Research Article

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Harnessing soybean hulls for improved polygalacturonase production by *Aspergillus sojae* through fine-tuning of ambient pH

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Abstract

BACKGROUND: Soybean hulls result from the processing of the bean for producing oil and protein products. This by-product generated massively in America has virtually no commercial value, so substantial effort is being applied to its exploitation for generating value-added goods. This work evaluates soybean hulls as inducer of the production of pectinolytic enzymes, through optimization studies regarding polygalacturonase production by *Aspergillus sojae* in submerged cultures.

RESULTS: A 2-fold improvement in polygalacturonase yield was found by varying the initial pH of the culture in a very narrow acid pH range (2.40–2.80). The optimized fermentation process was successfully transferred to stirred-tank bioreactors in terms of volumetric productivity, and final polygalacturonase yields were 42 U mL^{-1} and 1.39 U g^{-1} soybean hulls, which are among the highest reported with this by-product. Morphological characterization of *A. sojae* during cultivation showed that the fungus mainly developed in dispersed mycelia at initial pH of 2.40–2.80 while, conversely, fungal pellets predominated in cultures performed at initial pH of 5.40.

CONCLUSION: High enzyme titers are possibly connected to the formation of dispersed mycelia, as well as to acid-induced expression of the respective gene/s. It is foreseen that this data will be helpful regarding the production of fungal pectinases or other acid-induced enzymes.

Supporting information may be found in the online version of this article.

Keywords: soybean hull; polygalacturonase; *Aspergillus sojae*; initial culture pH

INTRODUCTION

Enzymes for application in food processing remain the largest segment of bulk industrial enzymes.[1] Among these, pectinases constitute a major group, especially – but not only – due to their use within the fruit juice industry for juice clarification. Pectinases is the term commonly used to refer to a collective of enzymes responsible for breaking down pectin and pectic substances, which are complex hetero-polysaccharides present in the middle lamella and primary cell wall of higher plants. Pectin plays an important role in the formation of these cell walls, lending strength and integrity to a structure which completes its core by cellulose and hemicellulose. The fine chemical structure of a pectin molecule remains unelucidated, several models having been proposed throughout the years to accommodate all the available information.[2] What is clear is that pectin is composed of the same repeating structural polysaccharide elements regardless of the pectin source, and of these elements, homogalacturonan is the most abundant.[2] A homolgalacturonan molecule basically consists of a linear backbone of galacturonic acid residues linked by α(1-4) glycosidic bonds, whose hydrolysis is catalyzed by polygalacturonases (PGs). Besides its application in fruit juice clarification, PGs as well as other pectinases are used for the maceration of vegetable tissue to produce pulpy products as base ingredients for purées destined for babies and elderly people.[3] Moreover, these enzymes are employed within the winemaking industry to promote the clarification of white wine and must, and to improve color extraction during red wine elaboration.[4]

Aspergillus sojae is a mesophilic filamentous fungus closely related to *A. oryzae*, both of which have a history of safe use throughout Asia for the production of fermented foods such as miso (fermented soybean paste) and soyu (soy sauce).[5] Centuries of use of these non-toxigenic aspergilli in food fermentation suggest their biosafety, so it can be argued that metabolites derived from *A. sojae*, such as enzymes, could be safely used as food additives. Currently, industrial production of pectinases is mainly performed by *A. niger*, partly due to the high titers obtained, although great efficiency in the production of extracellular enzymes is not a hallmark restricted to this species. In fact, within recent years

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several studies have been performed that report the effective production of PGs by *A. sojae*.[6–8]

Soybean hull is an agricultural by-product that results from the de-hulling of soybeans during its processing to make vegetable oil for human consumption and protein-rich meal as ingredient for food and feed uses.[9] Global soybean production has increased 41% in the last 10 years, reaching 313 million tons in 2015/2016 according to the United States Department of Agriculture. Nearly 90% of the total production comes from America, specifically Brazil, the USA and Argentina, the latter accounting for 18% of the total share. The seed coat, which represents around 8% of the bean,[10] has low commercial value as it is sold as feed for cattle. Interestingly, compared with other waste lignocellulosic biomass, soybean hulls typically contain 6–15% pectin and are poorly lignified, which contributes to raising the question whether this by-product generated massively in America could be exploited for pectinases production. Recently, substantial attention has been paid to different pretreatment methods, such as physico-chemical or enzymatic hydrolysis, to yield fermentable carbohydrates for bioethanol production.[10–12] Enzyme production from soybean hull has received some attention, although research was directed almost entirely to cellulases and xylanases.[9] The rationale behind this approach lies in the relatively higher content of cellulose and hemicellulose compared with pectin in soybean hulls,[10] which is in turn apparent due to the nature of the by-product. Nevertheless, since *A. sojae* was demonstrated to be an outstanding producer of pectinolytic activity employing agricultural residues rich in pectic substances such as orange peel and apricot pomace, [6–8] a pectinases production study linking this efficient microbial strain and soybean hulls as both substrate and inducer can certainly be of interest. It is worth mentioning that soybean hulls are produced mainly in the Pampean region in Argentina, which is also the place where this investigation was carried out in an effort to valorize the residue.

