Control of agitation rate and aeration for enhanced polygalacturonase production in submerged fermentation by Aspergillus sojae using agro-industrial wastes

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Abstract

BACKGROUND: The koji mold *Aspergillus sojae*, an industrially important microorganism, can produce high levels of pectinases utilizing agro-industrial wastes. This study introduces apricot and peach pomace, two agro-industrial wastes barely considered as raw material for the generation of value–added products, and focuses on its utilization together with orange peel for polygalacturonase production in submerged cultures using A. sojae.

RESULTS: A Doehlert response surface methodology design conducted in shake flasks and applied individually with these three by-products led to 60 – 80 U/ml polygalacturonase activity. In bioreactor studies performed with a mixture of apricot pomace and orange peel, by fixing stirrer speed to 600 rpm and cascading airflow to the dissolved oxygen tension up to 1.7 vvm, oxygen limitation problems were overcome and polygalacturonase activity values of 380 U/ml were achieved.

CONCLUSION: A simple and efficient strategy to minimize oxygen limitation with the lowest possible shear stress is provided for stirred-tank bioreactors working with highly viscous broths, so as to ultimately enhance microbial enzyme production. The polygalacturonase activity yields obtained in our study are among the highest reported in the literature.

Keywords: Batch fermentation; Oxygen limitation; Response surface design; Agro-industrial wastes

1. Introduction

Pectin and pectic substances are high molecular weight glycosidic macromolecules present in the middle lamella and primary cell wall of higher plants, structures which are responsible for providing integrity to the plant tissues. These complex polysaccharides are degraded by a heterogeneous group of related enzymes called pectinases. By 2011, the world industrial market for pectinases had revenues of 70 million dollars [1], accounting for at least 17% of a 1200 million dollars share of the food and beverages enzymes, a segment which in turn is expected to reach 1600 million dollars by 2016 [2]. The large stake pectinases hold in the enzyme market has to do with its wide application range, spanning any process that involves pectin degradation. Applications include primarily fruit juice extraction and clarification, viscosity reduction of cloudy citrus juice, mash treatment, clarification of white wine must and color extraction during red wine elaboration [3].

Industrial scale production of pectinases is mainly performed by filamentous fungi, particularly species from the *Aspergillus* genre. *A*. *niger* remains the main documented producer of commercial pectinases, partly due to the high amounts of enzymes excreted to the culture medium and also to its GRAS (Generally Regarded As Safe) status, which allows the use of its metabolites in food industry [4]. Metabolites derived from the traditional koji molds *A. sojae* and *A. oryzae* also posses GRAS status [5], implying that these

fungi can be considered as potential producers of pectinolytic enzymes suitable for food applications.

Fungal pectinases have been produced utilizing many agro-industrial wastes, such as wheat bran [6], orange processing wastes [7], lemon peel pomace [8], grape pomace [9], and apple pomace [10], among others. Apricot pomace, a primary by-product of the apricot processing industry, represents 10% of the total apricot processed for juice and puree [11]. According to FAO statistics, Turkey is the leading apricot producing country with a total of 0.79 million tonnes, accounting for 20% share of the world production in 2012. Another stone fruit residue, peach pomace, is generated in Turkey in approximately the same amount as that of apricot. Fresh and cold-stored peach pomace are particularly rich in pectin, presenting yields that can be as high as 18% (w/w)[12]. Due to the high pectin content, peach and apricot pomaces hold a great potential to be used for microbial production of pectinolytic enzymes.

Thus, the main purpose of this work was to study apricot and peach pomace utilization as carbon and energy sources (CES) for the production of polygalacturonase (PGase) activity by *A. sojae* in submerged fermentations. Orange peel utilization was also targeted, since this considerable by-product contains approximately 23% pectin (determined as galacturonic acid content, [13]) and proved to be an efficient inducer of PGase expression in *A. sojae* [14].

2. Materials and methods

2.1. Chemicals

Polygalacturonic acid and D-galacturonic acid monohydrate were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All other chemicals used were of analytical grade.

2.2. Microorganism and inoculum

A mutant *Aspergillus sojae* strain obtained from *A. sojae* ATCC 20235 by UV light exposure was kindly provided by Dr. Marcelo Fernández Lahore from Jacobs University gGmbH, Bremen, Germany. The propagation of the cultures was done on YME plates, containing malt extract (10 g/L), yeast extract (4 g/L), glucose (4 g/L) and agar (20 g/L), and incubated at 30° C until sporulation. Stock cultures of these strains were prepared using 20% (v/v) glycerol and stored at -80° C.

Molasses agar slants were used to obtain the spore suspensions for the seed inoculum [15] Spores were harvested from the slants with 0.02% Tween 80-water after incubation for 1 week at 30° C.

2.3. Submerged fermentations (SmF)

2.3.1. Shake flask cultures

The capacity of *A. sojae* to produce PGase activity from fruit processing industry wastes was assayed in 50 ml batch cultures performed in 250 ml Erlenmeyer flasks. Apricot pomace, peach pomace and orange peel were used for this purpose. Orange peel,

purchased in a local market in Izmir, Turkey, was milled to powder with a laboratory mill and stored at room temperature until use. Apricot and peach were purchased in a local market in Izmir, Turkey, and pressed to obtain the respective pomaces, which were subjected to hydrolysis with 4% phosphoric acid at 110° C for 40 minutes [16]. Hydrolysates obtained were filtered and adjusted to pH 5.0 with 6 N NaOH.

Hydrolyzed peach pomace, hydrolyzed apricot pomace and powdered orange peel were independently tested according to a Doehlert design (see Section 2.5) and run at 30° C and 120 rpm in an orbital shaker. Cultures were inoculated with 1.4 x 10^5 spores/ml. Samples were taken at different times during the incubation period and centrifuged for 15 minutes at 3500×g. The supernatant obtained from the culture broth was kept frozen at -20° C until used for the enzyme activity assay.

