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Evaluation of agro-industrial wastes, their state, and mixing ratio for maximum polygalacturonase and biomass production in submerged fermentation

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The potential of important agro-industrial wastes, apple pomace (AP) and orange peel (OP) as C sources, was investigated in the maximization of polygalacturonase (PG), an industrially significant enzyme, using an industrially important microorganism *Aspergillus sojae*. Factors such as various hydrolysis forms of the C sources (hydrolysed-AP, non-hydrolysed-AP, hydrolysed-AP + OP, non-hydrolysed-AP + OP) and N sources (ammonium sulphate and urea), and incubation time (4, 6, and 8 days) were screened. It was observed that maximum PG activity was achieved at a combination of non-hydrolysed-AP + OP and ammonium sulphate with eight days of incubation. For the pre-optimization study, ammonium sulphate concentration and the mixing ratios of AP + OP at different total C concentrations (9, 15, 21 g l^{-1}) were evaluated. The optimum conditions for the maximum PG production (144.96 U ml^{-1}) was found as 21 g l^{-1} total carbohydrate concentration totally coming from OP at 15 g l^{-1} ammonium sulphate concentration. On the other hand, 3:1 mixing ratio of OP + AP at 11.50 g l^{-1} ammonium sulphate concentration also resulted in a considerable PG activity (115.73 U ml^{-1}). These results demonstrated that AP can be evaluated as an additional C source to OP for PG production, which in turn both can be alternative solutions for the elimination of the waste accumulation in the food industry with economical returns.

Keywords: agro-industrial waste; polygalacturonase; apple pomace; orange peel; *Aspergillus sojae*

1. Introduction

Over the recent years, it has been observed that there is an increasing interest around the world towards efficient utilization of agro-industrial wastes, which could be bioconverted into different value-added products.[1,2] Several million tons of apple and orange juice processing wastes such as peel, pulp, seeds, etc., are produced annually all over the world. Although they are highly biodegradable, their disposal generates a serious environmental problem and finally leads to pollution.

Among these wastes, apple pomace (AP) wastes have been proposed as a substrate for the production of different value-added products including enzymes,[3,4] organic acids,[5] ethanol,[1,6] and natural antioxidants.[7] The world production of apples in 2010 was 69.5 million tons,[8] around 30% of this amount was used in the production of different products such as juice, concentrates, jelly, pulp, canned slices, wine, cider, etc. AP, which represents around 25–35% of the processed apples, is one of the main by-product of the fruit processing industry containing peel, seed, core, calyx, stem, and soft tissue.[2,9] AP is an excellent substrate for bioprocesses in terms of its high water content and composition containing polysaccharides such as cellulose, hemicellulose, and lignin. It is rich in galacturonic acid, arabinose, and galactose with minor amounts of rhamnose, xylose, and glucose, as well as small amounts of minerals, proteins, and vitamins. Also, AP is a natural source of pectic substances.[1,10,11]

On the other hand, oranges contribute around 10% of the world fruit production according to the Food and Agriculture Organization of the United Nations Statistical Databases (FAOSTAT).[8] During orange juice production only approximately half of fresh orange weight is transformed into juice, while the other half is considered as production waste.[12] Therefore, orange peel (OP) holds a great potential to be used as a substrate and an inducer for the production of polygalacturonases (PGs) by microorganisms due to its appreciable amount of pectin content.

PGs are a part of pectinases involved in pectin degradation. These enzymes are utilized in the fruit juice industry and in winemaking to increase the juice yield, facilitate pressing and filtration, and provide clarification. Pectinolytic enzymes used in food processing are mostly derived from fungi because the pH optima of these enzymes are in the range of natural pH of materials to be processed.[13] The utilization of OP and APs in enzyme production has also several advantages such

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as easy availability of cheaper raw material, reducing the cost of the enzyme, and resulting in the reduction of environmental pollution.[14]

Therefore, the goal of this study was to investigate the potential of important agro-industrial wastes, AP and OP as C sources, using an industrially important microorganism, *Aspergillus sojae*, in order to maximize the PG production under submerged fermentation using statistical tools. A final low cost media formulation that could be of industrial significance was attempted to be developed besides the goal of providing an alternative solution for the elimination of waste accumulation in the food industry that can lead to economical returns.

2. Materials and method

2.1. Microorganism

A. sojae ATCC 20235 was purchased from Procochem Inc., an international distributor of American Type of Culture Collection (ATCC) in Europe. This wild-type culture was randomly mutated using ultraviolet light exposure by Jacobs University gGmbH, Bremen, and used as the mutant strain in this study. The propagation of the culture was done on yeast malt extract plates containing (g l^{-1}) malt extract, 10; yeast extract, 4; glucose, 4; and agar, 20; and molasses agar slant medium containing (g l^{-1}) glycerol, 45; molasses, 45; peptone, 18; NaCl, 5; and agar, 20; and stock solutions (mg l^{-1}) $FeSO_4 \cdot 7H_2O$, 15; KH_2PO_4, 60; $MgSO_4$, 50; $CuSO_4 \cdot 5H_2O$, 12; and $MnSO_4 \cdot H_2O$, 15. Spores were harvested using 5 ml of Tween 80–water (0.02% v/v).

2.2. AP and OP

Fresh apple and orange peel were purchased from a local market in Buenos Aires, Argentina. AP obtained after pressing apples, composed of almost just peels of approximately 1 cm^2-sized particles, was stored at -20°C in plastic packages until needed. OP was ground by a laboratory mill and stored at room temperature.

2.3. Hydrolysation of AP

Based on our previous experiments, temperature of 110°C, 40 min, 4% phosphoric acid, and 10% solid liquid ratio were determined as the optimum hydrolysis conditions.[15] AP hydrolysates were filtered, pH adjusted to 5.0, using 6 N NaOH, and sterilized at 121°C for 15 min.

2.4. Fermentation

A. sojae was grown in 250 ml Erlenmeyer flasks containing 50 ml submerged medium given by the statistical design. Initial spore count was adjusted to approximately 2.8×10^3 spore ml^{-1} and used for the inoculation of the flasks, which were incubated at 30°C in a 250 rpm rotary shaker.

