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Evaluation of orange peel, an industrial waste, for the production of *Aspergillus sojae* polygalacturonase considering both morphology and rheology effects

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Abstract: Orange peel is an agroindustrial waste rich in pectin and known to be an inducer for pectinase production. The use of this low-cost substrate for the production of an industrially important enzyme, polygalacturonase (PG), can be an alternative way to turn this waste into a value-added product, contributing to the reduction of environmental waste disposal problems. Enzyme productions by fungal microorganisms are affected by environmental and nutritional factors, demanding the determination of optimum conditions for maximum enzyme production with the desired fungal morphology and broth rheology. Therefore, complex and additional carbon sources were optimized with respect to PG production by *Aspergillus sojae* using statistical approaches. Effect of pH, another significant parameter affecting the rheology and morphology of the strain, was investigated in the serial bioreactor system using the optimized medium composition. Highest PG enzyme yield and productivity together with the maximum PG enzyme production (93.48 U/mL) were obtained under uncontrolled pH conditions. Under these conditions, morphologically, pellet sizes exhibited a normal distribution ranging between 0.5–1.0 mm and 1.0–1.5 mm, and rheological measurements revealed that fermentation broths showed non-Newtonian flow. The low pH trend observed during the course of the fermentation was another important positive outcome for industrial fermentations, prone to contamination problems.

Key words: Polygalacturonase, *Aspergillus sojae*, orange peel, broth rheology, medium pH, fungal morphology, agroindustrial waste

1. Introduction

Agroindustrial wastes were generally evaluated in fermentation processes in order to produce precious products such as enzymes. For example, Birhanlı and Yeşilada (2013) produced laccase enzymes from white rot fungi by using various lignocellulosic wastes under a semisolid state and submerged fermentation conditions. Citrus peel is an important agroindustrial by-product that can be a rich carbon source for microbial growth and simultaneous polygalacturonase (PG) and xylanase production (Mamma et al., 2008). Citrus peel solid by-product composes approximately 50% of the fresh fruit weight (Rodriguez-Fernandez et al., 2011). The orange juice industry generates large amounts of residues, mainly the peel and segment membranes, which are rich in soluble and insoluble carbohydrates (Zhou et al., 2011). The disposal of these residues poses a challenge for many factories, which often pelletize the peel and use it as animal feed or as a pectin precursor (Rodriguez-Fernandez et al., 2011). In our previous study it was proven that orange peel and HCl concentrations and incubation time were significant factors affecting the activity of PG enzyme produced from *Aspergillus sojae* by solid-state fermentation (Demir et al., 2012). However, orange peel is rich in pectin, which is the inducer for pectinases. Therefore, in the current study, dry orange peel, which is rich in pectin, cellulose, and hemicellulose, was used as a low-cost fermentation substrate for enzyme production.

In recent years, fungal enzyme production is an especially crucial and fast-growing sector in the fermentation industry. According to Global Industry Analysts Inc., the global market for industrial enzymes is forecast to reach US$ 3.74 billion by the year 2015 (http://prweb.com/releases/industrial_enzymes/proteases_carbohdrases/prweb8121185.htm). Among these enzymes, pectinases are one of the most important type of industrial enzymes, and their production accounts for about 10% of the overall manufacturing of enzyme

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preparations (Pedrolli et al., 2009). They have wide applications in textile processing, degumming of plant fibers, pectic wastewater treatment, papermaking, and the food industry (coffee and tea fermentations, fruit juice and oil extraction, improvement of chromaticity and stability of red wines) (Rangarajan et al., 2010; Gomes et al., 2011). PG attracts the most attention among the family of pectinolytic enzymes, being responsible for the hydrolysis of the pectic substances present in most plants (Jayani et al., 2010; Gomes et al., 2011; Zhou et al., 2011). It is advantageous to produce enzymes using microorganisms since they are not influenced by climatic and seasonal factors and can be subjected to genetic and environmental manipulations to increase product yield (Dhillon et al., 2004). In industry, mostly molds like *A. niger*, *Coniothyrium diplodiella*, *Penicillium*, and *Rhizopus* species are used for PG production (Blandino et al., 2001; Souza et al., 2003; Gomes et al., 2011). In this study, a mutant strain of *A. sojae*, an industrially important species that has been used in food fermentations such as for soy sauce and bean paste, was used for PG production for the first time by our group (Gögus et al., 2006). It is known that in submerged fermentations, fungal pectinase production is affected by different process parameters such as the type of strain, cultivation conditions (pH, temperature, aeration, stirring rate, and incubation time), and growth medium composition (particularly carbon and nitrogen sources) (Gomes et al., 2011). Therefore, it is essential to optimize PG production in terms of these parameters. In particular, the type and concentration of the carbon sources in the fermentation media were the focus of many researchers (Teixeira et al., 2000; Malvessi and Silveira, 2004; Martinez-Trujillo et al., 2009). In a study performed by Sargın et al. (2013), several fermentation parameters such as effects of various nitrogen sources, initial moisture content, initial pH, incubation temperature, and incubation time to obtain maximum micropropagule production by *Trichoderma harzianum* EGE-K38 in solid state fermentation were evaluated.

Another critical environmental parameter that affects growth and activity in submerged fermentations is the pH of the submerged culture media. Generally, cells can only grow in a specific pH range and the metabolite production is also affected by certain pH values. Furthermore, the pH of the growth media plays an important role in pellet formation and coagulation of spores (Metz and Kossen, 1977; Fang and Zhong, 2002; Papagianni, 2004; Shu and Lung, 2008).