2.3.2. Bioreactor cultures

Batch cultures with a mixture of hydrolyzed apricot pomace and powdered orange peel as CES were performed in duplicates in a 1-L Sartorius BIOSTAT QPlus-6 MO serial bioreactor (Sartorius Stedim, Gottingen, Germany) with 700 ml of working volume. Total carbohydrate concentration was 21 g/L (14 g/L and 7g/L total carbohydrate coming from orange peel and apricot pomace, respectively), whereas ammonium sulphate (AS) concentration was 11.5 g/L. Bioreactors were directly inoculated with spores (1.4 x 10^5spores/ml), prepared as stated above.

Culture pH was measured with a glass electrode (Mettler Toledo), and allowed to change freely along the process. Dissolved oxygen tension was monitored by means of a polarographic-type electrode (Mettler Toledo).

Samples were periodically taken from the bioreactors, centrifuged at 3500×g for 15 minutes, and the supernatants were assayed for enzymatic activity and substrate consumption, the latter determined as total carbohydrates and reducing sugars.

2.4. Experimental design

Screening of the media formulation for the submerged cultures was performed by statistical analysis. A Uniform shell design proposed by Doehlert was built to maximize the PGase activity production (response) varying the total carbohydrate concentration of the CES (factor A) and the AS amount (factor B). Such a design with two independent factors has six points that all together draw a two-dimensional regular hexagon plus a central point [17]. In order to determine the experimental error the central point was replicated three times, so a total of nine runs were performed for each CES assayed (Table 2). Analysis of data and generation of response surface graphics was performed using Statgraphics Plus 5.1 software.

2.5. Analytical techniques

2.5.1. Enzyme assay

PGase activity was determined according to Cavalitto et al. [18] by measuring the release of reducing end groups with the colorimetric Nelson-Somogyi method, using polygalacturonic acid as substrate (0.2% in 20 mM acetate buffer, pH 5.0) and D-galacturonic acid as standard. The reaction temperature was 35° C, and the enzyme substrate ratio was 1/10 (v/v). One unit of enzyme activity (U) was defined as the amount of enzyme that produces one µmol of reducing sugar as D-galacturonic acid per minute under the given assay conditions.

2.5.2. Total soluble carbohydrate assay

Total carbohydrate concentration was measured by the phenol-sulfuric method, using sucrose as standard. For this assay, 0.5 ml of an appropriate dilution of the sample was mixed with 0.5 ml of 5% (w/v) phenol solution and 2.5 ml of 96% H_2SO_4. Solutions were left at room temperature for 20 minutes after which absorbance at 490 nm was measured.

3. Results and discussion

3.1. PGase production from orange peel, apricot and peach pomace in shake flasks

Three individual Doehlert designs with 9 runs each were employed to maximize PGase activity production with orange peel, apricot pomace and peach pomace, respectively. Total soluble carbohydrate (TSC) and AS values used in the Doehlert design are shown, together with the coded values, in Table 1. In order to detect the moment of maximum

PGase activity, response surface graphics were generated for different sampling times (from day 1 to day 6). For all three CES analyzed, the best results were obtained at an incubation time of 6 days.

Data from Table 1 were converted into second-order polynomial equations. ANOVA values of the linear, quadratic and interaction effects are shown in Table 2.

The R^2 values obtained were all higher than 0.98, which means that at most, only 2% of the total variation in PGase activity could not be explained by the models. The high P-values for the lack of fit obtained in all cases indicate that the fitted models adequately describe the experimental data observed.

The contour plots for PGase expression as a response to the interaction of TSC and AS are shown in Fig. 1 for culture media with apricot pomace, orange peel and peach pomace. According to these graphics, high PGase activity values can be attained at high values of TSC. Referring to cultures with apricot pomace, the maximum PGase activity (62.5 U/ml) was obtained with 17 g/L TSC and 1 g/L AS. As for cultures with peach pomace and orange peel, even though absolute maxima were not reached, a clear trend to higher TSC concentration values than the ones here assayed is observed. It is worth mentioning that high PGase activity values in cultures with apricot and peach pomace (around 60 U/ml) were obtained at low AS concentrations, whereas for orange peel cultures high AS concentrations are needed for high PGase expression (70-80 U/ml). These results for

orange peel cultures are consistent with those from Gogus et al. [19], in which PGase expression by *A. sojae* increased together with the increment in AS concentration in the fermentation medium. The differential behavior of PGase activity with AS in cultures with orange peel versus cultures with peach and apricot pomace could be due to different nitrogen content of the agro-industrial residues used. Information on nitrogen content of these wastes can be found in literature [20,12,16], although data is rather variable. Actually, composition of pomaces can change according to the fruit variety and the type of pre-treatment applied for juice extraction [21].

3.2. PGase production from apricot pomace and orange peel in bioreactors

According to the latter results and since all three wastes proved to be appropriate for PGase production yielding comparable enzyme expression levels at the assayed conditions, a culture medium consisting of a mixture of orange peel and apricot pomace was chosen to study PGase expression in a serial bioreactor system. In fungal fermentations, oxygen limitation is a very important aspect to be taken into account due to highly viscous fungal broths that result in a much lower gas-liquid mass transfer coefficient (k_La) in comparison to single cell microorganism fermentations [22]. Considering this aspect, fermentations were carried out at three different operational conditions, which were defined by configurations of stirrer speed and airflow (Table 3).

PGase activity, dissolved oxygen tension (DOT), TSC, reducing sugar concentration, agitation speed, and airflow as a function of the time course of fermentations are shown in Fig. 2. Total soluble carbohydrates as well as reducing sugars decreased from 20 g/L to less than 2 g/L after 240 hours in all three fermentations, indicating efficient sugar utilization from orange peel and apricot pomace in all cases.