This study intends to set out optimum culture conditions for efficient production of PG activity by *A. sojae* when growing on soybean hulls. No carbon and energy sources other than soybean hulls were utilized, aiming to formulate a cost-effective culture medium that would render process scale-up feasible. After a shake-flask-scale screening procedure by means of statistical analysis, the optimized culture conditions were assayed on bench-scale bioreactors to test the reproducibility of the PG yields obtained. Microscopic image analysis was used to come to a better understanding of the morphological changes experienced by *A. sojae* under some of the different culture conditions assayed and the likelihood of these changes being linked with the enzymatic levels achieved.

MATERIAL AND METHODS

Material

Soybean hulls, kindly supplied by a local oilseed processing company, were ground to powder using a laboratory mill and stored at room temperature until use. Polygalacturonic acid, xylan from birchwood and galacturonic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Microorganism and inoculum

Aspergillus sojae ATCC 20235 was obtained from the American Type of Culture Collection. The strain was propagated on YME plates (4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 4 g L^{-1} glucose, 20 g L^{-1} agar) by incubation at 30°C until sporulation, and stored at −80°C as stock cultures using 20% glycerol as cryoprotectant.

Conidia suspensions used to prepare the seed inocula were extracted from molasses agar slants after incubation for 1 week at 30°C.[13] Conidia were harvested from the slants using 0.02% Tween 80-water and counted microscopically in a Neubauer Chamber (Marienfeld, Germany).

Shake flask cultures

Medium composition and culture conditions studies were performed in 250 mL Erlenmeyer flasks containing 30 g L^{-1} of ground soybean hulls and inorganic salts in distilled water up to 50 mL working volume. The pH of the culture medium was adjusted to the desired value with HCl and the flasks were autoclaved at 121°C for 20 min prior to inoculation with 2×10^5 conidia mL^{-1}, unless otherwise stated. Cultures were incubated at 30°C in an orbital shaker running at 150 rpm. After 110 h, samples were collected from the flasks, centrifuged at 3500×g for 15 min and the supernatants assayed for PG activity determination. On the other hand, samples for fungal growth determination were obtained by lyophilization of the pellets resulting from harvesting and centrifuging (3500×g, 15 min) the whole contents of each culture.

Experimental design

Parameters that significantly affect PG production were identified using a two-level fractional factorial design of resolution IV. For the screening, the following parameters and low and high level conditions (-/+) were tested: $(NH_4)_2SO_4$ (1/5 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.25/1 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.04/0.18 g L^{-1}), KH_2PO_4 (0.25/2 g L^{-1}), inoculums size ($5\times10^4/4\times10^5$ conidia mL^{-1}), initial culture pH (4.4/5.4). Upon experimental run, a half-normal plot was used to evaluate the significance of each of the effects studied. The experimental design was built and analyzed employing Design-Expert software trial version 7.0.0.

Once significant parameters were identified, their amounts were varied in two successive Doehlert uniform shell designs. Statgraphics Plus 5.1 software was used for experimental design and data analysis. After running the experiments, a second-order polynomial equation was used to fit the response data:

$$Y = \beta_\circ + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_{ij}$$

where Y is the response variable, $\beta_\circ$ the model constant, β_i, β_{ii}, β_{ij} the coefficients for the linear, quadratic and interaction effect, respectively.

In order to code the real values of the independent variables the following equation, which is based on the linear functionality between codified (X_{cod}) and actual values (X), was used:

$$X = X_{cod}\frac{\Delta X_r}{\Delta X_{cod}} + X_0$$

where ΔX_r and ΔX_{cod} are the difference between the highest and lowest values of the real and coded numbers, and X_0 is the real value of the central point of the design.

For each of the experimental designs applied, the central point was replicated three times to calculate the experimental error.

Table 1. Fractional factorial experimental design and PG activity determined at 108 h of culture

