fermentation process.

Validation of the constructed model for PG activity

In order to validate the models constructed for PG activity and spore count in the optimization step, 4 experiments were conducted with the optimum conditions suggested by the software (Design Expert 7.0.0) and with the highest desirability values (0.891-0.995). The conditions of validation experiments and the actual and predicted values and their error percentages are summarized in Table 3.

Table 3. Numerical optimization and results of validation experiments.

Criterion	X_1 (%)	X_2 (mM)	X_3 (°C)	X_4 (days) Predicted	PG activity (U/g substrate)			Spore count (spores/mL)		
					Actual	Error rate (%)	Predicted	Actual	Error rate (%)	
1	In range 2.0	Minimize 50	In range 22.0	In range 3.7	Maximize 42.3	40.5	4.2	In range 4.7×10^8	3.6×10^8	22.4
2	In range 2.0	Minimize 50	In range 26.6	In range 5.3	In range 32.1	28.1	12.1	Maximize 1.4×10^9	1.2×10^9	9.6
2	In range 9.7	Minimize 50	In range 26.6	In range 5.3	In range 32.1	36.5	−14.1	Maximize 1.4×10^9	1.3×10^9	6.7
3	In range 2.0	Minimize 50	In range 22.0	In range 4.3	Maximize 41.7	41.1	1.3	Maximize 6.4×10^8	5.5×10^8	13.6

The error percentages of the validation experiments were in the range of 1.3%-22.4%, which indicated good compatibility of the model with the experimental results. Moreover, the percentages of error in the predicted values given in Table 3 demonstrate the accuracy and reliability of the constructed models for PG activity and spore count in the optimization step of this study.

Additionally, one randomly chosen treatment forming the shaded area of the overlay plot given in Figure 3 was validated experimentally. The conditions of this treatment were: 2% orange peel and 50 mM HCl concentrations, 4.3 incubation days, and 26 °C incubation temperature. The predicted PG activity and spore count values were validated with errors of 13.9% and 2.9%, respectively.

Many researchers have reported on the SSF production of pectinases from a wide variety of fungal strains and agroindustrial substrates under optimized conditions. The current PG activity values are compatible with the enzymatic activity results of Freitas et al. (17), Martin et al. (29), and Silva et al. (30). The maximum PG activity in this study was nearly 2 times higher than the activity obtained by Botella et al. (9) (25 U/g solid) using grape pomace as the solid substrate. Moreover, Martin et al. (29) obtained a maximum PG activity of 27.0 U/g solid with 90% sugarcane bagasse and 10% wheat bran and of 34.0 U/g solid with 90% wheat bran and 10% sugarcane bagasse, levels lower than the maximum enzyme activity obtained in the current study. On the other hand, Taşkın and Eltem (31) focused on the production of PG from *Aspergillus foetidus* by SSF using agroindustrial residues. In their study, PG enzyme activity using wheat bran (moistened with sulfate and phosphate salt solutions) was around 50 U/g, which was slightly higher than the maximum PG activity obtained in our study. Patil and Dayanand (32) produced a maximum exopectinase activity of 17.2 U/g using sunflower head and 10.2 U/g using lemon peel (both moistened with sulfate and phosphate salt solutions) by an *Aspergillus niger* strain, which showed us that the wheat bran and orange peel combination was superior to these agroindustrial residues with respect to PG production.

At the validation step of this study, a maximum spore production of 1.4×10^9 spores/mL (equivalent to 1.2×10^{10} spores/g substrate) was obtained. This value is higher than that obtained by Nava et al. (33), which was 2.9×10^9 spore/g dry substrate in a study conducted on the spore production in SSF of coffee pulp from *Penicillium commune*. Additionally, *Penicillium roqueforti* was cultivated by Larroche and Gros (34) on buckwheat seeds and resulted in hourly spore productivity values of 3.1×10^7 spores/g dry substrate, which was lower than in the current study (hourly values of 9.4×10^7 spores g/dry substrate).

Although these comparisons may not provide direct information about PG and spore production capacities because of the different conditions studied, a rough overview can be gained. Therefore, the mutant *A. sojae* M3 utilizing orange peel under optimized conditions offers significant industrial potential for the production of PG enzyme and spores.

The PG enzyme and spore production potential of a mutant *Aspergillus sojae* strain in a fermentation

medium lacking the support of synthetic compounds, but including natural components such as orange peel and wheat bran, was investigated. The PG activity and spore count values obtained were higher than the activity values achieved in a previous SSF study of our group using corncob, maize meal, and crushed maize as substrates. These results not only signify the increase of the PG activity and spore counts but also emphasize the utilization of agricultural by-product mixtures including orange peel, which is an important waste material of the fruit juice industry. In addition, this study offers 3 options for optimum production conditions to manufacturers (end users) according to priority, PG enzyme or spore production.

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