The first bioreactor was operated at constant airflow (1 vvm) and agitation speed (600 rpm), yielding PGase activity values of 250 U/ml by the end of the culture period (Fig. 2A). At the beginning of the culture, a liquid medium with small dispersed substrate particles was observed. When *A. sojae* began to grow and the vegetable tissue started to be degraded, the culture medium turned into a highly viscous broth –which matches the DOT drop witnessed at this stage-. After 150 hours, PGase activity stabilized and later increased substantially together with the DOT, as seen in Fig. 2A. This behavior is explained by the gradual decrease in apparent viscosity noticed at the final stage of the process, most likely consequence of extensive degradation of the complex CES, and of a more pelleted morphology into which the fungus evolved. Overall, although high activity levels were reached, it can be seen that DOT dropped below 20% after 72 hours and remained at low levels during the intermediate phase of cultivation. This decrease in DOT values could be related with K_La, since this important parameter is inversely proportional to the viscosity [23]. Oxygen limitation is a well-known cause of low level production of aerobic products like enzymes. In order to avoid this nutrient limitation, two different strategies were

addressed: the effects of airflow and agitation speed were tested in separate fermentations for being the two process variables that most strongly affect K_La.

It can be seen that when airflow was kept constant at 1 vvm and agitation speed was cascaded to the DOT, after 100 hours, agitation speed reached 1200 rpm (Fig. 2B). Because of the possible deleterious shear effect of the impeller on proteins and mycelium, cascade configuration was disabled and agitation speed was manually fixed at 750 rpm, which led to a consequent decrease in the DOT. The comparatively lower levels of PGase activity reached in this condition are probably due to the combined effect of the shear stress caused by the impeller and the oxygen limitation. This behavior was previously reported regarding recombinant protein production by *A. niger* in stirred tank bioreactors [24] [25], where enzyme activity increased together with increments in agitation rates up to a threshold level from which shear stress caused damage to fungal hyphae and resulted in poor biomass development.

Bandaipeth and Prasertsan [23] report that increasing the airflow is a better choice than increasing agitation speed in order to get high K_La values. In line with the above observation, the best results were found by cascading airflow to the DOT from 0.1 vvm up to 1.7 vvm while keeping agitation speed at 600 rpm during the whole fermentation process (Fig. 2C). Under these operational conditions, DOT remained above 25% without shear stress throughout the process and maximum PGase activity of 380 U/ml was achieved after 216 hours of cultivation.

Comparing PGase activity achieved in the present study with the values reported in the literature in submerged fermentations, yields were clearly improved with respect to those previously obtained with *A. sojae* [26,14,27,19] [28]. PGase activity obtained when *A. sojae* was grown in shake flasks with maltrin as the main CES was nearly 17 U/ml [26]. The addition of orange peel to the latter yielded 93.5 U/ml and 110 U/ml in 250-ml Erlenmeyer flasks and 1-L stirred-tanks, respectively [27]. In another study, a pre-optimized culture medium containing orange peel and sugar beet syrup was tested in a 5-L bioreactor, resulting 145 U/ml of PGase activity [14]. Fed-batch cultivation of *A. sojae* in 5-L bioreactors using orange peel as CES further enhanced the PGase activity levels to 244 U/ml [28]. Hence, utilization of apricot pomace combined with orange peel in submerged cultures allowed at least a 1.5-fold increase in PGase activity from *A. sojae* with respect to previous works.

Studies on PGase expression in submerged cultures with other filamentous fungi, yeast and bacteria are listed in the literature [29]. For instance, an unidentified *Aspergillus* species grown on sugar beet and ammonium phosphate produced 6.5 U/ml in shake flasks [30]. In another example, *A. oryzae* produced 54 U/ml growing on pectin and wheat bran in a 5-L stirred-tank [31]. In contrast, Teixeira et al., [32] reported an *A. japonicus* strain that was able to generate PGase activity values as high as 500 U/ml by using pectin and glucose as the CES. However, considering microbial production of PGases in submerged cultures utilizing only wastes and residues as CES, the yields obtained in the present study are among the highest found in literature up to date. It is important to stress that comparisons were

made as units per milliliter of culture medium (U/ml) on the basis of PGase activity values defined in the same way in all cases, namely 1 U as 1 µmol of reducing sugars per minute.

4. Conclusions

Apricot and peach pomaces, two agro-industrial-wastes barely considered as raw-material for the generation of value–added products, were utilized together with orange peel for the production of PGase activity by *A. sojae* in submerged fermentations. PGase production studies in shake flasks with these three by-products allowed establishing the starting conditions to approach bioreactor studies. By adjusting the operational conditions of the reactors, *A. sojae* successfully degraded apricot pomace and orange peel and produced huge amounts of PGase activity (380 U/ml), surpassing previous yields obtained not only with this fungus but also with most of the microorganisms reported in the literature.

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REFERENCES

1 Alimardani-Theuil P, Gainvors-Claisse A, and Duchiron F, Yeasts: an attractive source of pectinases—from gene expression to potential applications: a review. *Process Biochem* **46:**1525-1537 (2011).

2 Adrio JL and Demain AL, Microbial enzymes: Tools for biotechnological processes. *Biomolecules* **4:**117-139 (2014).

3 Kashyap DR, Vohra PK, Chopra S, and Tewari R, Applications of pectinases in the commercial sector: a review. *Bioresource Technol* **77:**215-227 (2001).

4 Naidu G and Panda T, Application of response surface methodology to evaluate some aspects on stability of pectolytic enzymes from *Aspergillus niger*. *Biochem Eng J* **2:**71-77 (1998).

5 JØrgensen TR, Identification and Toxigenic Potential of the Industrially Important Fungi, *Aspergillus oryzae* and *Aspergillus sojae*. **70:**2916-2934 (2007).