2.5. Statistical design of experiments

Design Expert Software Version 7.0 (Stat Ease, Minneapolis, USA) was used for the statistical experimental design for all the fermentation experiments. Primarily screening of media formulation was performed with D-optimal design. The analysed factors were carbon source, nitrogen source, and incubation time with the levels shown in Table 1. Responses were PG activity (U ml^{-1}) and biomass (g ml^{-1}). Total carbohydrate contents of each experiment given by the software were adjusted to 9 g l^{-1}. Content of nitrogen sources were adjusted to 8 g l^{-1} based on previous experiments. In the mixture of AP (hyd) + OP and AP (non-hyd) + OP experiments, total carbohydrate contents were distributed equally.

In the first optimization study, D-optimal design was generated and conducted with two factors determined by the results of screening experiments, which were the amount of ammonium sulphate (numeric) and OP + AP mixing ratio (categoric) with a total of 39 runs including 3 replicates (Table 2). Responses were PG activity (U ml^{-1}) and biomass (g l^{-1}). The factor levels of ammonium sulphate were 1 and 8 g l^{-1}. The factor levels of OP + AP mixing ratio were performed at 3 different total carbohydrate concentrations (9, 15, and 21 g l^{-1}) with 5 different mixing ratios (0:4, 1:4, 3:4, 1:1, 4:0) giving 15 levels (9(0:4), 9(1:4), 9(3:4), 9(1:1), 9(4:0), 15(0:4), 15(1:4), 15(3:4), 15(1:1), 15(4:0), 21(0:4), 21(1:4), 21(3:4), 21(1:1), 21(4:0)). Ratios were decided so that the first number in the brackets referred to the ratio of OP and the second number to the ratio of AP.

Table 1. Factors and levels of screening experiments.

Factor	Actual factor levels			
Carbohydrate source	AP (hyd)	AP (hyd) + OP	AP (non-hyd)	AP (non-hyd) + OP
Nitrogen source	Ammonium sulphate	Urea	–	–
Incubation time	4	6	8	–
Design type	D-optimal (27 runs)			

Note: AP (hyd): apple pomace (hydrolysed); AP (hyd) + OP: apple pomace (hydrolysed) + orange peel; AP (non-hyd): apple pomace (non-hydrolysed); AP (non-hyd) + OP: apple pomace (non-hydrolysed) + orange peel.

Table 2. D-optimal experimental design and results of the pre-optimization study.

Run	Factor 1 A: ammonium sulphate	Factor 2 B: OP + AP	Response 1 PG activity (U ml^{-1})	Response 2 Biomass (g l^{-1})
1	1.0	9, (4:0)	43.98	6.86
2	4.5	9, (1:4)	12.55	4.40
3	1.0	9, (1:1)	37.72	6.02
4	4.5	15, (3:4)	26.10	9.64
5	1.0	21, (1:4)	23.49	8.98
6	4.5	15, (1:4)	18.72	8.24
7	1.0	15, (1:4)	25.98	7.88
8	1.0	21, (4:0)	5.29	20.46
9	8.0	21, (1:4)	21.01	10.22
10	8.0	15, (0:4)	21.85	7.50
11	8.0	21, (1:1)	49.47	14.24
12	1.0	21, (3:4)	14.71	10.54
13	8.0	15, (1:4)	22.37	7.26
14	8.0	21, (1:1)	33.27	15.02
15	1.0	15, (1:1)	34.76	8.64
16	8.0	9, (1:4)	21.33	3.48
17	1.0	21, (0:4)	19.40	7.42
18	1.0	15, (0:4)	10.34	5.56
19	8.0	15, (4:0)	43.30	7.70
20	8.0	9, (1:1)	14.35	6.10
21	8.0	21, (0:4)	28.78	5.60
22	8.0	15, (3:4)	82.78	10.60
23	8.0	21, (3:4)	98.54	12.80
24	1.0	15, (4:0)	54.32	17.00
25	4.5	9, (1:1)	96.29	6.20
26	8.0	9, (3:4)	72.56	4.80
27	1.0	21, (1:1)	70.31	15.40
28	4.5	9, (3:4)	62.90	4.40
29	1.0	9, (3:4)	73.40	4.40
30	8.0	21, (3:4)	84.30	14.60
31	8.0	21, (4:0)	143.39	24.40
32	8.0	15, (1:1)	120.90	10.20
33	4.5	15, (1:1)	117.98	9.60
34	8.0	9, (0:4)	29.38	2.00
35	1.0	15, (4:0)	40.97	16.60
36	1.0	9, (0:4)	32.75	1.60
37	1.0	9, (1:4)	55.12	2.60
38	8.0	9, (4:0)	112.57	8.00
39	1.0	15, (3:4)	87.23	8.40

At the end of the first optimization study, a complete optimization of the factors could not be achieved; therefore, a second optimization study was performed. In this optimization study, combined D-optimal design was applied in order to obtain a mixture of AP and OP (Table 3). Hence they were the components of the design and ammonium sulphate was a factor with enlarged levels (1, 15 g l^{-1}). The total carbohydrate content was fixed to 21 g l^{-1}, which was the optimum carbohydrate concentration in terms of PG activity determined in the first optimization study. The mixing ratios given by the software were 0:4, 1:3, 1:1, 3:1, and 4:0.

Analysis of data and generation of graphics were performed using Design Expert Version 7.0 software. The analysis of variance (ANOVA) tables were generated and the significances of all terms in the model were judged statistically according to the p-values (significance level of $p < .1$).

2.6. PG activity

PG activity was assayed according to the modified procedure of Panda et al. using 2.4 g l^{-1} of polygalacturonic acid as substrate at pH 4.8 and 40°C.[16] One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 micromole of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions.

2.7. Biomass determination

Biomass expressed as dry cell weight (g l^{-1}) was determined by means of gravimetric method. The fermentation broth was filtered through the pre-weighed filter paper, followed by drying to constant weight at 100°C, overnight.

