# ENHANCED PRODUCTION OF EXO-POLYGALACTURONASE FROM AGRO-BASED PRODUCTS BY *ASPERGILLUS SOJAE*

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*Aspergillus sojae* has been previously shown to produce exo-polygalacturonase (exo-PG) in synthetic media, where the potential of the organism to utilize agricultural substrates was not considered so far. In this study, the utilization of agro-based products was taken into account in the enhanced production of exo-PG using an *A. sojae* mutant by applying statistical design methods. Complex sources (orange peel, wheat bran, and corn meal), simple sugar sources (glucose, maltrin, and sugar beet syrup), and two phosphate salts were screened using D-optimal design method. Orange peel yielded the highest exo-PG activity with all simple sugars and phosphate sources. According to the results of response surface methodology (RSM), the optimum concentrations of orange peel, sugar beet syrup, and $(NH_4)_2SO_4$ were found to be 10, 60, and 8 g $L^{-1}$, respectively. The exo-PG activity under these conditions was 145.4 U m $L^{-1}$ in shake flask cultures. In bioreactor studies enzyme production was induced at low pH values; thus highest production was obtained under uncontrolled pH conditions, in which the pH dropped to 2.0 in 72 h. As a result high exo-PG could be produced by an *A. sojae* mutant using a cost-effective medium containing agro-industrial substrates. Another important advantageous outcome was the low optimal pH, which is especially desired in industrial fermentations prone to contamination problems. In fact this highlights the easy adaptation of this fermentation to industrial scales.

*Keywords: Aspergillus sojae; Exo-polygalacturonase; Agro-industrial substrates; Medium optimization*

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## INTRODUCTION

Pectin and other pectic substances, which are the components of the cell wall and middle lamella, give firmness and structure to plant tissues (Gummadi and Panda 2003). Pectin is a methyl esterified 1,4-D-galacturonan chain, while its unesterified form is known as pectic acid or polygalacturonic acid (Gummadi and Panda 2003; Hoondal et al. 2002). Pectinases on the other hand, are a heterogeneous group of enzymes that degrade pectic substances. Therefore, pectinases find wide applications in the food industry, such as facilitating fruit juice extraction and reducing cloudiness and bitterness of juice, clarification of wine, coffee and tea fermentations, oil extraction, and pectic waste treatment. Other applications of pectinases include retting of textiles and fibers and bleaching of paper (Hoondal et al. 2002; Jayani et al. 2005; Kashyap et al. 2001).

Depolymerizing pectinases, PGs (EC 3.2.1.15 (endo-) and EC 3.2.1.67 (exo-)), and lyases (pectin lyase (EC 4.2.2.10), pectate lyases (EC 4.2.2.2 (endo-) and EC 4.2.2.9

(exo-)) break the glycosidic bond. These are sub-grouped depending on the substrate (pectin, pectic acid (polygalacturonate), or oligogalacturonate) and on the mode of action. They can cleave the chain randomly (endo-) or act on the terminal residue (exo-) (Jayani et al. 2005). Pectin methyl esterase (EC 3.1.1.11) de-esterifies pectin by the removal of methoxyl groups. These enzymes are synthesized in plants, filamentous fungi, bacteria, and yeast. Pectin lyases are produced by fungi, such as *Aspergillus, Penicillium*, and *Fusarium*, while pectate lyases are produced mainly by bacteria belonging to the genus *Bacillus, Erwinia*, and *Streptomyces* (Gummadi and Kumar 2005). *Aspergillus, Penicillium, Erwinia, Bacillus, Saccharomyces, Kluyveromyces,* and *Fusarium* have been studied for PG production, where the first three are the main producers. *A. niger* was used for industrial production of pectinases owing to its GRAS (Generally Regarded As Safe) status, which allows its products to be used in food-related applications (Naidu and Panda 1998).

Several agricultural products, by-products, and wastes, including orange industry wastes (El-Sheekh et al. 2009; Giese et al. 2008; Nighojkar et al. 2006; Silva et al. 2002), deseeded sunflower head (Patil and Dayanand 2006), wheat bran (Malvessi and da Silveira 2004; Silva et al. 2002), sugar beet (Anuradha et al. 2010; Olsson et al. 2003), and sugar cane (Solis-Pereira et al. 1993), corn (Nair and Panda 1997; Palaniyappan et al. 2009), and wheat flour (Palaniyappan et al. 2009) were utilized for production of pectinases in either submerged or solid state fermentation systems (Martin et al. 2010; Patil and Dayanand 2006; Ustok et al. 2007). Utilization of such low-cost substrates not only decreases the process cost, but it also eliminates the environmental problems associated with its disposal.

It has previously been demonstrated that *A. sojae* can be utilized for exo-PG (poly-(1-4)-α-D-galactosiduronate glycanohydrolyse) production both in submerged and solid-state fermentations (Gogus et al. 2006; Ustok et al. 2007). In submerged fermentation using synthetic media containing maltrin and glucose as the carbon source, the wild type *A. sojae* exhibits pellet type morphology, which is advantageous and desired due to its lower operating cost and easy downstream processing in industrial fungal fermentations (Gogus et al. 2006; Tari et al. 2007). Since pectinases have been studied extensively, there are a lot of reports on the subject; however *A. sojae* (with a GRAS status) was introduced relatively recently by earlier studies. No low-cost medium, which is necessary for adapting the process to the industry, has so far been defined for exo-PG production by this organism in submerged culture.