Run	$MgSO_4 \cdot 7H_2O$ (g L^{-1})	$CaCl_2 \cdot 2H_2O$ (g L^{-1})	KH_2PO_4 (g L^{-1})	Inoculum (10^5/mL)	pHi[a]	$(NH_4)_2SO_4$ (g L^{-1})	PG activity (U mL^{-1})
1	0.25 (–)[b]	0.04 (–)	0.25 (–)	4.00 (+)	4.45 (–)	5.00 (+)	11.37
2	1.00 (+)	0.04 (–)	2.00 (+)	4.00 (+)	4.45 (–)	1.00 (–)	8.72
3	0.63 (0)	0.11 (0)	1.13 (0)	2.25 (0)	4.95 (0)	3.00 (0)	12.22
4	0.25 (–)	0.18 (+)	2.00 (+)	4.00 (+)	4.45 (–)	5.00 (+)	12.43
5	0.63 (0)	0.11 (0)	1.13 (0)	2.25 (0)	4.95 (0)	3.00 (0)	11.79
6	1.00 (+)	0.04 (–)	0.25 (–)	0.50 (–)	5.45 (+)	1.00 (–)	5.34
7	0.25 (–)	0.04 (–)	0.25 (–)	0.50 (–)	4.45 (–)	1.00 (–)	8.08
8	0.25 (–)	0.18 (+)	0.25 (–)	4.00 (+)	5.45 (+)	1.00 (–)	4.80
9	0.25 (–)	0.04 (–)	2.00 (+)	4.00 (+)	5.45 (+)	1.00 (–)	5.25
10	1.00 (+)	0.04 (–)	2.00 (+)	0.50 (–)	4.45 (–)	5.00 (+)	11.42
11	0.25 (–)	0.18 (+)	2.00 (+)	0.50 (–)	4.45 (–)	1.00 (–)	9.28
12	0.25 (–)	0.04 (–)	2.00 (+)	0.50 (–)	5.45 (+)	5.00 (+)	10.47
13	1.00 (+)	0.18 (+)	0.25 (–)	4.00 (+)	4.45 (–)	1.00 (–)	8.36
14	1.00 (+)	0.18 (+)	2.00 (+)	4.00 (+)	5.45 (+)	5.00 (+)	11.47
15	1.00 (+)	0.18 (+)	0.25 (–)	0.50 (–)	4.45 (–)	5.00 (+)	10.72
16	1.00 (+)	0.18 (+)	2.00 (+)	0.50 (–)	5.45 (+)	1.00 (–)	4.88
17	1.00 (+)	0.04 (–)	0.25 (–)	4.00 (+)	5.45 (+)	5.00 (+)	10.82
18	0.63 (0)	0.11 (0)	1.13 (0)	2.25 (0)	4.95 (0)	3.00 (0)	11.49
19	0.25 (–)	0.18 (+)	0.25 (–)	0.50 (–)	5.45 (+)	5.00 (+)	11.25

[a] Initial pH of the culture medium.
[b] Coded factors.

Bioreactor cultures

A 2.5 L stirred-tank bioreactor (BioFlo 310; New Brunswick Scientific, Edison, NJ, USA) with 1.5 L working volume was employed to test the optimized culture medium. Agitation was provided by a standard Rushton impeller, whereas a second impeller was positioned in the headspace to work as foam-breaker; in addition, antifoam was used when necessary. Bioreactors were inoculated with 2×10^5 conidia mL^{-1} and operated at 30°C and 0.6 vvm; stirring was fixed at 450 rpm. The dissolved oxygen tension (DOT) was monitored using a polarographic-type electrode (Mettler-Toledo, Columbus, OH, USA), while pH variations were registered with a glass electrode (Mettler-Toledo). Outlet oxygen and carbon dioxide partial pressures were measured by means of oxygen (1100 series, Servomex) and carbon dioxide (PIR-2000, Horiba) gas analyzers. Batch cultures were performed in duplicate. Samples withdrawn regularly from the bioreactors were centrifuged (3500×g for 15 min) and the resulting supernatants analyzed for enzyme activity, total and reducing sugar concentration, and glucose content.

Analytical techniques

PG, xylanase (XYL) and endoglucanase activities were determined by measuring the reducing sugars released from the respective substrates, as stated below. PG activity measurements were assayed at 35°C according to Cavalitto *et al.*,[14] using 0.20% polygalacturonic acid (pH 5.0) as substrate and galacturonic acid as standard. XYL activity was determined at 50°C, employing 0.25% birchwood xylan (pH 5.3) as substrate and xylose as standard. For the endo-glucanase activity determination, the reaction was carried out at 50°C employing 0.25% carboxymethylcellulose (pH 4.0) and glucose as substrate and standard, respectively. Solutions were prepared in 12.5 mmol L^{-1} citric acid – 6.25 mmol L^{-1} Na_2HPO_4 buffer, and the ratio between enzyme and substrate was 1/10 (v/v). The reducing sugars released were quantified by the colorimetric Nelson-Somogyi method. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing groups from its substrate per min.

Total sugar concentration was determined by the phenol-sulfuric acid method as described elsewhere.[8] As standard, a 2/2/1 solution of xylose, arabinose and mannose was prepared so as to imitate approximately the soluble monosaccharide composition that is found due to acid hydrolysis of the soybean hulls' polysaccharides after autoclaving.[15] Glucose was measured with a glucose oxidase/peroxidase enzymatic kit,[16] following the manufacturer's instructions (Wiener, Argentina).

Fungal growth was estimated by conversion of fungal chitin to glucosamine via alkaline degradation, followed by glucosamine deamination with nitrous acid and a colorimetrical determination of the resulting aldehyde using 3-methyl-2-benzothiazolone hydrazone.[17]

Image analysis by microscopy

Morphological analyses were performed using a confocal laser scanning microscope (Leica TCS SP5; Leica, Germany). The size of the hyphal agglomerates, where suitable, was determined through manual photographic examination of at least 10 pellets per culture sample, by measuring the two-dimensional projections' diameter corresponding to the pellets, employing the ImageJ processing software (NIH, Bethesda, MD, USA, http://imagej.nih.gov/ij/).