The relationship between rheology and morphology has particular importance in fungal fermentations (Rodriguez Porcel et al., 2005). Different growth morphologies varying from compact pelleted to filamentous forms can be obtained under submerged culture conditions (El-Enshasy et al., 2006). Pelleted growth has an advantage of decreasing the viscosity of the broth, which results in the improving of mixing and mass transfer properties. It is also known that pelleted growth facilitates downstream processing by simplifying solid–liquid separation (El-Enshasy et al., 2006). On the other hand, dispersed filamentous morphology is developed when fungus grows on rapidly metabolized substrates, which may decrease product formation yield and impede oxygen transfer by increasing the viscosity of the culture fluid (Casas López et al., 2005). Therefore, broths produced by pelleted growth are more easily mixed and aerated as compared to filamentous growth (Rodriguez Porcel et al., 2005).

Considering these facts, the optimization of the fermentation conditions and development of a low-cost industrial medium formulation for the production of PG enzyme by *A. sojae* using statistical tools was initiated in this study. Furthermore, the effect of pH, one of the key parameters, on PG activity, biomass, fungal morphology, and broth rheology was evaluated using the optimized medium formulation in a 750-mL scale serial bioreactor system. The goal was to define the optimum pH condition in the serial bioreactor system with a low-cost medium formulation, which would result in a pellet type of morphology preferred by the industry and ease the subsequent downstream processing.

2. Materials and methods

2.1. Materials

All chemicals were supplied by Sigma-Aldrich (www.sigmaaldrich.com), AppliChem (www.applichem.com), Merck (www.merck.com), or Riedel-de Haën (www.riedeldehaen.com).

2.2. Microorganism

A. sojae ATCC 20235 was purchased from LGC Promochem Inc. (Teddington, Middlesex, UK), an international distributor of the American Type of Culture Collection (ATCC). This wild-type culture was randomly mutated using ultraviolet light exposure by Jacobs University GmbH, Bremen, Germany, according to a modified procedure of De Nicolás-Santiago et al. (2006).

The propagation of the cultures was done on YME agar slant medium according to the procedure given by Gögus et al. (2006). Stock cultures of these strains were prepared with 20% glycerol-water and stored at –80 °C.

2.3. Preparation of inoculum

The inoculum for either shake flasks or bioreactor was obtained on molasses agar slants as optimized by Gögus et al. (2006) after a preactivation step performed on YME agar using the stock cultures. After 1 week of incubation at 30 °C, spores were harvested by the addition of 5 mL of Tween 80 and water (0.01% (v/v)) per slant. The spore suspension was counted using a Thoma hemocytometer

(Marienfeld, Germany) and the suspensions were stored at 4 °C until inoculation. Inoculation rate was 2.8×10^3 spores/mL for both bioreactor and shake flask experiments.

2.4. Production medium and fermentation

For the development of a low-cost industrial medium formulation, shake flask experiments were conducted in 250-mL Erlenmeyer flasks each containing 50 mL of sterilized medium composed of orange peel or corn meal as the complex carbon source and Maltrin (purchased from Pendik Nişasta İstanbul, Turkey) or glucose as the additional carbon source at different concentrations according to the experimental design. Orange peel was purchased from a local market in Bremen, Germany. Ammonium sulfate as the nitrogen source and sodium dihydrogen phosphate and disodium hydrogen phosphate as the phosphate sources were added to the medium at the constant amounts of 8 g/L, 3.3 g/L, and 3.2 g/L, respectively. The agitation speed ranged between 150 and 350 rpm and incubation time ranged between 5 and 7 days, according to the experimental design. Temperature was maintained at 30 °C.

In order to determine the effect of pH on the PG production, a set of bioreactor experiments were conducted with the optimized medium formulation containing orange peel (34 g/L), Maltrin (142 g/L), ammonium sulfate (8 g/L), monosodium phosphate (3.3 g/L), and disodium phosphate (3.2 g/L). The fermentation was carried out in a 1-L Sartorius BIOSTAT Qplus-6 MO serial bioreactor (Sartorius Stedim, Gottingen, Germany) with a working volume of 750 mL at 30 °C, 600 rpm agitation speed, and 1 vvm aeration rate for 120 h (optimized in shake flask experiments). At the end of fermentation, the collected samples were filtered for biomass determination, and their enzyme activity and total protein and total carbohydrate contents were determined from supernatant obtained by centrifugation of the broth at 6000 rpm for 15 min.

2.5. Enzyme assay

PG activity was assayed according to the modified procedure given by Panda et al. (1999) using 2.4 g/L of polygalacturonic acid as a substrate at pH 4.8 and 40 °C. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of galacturonic acid per unit volume of culture filtrate per unit time under standard assay conditions.

2.6. Protein and carbohydrate assay

The total protein contents of the samples were determined according to the method described by Bradford (1976) using bovine serum albumin as a standard. The phenol-sulfuric acid method was used to determine the total carbohydrate content (DuBois et al., 1956).

2.7. Biomass determination

The biomass, expressed as dry cell weight (g/L), was determined by means of the gravimetric method. The fermentation broth was filtered through preweighed Whatman No. 1 filter paper, followed by drying to a constant weight at 37 °C for approximately 24 h.

2.8. Yield and productivity calculation

Enzyme production yield coefficients $Y_{P/S}$ (U activity/mg substrate) were calculated by:

$$Y_{P/S} = -\Delta P/\Delta S. \quad (1)$$

Volumetric production rates for enzymatic activities (q_{xE}) were calculated by (Shuler and Kargi, 2002; Martinez-Trujillo et al., 2009):

$$q_{xE} = \frac{E_2 - E_1}{t_2 - t_1} = \frac{U}{mL.h}. \quad (2)$$

where E is the enzyme activity (U/mL) produced and t is the time (h).