6 Blandino A, Iqbalsyah T, Pandiella S, Cantero D, and Webb C, Polygalacturonase production by *Aspergillus awamori* on wheat in solid-state fermentation. *Appl Microbiol Biotechnol* **58:**164-169 (2002).

7 Giese EC, Dekker RF, and Barbosa AM, Orange bagasse as substrate for the production of pectinase and laccase by Botryosphaeria rhodina MAMB-05 in submerged and solid state fermentation. *BioResourses* **3:**335-345 (2008).

8 Ruiz HA, Rodríguez-Jasso RM, Rodríguez R, Contreras-Esquivel JC, and Aguilar CN, Pectinase production from lemon peel pomace as support and carbon source in solid-state fermentation column-tray bioreactor. *Biochem Eng J* **65:**90-95 (2012).

9 Botella C, Diaz A, de Ory I, Webb C, and Blandino A, Xylanase and pectinase production by Aspergillus awamori on grape pomace in solid state fermentation. *Process Biochem* **42:**98-101 (2007).

10 Hours RA, Voget CE, and Ertola RJ, Apple pomace as raw material for pectinases production in solid state culture. *Biological Wastes* **23:**221-228 (1988).

11 Sanal IS, Güvenç A, Salgin U, Mehmetoglu Ü, and Calimli A, Recycling of apricot pomace by supercritical CO2 extraction. *The Journal of Supercritical Fluids* **32:**221-230 (2004).

12 Pagán J, Ibarz A, Llorca M, Pagán A, and Barbosa-Cánovas GV, Extraction and characterization of pectin from stored peach pomace. *Food Research International* **34:**605-612 (2001).

13 Yapo BM, Lerouge P, Thibault JF, and Ralet MC, Pectins from citrus peel cell walls contain homogalacturonans homogenous with respect to molar mass, rhamnogalacturonan I and rhamnogalacturonan II. *Carbohydr Polymers* **69:**426-435 (2007).

14 Buyukkileci AO, Tari C, and Fernandez Lahore M, Enhanced production of exo-polygalacturonase from agro-based products by Aspergillus sojae. *BioResourses* **6:**3452-3468 (2011).

15 Gögus N, Tari C, Oncü S, Unluturk S, and Tokatli F, Relationship between morphology, rheology and polygalacturonase production by Aspergillus sojae ATCC 20235 in submerged cultures. *Biochem Eng J* **32:**171-178 (2006).

16 Ucuncu C, Tari C, Demir H, Buyukkileci AO, and Ozen B, Dilute-Acid Hydrolysis of Apple, Orange, Apricot and Peach Pomaces as Potential Candidates for Bioethanol Production. **7:**376-389 (2013).

17 Doehlert DH, Uniform shell designs. *Applied Statistics*231-239 (1970).

18 Cavalitto SF, Hours RA, and Mignone CF, Quantification of protopectinase SE, an endopolygalacturonase with pectin-releasing activity from *Geotrichum klebahnii*. *Biotechnol Tech* **13:**385-390 (1999).

19 Gogus N, Evcan E, Tari C, and Cavalitto SF, Evaluation of agro-industrial wastes, their state, and mixing ratio for maximum polygalacturonase and biomass production in submerged fermentation. *Environmental Technology*1-11 (2015).

20 Martín MA, Siles JA, Chica AF, and Martín A, Biomethanization of orange peel waste. *Bioresource Technol* **101:**8993-8999 (2010).

21 Paganini C, Nogueira A, Silva NC, and Wosiacki G, Utilization of apple pomace for ethanol production and food fiber obtainment. *Ciência e Agrotecnologia* **29:**1231-1238 (2005).

22 Cui YQ, van der Lans RGJM, and Luyben KC, Effects of dissolved oxygen tension and mechanical forces on fungal morphology in submerged fermentation. *Biotechnol Bioeng* **57:**409-419 (1998).

23 Bandaiphet C and Prasertsan P, Effect of aeration and agitation rates and scale-up on oxygen transfer coefficient, kLa in exopolysaccharide production from Enterobacter cloacae WD7. *Carbohydr Polymers* **66:**216-228 (2006).

24 Wang L, Ridgway D, Gu T, and Moo-Young M, Effects of process parameters on heterologous protein production in *Aspergillus niger* fermentation. *J Chem Tech Biotechnol* **78:**1259-1266 (2003).

25 Kelly S, Grimm L, Hengstler J, Schultheis E, Krull R, and Hempel D, Agitation effects on submerged growth and product formation of Aspergillus niger. *Bioprocess Biosyst Eng* **26:**315-323 (2004).

26 Tari C, Gogus N, and Tokatli F, Optimization of biomass, pellet size and polygalacturonase production by Aspergillus sojae ATCC 20235 using response surface methodology. *Enzyme Microb Technol* **40:**1108-1116 (2007).

27 Gogus N, Taze BH, Demir H, Tari C, Unluturk S, and Fernandez-Lahore M, Evaluation of orange peel, an industrial waste, for the production of Aspergillus sojae polygalacturonase considering both morphology and rheology effects. *Turkish Journal of Biology* **38:**537-548 (2014).

28 Buyukkileci AO, Fernandez-Lahore M, and Tari C, Utilization of orange peel, a food industrial waste, in the production of exo-polygalacturonase by pellet forming Aspergillus sojae. *Bioprocess Biosyst Eng* **38:**749-760 (2014).

29 Favela-Torres E, Volke-Sepúlveda T, and Viniegra-Gonzalez G, Production of hydrolytic depolymerising pectinases. *Food Technol Biotech* **44:**221-227 (2006).

30 Galiotou-Panayotou M, Kapantai M, and Kalantzi O, Growth conditions of Aspergillus sp. ATHUM-3482 for polygalacturonase production. *Appl Microbiol Biotechnol* **47:**425-429 (1997).

31 Malvessi E and Silveira MMd, Influence of medium composition and pH on the production of polygalacturonases by Aspergillus oryzae. *Brazilian Archives of Biology and Technology* **47:**693-702 (2004).