3. Results and discussion

For any industrial fermentation, medium optimization is of utmost importance. The classical method of changing the medium variables one at a time in order to optimize the performance is impractical. Therefore, the need for efficient methods for screening large number of variables has led to the adaptation of statistical experimental designs.[17]

Among related researches, Sathishkumar et al. optimized culture conditions for laccase production from fungus *Pleurotus florida* by statistical experimental design using agro-industrial wastes such as banana peel.[18] Also apricot and peach pomaces were used to produce gibberellic acid from *Aspergilus niger* by Cihangir and Aksöz.[19] Furthermore Carchesio et al. compared biomethane production of some selected agricultural substrates such as grape seeds and plum stones.[20]

3.1. Screening experiments

Factors such as carbon and nitrogen sources and their concentrations have always been of great interest to the researchers in the industry for the low cost media design since 30–40% of the production cost of industrial enzymes is estimated to be the cost of growth medium.[21] In the literature various agro-industrial wastes including OP and AP have been searched for the PG production for low cost media design.[22–24] It is generally agreed that the optimum medium for the enhanced production of PG is that containing pectic materials as an inducer.[25] In the current study, the effects of C source (AP (hyd), AP (hyd) + OP, AP (non-hyd), and AP (non-hyd) + OP), N source (ammonium sulphate and urea), and incubation time (4, 6, and 8 days) were screened in terms of PG activity and

Table 3. Combined D-optimal experimental design and results of the optimization study.

Run	Component 1 A: OP	Component 2 B: AP	Factor 3 C: ammonium sulphate	Response 1 PG activity (U ml^{-1})	Response 2 Biomass (g l^{-1})
1	10.5	10.5	15.0	74.40	19.60
2	21.0	0	4.5	110.20	17.80
3	21.0	0	1.0	79.17	19.80
4	10.5	10.5	15.0	89.28	19.80
5	0	21.0	1.0	18.80	7.40
6	21.0	0	15.0	92.92	26.20
7	5.25	15.75	11.5	27.78	14.40
8	0	21.0	8.0	20.08	7.80
9	10.5	10.5	1.0	41.33	10.80
10	0	21.0	15.0	19.56	8.60
11	10.5	10.5	8.0	36.96	15.60
12	15.75	5.25	11.5	115.73	17.80
13	5.25	15.75	4.5	32.07	13.20
14	0	21.0	15.0	31.15	7.20
15	21.0	0	1.0	10.38	18.00
16	21.0	0	8.0	127.96	24.80
17	15.75	5.25	4.5	102.66	17.60
18	0	21.0	1.0	21.41	6.20
19	21.0	0	15.0	144.96	20.60

biomass. AP was screened in the form of hydrolysed and non-hydrolysed.

With the hydrolysation process, the aim was to open the accessible areas in the cellulose structure of AP. Hydrolysis affects lignocelluloses, creating larger accessible surface area and pore size. Moreover, hydrolysis was expected to improve the formation of sugars, avoid the degradation or loss of carbohydrate and the formation of inhibitory by-products for subsequent fermentation, and be cost effective.[26–29] After pretreatment, water insoluble solids were filtered in order to obtain the majority of cellulose where lignin and the hemicellulosic sugars remained in the filtrate. AP was pretreated with phosphoric acid (H_3PO_4) since after neutralization of hydrolysates with NaOH the salt formed was sodium phosphate, which could be used as a nutrient by microorganisms.[30,31]

As a result, it was seen from the ANOVA that the effect of C source (A), N source (B), and their interaction (AB) had a significant effect on the PG activity ($p \ll .1$). But the effect of incubation time and its interactions with the other factors were insignificant on PG activity ($p > .1$). Furthermore, the lack of fit of the model was insignificant, indicating that the model could be used with confidence. From the AB interaction plot shown in Figure 1(a), it can be observed that the highest PG activity (64.39 U ml^{-1}) was achieved using AP (non-hyd) + OP level as C and ammonium sulphate as N sources at eight days of incubation.

In terms of biomass production, ANOVA results showed that all the determined factors, C sources (A), N sources (B), incubation time (C), and their interactions had a significant effect ($p < .1$). The highest biomass production (52.98 g l^{-1}) was obtained with AP (hyd) as shown in Figure 1(b). Similar to PG production, ammonium sulphate as nitrogen source also resulted in maximum biomass production. In terms of incubation time, there was no significant difference between the sixth and eighth days of incubation, but on the fourth day biomass production was very low (Figure 1(c)).

Lower PG activity but higher biomass were obtained with hydrolysed AP (Figure 1(a) and 1(b)). This result might be due to the consumption of small sugars formed after hydrolysation towards biomass production instead of PG. During the hydrolysis process pectin was not hydrolysed; therefore, there were no galacturonic acid units in the hydrolysate as was confirmed in previous unpublished results. Probably the glycosidic bonds between galacturonic acid units were too resistant to acid hydrolysis. In the hydrolysate used as fermentation medium there were no apple peels, but in the non-hydrolysed AP there were also apple peels in the medium. Absence of peels in the medium might have reduced the pectin content that induced the PG production.

In the literature, AP has been utilized solely [32] or in combination with various agro-industrial wastes for pectinolytic enzyme productions.[33] However, to the best of our knowledge, this media composition, the mixture of AP and OP, has not been previously considered for this purpose. As a conclusion, one can prefer the use of hydrolysed AP for optimum biomass production and non-hydrolysed AP + OP for optimum PG production in the presence of ammonium sulphate and eight days of incubation. Since the goal in the current study was to achieve maximum PG production, the optimization study continued with non-hydrolysed AP + OP as the fermentation medium.