2.9. Experimental design and statistical analysis

Experiments were started with the screening of the process variables and then continued with the preoptimization and optimization experiments. Contour plots and data analysis were done with Design Expert 7.0.0 Trial Version (Stat-Ease Inc., Minneapolis, MN, USA) in both screening and optimization stages. The following equation was used for coding of the actual values of the factors in the range of (–1) to (+1):

$$\begin{aligned} x = [&\text{actual} - (\text{low level} + \text{high level}) / 2)] / \\ &[(\text{high level} - \text{low level}) / 2]. \end{aligned} \quad (3)$$

In the screening part of this study, a general factorial design with 24 runs was used in order to determine the effects of additional carbon sources, complex carbon sources, and incubation time on the PG activity.

In the preoptimization step, according to the results of screening experiments, orange peel, Maltrin, and glucose concentration levels were determined in order to be used in the optimization study. Agitation rate, another important parameter of submerged fermentations, was also included in the preoptimization process as a factor in order to screen its effect on the PG activity. Hence, 30 experimental combinations as determined by face-centered central composite design were employed using the given factors.

In the optimization step, a face-centered central composite design was used with the enlarged factor levels, and optimum Maltrin and orange peel concentrations were determined for the maximum PG activity. Finally, a second-order polynomial regression equation was fitted to the response data:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j + \varepsilon, \quad (4)$$

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

2.10. Morphological and rheological measurements

At the end of the fermentation, in order to investigate the pellet morphology, 1 mL of fermentation broth was transferred to a petri dish. Images were captured with a digital camera (Sony Cybershot, Sony Electronics Inc., San Diego, CA, USA). Pellet morphology was characterized by image analysis using Image Pro Plus software package 4.5.1. (Media Cybernetics Inc., Silver Spring, MD, USA). The size of the pellet was quantified using the diameter corresponding to a circular area equivalent to the pellet's projected area (Metz and Kossen, 1977). Pellet numbers per given volume, pellet diameter, and pellet size distributions were determined. Rheological measurements were carried out with a concentric cylinder viscometer (Haake VT 550 Viscotester, Thermo Inc., Germany) equipped with an immersion vane spindle adapter.

In order to calculate flow behavior index (*n*) and consistency coefficient (*K*), the following equation was used (Sahin and Sumnu, 2006). τ represents the shear stress (Pa), and $\dot{\gamma}$ indicates the shear rate (1/s).

$$\tau = K\dot{\gamma}^n \tag{5}$$

The power law model was applied to the data, and *K* and *n* values were determined. Root square mean error (RSME) was also calculated in order to assess the compatibility of the model and the data. The formula for RSME calculation is given below.

$$\sqrt{\frac{\sum (f(x_i) - y_i)^2}{n}} \tag{6}$$

3. Results and discussion

3.1. Screening of the medium composition and incubation time

In this study, a general factorial design with 24 runs was employed in order to determine the effects of additional carbon sources, complex carbon sources, and incubation time on the PG activity. Analyzed factors and their levels were decided by considering our previous experiments (unpublished results). Consequently, due to their high PG activity potential, Maltrin (100 g/L) and glucose (20 g/L) as additional carbon sources, orange peel (10 g/L) and corn meal (10 g/L) as complex carbon sources, and incubation time (5, 6, and 7 days) were screened using statistical techniques to investigate their interactive effects.

Figures 1A and 1B show that the maximum PG activity (90.66 U/mL) was achieved when orange peel was used as the complex carbon source and harvested at the end of 6 days of incubation. ANOVA analysis showed that the generated model was significant for the determination of the effects of examined factors on the PG activity ($P < 0.1$). Among the investigated factors, the most important factor affecting the PG activity was complex carbon sources ($P < 0.001$). However, additional carbon sources and incubation time had no significant effect on the PG activity at the studied levels ($P > 0.1$). The lack-of-fit value was also insignificant ($P > 0.1$), indicating that the investigated factors were efficient for the model construction.

Since additional carbon sources (Maltrin and glucose) at the studied levels did not result in major differences in PG activity and were close to each other in terms of their results, both of their concentrations were taken as additional factor variables with orange peel in the preoptimization step. The incubation time, on the other hand, was fixed at 6 days, when the maximum PG activity was observed.

3.2. Preoptimization study for the determination of the levels of the medium components and agitation rate

According to the results of screening experiments, a preoptimization study, similar to another screening study, was performed considering the significant factors of orange peel, Maltrin, and glucose concentrations in order to gain an understanding about their factor levels and interactive effects on PG activity. Furthermore, agitation rate, another important parameter in submerged fermentations, was also included in the optimization process as a factor variable. Hence, 30 experimental combinations determined by face-centered central composite design was performed with factors of orange peel concentration (A), Maltrin concentration (B), glucose concentration (C), and agitation rate (D) at the levels of 3–30 g/L, 0–120 g/L, 0–30 g/L, and 150–350 rpm, respectively.

ANOVA indicated that the agitation rate (D) had no significant effect ($P = 0.91$) on the PG activity at the chosen levels. The interactions of the agitation rate (D) with orange peel (A) and Maltrin (B) were also insignificant ($P > 0.1$). On the other hand, orange peel (A) was found to be the most important factor on the PG activity, at $P < 0.001$. Glucose (C) and Maltrin (B) had insignificant effects on PG activity ($P > 0.1$), but as the P-value of Maltrin (B) was close to the significance level of $P > 0.1$, it was included in the optimization study.