32 Teixeira MFS, Lima Filho JL, and Durán N, Carbon sources effect on pectinase production from Aspergillus japonicus 586. *Braz J Microbiol* **31:**286-290 (2000).

Table 1: Codified values, actual values and enzyme activity obtained as response at 6 days of incubation in the Doehlert designs corresponding to cultures performed with orange peel, peach pomace and apricot pomace.

	Codified values		Actual values		Enzyme activity (U/ml)		
	TSC	AS	TSC (g/L)	AS (g/L)	Orange peel	Peach pomace	Apricot pomace
1	0	0	12	2.5	48.3	40.3	53.1
2	0	0	12	2.5	50.2	34.6	55.4
3	0	0	12	2.5	43.5	43.3	54.6
4	1	0	20	2.5	55.8	34.0	40.3
5	0.5	0.87	16	0	56.3	1.08	2.52
6	-0.5	0.87	8	0	26.8	0.15	2.08
7	-1	0	4	2.5	10.8	4.67	9.78
8	-0.5	-0.87	8	5	0	9.34	18.1
9	0.5	-0.87	16	5	0	50.6	48.6

Table 2: ANOVA values of the regression coefficients and their significance level, obtained in the full quadratic models built according to the Doehlert designs.

	Regression coefficient			P-value		
	Orange peel	Peach pomace	Apricot pomace	Orange peel	Peach pomace	Apricot pomace
Constant	47.33	39.4	54.41	-	-	-
Linear: TSC	19.92	16.81	15.32	0.0099	0.0233	0.0018
Linear: AS	23.96	-16.93	-17.91	0.0068	0.0219	0.0013
Quadratic: TSC	-14.13	-20.06	-29.38	0.0463	0.0381	0.0013
Interaction: TSC•AS	17.01	-23.26	-17.29	0.0507	0.0448	0.0057
Quadratic: AS	-30.63	-25.39	-38.89	0.0104	0.0242	0.0007
Lack of fit	-	-	-	0.2083	0.3568	0.9143

R^2 (Orange peel) = 0.985

R^2 (Peach pomace) = 0.979

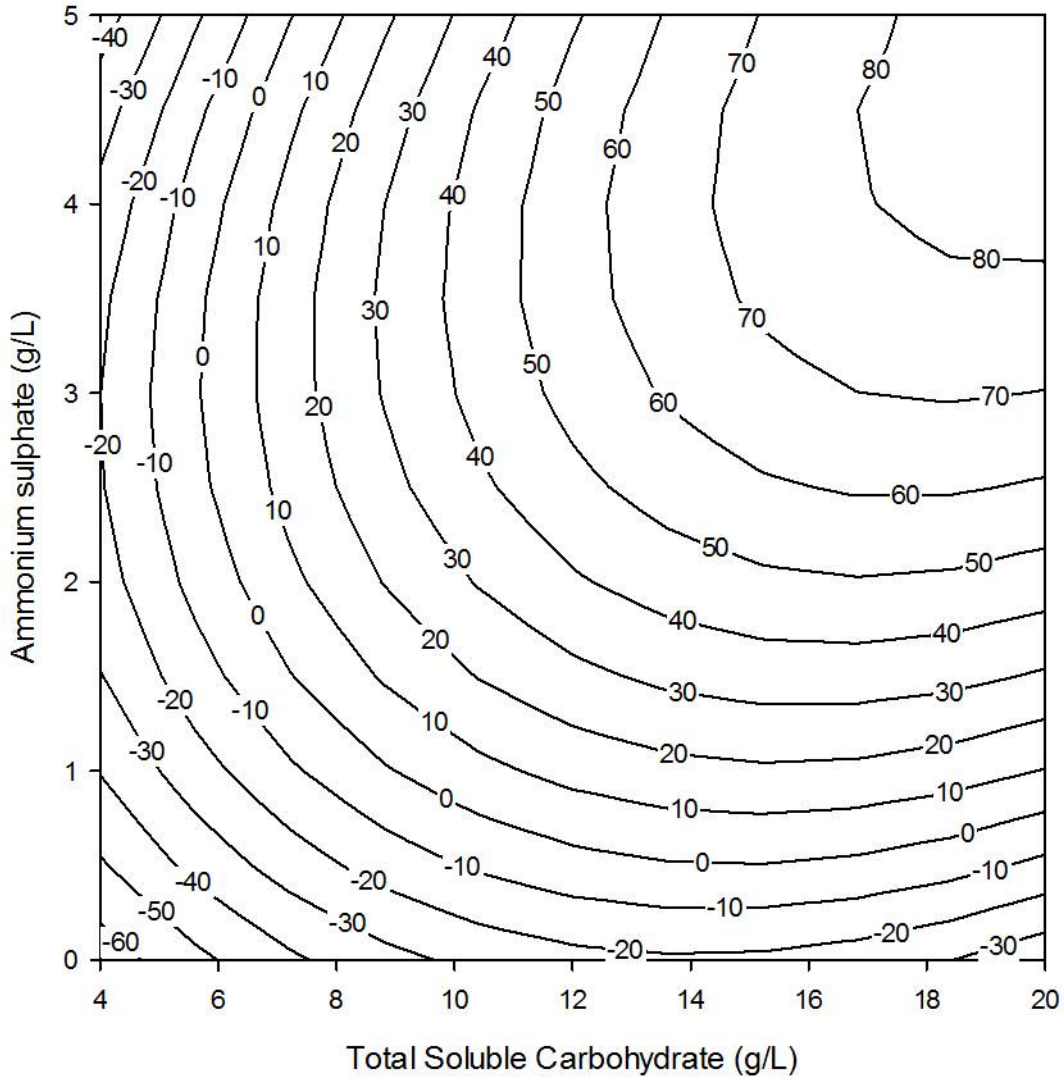
R^2 (Apricot pomace) = 0.999

Table 3: Operational conditions tested in a serial bioreactor system used to perform batch cultures of *A. sojae* with a mixture of apricot pomace and orange peel as CES.