RESULTS

Optimization studies

The fractional factorial design implied 19 experimental runs, with the highest and lowest PG activity values obtained from the conditions tested in the fourth (12.43 U mL^{-1}) and eighth run (4.80 U mL^{-1}), respectively (Table 1). The normal probability plot exhibited in Fig. 1 points out to initial pH (pHi) and $(NH_4)_2SO_4$ as parameters that modify PG activity significantly, since their main

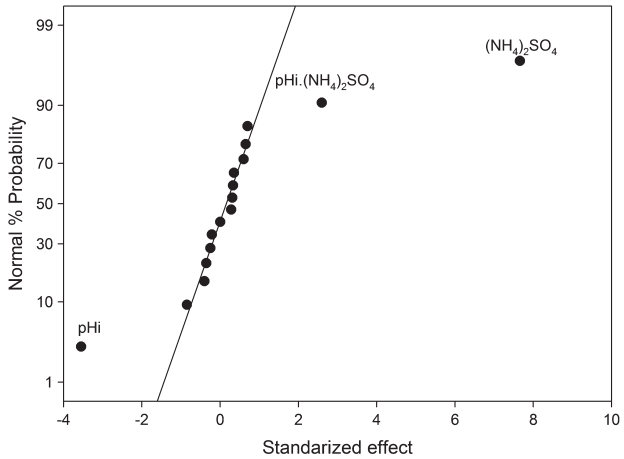

Figure 1. Normal probability plot for assessment of the effect of the parameters tested in the fractional factorial design on PG production. Labeled effects correspond to those that influence enzyme production significantly

Table 2. First Doehlert design and PG activity determined at 108 h of culture

Run	pHi	$(NH_4)_2SO_4$ (g L^{-1})	PG activity (U mL^{-1})
1	5.60 (1)[a]	7 (0)	8.22
2	4.85 (0.5)	13 (0.867)	7.22
3	3.35 (−0.5)	13 (0.867)	12.53
4	2.60 (−1)	7 (0)	26.94
5	3.35 (−0.5)	1 (−0.867)	10.66
6	4.85 (0.5)	1 (−0.867)	3.67
7	4.10 (0)	7 (0)	9.08
8	4.10 (0)	7 (0)	8.97
9	4.10 (0)	7 (0)	9.48

[a] Coded factors.

effects diverge clearly from linear behavior. Further, from their position relative to the straight line it follows that production is favored by lower pH values and higher $(NH_4)_2SO_4$ concentrations with respect to the central point of the design. A third significant effect appears to be the two-factor interaction between pHi and $(NH_4)_2SO_4$ since, albeit confounded with other two-factor interactions due to the resolution power of the design, it is the most reasonable alternative given the other relevant effects. The aptness of the model obtained was confirmed by ANOVA (Supplementary Table S1).

Based on the latter results, lower values of culture pHi and higher concentrations of $(NH_4)_2SO_4$ were tested in a nine run-Doehlert design (Table 2). The most acid condition yielded 26.94 U mL^{-1} and at least doubled the PG activity levels measured with the other conditions. Significant effects were selected by the ANOVA test and included in the model, which accounts for 97.7% of the total variability in PG activity (Supplementary Table S2). Coefficients of the polynomial equation suggest a strong effect of pH within the screened space, being better appreciated in the contour plot (Fig. 2(A)), where it can also be seen that a true peak in PG production apparently has not been reached.

Therefore, a second Doehlert design centered in the pH condition that rendered the highest PG levels was performed; pHi values were tested from 2.40 to 2.80, whereas the $(NH_4)_2SO_4$ concentration range was slightly broadened towards higher levels (Table 3). The model created with the experimental results was adequate to represent the data and explain more than 97% of the results (Supplementary Table S3). A peak in PG activity pHi 2.60 is depicted in the respective contour plot (Fig. 2(B)), along with a fairly steep descent in enzymatic production towards higher or lower pH values. Regarding $(NH_4)_2SO_4$, its effect on the response is comparatively smaller as judged by the ANOVA test (Supplementary Table, S3). The highest PG activity measured, around 26 U mL^{-1}, came from cultures performed following the central point of the design, and matched that obtained at practically the same condition in the first Doehlert design.

Fungal development and morphology

Fungal growth was evaluated by performing, with the optimal culture medium (30 g L^{-1} soybean hulls, 7 g L^{-1} $(NH_4)_2SO_4$, 1 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 g L^{-1} KH_2PO_4, 0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$), two sets of cultures at pHi values of 2.60 and 5.40. In both cases, a plateau was reached simultaneously near 110 h, and cultures run at pHi 5.40 showed comparatively higher glucosamine contents; as a consequence, *A. sojae* grown at pHi 2.60 presented the highest levels of PG production per unit of fungal biomass (Table 4). Microscopic examination of the culture broth revealed that the fungus developed in highly entangled and dense spherical agglomerates of hyphae (pellets) when cultivated at pHi 5.40, whereas for cultures performed at pHi close to 2.60 (i.e. 2.40, 2.60, 2.80) rather dispersed mycelia were witnessed (Fig. 3). Further, control cultures performed at the same initial acidity conditions but replacing soybean hulls with glucose as carbon source rendered fungal pellets in all cases. The mean pellet's diameter developed with soybean hulls at pHi 5.40 (341±155 μm) was two-fold smaller compared with pellets obtained with glucose at pHi around 2.60 (670±131 μm). Regarding *A. sojae* grown with glucose at pHi 5.40, the fungus developed in pellets that in some cases reached diameters of 5 mm (Supplementary Fig. S1).