This paper demonstrates attempts to use agricultural products as inducers of exo-PG production by *A. sojae* and its effect on the morphology in submerged culture. At the beginning of the study, the wild type *A. sojae* and two mutants were tested for exo-PG production, where the inducing effect of corn meal and pectin was considered. In the screening part, the selected mutant was grown on several complex substrates and simple sugars in order to find the most effective ones for the production of this enzyme. After optimizing the concentrations of these components, the optimal medium was extended to bioreactor studies (5 L), where the effect of pH on the exo-PG production in combination to total sugar utilization was investigated. Statistical methods, which allow the user to investigate the effect of several factors simultaneously rather than one factor at a time, were utilized to screen the substrates and optimize the concentrations. In pectinase

production studies, factorial (Bari et al. 2010; Gogus et al. 2006) and response surface methodology (RSM) (Gogus et al. 2006; Nair and Panda 1997; Panda et al. 1999; Tari et al. 2007) designs, which were used in this study, have been shown to be powerful tools for screening several factors and optimizing the levels of factors, respectively.

## EXPERIMENTAL

### Materials

Bitter orange peel (*Aurantii amari epicarpium et mesocarpium*) (Bombastus-Werke AG, Freital-Germany) and sugar beet syrup (Grafschafter Krautfabrik, Mackenheim, Germany) were purchased from a local market in Bremen, Germany. Orange peel with a moisture content of 10% was ground to fine powder using a coffee grinder. According to the manufacturer's specifications, the total sugar concentration in the sugar beet syrup was 69% (sucrose 33%, glucose 17%, and fructose 16%). Wheat bran was obtained from a local farm in Bremen. Maltrin with a dextrose equivalent value of 42 was purchased from Pendik Nisasta (Istanbul, Turkey). All other chemicals were analytical grade and from Applichem (Darmstadt, Germany), Sigma (St. Louis, MO, USA), or Fluka (Steinheim, Germany).

### Microorganisms and Culture Conditions

*A. sojae* ATCC 20235 and its UV mutants M5/6 and M3 were evaluated in this study. Mutants were generated and selected in Prof. Lahore's laboratory at Jacobs University Bremen (unpublished results). Fungi were maintained on solid media composed of (g $L^{-1}$) glycerol (45), sugar beet syrup (45), peptone (18), NaCl (5), agar (20), $FeSO_4 \cdot 7H_2O$ (0.015), $KH_2PO_4$ (0.060), $MgSO_4 \cdot 7H_2O$ (0.050), $CuSO_4 \cdot 5H_2O$ (0.012), and $MnSO_4 \cdot H_2O$ (0.015) (Gogus et al. 2006). After five days of incubation of the plates at 30 $^oC$, spores were collected in 0.01 g $L^{-1}$ Tween 80.

Comparison of wild type and mutants and screening and optimization of medium components were carried out in shake flasks. The cultivations were carried out in a 250 mL flask containing 50 mL of culture media (see Results and Discussion Section for medium compositions). The flasks were sterilized in an autoclave at 121 $^oC$ for 20 min. These were inoculated to a final spore concentration of $4x10^5$ spore $mL^{-1}$ and incubated in shaking incubators (New Brunswick Scientific, NJ, USA) at 30 $^oC$ and 250 rpm.

The optimum medium obtained in the optimization study was tested in a five liter bioreactor with four liters of working volume (Biostat B plus, Sartorius, Gottingen, Germany) at 30 $^oC$, 350 rpm, and 0.5 vvm aeration. Antifoam (Sigma Antifoam A, St. Louis, MO, USA) was used to prevent foaming. The bioreactor was inoculated directly with spores, as in the shake flask cultures. The pH was allowed to drop to the set value spontaneously before pH control was initiated, in order to not restrict the growth of the organism due to low pH in the initial phase of cultivation. Thereafter, in the constant pH runs, pH was maintained at the set values by automatic addition of 4N NaOH or 4N $H_2SO_4$. Bioreactor runs were done in duplicates and were highly reproducible. Representative examples are shown. Bacterial contamination in the bioreactor was checked daily under microscope. No contamination was detected under any of the conditions.

## Analytical Methods

Two mL samples were taken from the shake flasks and the bioreactor in intervals and centrifuged at 10000 g for 10 minutes. Supernatants were kept at 4 $^{o}$C until exo-PG was assayed. Samples for total sugar analysis were kept frozen until analysis was ready to be carried out. Exo-PG activity was assayed according to the procedure provided by Panda et al. (1999) with slight modifications, which is based on measuring the reducing sugar concentration after incubation of enzyme with the substrate. Culture filtrate (containing exo-PG enzyme) in the amount of 86 µL was mixed with 400 µL of 2.4 g $L^{-1}$ polygalacturonic acid solution in 100 mM acetate buffer at a pH of 4.8. This mixture was incubated at 40 $^{o}$C for 20 minutes. The reducing sugar released was measured using the Nelson-Somogyi method calibrated with galacturonic acid (Nelson 1944). One unit of exo-PG activity was defined as the enzyme that catalyzes the release of 1 µmol of product per unit volume of culture filtrate per unit time under standard assay conditions (at 40 $^{o}$C and a pH of 4.8).

Total sugar concentration was measured by the phenol-sulfuric acid method using sucrose as the standard (DuBois et al. 1956). Two hundred µL of culture filtrate was mixed with the same amount of 50 g $L^{-1}$ phenol, and one mL of concentrated $H_2SO_4$ was added rapidly. Absorbance was measured at 492 nm after 10 minutes.

Biomass could not be quantified by gravimetric methods due to interference of non-soluble complex substrates used in the study.

## Experimental Design

Experiments were designed and data were analyzed using the trial version of Design of Experiments 7.1.5 statistical software (Stat-Ease, Inc, Minneapolis, USA). D-optimal factorial design was used in screening the medium components. Categorical factors such as complex C sources (orange peel, wheat bran, and corn meal) and simple sugar sources (glucose, maltrin, and sugar beet syrup) with two different phosphate sources were used. Optimization of concentrations of medium components was done using face centered composite RSM design with four factors. This design required 30 runs with six replicates at center points. Exo-PG activity was taken as the response. A second order polynomial of the following form was fitted,

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_j X_i X_j + \varepsilon \quad (1)$$

where $Y$ is the predicted response, $k$ is the number of factor variables, $\beta_0$ is the model constant, $\beta_i$ is the linear coefficient, $X_i$ is the factor variable in its coded form, $\beta_{ii}$ is the quadratic coefficient, $\beta_{ij}$ is the interaction coefficient, and $\varepsilon$ is the error factor. The variables were coded according to Eq. (2):

$$x = \frac{actual - (low level + high level)/2}{(high\ level - low\ level)/2} \quad (2)$$

The critical significance level was selected as 5% ($\alpha$=0.05) for both designs. Factors having a p-value > 0.05 in the ANOVA of the model were considered insignificant.