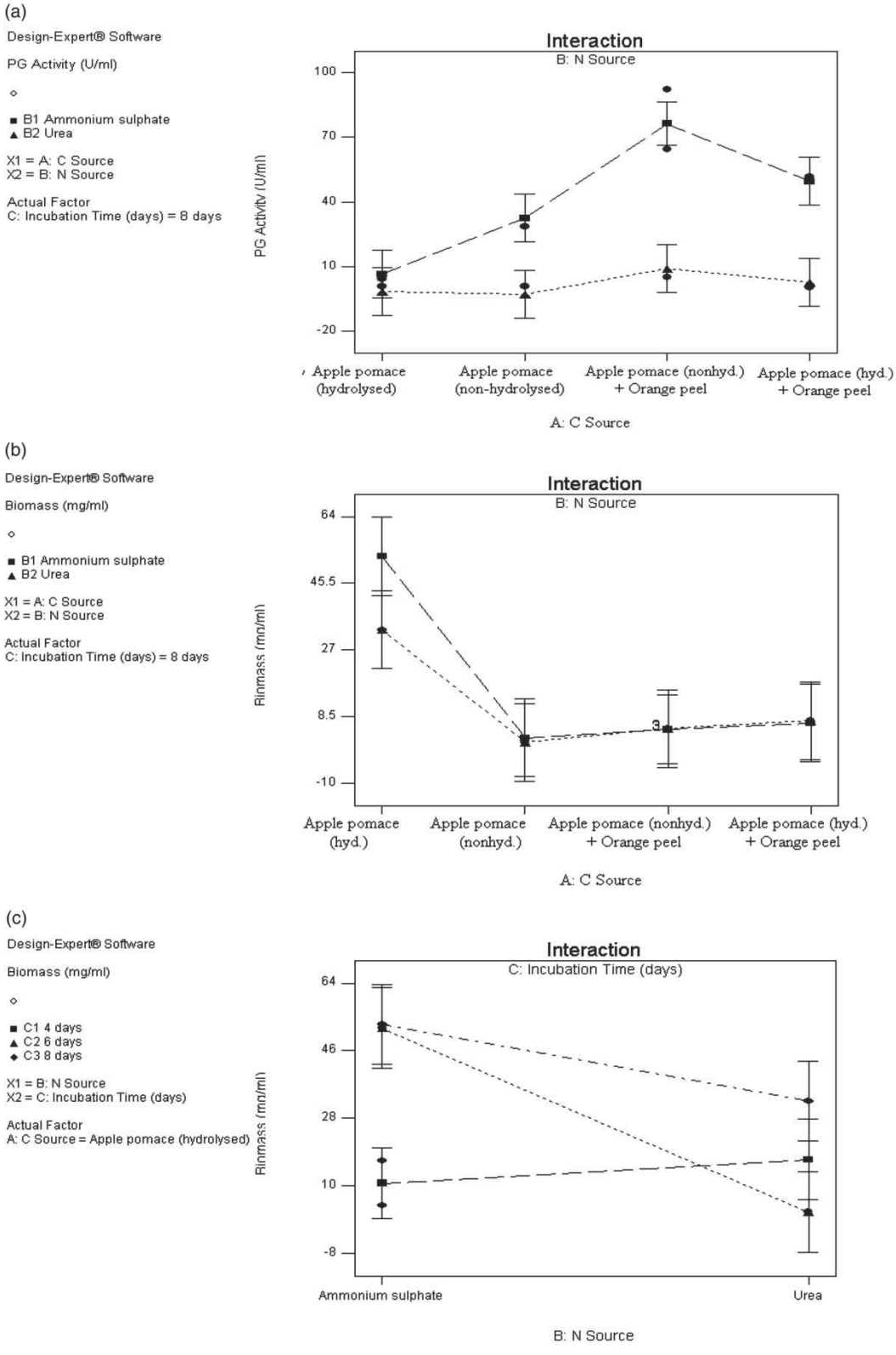

Figure 1. (a) Effect of interaction of carbon and nitrogen sources (AB) on PG activity, (b) interaction of carbon source and nitrogen source (AB) and (c) interaction of nitrogen source and incubation time (BC) on biomass production.

3.2. Optimization experiments

Based on the initial screening experimental results, D-optimal design with two factors, amount of ammonium sulphate (numeric) and OP + AP mixing ratio (categoric), was performed (Table 2).

ANOVA results indicated that both ammonium sulphate amount (A) and OP + AP mixing ratio (B) were the significant factors ($p < .1$). Furthermore, their interaction (AB) and their quadratic interaction (A^2B) were also significant terms with respect to PG activity ($p < .1$).

One factor plot of OP + AP mixing ratio indicated that maximum PG activity (143.39 U ml^{-1}) was achieved, in the presence of maximum total carbohydrate concentration, coming totally from OP (21, (4:0)), and maximum ammonium sulphate concentration (8 g l^{-1}) (Figure 2(a) and 2(b)). Additionally, the data in Figure 2(b) are summarized with three different figures given in Figure 3(a)–(c). These plots illustrate the PG activity change by a change in OP + AP mixing ratio for different total carbohydrate concentrations (9, 15, and 21 g l^{-1}) at three different ammonium sulphate concentrations (1, 4.5, and 8 g l^{-1}). The axis of the plots showing the OP + AP mixing ratio is in the order of ascending AP and descending OP ratio (4:0, 1:1, 3:4, 1:4, 0:4). The plots indicated that in the presence of only AP, PG activity was very low for all of the ammonium sulphate concentrations (Figure 3(a)–(c)). Comparing Figure 3(b) and 3(c), an increase in ammonium sulphate concentration from only 4.5 to 8 g l^{-1} resulted in a decrease in PG at 9 g l^{-1} total concentration of carbon source at 1:1 ratio of orange to apple (> 90 to < 20). This could be explained by the non-significant effect of AP on PG activity. As it was stated before, 3:4, 1:4, and 0:4 conditions hold higher AP pomace concentrations which were not effective on PG activity. Therefore, an increase in ammonium sulphate concentration could only cause a drastic decrease in PG activity at 1:1 condition at which OP and AP concentrations were the same, and OP concentration was more robust than at the other conditions.

Another view point in discussing this issue would be to consider the C/N ratio. In this particular case it was observed that the C/N ratio was 2 at 9 g l^{-1} carbohydrate concentration in Figure 3(b) (4.5 g l^{-1} ammonium sulphate) and dropped to 1.125 in Figure 3(c) (8 g l^{-1} ammonium sulphate) for the same 1:1 ratio of OP to AP. However, this ratio was 3.33 in Figure 3(b) at 15 g l^{-1} carbohydrate concentration and dropped to 1.875 in Figure 3(c) when ammonium sulphate concentration was increased to 8 g l^{-1}. Since C/N ratios of 2 and 1.875 were