Bioreactor cultures

The time-course of enzymatic production, soluble sugars consumption and DOT is displayed in Fig. 4 for *A. sojae* growing under the optimal conditions in a stirred-tank bioreactor. It can be seen that the DOT decreased exponentially from approximately 18 to 26.5 h of cultivation, time at which an instant step-like rise from 21 to 60% DOT was evidenced that, in turn, matched an abrupt

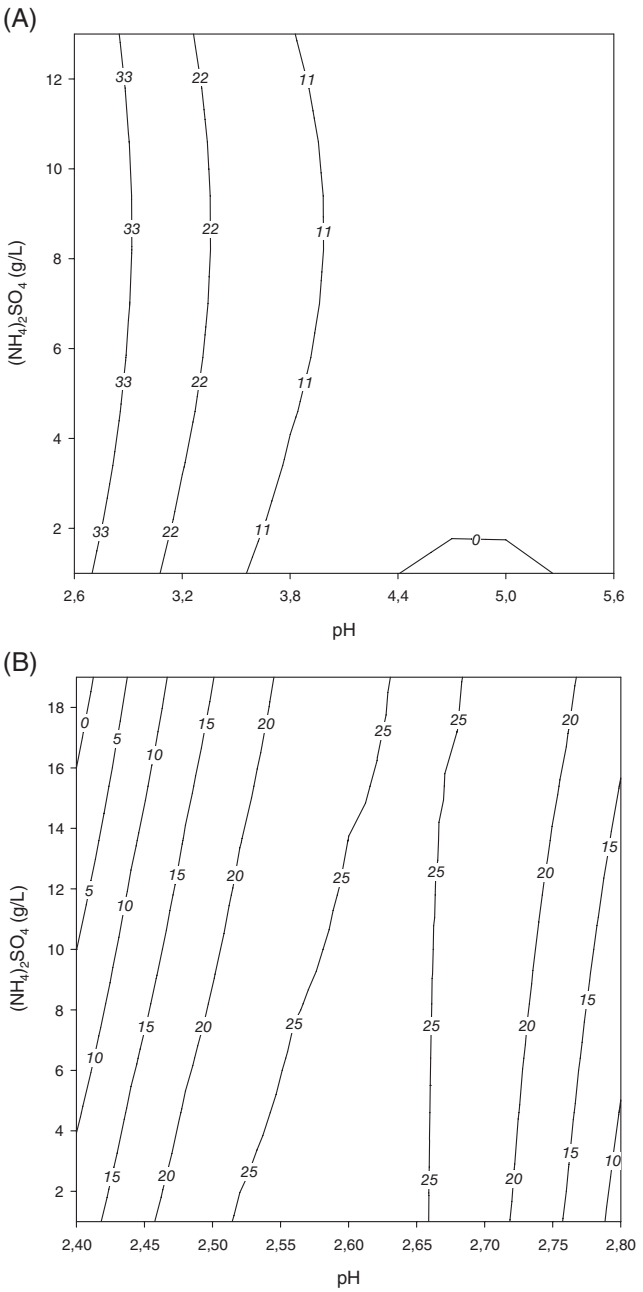

Figure 2. Contour plots obtained through Doehlert designs. Each plot shows the effects of $(NH_4)_2SO_4$ concentration and initial pH on PG yield in the first (A) and second (B) optimization studies.

descent in the outlet CO_2 pressure (Supplementary Fig. S2). From here on, the DOT increased slowly until the end of the cultivation period. Total soluble sugars decreased from 7.5 to less than 4 g L^{-1} during the first 72 h. The reducing sugars concentration hit a low at 192 h, but from 27 to 44 h incremented noticeably before resuming the expected consumption trend. No glucose was detected at any point throughout the fermentation and, consistently, neither was endo-glucanase activity. PG activity was not evidenced until the inflection point in the DOT and outlet gases' trend, and reached a maximum of 42 U mL^{-1} at 240 h of cultivation. Regarding other glucan-hydrolases, XYL production delayed in comparison, but followed similar kinetics to those of PG, reaching 1.3 U mL^{-1} after 288 h of cultivation.

Table 3. Second Doehlert design and PG activity determined at 108 h of culture

Run	pHi	$(NH_4)_2SO_4$ (g L^{-1})	PG activity (U mL^{-1})
1	2.80 (1)[a]	10 (0)	18.63
2	2.70 (0.5)	19 (0.867)	25.45
3	2.50 (−0.5)	19 (0.867)	19.22
4	2.40 (−1)	10 (0)	15.97
5	2.50 (−0.5)	1 (−0.867)	24.45
6	2.70 (0.5)	1 (−0.867)	23.94
7	2.60 (0)	10 (0)	26.04
8	2.60 (0)	10 (0)	25.10
9	2.60 (0)	10 (0)	26.70

[a] Coded factors.