## RESULTS AND DISCUSSION

### Comparison of Mutants and Wild Type Strains

In order to determine the effectiveness of the mutations and to select the best strain, wild type and mutants M3 and M5/6 were grown for 120 h in a medium composed of (g $L^{-1}$) glucose (20), $(NH_4)_2SO_4$ (8), and $KH_2PO_4$ (2) and containing 20 g $L^{-1}$ of either corn meal or pectin (Nair and Panda 1997). In fact, another aim was to determine the effect of a possible induction on the exo-PG synthesis by using these two components (corn meal and pectin).

Based on the results obtained, exo-PG activities were considerably higher with mutants than with the wild type. Under these conditions corn meal was a better inducer of exo-PG than purified pectin for both mutants. The highest exo-PG activity was 98.6 U $mL^{-1}$ and was obtained in corn meal using M5/6 mutant, while this mutant produced 56.8 U $mL^{-1}$ of enzyme in pectin. The activities obtained using M3 mutant were 50.1 and 7.7 U $mL^{-1}$ in corn meal and pectin, respectively. Wild type *A. sojae* could only produce around 2 U $mL^{-1}$ of exo-PG in the presence of both substrates. Based on these results, *A. sojae* M5/6 was selected as the best strain for further screening and optimization studies.

M5/6 was a mutant generated in a further mutation cycle from the M3 mutant (unpublished results). The mutations carried out may have enhanced the utilization of this complex source by possibly increasing the activities of hydrolytic enzymes other than exo-PG, which could have contributed to the production of exo-PG. A synergistic effect may have occurred between the mutations and the nature of the complex.

### Screening of the Medium Components

In order to develop an industrially-significant low-cost medium, some agricultural products and simple sugar sources together with the phosphate sources were screened for potential high exo-PG activity. Corn meal, wheat bran, and orange peel, at concentrations of 20 g $L^{-1}$ (dry basis), were selected as the complex substrates, considering that a potential to induce pectinase production had been shown for other organisms (Cui et al. 1998; El-Sheekh et al. 2009; Fontana et al. 2009; Malvessi and da Silveira 2004; Martin et al. 2004; Martin et al. 2010; Nair and Panda 1997; Nighojkar et al. 2006; Panda et al. 2004).

One of the simple sugar sources, such as glucose (20 g $L^{-1}$), maltrin (100 g $L^{-1}$), or sugar beet syrup (40 g $L^{-1}$), was added in order to stimulate the growth of the organism. In the preliminary runs, different effects of K and Na phosphate on the exo-PG production were observed, so that phosphate source type was included in the screening design. The $KH_2PO_4$ concentration was 6.8 g $L^{-1}$, while sodium phosphate was supplied as a mixture of $NaH_2PO_4 \cdot H_2O$ (3.8 g $L^{-1}$) and $Na_2HPO_4 \cdot 2H_2O$ (4.0 g $L^{-1}$) (Gogus et al. 2006). $(NH_4)_2SO_4$ was used as the sole nitrogen source in all runs at a level of 8 g $L^{-1}$.The culture media did not contain any other salts.

Selection of the best combination was done using a D-optimal factorial design composed of 24 runs (Table 1). Activities were measured after 120 h of cultivation. This time point was determined as the optimum for the screening study based on the results of the preliminary experiments.

**Table 1.** D-optimal Design and Experimental Results of exo-PG Activity*

| Run Order | Factor 1, A: Complex C source | Factor 2, B: Simple sugar source | Factor 3, C: Phosphate source | Response: Exo-PG Activity (U $mL^{-1}$) |
|---|---|---|---|---|
| 1 | Orange Peel | Maltrin | K phosphate | 67.4 |
| 2 | Corn Meal | Sugar Beet Syrup | Na phosphate | 27.8 |
| 3 | Corn Meal | Glucose | Na phosphate | 20.1 |
| 4 | Orange Peel | Sugar Beet Syrup | K phosphate | 132.7 |
| 5 | Wheat Bran | Glucose | Na phosphate | 15.2 |
| 6 | Corn Meal | Glucose | K phosphate | 79.6 |
| 7 | Wheat Bran | Sugar Beet Syrup | K phosphate | 29.1 |
| 8 | Orange Peel | Glucose | Na phosphate | 60.5 |
| 9 | Orange Peel | Sugar Beet Syrup | Na phosphate | 95.9 |
| 10 | Corn Meal | Maltrin | K phosphate | 66.7 |
| 11 | Wheat Bran | Maltrin | K phosphate | 46.0 |
| 12 | Wheat Bran | Glucose | K phosphate | 26.1 |
| 13 | Wheat Bran | Sugar Beet Syrup | Na phosphate | 41.6 |
| 14 | Corn Meal | Sugar Beet Syrup | Na phosphate | 31.2 |
| 15 | Corn Meal | Maltrin | Na phosphate | 11.2 |
| 16 | Orange Peel | Maltrin | Na phosphate | 100.4 |
| 17 | Orange Peel | Sugar Beet Syrup | Na phosphate | 118.5 |
| 18 | Wheat Bran | Maltrin | Na phosphate | 34.8 |
| 19 | Corn Meal | Maltrin | K phosphate | 49.3 |
| 20 | Wheat Bran | Glucose | K phosphate | 22.0 |
| 21 | Orange Peel | Glucose | K phosphate | 82.9 |
| 22 | Corn Meal | Sugar Beet Syrup | K phosphate | 38.7 |
| 23 | Orange Peel | Glucose | Na phosphate | 93.1 |
| 24 | Wheat Bran | Maltrin | K phosphate | 39.9 |