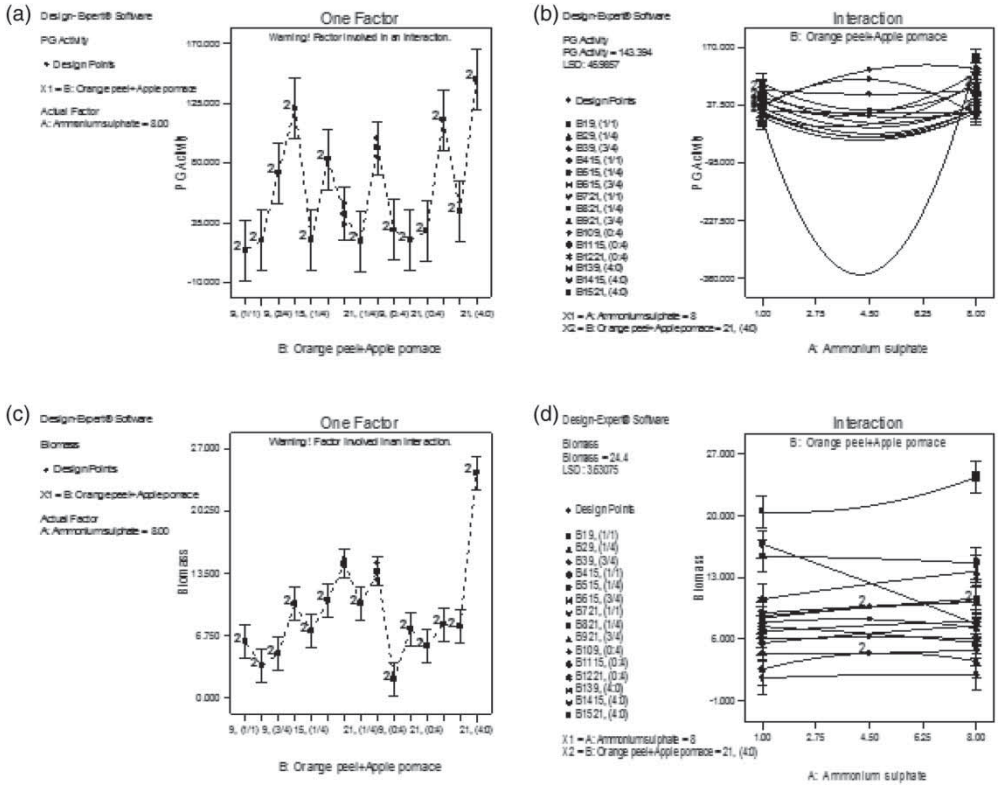

Figure 2. (a) Effect of OP + AP mixing ratio (B), (b) the interaction of ammonium sulphate amount and OP + AP mixing ratio (AB) on PG production, (c) effect of OP + AP mixing ratio (B), and (d) the interaction of ammonium sulphate amount and OP + AP mixing ratio (AB) on biomass production, respectively, at different total carbohydrate concentrations.

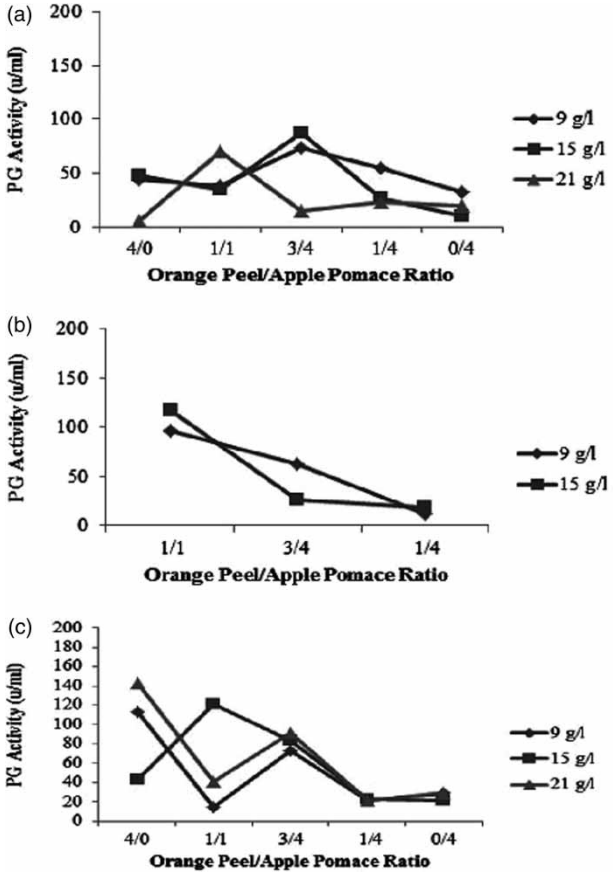

Figure 3. Effect of OP + AP mixing ratio at different total carbohydrate concentrations and at constant ammonium sulphate concentrations of (a) 1 g l^{-1}, (b) 4.5 g l^{-1}, and (c) 8 g l^{-1}.

close values, this decrease was not as drastic for 15 g l^{-1} carbohydrate concentration compared to 9 g l^{-1} at high ammonium sulphate concentrations (8 g l^{-1}). In this particular case, the critical C/N ratio seemed to be below 1.875. Similarly, at low ammonium concentration of 1 g l^{-1} at the same OP:AP ratio of 1:1, the C/N ratios were 15 and 9 at both 15 and 9 g l^{-1} carbohydrate concentrations, respectively (Figure 3(a)), which were quite high. Since again there seemed not to be a balance, the PG activities were low compared to the intermediate ammonium concentration of 4.5 g l^{-1}. Therefore, one should pay attention to this ratio when making choices of adjusting the carbohydrate and ammonium sulphate concentrations. Thus, there should be a balance between C and N sources which will determine the route of the metabolic pathways.

At the 8 g l^{-1} ammonium sulphate concentration, which was the optimum concentration for PG production, the presence of only OP in the medium with the maximum total carbohydrate concentration (21 g l^{-1}) resulted in the maximum PG production (Figure 3(c)). Additionally, as an alternative combination, 15 g l^{-1} total carbohydrate concentration gave reasonable PG activity (98 and 120 U ml^{-1}) at both ammonium sulphate concentrations of 1 and 8 g l^{-1} with 3:4 and 1:1 OP + AP mixing ratios, respectively (Figure 3(a) and 3(c)), which can enable the use of AP with OP. With these results, the possible use of another agro-industrial waste such as AP in PG production besides OP was proved. As the factor levels of OP + AP mixing ratio were categoric, the response surface plots could not be determined for ammonium sulphate amount and OP + AP mixing ratio. Their interactions (AB) made it difficult to observe the optimum conditions (Figure 2(b)). Therefore, in order to determine the optimum conditions, an additional optimization study was decided to be performed.

According to ANOVA results, considering biomass production, ammonium sulphate was insignificant ($p > .1$), whereas OP + AP mixing ratio and their interactions were significant terms ($p < .1$) at the determined levels. The maximum biomass (24.4 g l^{-1}) was also achieved at the maximum concentration of carbohydrate of 21 g l^{-1} with (4:0) mixing ratio and 8 g l^{-1} ammonium sulphate amount as in PG production (Figure 2(c)). The interaction plot of ammonium sulphate amount and OP + AP mixing ratio supported the data that ammonium sulphate had no significant effect on biomass production between the current studied levels (Figure 2(d)).