The contour plot given in Figure 2A shows that high concentrations of both Maltrin (60–120 g/L) and orange peel (25–30 g/L) gave the maximum PG activity. However, it is clear from Figure 2A that for both Maltrin and orange peel concentrations beyond the selected levels, PG activity still had an increasing trend. Therefore, in order

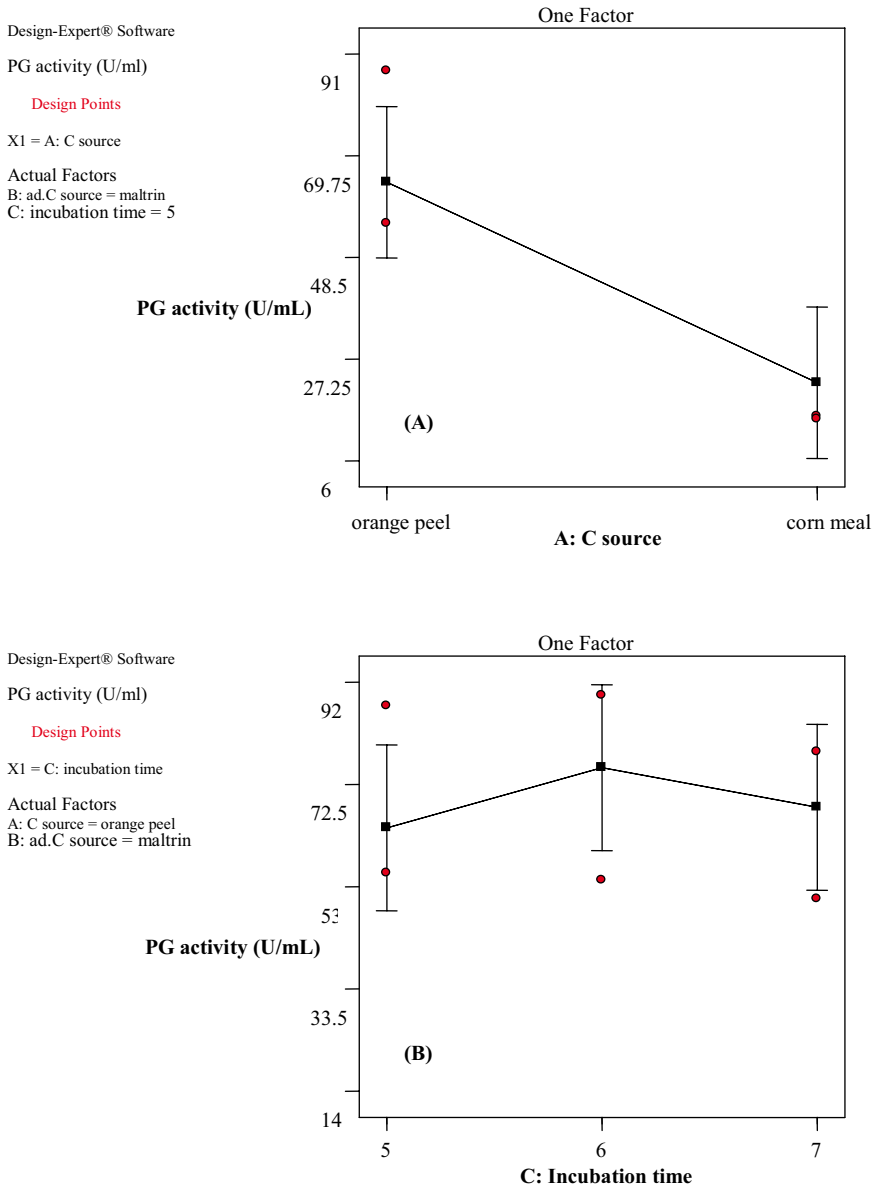

Figure 1. A) Effect of complex carbon sources on the PG activity (held at midlevels; additional C source: Maltrin; incubation time: 5 days). **B)** Effect of incubation time on the PG activity (held at midlevels; complex C source: orange peel; additional C source: Maltrin).

to determine the exact optimum levels of the factors and to construct a model with these factors, an optimization process with enlarged factor levels was conducted.

3.3. Optimization study of medium components

In order to determine the optimum Maltrin and orange peel concentrations for the maximum PG activity, face-centered central composite design was used with the enlarged factor levels of orange peel (A) and Maltrin (B) at concentrations of 20–80 g/L and 0–150 g/L, respectively. The other medium components were the same as in the previous screening and preoptimization experiments.

Variance analysis of the PG activity values showed that the quadratic form of Maltrin (B^2) had an insignificant effect on PG activity with a P-value of 0.88 ($P > 0.1$); thus, it was not included in the model. Moreover, although orange peel seemed to be an insignificant factor at the studied level, it was included in the model due to its significant interaction with Maltrin and its significant effect seen in the preoptimization process. According to ANOVA, the constructed model was significant ($P < 0.001$) in describing the PG activity with a R^2 value of 0.90, expressed in the following equation.