Table 4. Fungal growth and PG production per g of glucosamine, of *A. sojae* cultured in the optimal media pre-adjusted to pH 5.40 or 2.60 before inoculation

	Fungal growth (mg glucosamine mL^{-1})		PG production (U g^{-1} glucosamine)	
Time (h)	pHi 5.40	pHi 2.60	pHi 5.40	pHi 2.60
60	173.8±51	189.2±7.7	62.1±20	69.3±5.3
84	275.1±10	232.4±22	43.9±4.8	101.9±13
110	378.0±93	322.6±18	40.0±9.8	84.4±10
135	381.4±2.2	306.9±39	36.2±3.7	102.9±16

Values are the mean of two independent experiments ± standard deviation.

DISCUSSION

Effects of each of the inorganic salts added to the culture medium, plus the size of the inoculum and the culture's pHi were examined through experimental designs. The positive effect of higher amounts of $(NH_4)_2SO_4$ concerning PG production is most probably a consequence of metabolic limitations due to shortage in simple nitrogen sources, since after increasing $(NH_4)_2SO_4$ concentration ranges on the subsequent experimental designs, the influence of ammonium on enzyme production decreased significantly. Of a more complex nature is the effect of pHi on PG production, as it appears to involve more than one phenomenon.

The grinding and autoclaving processes to which soybean hulls are subjected disrupts the micro-structures and increases the concentration of soluble pentose sugars while exposing the cellulose microfibers as a result of hydrolysis of the outer layer of the fiber.[11] Hydrolysis is further promoted by the action of acids, as was also witnessed in this work (Supplementary Fig. S3). Nevertheless, a 50% increase in soluble sugars did not boost fungal growth at pHi 2.60, which would have finally led to higher enzyme yields. Lower pH values may have caused more efficient enzyme production, partly, due to the hyphal morphology *A. sojae* developed into, that assures adequate nutrient intake while preventing diffusion limitations associated with pellet-like morphology.[18] Despite difficulties establishing general relationships between fungal morphology and process variables, there is consensus that pellet formation is favored as ambient pH rises.[19] Among different mechanisms of pellet formation by filamentous fungi, the coagulating type,