As a result in this pre-optimization study, it was seen that the optimum conditions for PG and biomass production were the same at the maximum levels. Therefore using these conditions in *A. sojae* fermentation, one can ensure both maximum PG and biomass production at the same time, which can be a great advantage for the industry.

In the second part of the optimization, since true optimum values could not be determined in the pre-optimization study, a combined D-optimal design was applied in order to obtain a mixture of AP and OP (Table 3). Hence they were the components of the design and ammonium sulphate was a factor with enlarged levels (1, 15 g l^{-1}). The total carbohydrate content was fixed to 21 g l^{-1}, which was the optimum carbohydrate content in terms of PG activity in the first optimization study. The mixing ratios given by the software were 0:4, 1:3, 1:1, 3:1, and 4:0.

According to the ANOVA results of PG activity, the applied model was significant with a p value of .0361 ($p \ll .1$). The lack of fit F value of 0.43 implied that the lack of fit was not significant ($p = .6705$). Additionally, the linear mixture meant that the mixture of OP + AP (A + B) was the significant factor ($p \ll .1$).

The model equation of the PG activity (Equation (1)) in terms of coded factors is as follows:

$$\begin{aligned}\text{PG activity (U ml}^{-1}) = {} & +131.71^{*}\text{A} + 23.23^{*}\text{B} \\ & - 86.64^{*}\text{A}^{*}\text{B} + 14.16^{*}\text{A}^{*}\text{C} \\ & - 16.29^{*}\text{B}^{*}\text{C} - 0.93^{*}\text{A}^{*}\text{B}^{*}\text{C} \\ & - 49.54^{*}\text{A}^{*}\text{C}^{2} - 0.23^{*}\text{B}^{*}\text{C}^{2}\end{aligned}$$

$$+ 166.36^{*}A^{*}B^{*}(A - B) + 131.93^{*}A^{*}B^{*}C^{2} + 22.88^{*}A^{*}C^{3} + 18.88^{*}B^{*}C^{3}. \quad (1)$$

It is clear from Figure 4(a) that as the concentration of OP in the linear mixture of OP and AP increased, the PG activity increased and the maximum PG activity (144.96 U ml^{-1}) was achieved in the presence of only OP in the fermentation medium. It can also be deduced that at the highest ammonium sulphate concentration, the presence of low amount AP ratio in the medium also promoted a reasonable PG activity (Figure 4(a)). Additionally like the linear mixture, as the ammonium sulphate concentration increased in the fermentation medium, PG activity increased, too. The optimum conditions for the maximum PG production (144.96 U ml^{-1}) was 21 g l^{-1} total carbohydrate concentration totally coming from OP at 15 g l^{-1} ammonium sulphate concentration. Moreover, 3:1 mixing ratio of OP + AP at 11.50 g l^{-1} ammonium sulphate concentration also resulted into a considerable PG activity (115.73 U ml^{-1}).

According to the ANOVA results of biomass, the applied model was significant with a F value of 16.77, and there was only 0.12% chance that a model F value this large could occur due to noise ($p = .0012$). In this case linear mixture components (A + B), AC, BC, and BC^3 were significant model terms ($p < .1$). The lack of fit F value of 0.013 implied that the lack of fit was not significant ($p = .9152$). The model equation of the biomass (Equation (2)) in terms of coded factors is as follows:

$$\text{Biomass}\ (g\,l^{-1}) = +24.83^{*}A + 7.83^{*}B - 2.27^{*}A^{*}B + 15.54^{*}A^{*}C - 20.69^{*}B^{*}C + 12.17^{*}A^{*}B^{*}C - 3.67^{*}A^{*}C^{2} - 0.47^{*}B^{*}C^{2} - 22.40^{*}A^{*}B^{*}(A - B) + 6.34^{*}A^{*}B^{*}C^{2} - 13.29^{*}A^{*}C^{3} + 21.24^{*}B^{*}C^{3} - 78.92^{*}A^{*}B^{*}C^{*}(A - B). \quad (2)$$

From Figure 4(b) it can be concluded that an increase in the OP concentration in the linear mixture OP + AP

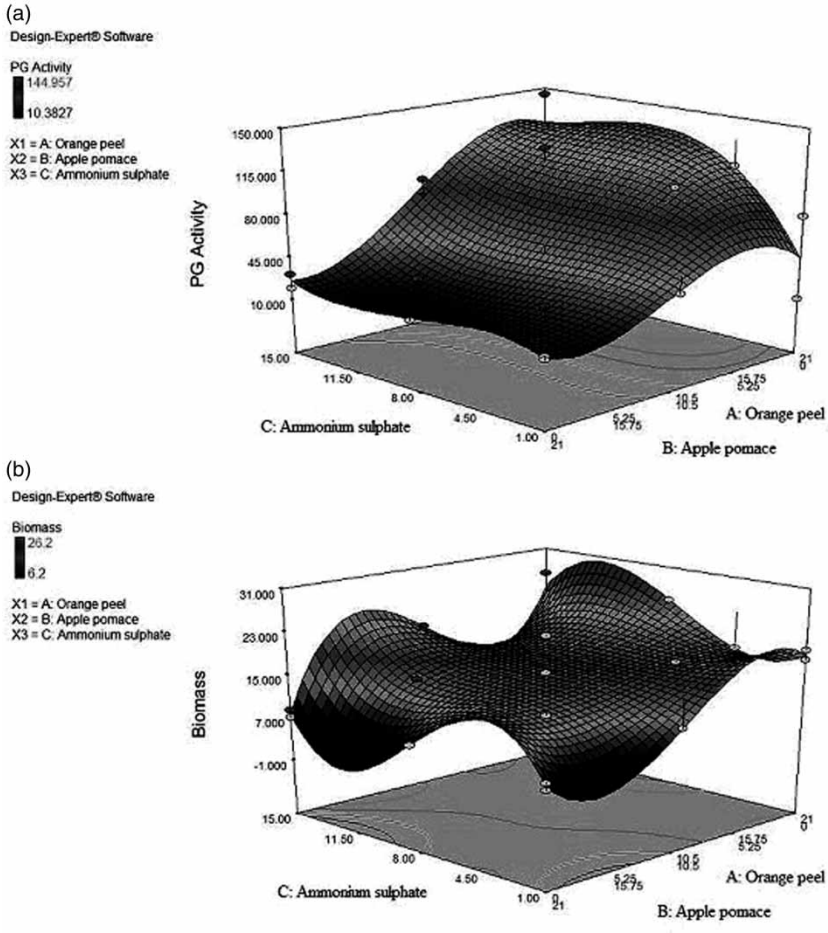

Figure 4. Response surface plots of the interaction of ammonium sulphate amount (C) and linear mixture (A + B) on (a) PG production and (b) biomass production.