*Experiments were carried out in 250 mL shake flask with a working volume of 50 mL incubated at 30°C and 250 rpm for 120 h

A significant model was obtained with an insignificant lack of fit (Table 2). Complex sources had significant effects on the exo-PG activity, while simple sugar sources and phosphate sources did not. All interaction effects were also insignificant. As seen in the interaction graphs of the model, orange peel induced more exo-PG production than the other complex substrates in the presence of either K or Na phosphates (Figs. 1 A and B). The high pectin content of orange peel may have induced the production of exo-PG (Nighojkar et al. 2006). The effect of simple sugar sources depended on the complex carbon source used. For example, glucose and maltrin were complimentary with corn meal and wheat bran, respectively. On the other hand, orange peel was more effective when used with sugar beet syrup. These findings could be partly related to the compositional differences of the complex sugars and to the stimulating effect of the simple sugars to produce certain enzymes, which might have contributed to the assimilation of the complex sources more efficiently. Similar trends were observed for

both phosphate sources, except when corn meal was the substrate; activities were higher with K phosphate than Na phosphate for all simple sugars. In the presence of corn meal, *A. sojae* may have needed additional potassium in the culture medium for growth and/or enzyme production. These can be explained by the complex mineral compositions of the agricultural substrates used in the study.

**Table 2.** Anova Table: Effect of the Parameters on the Exo-PG Activity

| Source | Sum of Squares | df | Mean Square | F Value | p-value | |
|---|---|---|---|---|---|---|
| Model | 2.41E+04 | 13 | 1.85E+03 | 6.89 | 0.0022 | significant |
| A: Complex C source | 1.91E+04 | 2 | 9.53E+03 | 35.5 | < 0.0001 | |
| B: Additional C source | 1.04E+03 | 2 | 5.20E+02 | 1.93 | 0.1950 | |
| C: Phosphate source | 1.07E+03 | 1 | 1.07E+03 | 3.98 | 0.0739 | |
| AB | 2.08E+03 | 4 | 5.21E+02 | 1.94 | 0.1809 | |
| AC | 1.51E+03 | 2 | 7.57E+02 | 2.82 | 0.1071 | |
| BC | 2.70E+02 | 2 | 1.35E+02 | 0.501 | 0.6202 | |
| Residual | 2.69E+03 | 10 | 2.69E+02 | | | |
| Lack of Fit | 1.72E+03 | 4 | 4.29E+02 | 2.65 | 0.1378 | not significant |
| Pure Error | 9.72E+02 | 6 | 1.62E+02 | | | |
| Total | 2.68E+04 | 23 | | | | |

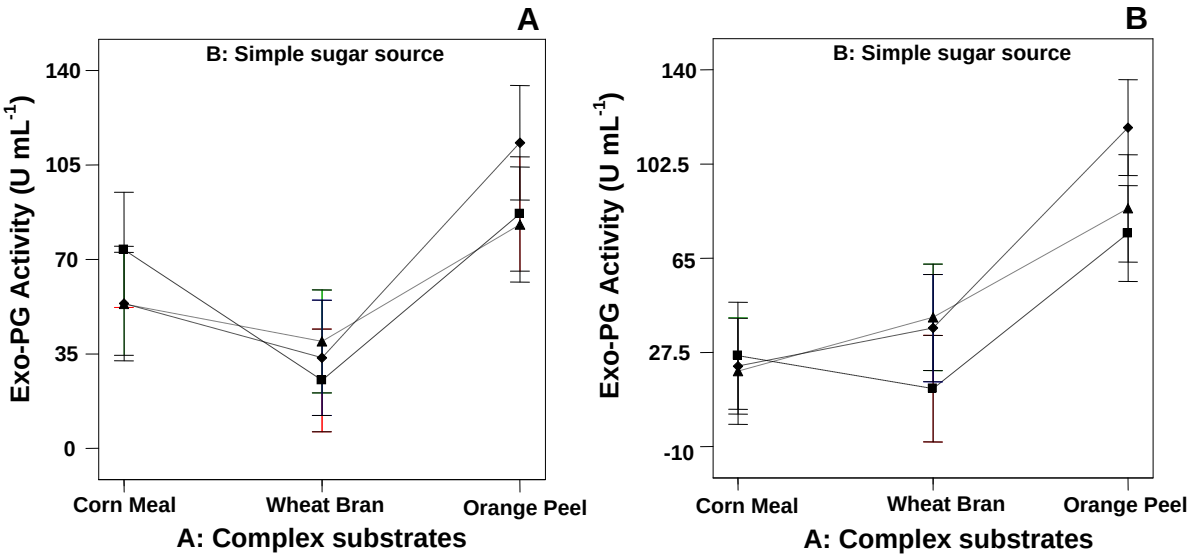


**Fig. 1.** Results of D-optimal design: Plots showing the interactive effect of complex C sources and the simple sugar sources (■: glucose, ▲: maltrin, and ♦: sugar beet syrup) on the exo-PG activity in the presence of K phosphate (A) and Na phosphate (B)

According to the model, the highest exo-PG activity was obtained by using orange peel and sugar beet syrup supplemented by either K or Na phosphates. In addition to the sugar content, the pectin in sugar beet syrup could be the reason for the high exo-PG production in this media (Phatak et al. 1988). However, with corn meal and wheat

bran, the effect of sugar beet syrup was not considerably different than glucose and maltrin (Figs. 1 A and B). Thus, orange peel, sugar beet syrup, $K_2HPO_4$, and $(NH_4)_2SO_4$ were selected as the medium components for the following optimization study.

