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

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 (hydrolyzed-AP, nonhydrolyzed-AP, hydrolyzed-AP+OP, nonhydrolyzed-AP+OP), N sources (ammonium sulphate and urea) and incubation time (4, 6, 8 days) were screened. It was observed that maximum PG activity was achieved at a combination of non-hydrolyzed-AP+OP and ammonium sulphate with 8 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 ammonium sulphate concentration also resulted into 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 bio-converted into different value-added products. [1, 2] Million tons of apple and orange juice processing wastes like peel, pulp, seeds, etc. are produced annually all over the world, being highly biodegradable where their disposal generates a serious environmental problem and finally leads to pollution.

Among these wastes, apple pomace wastes have been proposed as 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 tonnes, [8] around 30% of this amount was used in the production of different products like juice, concentrate, jelly, pulp, canned slices, wine, cider, etc. Apple pomace, which represents around 25–35% of the processed apples, is one of the main by-product of fruit processing industry containing peel, seed, core, calyx, stem, and soft tissue. [2, 9] Apple pomace 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, galactose with minor amounts of rhamnose, xylose and glucose, as well as small amounts of minerals, proteins, and vitamins. Also apple pomace 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 the half of fresh orange weight is transformed into juice while the other half is considered as production waste. [12] Therefore orange peel holds a great potential to be used as substrate and inducer for the production of polygalacturonases (PG) 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 fruit juice industry and wine making to increase the juice yield, facilitate pressing and filtration and to 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] Utilization of orange peel and

apple pomaces in enzyme production has also several advantages like easy availability of cheaper raw material, reducing the cost of the enzyme and resulting in reduction of environmental pollution. [14]

Therefore, the goal of this study was to investigate the potential of important agro-industrial wastes; apple pomace and orange peel 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*

Aspergillus sojae ATCC 20235 was purchased from Procochem Inc., an international distributor of ATCC (American Type of Culture Collection) 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 (YME) 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; agar, 20; and stock solutions (mg l^{-1}): $FeSO_4.7H_2O$, 15; KH_2PO_4, 60; $MgSO_4$, 50; $CuSO_4.5H_2O$, 12; and $MnSO_4.H_2O$, 15. Spores were harvested using 5 ml of Tween80-water (0.02% v/v).

2.2. *Apple pomace and orange peel*

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

2.3. *Hydrolyzation of apple pomace*

Based on our previous experiments temperature of 110°C, 40 minutes, 4% phosphoric acid and 10% solid liquid ratio were determined as optimum hydrolysis conditions. [15] Apple pomace hydrolysates were filtered, pH adjusted to 5.0, using 6N NaOH and sterilised at 121^0C for 15 minutes.

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 (nonhyd)+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 total of 39 runs including 3 replicates (Table 2). Responses were PG activity (U ml^{-1}) and biomass (g ml^{-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 three different total carbohydrate concentrations (9, 15, 21 g l^{-1}) with five 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 orange peel and the second number to the ratio of apple pomace.

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 apple pomace and orange peel (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, 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 $p<0.1$).

2.6. *Polygalacturonase (PG) activity*

Polygalacturonase (PG) activity was assayed according to the modified procedure of Panda et al. (1999) 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 preweight filter paper, followed by drying to constant weight at 100°C, overnight.

3. Results and discussion

For any industrial fermentation medium optimization is of outmost 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 2013 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 1997. [19] Furthermore Carchesio et al 2014 compared biomethane production of some selected agricultural substrates such as grape seeds and plum stones. [20]

3.1. Screening experiments

Factors like 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% to 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 orange peel and apple pomace 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 polygalacturonase is that containing pectic materials as inducer. [25] In the current study the effect of C source (AP (hyd), AP (hyd)+OP, AP (nonhyd) and AP (nonhyd)+OP), N source (Ammonium sulphate and urea) and incubation time (4, 6 and 8 days) were screened in terms of PG activity and biomass. AP was screened in the form of hydrolyzed and non-hydrolyzed.

With the hydrolyzation process the aim was to open the accessible areas in the cellulose structure of apple pomace. Hydrolysis affects lignocelluloses, creating larger accessible surface area and pore size. Moreover, hydrolysis was expected to improve the formation of sugars and avoid degradation or loss of carbohydrate and formation of inhibitory by-products for subsequent fermentation and be cost effective. [26-29] After pre-treatment, water insoluble solids were filtered in order to obtain the majority of cellulose where lignin and the hemicellulosic sugars remained in the filtrate. Apple pomace was pre-treated with the phosphoric acid (H_3PO_4) since after neutralization of hydrolysates with NaOH the salt formed was sodium phosphate, which could be used as 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 significant effect on the PG activity ($p<<0.1$). But the effect of incubation time and its interactions with the other factors were insignificant on PG

activity ($p>0.1$). 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 1a, it can be observed that the highest PG activity (64.39 U ml^{-1}) was achieved using AP (nonhyd.)+OP level as C and ammonium sulphate as N sources at 8 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 significant effect ($p<0.1$). The highest biomass production (52.98 g l^{-1}) was obtained with AP (hydr.) as shown in Figure 1b. 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 6^{th} and 8^{th} days of incubation but on the 4^{th} day biomass production was very low (Figure 1c).