Reactor	**Agitation speed**	**Aeration**	**Initial operational conditions**
1	600 rpm	1 vvm	Constant
2	Cascade	1 vvm	300 rpm / 1 vvm
3	600 rpm	cascade	600 rpm / 0.1 vvm

Figure Captions

(A)



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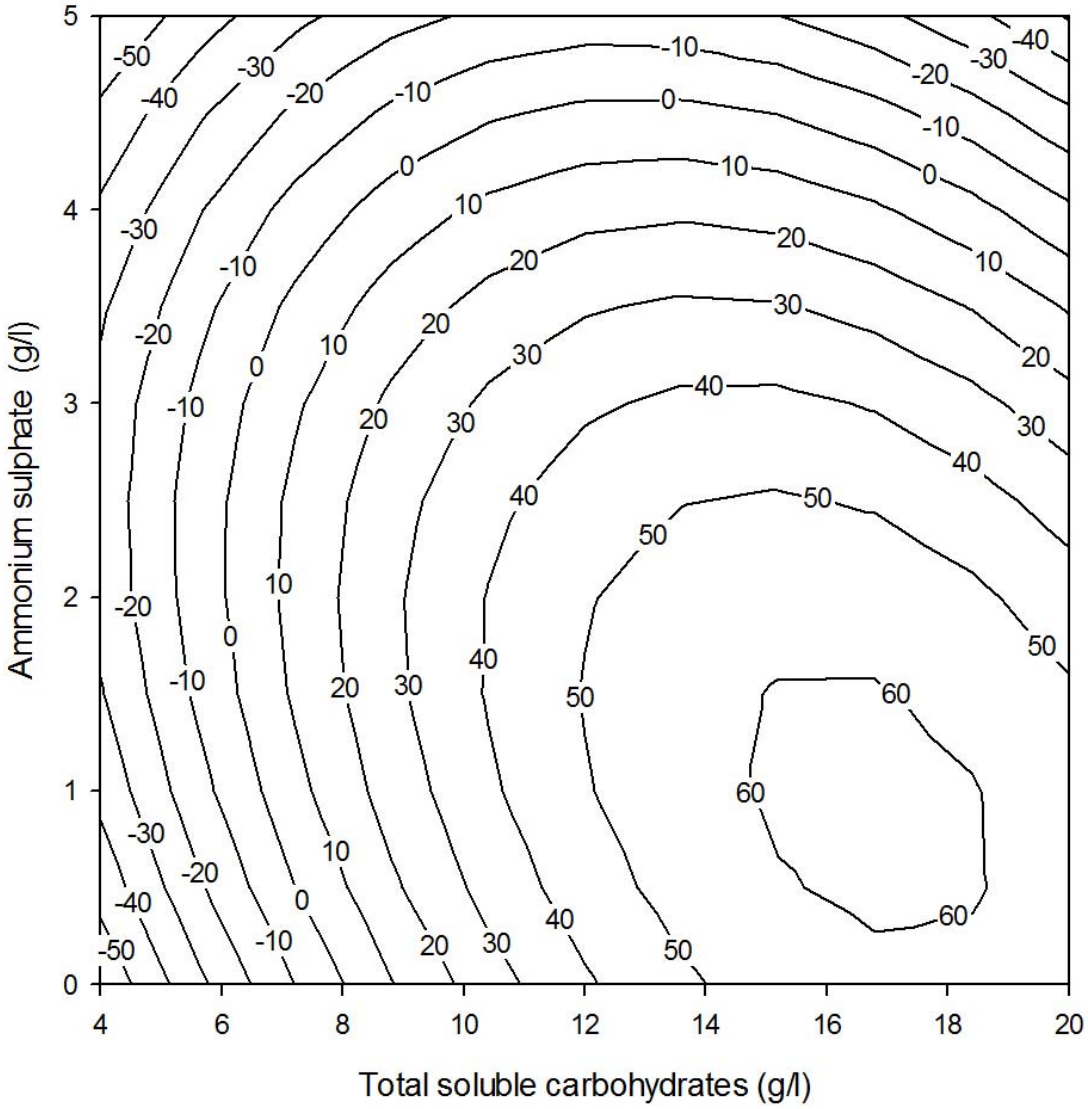