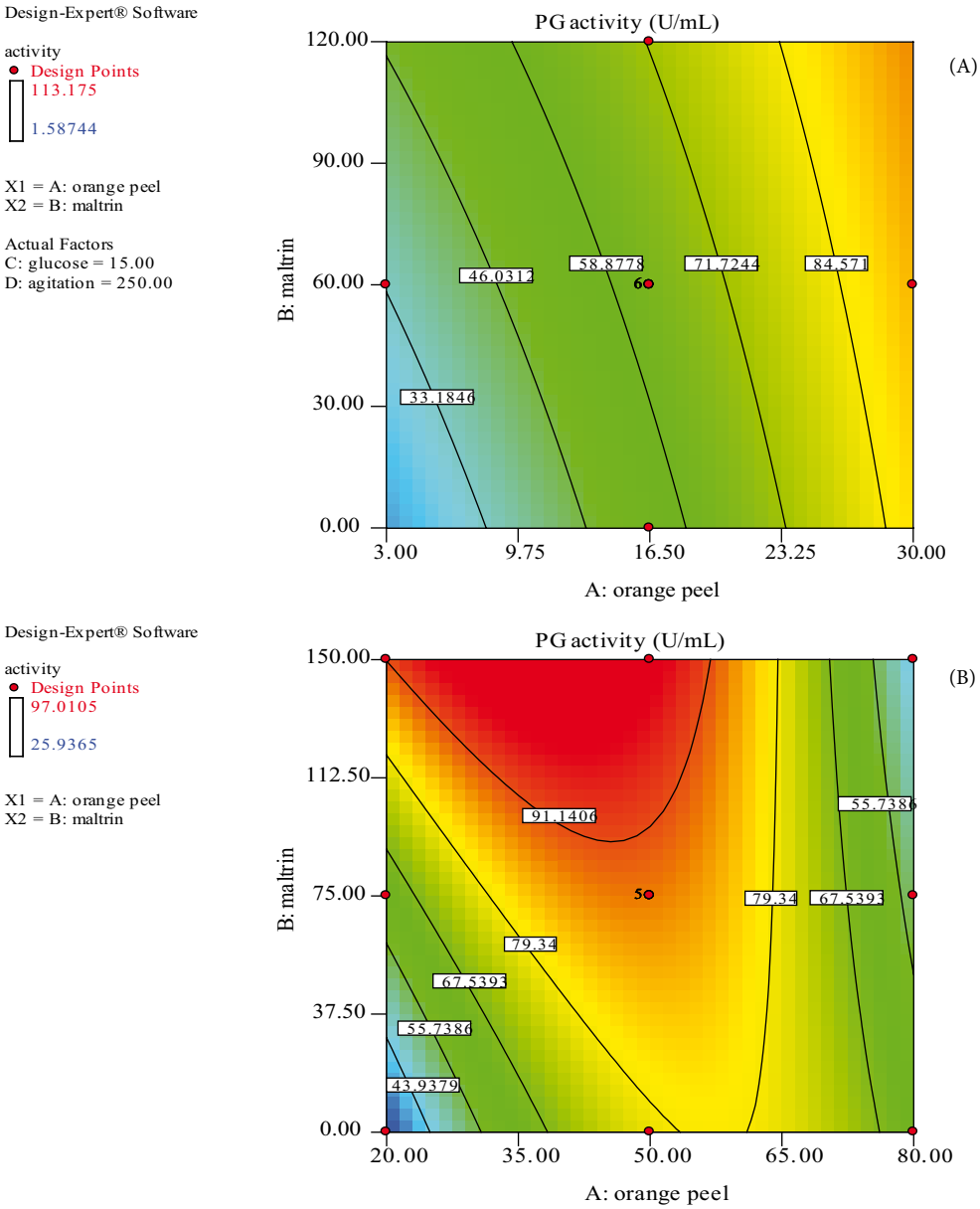

Figure 2. Interaction of orange peel concentration and Maltrin concentration factors: **A)** in the preoptimization study (glucose: 15 g/L, agitation speed: 250 rpm), **B)** in the optimization study.

$$\text{PG activity} = 88.17 - 4.55A + 10.12B - 19.48AB - 30.99A^2 \quad (7)$$

The contour plot given in Figure 2B indicates that maximum PG activity was achieved at a high level of Maltrin (B) and medium level of orange peel (A) concentration. As a result, optimum orange peel and Maltrin concentrations for the production of maximum PG activity (97.01 U/mL) were determined as 42.35 g/L and 137.09 g/L, respectively. This result was obtained at 30 °C with 250 rpm agitation and 6 days of incubation.

3.4. Validation of the constructed model for PG activity

A model was constructed for maximum PG activity in the optimization process. This model was validated by performing 3 experiments at the optimum conditions given by Design Expert 7.0.0 software. These experimental conditions and the actual and predicted PG activity values are summarized in Table 1.

There was a 5.31% difference between the predicted PG activity values given by the software (99.51 ± 1.63 U/mL) and the actual PG activity values obtained by the validation experiments (104.79 ± 7.58 U/mL). Validation experiments

Table 1. Validation experiment combinations of optimization study.

Run	Orange peel (g/L)	Maltrin (g/L)	Predicted PG activity (U/mL)	Actual PG activity (U/mL)
1	38.01	140.84	100.75	107.13
2	33.98	142.06	100.11	110.93
3	44.19	127.78	97.66	96.31

also showed that the empirical model constructed in the optimization process was a reliable and accurate model for the prediction of the maximum PG activity within the studied concentration ranges. Indeed, this validated optimum medium formulation (orange peel: 33.98 g/L; Maltrin: 142.06 g/L) was selected for use in bioreactor studies for further determination of the optimum pH. In our previous studies (Oncu et al., 2007), we found maximum PG activity as 6.73 U/mL, which was far below that of the current study. Gomes et al. (2011) achieved maximum PG activity of 51.82 U/mL with *A. niger* using pectin as a carbon source, which was again lower than our current findings.

3.5. Determination of the effect of pH on PG activity, biomass, morphology, and rheology in a serial bioreactor system

In order to determine the effect of pH, 4 different fermentation studies were performed at uncontrolled pH, pH 6, pH 5, and pH 4. pH was fixed at pH 6, 5, and 4 by automatically adding 4 N NaOH and 4 N H_2SO_4 using a serial bioreactor system.