## Optimization of the Medium Components

The concentrations of orange peel (A), sugar beet syrup (B), $KH_2PO_4$ (C), and $(NH_4)_2SO_4$ (D) were optimized using a face centered central composite design. The low and high values for the factors were defined in g $L^{-1}$ as: 10 and 70 for orange peel, 10 and 70 for sugar beet syrup, 0.50 and 10.00 for $(NH_4)_2SO_4$, and 0 and 8 for $KH_2PO_4$. The design was composed of 30 runs, six of which were the center points. The design and the results are given in Table 3. The $KH_2PO_4$ term and its interactions (D, $D^2$, AD, BD, and CD) as well as the quadratic term of orange peel ($A^2$) had insignificant p-values ($p>0.05$), so they were excluded from the model for a better fit. The p-value of the resulting model was less than 1 x $10^{-4}$ (significant) and 0.330 for the lack of fit (insignificant). All other terms included in the model were significant at the 5% significance level except for the interaction of orange peel and sugar beet syrup, which had a p-value of 0.069 (Table 4). The final equation of the model in terms of coded factors was:

$$Activity = 121.8 - 13.52\,A + 16.5\,B + 31.6\,C - 11.7\,A\,B - 15.4\,A\,C + 14.9\,B\,C - 29.1\,B^2 - 45.2\,C^2 \quad (3)$$

Three-dimensional models showing the interactions between the factors are given in Fig. 2. Orange peel concentration had an inverse effect on the exo-PG activity. Activity decreased linearly as the concentration increased from 10 to 70 g $L^{-1}$. At 8 g $L^{-1}$ of $(NH_4)_2SO_4$, maximum exo-PG activity was obtained at around 60 g $L^{-1}$ of sugar beet syrup and 10 g $L^{-1}$ of orange peel (Fig. 2 A). Similarly, at 10 g $L^{-1}$ of orange peel, exo-PG activity was maximized at around 60 g $L^{-1}$ of sugar beet syrup and 8 g $L^{-1}$ of ammonium sulfate (Fig. 2 B). Since the potassium phosphate concentration was excluded from the model, it did not have an effect on the response. The validity of the model was checked at varying concentrations of the factors in the range of the study including the optimum condition (Table 5). The measured activities deviated 9 to 21% from the values predicted by the model. It was concluded that, the optimum medium for exo-PG production under these conditions was composed of orange peel (10 g $L^{-1}$), sugar beet syrup (60 g $L^{-1}$), and $(NH_4)_2SO_4$ (8 g $L^{-1}$). In this medium pH was around 2.0 after 120 h of cultivation in the shake flask.

Since orange peel had a negative effect on the exo-PG activity in the range of this study (10-70 g $L^{-1}$), an additional run was carried out in which orange peel was omitted from the optimum medium. In the presence of 60 g $L^{-1}$ of sugar beet syrup and 8 g $L^{-1}$ of $(NH_4)_2SO_4$, *A. sojae* M5/6 produced 43.0 U $mL^{-1}$ of exo-PG in 96 h. This showed that orange peel was necessary to obtain high exo-PG activity. It was previously reported that orange peel powder above 15 g $L^{-1}$ inhibited the PG activity obtained by *A. niger* (Nighojkar et al. 2006). Inhibition of exo-PG was also observed for *A. japonicus* when the pectin concentration was increased from 5 to 10 g $L^{-1}$ (Teixeira et al. 2000). In these reports, the authors explained the inhibition by the possible presence of pectin degradation products, which may have acted as enzyme inhibitors.

**Table 3.** Face Centered Central Composite Design (Face Centered CCD) and Experimental Results of Exo-PG Activity*

| Run Order | Factor 1, A: Orange Peel (g $L^{-1}$) | Factor 2, B: Sugar Beet Syrup (g $L^{-1}$) | Factor3, C: Ammonium sulfate (g $L^{-1}$) | Factor 4, D: $KH_2PO_4$ (g $L^{-1}$) | Response: Exo-PG Activity (U $mL^{-1}$) |
|---|---|---|---|---|---|
| 1 | 10 (-1) | 70 (+1) | 0.50 (-1) | 8 (+1) | 14.3 |
| 2 | 70 (+1) | 10 (-1) | 10.00 (+1) | 8 (+1) | 111.9 |
| 3 | 10 (-1) | 70 (+1) | 10.00 (+1) | 0 (-1) | 157.9 |
| 4 | 40 (0) | 40 (0) | 5.25 (0) | 4 (0) | 101.6 |
| 5 | 40 (0) | 40 (0) | 5.25 (0) | 4 (0) | 112.4 |
| 6 | 70 (+1) | 70 (+1) | 0.50 (-1) | 0 (-1) | 17.6 |
| 7 | 70 (+1) | 10 (-1) | 0.50 (-1) | 0 (-1) | 9.0 |
| 8 | 40 (0) | 40 (0) | 5.25 (0) | 4 (0) | 133.2 |
| 9 | 10 (-1) | 10 (-1) | 0.50 (-1) | 0 (-1) | 8.9 |
| 10 | 10 (-1) | 70 (+1) | 0.50 (-1) | 0 (-1) | 11.2 |
| 11 | 10 (-1) | 70 (+1) | 10.00 (+1) | 8 (+1) | 146.1 |
| 12 | 40 (+1) | 40 (0) | 5.25 (0) | 4 (0) | 119.0 |
| 13 | 10 (-1) | 10 (-1) | 10.00 (+1) | 0 (-1) | 56.2 |
| 14 | 10 (-1) | 10 (-1) | 0.50 (-1) | 8 (+1) | 7.0 |
| 15 | 10 (-1) | 10 (-1) | 10.00 (+1) | 8 (+1) | 30.2 |
| 16 | 70 (+1) | 10 (-1) | 0.50 (-1) | 8 (+1) | 23.8 |
| 17 | 70 (+1) | 70 (+1) | 0.50 (-1) | 8 (+1) | 19.3 |
| 18 | 70 (+1) | 10 (-1) | 10.00 (+1) | 0 (-1) | 18.4 |
| 19 | 70 (+1) | 70 (+1) | 10.00 (+1) | 8 (+1) | 72.6 |
| 20 | 70 (+1) | 70 (+1) | 10.00 (+1) | 0 (-1) | 31.0 |
| 21 | 40 (0) | 10 (-1) | 5.25 (0) | 4 (0) | 83.0 |
| 22 | 40 (0) | 40 (0) | 5.25 (0) | 8 (+1) | 114.1 |
| 23 | 40 (0) | 70 (+1) | 5.25 (0) | 4 (0) | 113.2 |
| 24 | 40 (0) | 40 (0) | 10.00 (+1) | 4 (0) | 140.4 |
| 25 | 40 (0) | 40 (0) | 5.25 (0) | 4 (0) | 91.8 |
| 26 | 10 (-1) | 40 (0) | 5.25 (0) | 4 (0) | 142.0 |
| 27 | 70 (+1) | 40 (0) | 5.25 (0) | 4 (0) | 88.6 |
| 28 | 40 (0) | 40 (0) | 5.25 (0) | 4 (0) | 137.1 |
| 29 | 40 (0) | 40 (0) | 5.25 (0) | 0 (-1) | 188.3 |
| 30 | 40 (0) | 40 (0) | 0.50 (-1) | 4 (0) | 23.5 |