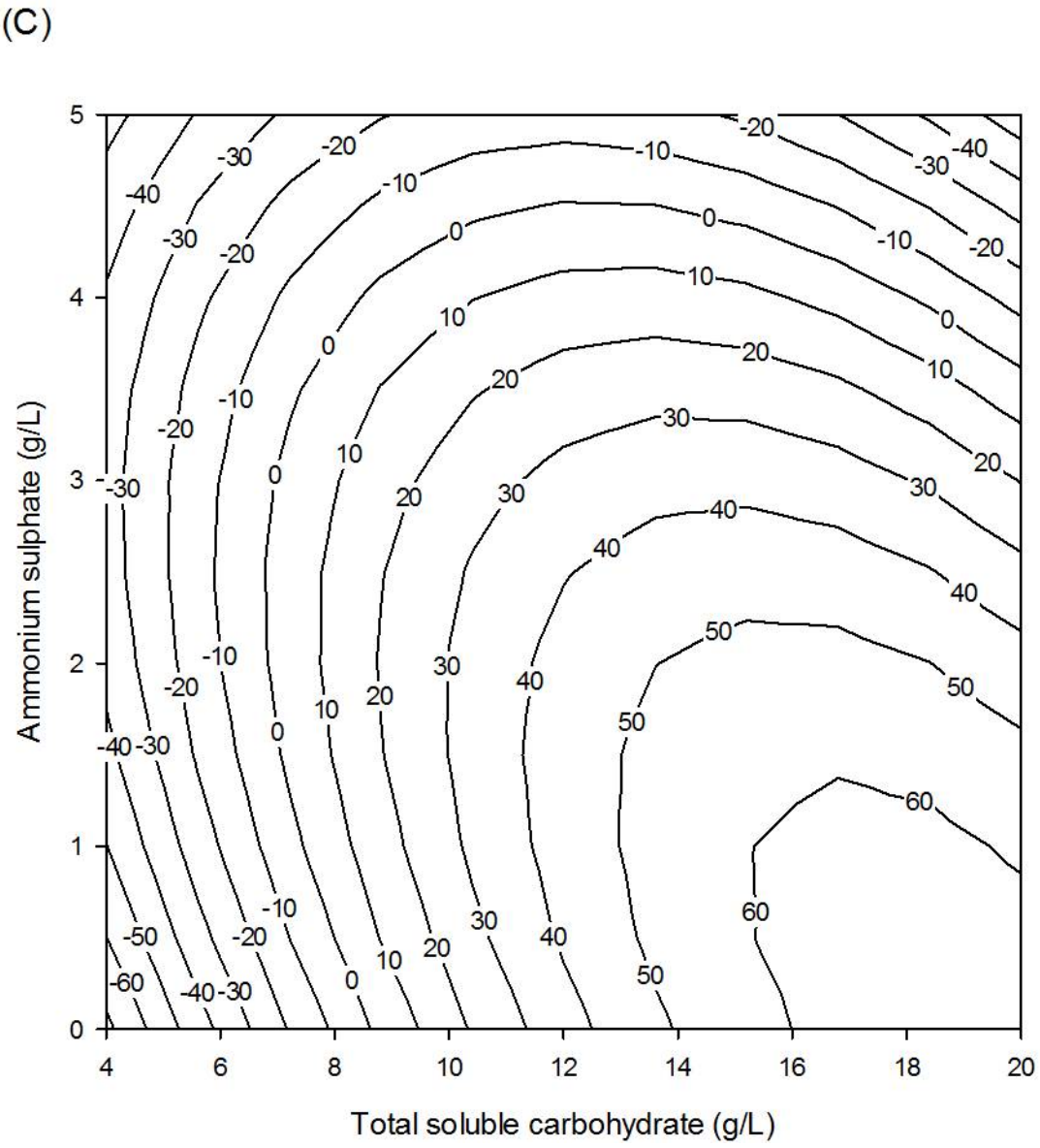
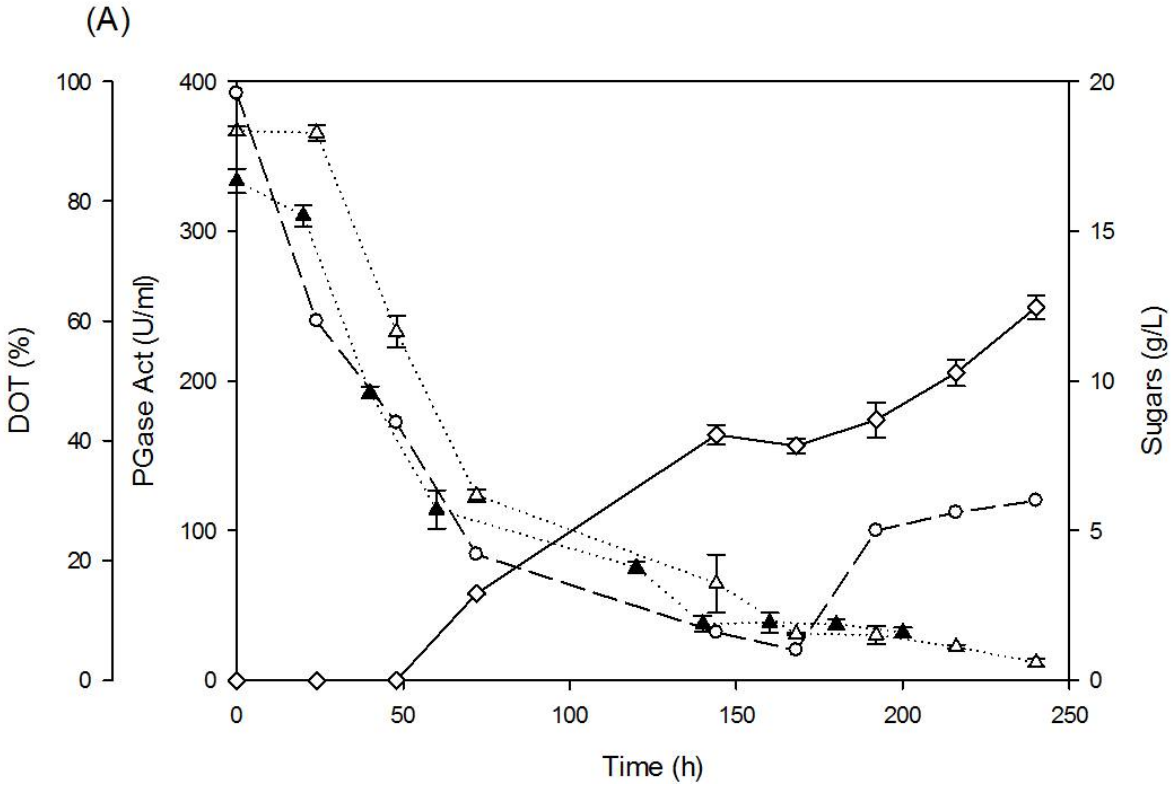
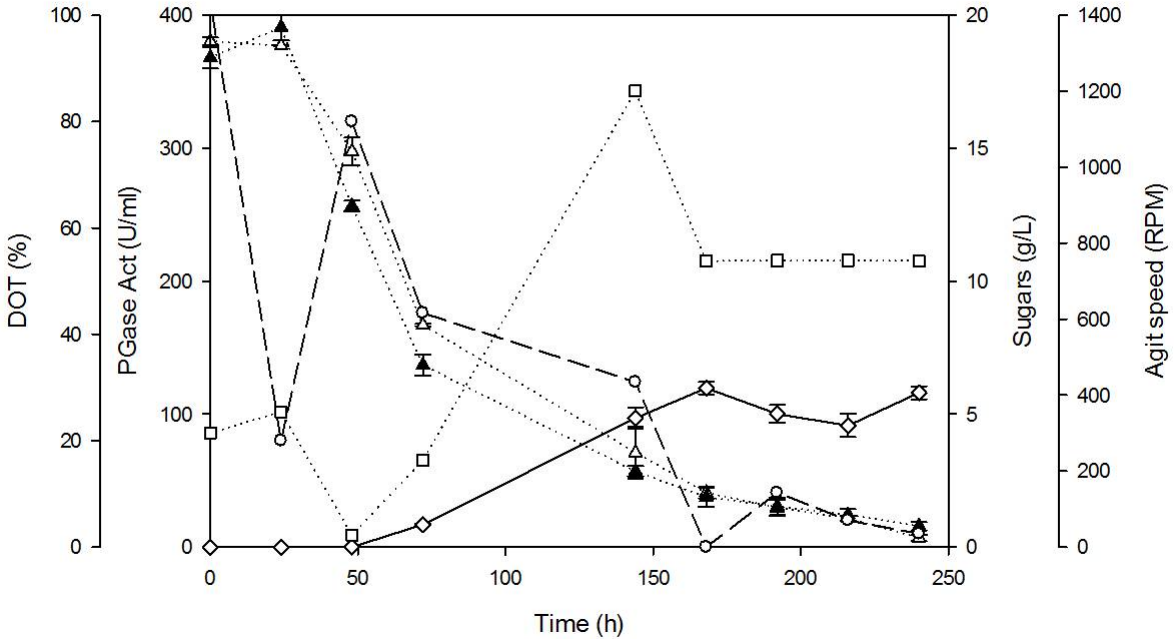