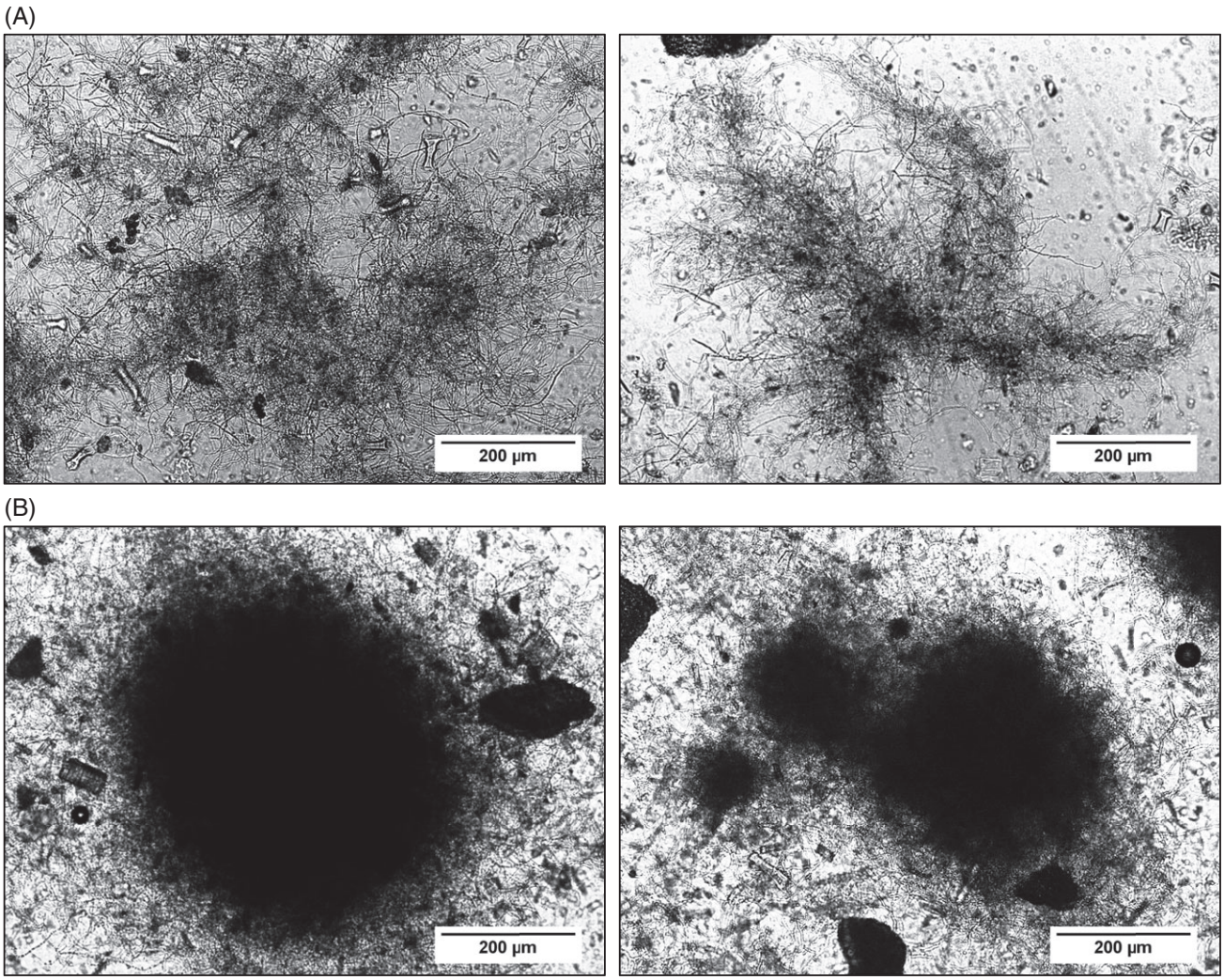

Figure 3. Morphological development of *A. sojae*, 72 h after inoculation, in submerged cultures with soybean hulls as carbon source at different initial pH values. (A) Typical dispersed morphology resulting from fungal development at pHi 2.60. (B) Typical pelleted morphology resulting from fungal development at pHi 5.40.

predominant in species of *Aspergillus*, involves conidial aggregation, first at the early stage of cultivation and second by spore adhesion to the germinated hyphal tubes.[20,21] Changes in the surface properties of conidia, mediated by pH, would negatively influence conidial aggregation, thus limiting pellet formation.[19] Soybean hull particles used in this study probably contributed to restrict pellet formation by reducing random encounters between conidia the same way modern morphological engineering techniques such as addition of inert microparticles to the media do.[22] This hypothesis is supported by the different pellet sizes obtained in cultures performed with glucose instead of soybean hulls at pHi 5.40 (Fig. 3(B) and Supplementary Fig. S2B).

On the other hand, several studies point out to pectinases expression or secretion in ascomycetes being modulated by ambient pH. Rollins and Dickman found that the number of transcripts of an endopolygalacturonase–encoding gene (*pg1*) from *Sclerotinia sclerotiorum* peaked at pH 3.0–3.8 and decreased significantly at pH 4.2; [23] mutants of this fungus with loss-of -function alleles of the *pac1* locus that codifies for a pH-responsive transcription factor showed a shift in maximal accumulation of *pg1* transcripts towards higher pH values.[24] First described in *A. nidulans*, ambient pH-responsive regulation mediated by the transcription factor PacC is also found in other aspergilli,[25] but regarding pectinases, even though putative PacC homolog-recognition sites have been described in promoters of pectinolytic genes from *A. niger*, no functionality studies have been performed so far. As for post-translational regulation, Teixeira *et al.* reported different levels of PG secretion in recombinant strains of *Penicillium griseoroseum* cultivated at different pH conditions with no change in the level of the respective gene transcript.[26]

Altogether, the results presented in the current study suggest that the enhanced PG production under acidic conditions is a consequence of the combined effect of the predominant hyphal morphology and a higher gene expression and/or effective protein secretion. In addressing the possible cause of the finding specifically within the narrow window of pH shift, morphological changes can be ruled out since no visible microscopic differences were found between culture samples of pHi 2.80, 2.60 or 2.40 (data not shown), the most plausible reason being a refined modulation of PG production at transcript or protein level. As the pH conditions where the PG yield improvement was obtained are considerably acidic and therefore potentially harsh to the fungus, a higher pectinases production under this environmental

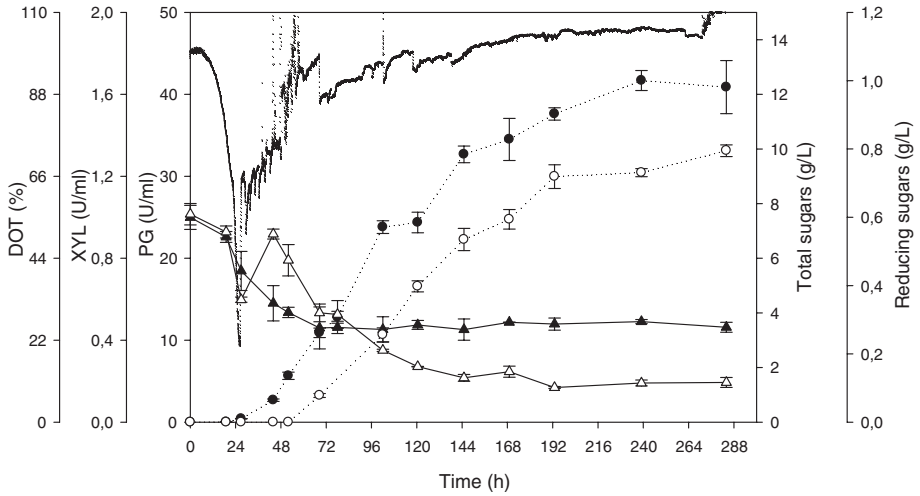

Figure 4. Cultivation profile of *A. sojae* growing on soybean hulls as carbon source, in a 2.5 L stirred tank bioreactor operated at 450 rpm and 0.5 vvm. PG activity (●), XYL activity (○), DOT (_), total sugars (▲), reducing sugars (△). Values are the mean of three different determinations. Error bars represent the standard deviation.

conditions could be a stress-mediated response aimed at assuring proper nutrient acquisition to cope with a non-favorable environment. Investigations into transcriptional regulation of the pectinolytic genes and PacC/Pal system components under pH conditions similar to those covered in this work are interesting topics for future research.