Table 4. Results of validation experiments.

Run	Carbohydrate concentration coming from OP (g l^{-1})	Carbohydrate concentration coming from AP (g l^{-1})	Ammonium sulphate (g l^{-1})	Predicted PG activity (U ml^{-1})	Actual PG activity (U ml^{-1})	Error (%)
1	21	–	9.13	132.80	109.64	17.44
2	10.27	10.73	15	81.51	49.19	39.65
3	–	21	4.13	28.97	32.67	12.77

resulted in an increase in the biomass production at the higher ammonium sulphate concentrations. The maximum biomass production (26.2 g l^{-1}) was achieved at 21 g l^{-1} total carbohydrate concentration totally coming from OP similar to the maximum PG production.

3.3. Validation

In order to validate the adequacy of the model equations, a total of three verification experiments were carried out at the predicted optimum conditions for PG production. As a result 17.44%, 39.65%, and 12.77% deviation were observed for each of the validation experiments (Table 4). The overall margin of error was 23.29%.

Moreover, maximum PG activity in the validation experiments was experimentally determined as 21 g l^{-1} carbohydrate concentration totally coming from OP at 9.13 g l^{-1} ammonium sulphate concentration, giving 109.64 U ml^{-1} PG activity with 17.44% deviation from the predicted PG activity (132.80 U ml^{-1}).

The fermentation yields mostly depend on each substrate type and concentration used. Therefore, it is crucial to choose the optimum substrate type and concentration by optimizing fermentation techniques for each substrate. This is primarily due to the reason that each organism reacts differently to each substrate. The utilization rates of various nutrients differ in each substrate, which in turn affects productivity and yield. Mostly agro-industrial wastes such as wheat bran, orange bagasse, coffee pulp, and sugar cane bagasse are used in solid state fermentations.[34–36] Hence, the current study will serve as a starting point for the use of cost-effective substrates, agro-industrial wastes, in further submerged fermentation studies.

Many researchers have reported on the production of PGs from a wide variety of fungal strains and agro-industrial wastes under optimized conditions. The maximum PG activity in this study was nearly nine times higher than the activity obtained by Anuradha et al. (16 U ml^{-1}) using OP.[24] Moreover, Mohamed et al. obtained a maximum PG activity of 10 U ml^{-1} with *Trichoderma harzianum* grown on mandarin *Citrus reticulata* peel as the culture medium, levels lower than the maximum enzyme activity obtained in the current study.[23] On the other hand, Pedrolli et al. focused on the production of PG from *Aspergillus giganteus* by submerged fermentation using agro-industrial wastes such as wheat bran, lemon peel, sugar beet, apple, and orange bagasse.[22] In their study, using citrus pectin as the sole carbon source, the highest extracellular enzyme activity was 9.5 U ml^{-1}, while using orange bagasse, the highest extracellular activity was 48.5 U ml^{-1}, which were lower than the maximum PG activity obtained in our study. Favela-Torres et al. reviewed some PG activities by submerged fermentation with different microorganisms using various substrates.[36] Fontana and da Silveira performed submerged fermentation by using non-hydrolysed and partially hydrolysed pectin as the C source for the cultivation of *Aspergillus oryzae* in stirred tank bioreactor and found a maximum exo-PG activity of 80 ± 0.2 U ml^{-1}, which was quite lower than the one found in the current study (109.64 U ml^{-1}).[37] Moreover, the maximum PG activity was found to be 51.82 U ml^{-1} in submerged fermentation by *A. niger* ATCC 9642 using pectin as the C source in the study performed by Gomes et al.[38] The PG activity obtained in the current study was considerably higher than the PG activities obtained by other researchers. However, up to date there is no report about the use of the mixture of OP and AP as substrate in order to obtain optimum PG production conditions. Data obtained in this study showed us that the AP and OP combination was superior to these agro-industrial residues with respect to PG production.

4. Conclusion

The potential of important agro-industrial wastes, AP and OP, as C sources using an industrially important microorganism *A. sojae* was used in the maximization of the PG production. In the screening experiments, it was observed that maximum PG activity was achieved with a combination of non-hydrolysed-AP + OP and ammonium sulphate at the end of eight days of incubation. The optimum conditions for the maximum PG production (144.96 U ml^{-1}) was found to be 21 g l^{-1} total carbohydrate concentration totally coming from OP at 15 g l^{-1} ammonium sulphate concentration. On the other hand, a 3:1 mixing ratio of OP + AP at 11.50 g l^{-1} ammonium sulphate concentration resulted in a considerable PG activity (115.73 U ml^{-1}) as well. These results demonstrated that AP can be evaluated as an additional C source to OP for PG production. In

fact, both can serve as alternative solutions for the elimination of waste accumulation in the food industry with economical returns.