*The actual levels of the factors are given with coded levels in parentheses
Experiments were carried out in 250 mL shake flask with a working volume of 50 mL incubated at 30°C and 250 rpm for 120 h

**Table 4.** Anova Table: Effect of the Parameters on the Exo-PG Activity

| Source | Sum of Squares | df | Mean Square | F Value | p-value | |
|---|---|---|---|---|---|---|
| Model | 5.70E+04 | 8 | 7.12E+03 | 11.9 | < 0.0001 | significant |
| A: Orange Peel | 3.29E+03 | 1 | 3.29E+03 | 5.52 | 0.0292 | |
| B: Sugar Beet Syrup | 4.89E+03 | 1 | 4.89E+03 | 8.20 | 0.0096 | |
| C: Ammonium Sulfate | 1.79E+04 | 1 | 1.79E+04 | 30.1 | < 0.0001 | |
| AB | 2.20E+03 | 1 | 2.20E+03 | 3.70 | 0.0689 | |
| AC | 3.80E+03 | 1 | 3.80E+03 | 6.38 | 0.0201 | |
| BC | 3.57E+03 | 1 | 3.57E+03 | 5.99 | 0.0238 | |
| $B^2$ | 2.69E+03 | 1 | 2.69E+03 | 4.51 | 0.0464 | |
| $C^2$ | 6.48E+03 | 1 | 6.48E+03 | 10.9 | 0.0036 | |
| Residual | 1.19E+04 | 20 | 5.96E+02 | | | |
| Lack of Fit | 1.04E+04 | 16 | 6.49E+02 | 1.68 | 0.3299 | not significant |
| Pure Error | 1.55E+03 | 4 | 3.87E+02 | | | |
| Total | 8.92E+04 | 29 | | | | |

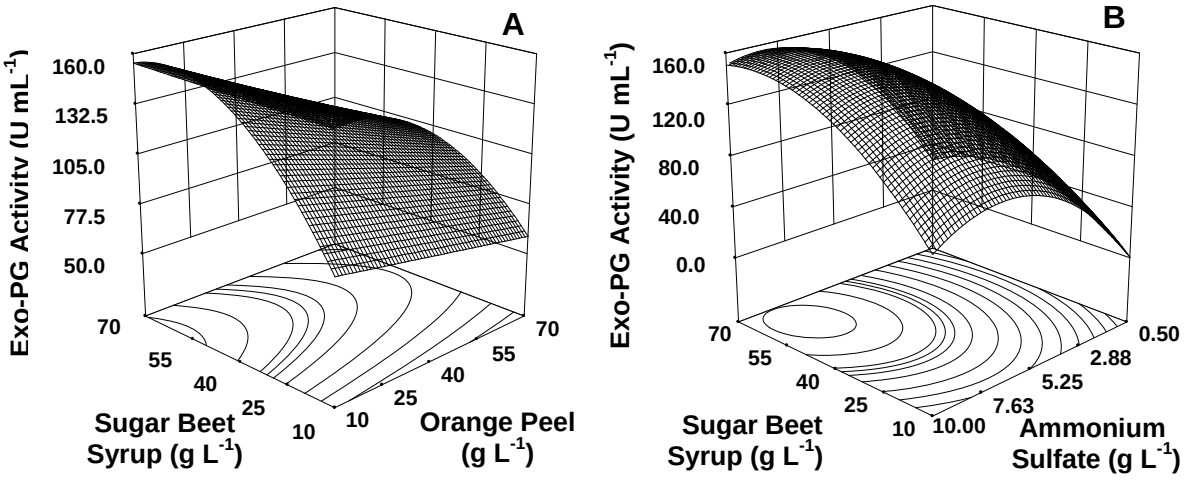


**Fig. 2.** Results of Face Centered CCD: Response surface plots showing the interactive effect of orange peel and sugar beet syrup concentrations in the presence of 8 g $L^{-1}$ of ammonium sulfate (A) and sugar beet syrup and ammonium sulfate in the presence of 10 g $L^{-1}$ of orange peel (B) on the exo-PG activity

Under the conditions of this study, high orange peel concentrations caused viscous broths, which may have had adverse effects on stirring and aeration of the culture broth. Nevertheless, this combination, when considered by an industrial point of view, is still economical, since it utilizes orange peel as the waste product of the fruit juice industry, solving an important pollution problem. The required medium composition is not very complex, composed of only three main components, making the medium preparation step easier and thus reducing the operational cost.