From a bioprocess point of view, studies dealing with PG production by *A. sojae* utilizing ground orange peels in submerged fermentation have demonstrated the advantage of cultivating the fungus under uncontrolled pH conditions from above any other controlled pH value (e.g. pH 3, pH 4, pH 5, pH 6), to obtain high enzyme titers.[6,27] In the present study, however, the 2-fold improvement in PG yields accomplished by changing pHi over such a narrow range further shows the importance of fine-tuning initial pH conditions in order to enhance PG production.

Besides testing the effect of scale-up on PG production, stirred-tank bioreactor runs allowed a more profound characterization of the culture mainly by monitoring the DOT and O_2 and CO_2 outlet pressures throughout the cultivation. An almost identical but reversed shape of the time-course of the O_2 and CO_2 outlet pressures plainly signals a respiratory metabolism in *A. sojae*, which is common in filamentous fungi (Supplementary Fig. S2). The exponential decrease in the DOT during the first 24 h is consistent with fast consumption of the soluble sugars present in the medium. Monosaccharides present in soybean hull hydrolysates comprise mainly xylose, mannose and arabinose, the first two due to hemicelluloses decomposition, whereas arabinose is released from pectin; other main structural sugars of pectin are apparently not hydrolyzed during autoclaving.[15] As glucose was not detected during the present studies, PG production is expected to be repressed by the aforementioned monosaccharides. An abrupt decrease in the CO_2 outlet pressure at 26.5 h indicates a slower metabolic activity probably related to shortage of easily assimilating-sugars, and matched the beginning of PG production (Supplementary Fig. S2 and Fig. 4), suggesting a shift in carbon utilization by *A. sojae* to the soybean hull suspended particles; the increase in reducing sugars between 27 and 44 h supports this assumption (Fig. 4). The inflection point in the DOT trend, consistent with the above explanation, has been reported previously for *A. sojae* growing in submerged cultures utilizing other by-products such as orange peels and apricot pomace.[7,8] In this type of submerged fermentation with filamentous fungi where powdered, vegetable raw material is used as carbon source, oxygen limitation has proved to be a factor that affects enzyme yields significantly. For instance, by preventing the DOT from dropping below 20% a 1.5-fold improvement in PG yields was achieved.[8] In the present study, since the DOT varied above 20% during the process, no oxygen limitation is assumed to have occurred.

Regarding PG production, activity values measured around 110 h in the bioreactor cultures (23 U mL^{-1} at 102 h and 25 U mL^{-1} at 120 h) were almost the same as the PG values obtained in the shake-flask studies, indicating adequacy of the bioprocess from Erlenmeyer flasks to stirred tank bioreactors. Enzyme production can be divided into a first phase from 24 to 144 h, followed by a second phase from 144 h onwards where the production rate was comparatively lower. The overall productivity at 240 h (i.e. where maximum PG levels were reached) was 0.174 U mL^{-1} h^{-1}; productivity increases 30 % (0.225 U mL^{-1} h^{-1}) if calculated at 144 h, which is a relevant aspect to be considered when deciding the length of the process. Li *et al.* compared 15 different *Aspergillus* species and strains as carbohydrase producers using soybean hulls, and highlighted an *A. niger* strain as the average top performer, [28] producing in 15.6 U mL^{-1} PG in 72 h. Since PG activity determination in the latter case was performed, in comparison, at more favorable conditions for enzymatic catalysis (50°C), the similar yields obtained in the present study (11 U mL^{-1} at 68 h) stress the potential of *A. sojae* as pectinases producer exploiting soybean hulls. The final PG yield per gram of soybean hulls calculated was 1. 39 U g^{-1} hulls, which is comparatively higher than the 0.58 U g^{-1} hulls obtained by culturing *Trichoderma reesei* in shake flasks on the same substrate as the main carbon source.[29] Soybean hulls also induced production of XYL activity in *A. sojae*, although yields were considerably lower compared with PG and with other literature reports.[28,29] Ambient pH is known to play an important role in XYL production in filamentous ascomycetes,[30] but no further studies concerning xylan-degrading enzymes were conducted in the present work.

CONCLUSIONS

Efficient induction of PG activity by *A. sojae* was achieved by utilizing soybean hulls as the sole carbon source. After

optimizing the culture conditions for PG production by a design-of-experiments approach, the process was successfully transferred to bioreactors, where the PG yields obtained per gram of soybean hulls were, to the best of our knowledge, among the greatest reported. The evidence provided in the present study of the significant improvement in PG production through small pH changes to the culture medium that are prone to go unnoticed due to the fine adjustments required, may be helpful with a view to improving production processes in which PGs or other acid-induced enzymes are involved, both at laboratory and industrial scale.

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Supporting Information

Supporting information may be found in the online version of this article.

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