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Disclosure statement

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References

[1] Bhushan S, Kalia K, Sharma M, Singh B, Ahuja PS. Processing of apple pomace for bioactive molecules. Crit Rev Biotechnol. 2008;28:285–296.
[2] Vendruscolo F, Albuquerque PM, Streit F, Esposito E, Ninow JL. Apple pomace: a versatile substrate for biotechnological applications. Crit Rev Biotechnol. 2008;28:1–12.
[3] Botella C, Diaz A, Ory I, Webb C, Blandino A. Xylanase and pectinase production by *Aspergillus awamorion* grape pomace in solid state fermentation. Process Biochem. 2007;42:98–101.
[4] Shankar SK, Mulimani VH. α-Galactosidase production by *Aspergillus oryzae* in solid-state fermentation. Bioresour Technol. 2007;98:958–961.
[5] Shojaosadati SA, Babaeipour V. Citric acid production from apple pomace in multi-layer packed bed solid-state bioreactor. Process Biochem. 2002;37:909–914.
[6] Chatanta DK, Attri C, Gopal K, Devi M, Gupta G, Bhalla TC. Bioethanol production from apple pomace left after juice extraction. Internet J Microbiol. 2007;5:2.
[7] Cao X, Wang C, Pei H, Sun B. Separation and identification of polyphenols in apple pomace by high-speed countercurrent chromatography and high-performance liquid chromatography coupled with mass spectrometry. J Chromatogr A. 2009;1216:4268–4274.
[8] Fruit production statistics – Food and Agriculture Organization (FAO) of the United Nations [Internet]. Argentina [cited 2012 Dec 20]. Available from: http://faostat.fao.org.
[9] Joshi V, Devender A. Solid state fermentation of apple pomace for the production of value added products. Nat Prod Rad. 2006;5:289–296.
[10] Paganini C, Nogueira A, Silva NC, Wosiacki G. Utilization of apple pomace for ethanol production and food fiber obtainment. Cienc Agrotec. 2005;29:1231–1238.
[11] Pirmohammadia R, Rouzbehan Y, Rezayazdi K, Zahedifar M. Chemical composition, digestibility and in situ degradability of dried and ensiled apple pomace and maize silage. Small Ruminant Res. 2006;66:150–155.
[12] Garcia-Castello EM, Mayor L, Alcaraz N, Gras ML, Argüelles A, Vidal-Brotóns D. Orange solid waste valorization: optimization of pectinase extraction and enzymatic treatment of orange press liquor. Chem Eng Trans. 2012;29:823–828.
[13] Nighojkar S, Phanse Y, Sinha D, Nighojkar A, Kumar A. Production of polygalacturonase by immobilized cells of *Aspergillus niger* using orange peel as inducer. Process Biochem. 2006;41:1136–1140.
[14] Joshi VK, Parmar M, Rana NS. Pectin esterase production from apple pomace. Food Technol Biotechnol. 2006;44:253–256.
[15] Üçüncü C. Chemical composition analysis of agroindustrial waste and their potential usage in bio-ethanol production [dissertation]. Izmir: Izmir Institute of Technology; 2011.
[16] Panda T, Naidu GSN, Sinha J. Multiresponse analysis of microbiological parameters affecting the production of pectolytic enzymes by *Aspergillus niger*: a statistical view. Process Biochem. 1999;35:187–195.
[17] Djekrif-Dakhmouche S, Gheribi-Aoulmi Z, Meraihi Z, Bennamoun L. Application of a statistical design to the optimization of culture medium for a-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder. J Food Eng. 2006;73:190–197.
[18] Sathishkumar P, Palvannana T, Murugesan K, Kamala-Kannanc S. Detoxification of malachite green by *Pleurotus florida* laccase produced under solid-state fermentation using agricultural residues. Environ Technol. 2013;34:139–147.
[19] Cihangir N, Aksöza N. Evaluation of some food industry wastes for production of gibberellic acid by fungal source. Environ Technol. 1997;18:533–537.
[20] Carchesio M, Tatàno F, Lancellotti I, Taurino R, Colombo E, Barbieri L. Comparison of biomethane production and digestate characterization for selected agricultural substrates in Italy. Environ Technol. 2014;35:2212–2226.
[21] Tari C, Gogus N, Tokatli F. Optimization of biomass, pellet size and polygalacturonase production by *Aspergillus sojae* ATCC 20235 using response surface methodology. Enzyme Microb Technol. 2007;40:1108–1116.
[22] Pedrolli D, Gomes E, Monti R, Carmona E. Studies on productivity and characterization of polygalacturonase from *Aspergillus giganteus* submerged culture using citrus pectin and orange waste. Appl Biochem Biotechnol. 2008;144:191–200.
[23] Mohamed S, Al-Malki A, Kumosani T. Characterization of a polygalacturonase from *Trichoderma harzianum* grown on citrus peel with application for apple juice. Aust J Basic Appl Sci. 2009;3:2770–2777.
[24] Anuradha K, Padma P, Venkateshwar S, Reddy G. Fungal isolates from natural pectic substrates for polygalacturonase and multienzyme production. Indian J Microbiol. 2010;50:339–344.
[25] Mamma D, Kourtoglou E, Christakopoulos P. Fungal multienzyme production on industrial by-products of the citrus-processing industry. Bioresour Technol. 2008;99:2373–2383.
[26] Chandel AK, Chan E, Rudravaram R, Narasu ML, Rao LV, Ravindra P. Economics and environmental impact of bioethanol production technologies: an appraisal. Biotechnol Mol Biol. 2007;2:14–32.
[27] Balat M, Balat H, Öz C. Progress in bioethanol processing. Prog Energy Combust. 2008;34:551–573.
[28] Sanchez OJ, Cardona CA. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol. 2008;99:5270–5295.

[29] Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F. New improvements for lignocellulosic ethanol. Curr Opin Biotechnol. 2009;20:372–380.
[30] Gamez S, Gonzales-Cabriales JJ, Ramirez JA, Garrote G, Vazquez M. Study of the hydrolysis of sugarcane bagasse using phosphoric acid. J Food Eng. 2006;74:78–88.
[31] Cardona CA, Quintero JA, Paz IC. Production of bioethanol from sugarcane bagasse: status and perspectives. Bioresour Technol. 2009;101:4754–4766.
[32] Hours R, Voget C, Ertola R. Some factors affecting pectinase production from apple pomace in solid-state cultures. Biol Waste. 1988;24:147–157.
[33] Berovic' M, Ostrovers'nik H. Production of *Aspergillus niger* pectolytic enzymes by solid statebioprocessing of apple pomace. J Biotechnol. 1997;53:47–53.
[34] Maldonado MC, Saad AMS. Production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid state systems. J Ind Microbiol Biotechnol. 1998;20:34–38.
[35] Pandey A, Selvakumar P, Soccol CR, Nigam P. Solid state fermentation for the production of industrial enzymes. Curr Sci. 1999;77:149–162.
[36] Favela-Torres E, Volke-Sepúlveda T, Viniegra-González G. Production of hydrolytic depolymerising pectinases. Food Technol Biotechnol. 2006;44:221–227.
[37] Fontana RC, da Silveira MM. Production of polygalacturonases by *Aspergillus oryzae* in stirred tank and internal- and external-loop airlift reactors. Bioresour Technol. 2012;123:157–163.
[38] Gomes J, Zeni J, Cence K, Toniazzo G, Treichel H, Valduga E. Evaluation of production and characterization of polygalacturonase by *Aspergillus niger* ATCC 9642. Food Bioprod Process. 2011;89: 281–287.