**Table 5.** Validation Experiments of the Optimized Conditions with the Experimental (Measured After 120 h) and Predicted Exo-PG Activities*

| Run no | Orange Peel ($g\ L^{-1}$) | Sugar Beet Syrup ($g\ L^{-1}$) | $(NH_4)_2SO_4$ ($g\ L^{-1}$) | Predicted Exo-PG Activity ($U\ mL^{-1}$) | Measured Exo-PG Activity ($U\ mL^{-1}$) |
|---|---|---|---|---|---|
| 1 | 40 | 10 | 5.0 | 75.2 | 95.9 (6.9) |
| 2 | 70 | 10 | 10.0 | 30.3 | 33.7 (2.5) |
| 3 | 10 | 60 | 8.0 | 159.0 | 145.4 (1.5) |
| 4 | 29 | 38 | 3.2 | 101.7 | 133.6 (11.3) |

*Standard deviation of two runs is given in parenthesis
Experiments were carried out in 250 mL shake flask with a working volume of 50 mL at 30°C and 250 rpm

## Bioreactor Studies

The optimal medium (orange peel (10 g $L^{-1}$), sugar beet syrup (60 g $L^{-1}$), and $(NH_4)_2SO_4$ (8 g $L^{-1}$)) was tested in a five-liter bioreactor in order to evaluate the effect of scale up and pH on the exo-PG production. In the first run, in which pH was not controlled, 115.5 U $mL^{-1}$ of exo-PG activity was obtained in 96 h, while the activity increased slightly to 120.0 U $mL^{-1}$ in 16 h (Fig. 3 A). This value was around 20% lower than the one obtained in the flask culture. The sugar concentration at the beginning of the culture was 43 g $L^{-1}$; where after 96 h of incubation, a substantial amount (22 g $L^{-1}$) remained that corresponded to a substrate utilization rate of 0.14 g $L^{-1}$ $h^{-1}$ (Fig. 3 B). In fact, the slow utilization of sugars can be explained by the slow growth and metabolism of *A. sojae* at low pH values. The initial pH of the fermentation broth was around 4.2 and decreased to 2.0 in 66 h, where it remained at this value afterwards, confirming the explanation made above.

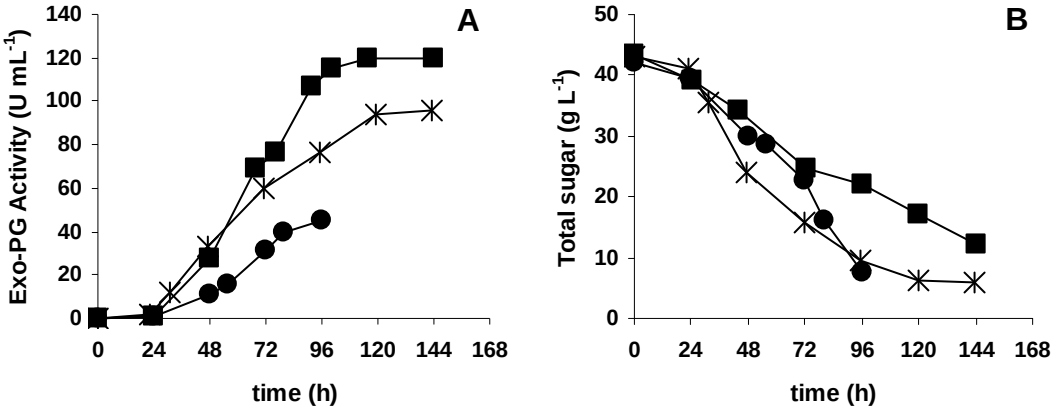


**Fig. 3.** Effect of culture pH on the exo-PG production (A) and sugar consumption (B) (■: no pH control, x: pH=3, and ●: pH=4). Experiments were carried out in the bioreactor with a working volume of 4 L at 30 °C and 350 rpm. Culture medium was composed of orange peel (10 g $L^{-1}$), sugar beet syrup (60 g $L^{-1}$), and $(NH_4)_2SO_4$ (8 g $L^{-1}$).

In order to eliminate the adverse effect of low pH, experiments where the *A. sojae* was grown at slightly higher pH values, i.e. at 3 and 4, were conducted (Fig. 3). In these

experiments, sugar utilization continued with an assimilation rate of 0.27 and 0.35 g $L^{-1}$ $h^{-1}$, at pH 3 and 4 respectively, considering the individual fermentation times of 96 and 120 h. The corresponding exo-PG activities were 45.2 U $mL^{-1}$ and 95.0 U $mL^{-1}$ at pH 3 and 4, respectively. Furthermore, the exo-PG activity was also very low in an experiment conducted at a pH of 6, namely below 5 U $mL^{-1}$ (data not shown). This fact showed that pH had a drastic effect on exo-PG production by *A. sojae* using sugar beet syrup and orange peel. Therefore, low pH values were necessary to induce exo-PG production. The induction of exo-PG production by *A. sojae* at low pH values is yet not clear and should be investigated with more detailed studies. However, this observation could be correlated to the activation of various enzymes at this pH responsible in the utilization of these resources. As a result, this could have deviated the metabolic pathways in favor of exo-PG production. Similarly, a pH of around 3 was reported to be necessary for enhanced endo-PG and exo-PG production in wheat bran-containing medium by *A. oryzae* (Malvessi and da Silveira 2004). *A. flavipes* FP-500 growing on pectin produced higher exo and endo-pectinase at an initial pH of 3.5 compared to a pH 4.2 and 5.0 despite the lower cell growth at a pH of 3.5. The composition of the pectinase pool of *Aspergillus* strains was also reported to be affected by pH in submerged (Martinez-Trujillo et al. 2009; Trejo-Aguilar et al. 1996) and solid-state fermentations (Cavalitto et al. 1996). Therefore, pH should be adjusted so that the growth and exo-PG production is balanced or exo-PG production is enhanced by decreasing the pH after substantial growth is obtained at higher pH values.