Changes in the amount of PG activity, specific enzyme activity, biomass, and total carbohydrates as a function of time are shown in Figure 3. Moreover, PG enzyme yields and productivities were calculated for each pH experiment and results are given in Table 2. Furthermore,

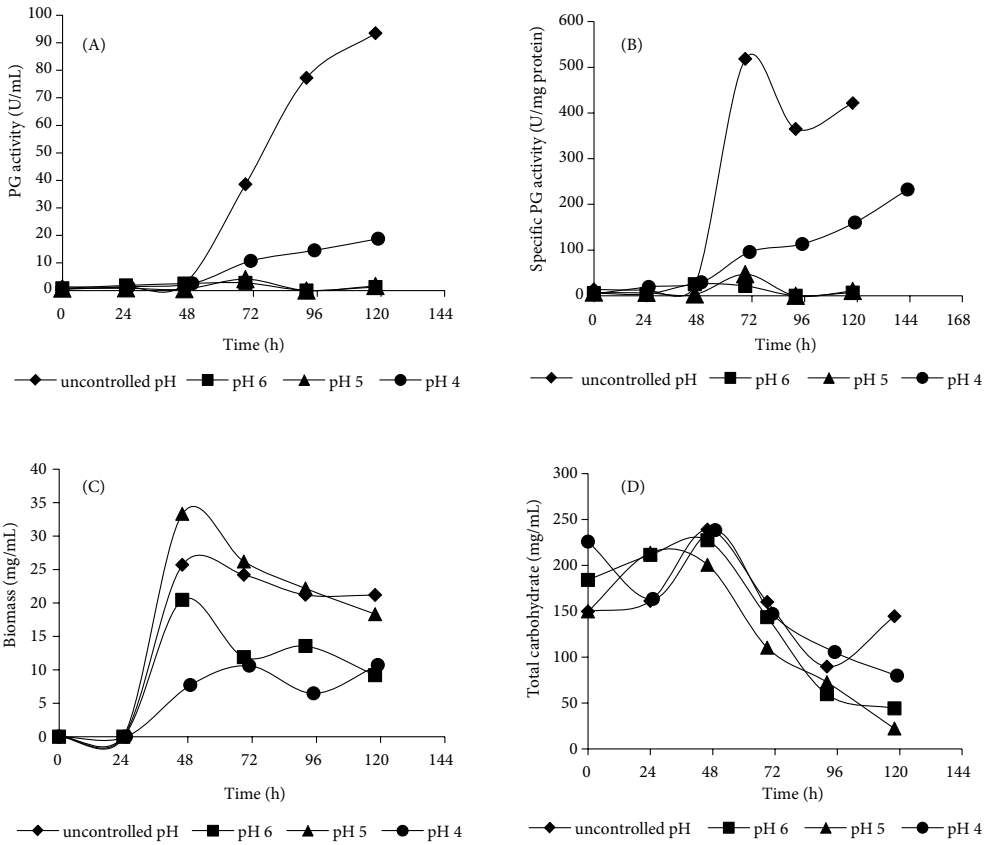

Figure 3. Profiles of pH experiment performed at 600 rpm, 30 °C, and 1 vvm aeration: **A)** PG activity, **B)** specific PG activity, **C)** biomass, **D)** total carbohydrate.

Table 2. Yield and productivity values of the pH experiments.

	Uncontrolled pH	pH 6	pH 5	pH 4
Yield ($Y_{P/S}$) (U activity/mg substrate)	17.660	0.003	0.007	0.282
Productivity (U/mL h)	0.781	0.004	0.008	0.191

morphological properties of obtained pellets are presented in Figures 4A and 4B. Flow behavior of the broths was also determined according to rheological measurements (Figure 5A). All the broths exhibited non-Newtonian flow behavior. As the shear rate increased, a decrease in the apparent viscosity values represented pseudoplastic flow behavior (Figure 5B). Therefore, a power law model was applied to the data, from which flow behavior index (*n*) and consistency coefficient (*K*) values were calculated (Table 3). All these results are discussed below considering each individual pH condition.

3.5.1. Effect of pH 4

Enzyme assay results showed that PG production remained at low levels at pH 4 (Figure 3A). Only 18.8 U/mL PG activity was obtained at this pH. On the other hand, the specific PG activity curve (Figure 3B) revealed a relatively higher value (232.49 U/mg protein), which indicated that nutrients were used for enzyme production rather than biomass formation. Figure 3C denotes a low amount of biomass (10.71 mg/mL) formation under this pH condition. It was also observed that carbohydrate was not completely consumed (Figure 3D). Therefore, it