Fig. 1. Contour plots corresponding to culture media with orange peel (A), apricot pomace (B) and peach pomace (C). Each graphic shows the interaction effect between total soluble carbohydrate and AS concentrations on PGase activity production.



(B)



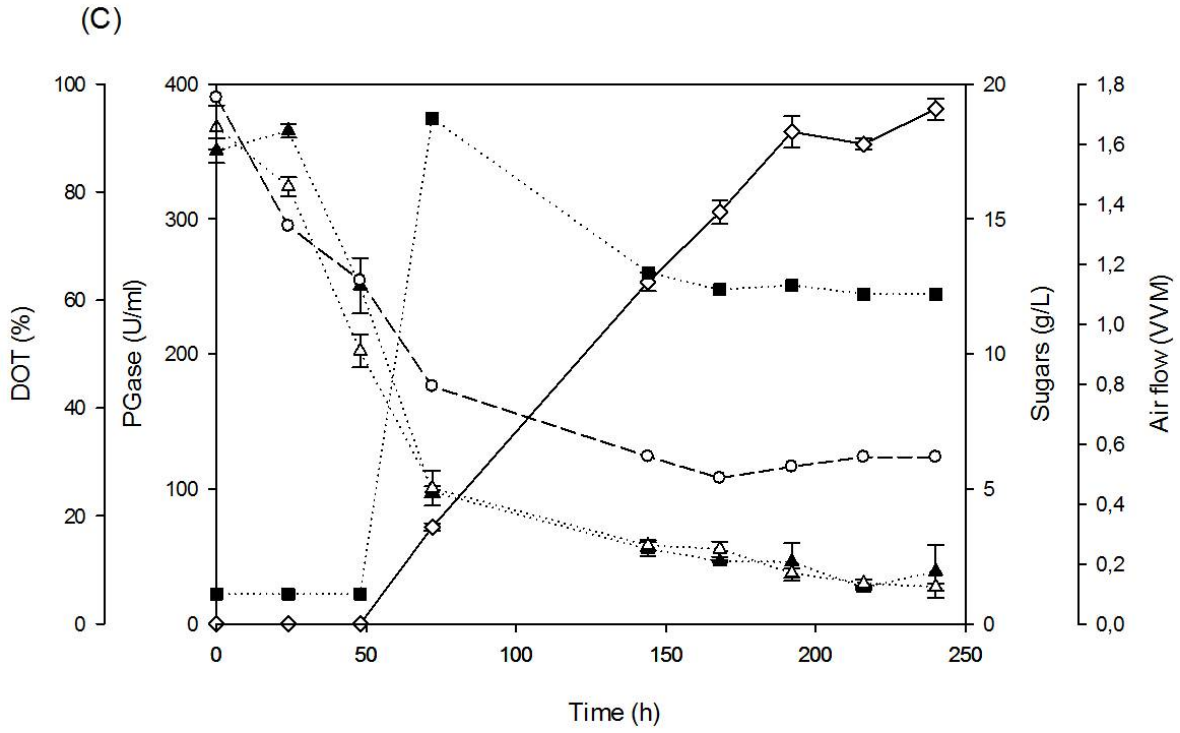


Fig. 2. Cultivation profile of *A. sojae* growing on orange peel and apricot pomace in 1-L bioreactors operated at (A) constant airflow and agitation speed (1 vvm, 600 rpm), (B) constant airflow (1 vvm) and cascading agitation speed, (C) constant agitation speed (600 rpm) and cascading airflow. Reducing sugars (▲), TSC (△), Air flow (■), DOT (○), PGase Act (◇), Agitation Speed (□). The observed values of PGase activity, TSC and reducing sugars are the mean values of triplicates with standard deviation (SD). Error bars represent SD.