The low pH value obtained after 24 h until the end of fermentation, where the maximum exo-PG was obtained, will be very suitable for large-scale fermentations of *A. sojae*, first, because it will not require pH control, reducing the demand for acid and base additions and, secondly, preventing the contamination problems. In both cases the operational and raw material costs will be reduced. It should not be overlooked, however, that not all industrial fungal fermentations provide such an advantage. With this perspective this study not only proposes an optimum low-cost industrial medium that results in very high exo-PG activity, but also proposes an easy operational bioprocess with almost no requirement for pH and contamination control. Thus, it can be considered as a self-regulating process with this regard mainly preferred in the industry.

Considering the morphology of *A. sojae* M5/6, it was observed that it grew in mycelial clumps in all current flasks and the bioreactor studies. Here, mycelial clumps are referred to as a network of entangled mycelia, forming small groups. This type of morphology yielded viscous fermentation broths and resulted in poorly mixed zones especially in the bioreactor. This situation was more apparent when pH was kept at higher values (3 and 4) due to a possibly higher amount of biomass formation at these pH values. The lower enzyme level in the bioreactor than the shake flask culture could partly be related to this observation, that is, different geometrical shapes of the particular systems may have resulted in different mixing and aeration efficiency, which could have influenced the enzyme production. Under the conditions of this study *A. sojae* did not form pellets, which were the morphology observed in the synthetic medium (Gogus et al. 2006). Pelleted growth is advantageous in most of the fungal fermentations since it does not cause a viscous broth, unlike filamentous growth. Therefore, the current study demonstrated that some more extensive work on the factors promoting pellet type of

growth without adversely affecting the exo-PG yields needs to be carried out in future studies.

In the comparison of the current results with the literature studies, the exo-PG activity values obtained in this study were higher than those obtained with wild type *A. sojae* in the previous research reports (Gogus et al. 2006; Oncul et al. 2007; Tari et al. 2007; Ustok et al. 2007) and also higher than the values in most of the reports in the literature (Favela-Torres et al. 2006). In synthetic medium containing glucose and maltrin as carbon sources, the maximum exo-PG activity obtained with wild type *A. sojae* in pellet form was 0.2 U $mL^{-1}$ (Gogus et al. 2006). Optimization of culture conditions for the same strain increased the exo-PG activity to 13.5 U $mL^{-1}$ (Tari et al. 2007). Thus, the use of mutants of *A. sojae* (M5/6) and complex carbon sources in this study resulted in marked increase (145.4 U $mL^{-1}$) in submerged exo-PG production.

Sugar beet syrup has not been used directly as a substrate for exo-PG production; however, sugar beet pulp was utilized for PG production by *Trichoderma reesei* (Olsson et al. 2003) and by *Mucor flavus* (Gadre et al. 2003) in submerged culture. Orange industry wastes like peel, bagasse, and pulp were successfully used for solid-state production of exo-PG or polymethylgalacturonase (PMG) by *F. moniliforme* (Niture and Pant 2004), *P. viridicatum* (Silva et al. 2002), and *Thermoascus aurantiacus* (Martins et al. 2002), while enzyme titers were generally lower in the submerged culture. The use of orange peel as inducer of exo-PG production by immobilized *A. niger* resulted in enzyme activity less than 1 U $mL^{-1}$ (Nighojkar et al. 2006). Similarly, the highest thermostable PMG activity obtained with *Sporotrichum thermophile* was 0.5 U $mL^{-1}$ in citrus peel containing medium (Kaur et al. 2004). *Thermomucor indicae-seudaticae* produced 13.6 U $mL^{-1}$ of exo-PG in submerged culture containing orange bagasse (10 g $L^{-1}$) and wheat bran (10 g $L^{-1}$), while in the solid-state fermentation exo-PG activity was higher (Martin et al. 2010). On the other hand, high PG activities were obtained in submerged fermentations utilizing purified pectin (Favela-Torres et al. 2006). For example, 221 U $mL^{-1}$ of exo-PG activity was obtained by a *P. occitanis* mutant using citrus pectin (Hadj-Taieb et al. 2002), while *A. japonicus* produced 500 U $mL^{-1}$ of exo-PG in a medium containing pectin (5 g $L^{-1}$) and glucose (5 g $L^{-1}$) (Teixeira et al. 2000).

Apart from those discussed above, exo-PG activity values reported in the literature were obtained using different substrates and fungal organisms in submerged culture and are mostly well below those obtained in this study (Favela-Torres et al. 2006). This study serves as a good example for medium formulations, where orange peel and sugar beet syrup can be used as important low-cost media sources in submerged fermentations.

Generally, materials like orange peel are considered in solid-state fermentations. Hence, this will create a new area of application for the production of various value added products using different fungal organisms. Moreover, low operational pH of this process is attractive from the industrial point of view, since it does not require pH control and reduces contamination risk. Furthermore, using specifically *A. sojae* mutant strain that is not very common in the literature, will further contribute to the field of pectinases produced using novel organism.

## CONCLUSIONS

1. *A. sojae* mutant M5/6 was able to utilize some agro-industrial sources, such as orange peel, wheat bran, and corn meal, efficiently to produce exo-PG, an enzyme that has a high economic value, in submerged culture.
2. A simple and low-cost medium composed of orange peel, sugar beet syrup, and $(NH_4)_2SO_4$ was optimized to obtain a considerably higher amount of the enzyme. *A. sojae* M5/6 produced 145.4 and 120.0 U $mL^{-1}$ of exo-PG in shake flask and bioreactor cultures, respectively.
3. Exo-PG production was highest at low pH values. Higher values decreased the enzyme levels significantly. Due to the low operational pH, no pH control is required and bacterial contamination risk may be reduced.
4. Although this organism was previously shown to grow as pellets in some media, the morphology under the conditions of this study was mycelial clumps. This morphology created mixing problems in the bioreactor.

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