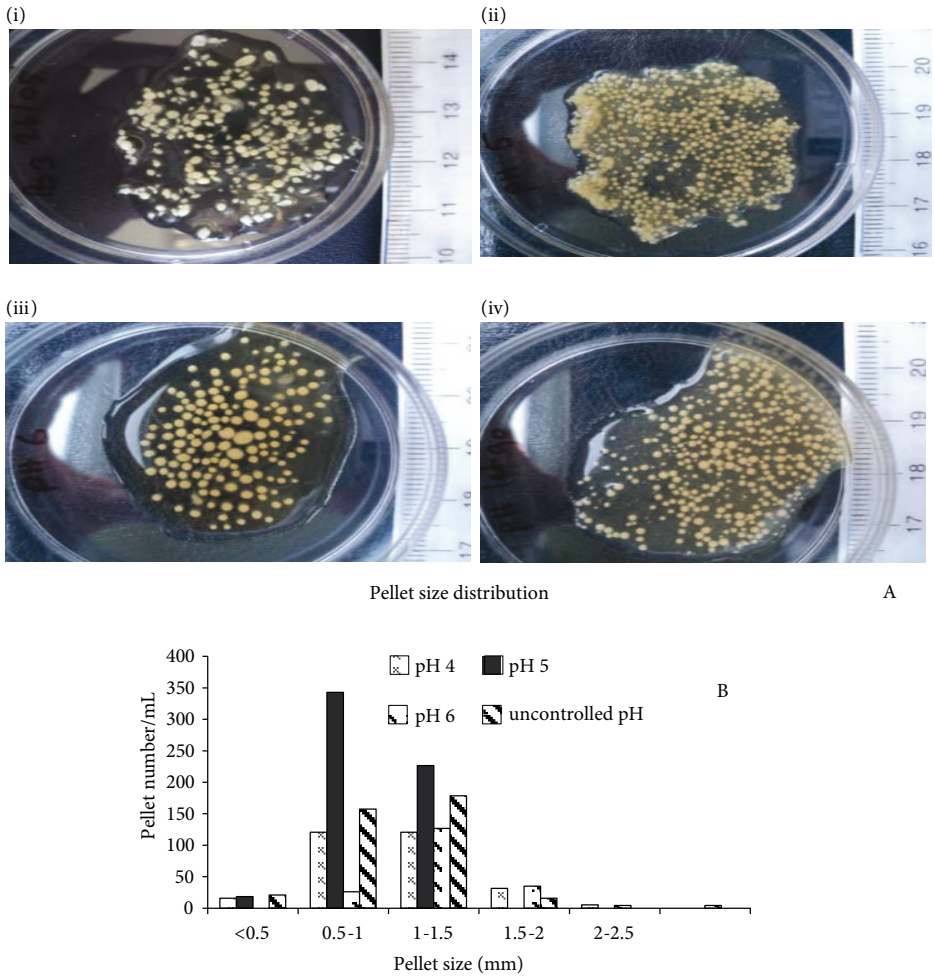

Figure 4. A) Sample photographs of (i) pH 4, (ii) pH 5, (iii) pH 6, and (iv) uncontrolled pH. **B)** Pellet size distribution under different pH values.

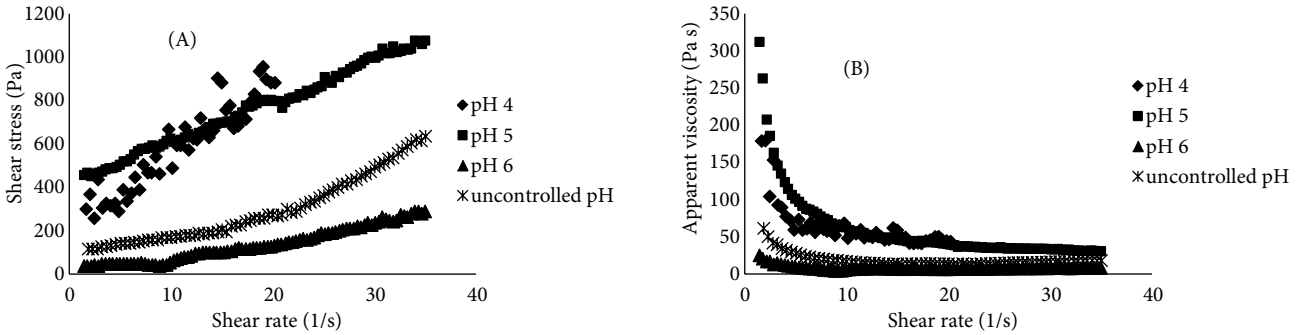

Figure 5. A) Shear stress vs. shear rate and **B)** apparent viscosity vs. shear rate graphs for pH 4, pH 5, pH 6, and uncontrolled pH.

Table 3. Application of power law model to the data.

	n	K (Pa s^n)	RSME	R^2
pH 4	0.519	174.68	0.064	0.841
pH 5	0.323	310.3	0.030	0.928
pH 6	0.809	12.691	0.104	0.868
Uncontrolled pH	0.643	45.331	0.087	0.843

can be concluded that insufficient transfer of nutrients to the cell interiors may have resulted in lower biomass and enzyme production. On the other hand, the second highest enzyme yield and productivity was observed at pH 4 (Table 2). According to morphological analysis, smaller amounts of pellets with irregular surfaces were detected in fermentation broth at this pH (Figures 4A and 4B). The pellet size distribution graph also indicates a wider range, mainly changing from 0.5 mm to 1.5 mm for pellet sizes (Figure 4B). Although the fermentation broth was clear, the consistency coefficient (*K*) of the broth was found to be high, which implies high viscosity under this condition (Table 3). Irregular pellet surfaces might have caused sticking of the pellets, which gave rise to increased viscosity by increasing resistance to applied shear stress during viscosity measurements. The RSME value for the applied model was low, as desired for the goodness of the fit (Table 3). Overall it can be concluded that pellet morphology in the form of irregular pellets, which increased the broth viscosity, might have limited the use of nutrients by the cells and resulted in low PG enzyme production.

3.5.2. Effect of pH 5

It was observed that PG activity remained at low levels like 1.6 U/mL when pH was set to 5 (Figure 3A). Furthermore, specific PG enzyme activity was found to be 10.55 U/mg protein (Figure 3B). Figures 3C and 3D show that efficient carbohydrate utilization contributed to higher amounts of biomass production. It can be deduced that the strain used carbohydrate for biomass production instead of enzyme production due to the fact that high acidity in the fermentation medium favors the production of PG enzyme rather than biomass. The lowest enzyme yield and productivity value were obtained due to the low amount of enzyme production when pH was set to 5 (Table 2). As can be seen from Figure 4A, a jelly-like broth occurred due to the high pellet formation at this pH. The pellet size distribution graph (Figure 4B) indicates the highest number of pellets (588 pellets/mL), whose sizes were in the range of 0.5–1 mm. Additionally, Table 3 shows that fermentation broth obtained at this condition had the lowest flow behavior index (*n*). Therefore, the consistency coefficient (*K*) was the highest, which indicated the highest viscosity due to the occurrence of jelly-like broth under this condition. Additionally, the applied mathematical model showed the best compliance with data at pH 5 (the lowest RSME and the highest R^2) (Table 3).

3.5.3. Effect of pH 6

Activity results revealed that enzyme production was the lowest (only 1.2 U/mL PG activity) at pH 6 (Figure 3A). Moreover, the specific PG activity and biomass formation were also found to be low (6.83 U/mg protein and 18.31 mg/mL, respectively) compared to the others (Figures 3B and 3C). However, carbohydrates were effectively consumed under this condition (Figure 3D). Due to the lowest enzyme production, enzyme yield and productivity values were also the lowest at this pH (Table 2). It was observed that pellets had smooth surfaces at pH 6 (Figure 4A). According to the literature, a reduction in the hyphal

length of *Penicillium chrysogenum* was observed due to the increase of medium pH. It was also declared that hyphal wall structure and hyphal length showed high sensitivity to pH levels (Papagianni, 2004). The lowest pellet number (197 pellets/mL) and the biggest pellets, whose sizes were in a wide range of mainly 1 mm to 2 mm, were obtained at pH 6 (Figure 4B). Papagianni (2004) reported that pellet size increased when pH increased. Furthermore, it was speculated that pellet formation at high pH levels resulted from accumulation of spores. Pazouki and Panda (2000) stated that surface properties of spores were affected by the medium pH level. Accordingly, coagulation of the spores was thought to be increased due to the electrostatic interaction between the spores themselves. An increased trend towards formation of mostly pellet structures, occurrence of short hyphae, and absence of mycelia at high pH levels resulted in the least viscous fermentation broth. It was found that the consistency coefficient was the lowest and the flow behavior index was approximately 1, showing a decrease in viscosity when pH was 6 (Table 3) and indicating the least viscous broth among all others.

3.5.4. Effect of uncontrolled pH

Considering the enzyme activity results, maximum PG activity (93.48 U/mL) was obtained at uncontrolled pH condition and this value was 78, 57, and 4.97 times higher than those obtained under pH 6, 5, and 4 conditions, respectively (Figure 3A). Figure 3B shows that specific PG activity exhibited the same profile as PG activity, indicating PG enzyme produced under uncontrolled pH condition did not contain impurities. Furthermore, high biomass production (21.18 mg/mL) was observed when pH was not controlled during the fermentation (Figure 3C). During the course of fermentation studies, the initial pH value of the fermentation medium was approximately 4.8–5.0. As stated earlier, because the initial pH was about 5, nutrient compounds were considered to dissolve better in the case of uncontrolled pH and pH 5. However, it was observed that carbohydrates in the medium were not used during the first 24 h of fermentation under uncontrolled pH condition while they were rapidly and efficiently used at pH 5 (Figure 3D). After the first 24 h of fermentation, the pH of the medium started to decrease along with initiation of microbial proliferation, and cell metabolism accelerated. Therefore, carbohydrate consumption also began. It was reported that low pH values affected cell permeabilization, and transfer of nutrients inside the cell was much more effective under this condition (Oncu et al., 2007). As a general observation, the amount of total carbohydrates showed an increase after initiation of microbial growth due to the decomposition of Maltrin by microbial cells. Afterwards, the amount of total carbohydrates in the medium was found to be lower as a result of the consumption of carbohydrates by the cells. It can be concluded that carbohydrates were not completely metabolized under the uncontrolled pH condition considering total sugar analysis results (Figure 3D). Obtaining the highest PG activity with an uncontrolled pH value indicated that nutrients were mostly used for enzyme production under this condition. Furthermore, the highest enzyme yield and productivity were reached under uncontrolled pH (Table 2). The best yield and productivities for both endo- and exo-PG activities were reported to be achieved under freely decreased pH conditions in the experiments conducted by Malvessi and Silveira (2004). This finding is similar to the results of this current study. However, Malvessi and Silveira (2004) reported lower exo-PG activity (54 U/mL) than that found in this research. Morphological observations included the formation of big pellets at uncontrolled pH (Figure 4A). Pellet counts indicated the second highest pellet formation, with sizes mainly ranging between 1 and 1.5 mm at this condition (Figure 4B). The fermentation broth was found to have a slightly viscous nature (Table 3), which is desired for the ease of downstream processes.

It was noted that the pH value of the medium decreased up to pH 2 at the 118th hour of fermentation, when the highest enzyme activity was obtained under the uncontrolled pH condition. Along with obtaining the highest enzyme activity, working under high acidic conditions reasonably reduces the risk of the microbial contamination that can occur in the fermentation medium. This is an important advantage of this medium and these processing conditions, which can be considered for industrial fermentations that are prone to contamination problems. Uncontrolled pH conditions will also significantly reduce the acid and base consumption, which is not required in this case. Therefore, the uncontrolled pH condition was selected for further fermentation studies.

In conclusion, the developed medium formulation (orange peel: 33.98 g/L; Maltrin: 142.06 g/L), resulting in the highest PG activity, constituted a simple and economic medium that enabled the production of PG from *A. sojae* in a serial bioreactor system. Moreover, giving the highest enzyme yield and productivity at the same time as the highest PG activity, uncontrolled pH was the optimum pH for the maximum PG production by this *A. sojae* strain. Uncontrolled pH was also found to be the optimum pH condition from the morphological and rheological points of view. It is hereby proven that low-cost carbon sources (orange peel) can be suitable for the production of considerable amounts of industrially important PG enzyme.

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