Lower PG activity but higher biomass was obtained with hydrolyzed AP (Figures 1a and b). This result might be due to the consumption of small sugars formed after hydrolyzation towards biomass production instead of PG. During the hydrolysis process pectin was not hydrolyzed therefore there was no galacturonic acid units in the hydrolysate which 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-hydrolyzed apple pomace 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 combined with various agro-industrial wastes for pectolytic enzyme productions. [33] However, to the best of our knowledge, this media composition, the mixture of apple pomace and orange peel, has not been previously considered for this purpose. As a conclusion one can prefer the use of hydrolyzed AP for optimum biomass production and non-hydrolyzed AP+OP for optimum PG production in the presence of ammonium sulphate and 8 days of incubation. Since the goal in the current study was to achieve maximum PG production, the optimization study continued with non-hydrolyzed AP+OP as fermentation medium.

3.2. *Optimization experiments*

Based on the initial screening experimental results, D-optimal design with 2 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 ($p<0.1$). Furthermore their interaction (AB), their quadratic interaction (A^2B) were also significant terms with respect to PG activity ($p<0.1$).

One factor plot of orange peel + apple pomace 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 orange peel (21, (4:0)) and maximum ammonium sulphate concentration (8 g l^{-1}) (Figure 2a and b). Additionally, the data in Figure 2b were summarized with 3 different figures given in Figure 3 (a,b,c). These plots illustrate the PG activity change by a change in OP+AP mixing ratio for different total carbohydrate concentrations (9, 15, 21 g l^{-1}) at three different ammonium sulphate concentrations (1, 4.5, 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 3a, b, c). Comparing Figure 3b and 3c, an increase in ammonium sulphate concentration from only 4.5 g l^{-1} 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 with 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 3b (4.5 g l^{-1} ammonium sulphate) and dropped to 1.125 in Figure 3c (8 g l^{-1} ammonium sulphate) for the same 1:1 ratio of orange peel to apple pomace. However, this ratio was 3.33 in Figure 3b at 15 g l^{-1} carbohydrate concentration and

dropped to 1.875 in Figure 3c when ammonium sulphate concentration was increased to 8 g l^{-1}. Since a C/N ratio of 2 and 1.875 were 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 ratio was 15 and 9 at both 15 and 9 g l^{-1} carbohydrate concentrations, respectively (Figure 3a) which were quite high. Since again there seemed not 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, presence of only OP in the medium with the maximum total carbohydrate concentration (21 g l^{-1}) resulted in the maximum PG production (Figure 3c). 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 c), which can enable the use of apple pomace with orange peel. With these results the possible use of another agro-industrial waste such as apple pomace was proved to be used in PG production besides orange peel. As the factor levels of OP+AP mixing ratio were categorical 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 2b). 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 ($p>0.1$) whereas OP+AP mixing ratio and their interactions were significant terms ($p<0.1$) at the determined levels. The maximum biomass (24.4 g l^{-1}) was also achieved at maximum concentration of carbohydrate 21 g l^{-1} with (4:0) mixing ratio and 8g l^{-1} ammonium sulphate amount as in PG production (Figure 2c). 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 2d).

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 *Aspergillus 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 apple pomace and orange peel (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, 4:0.

According to ANOVA results of PG activity, the applied model was significant with a p value of 0.0361 ($p<<0.1$). The lack of fit F value of 0.43 implied that the lack of fit was not significant ($p=0.6705$). Additionally linear mixture which meant the mixture of OP+AP (A+B) was the significant factor ($p<<0.1$).

The model equation of the PG activity (Eq. 1) in terms of coded factors is given below;

$$\text{PG activity (U ml}^{-1}) = +131.71*A+23.23*B-86.64*A*B+14.16*A*C-16.29*B*C-0.93*A*B*C-49.54*A*C^2-0.23*B*C^2+166.36*A*B*(A-B)+131.93*A*B*C^2+22.88*A*C^3+18.88*B*C^3 \quad (1)$$

It was clear from the Figure 4a, 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, presence of low amount AP ratio in the medium also promoted a reasonable PG activity (Figure 4a). 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 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=0.0012). In this case linear mixture components (A+B), AC, BC, BC^3 were significant model terms (p<0.1). The lack of fit F value of 0.013 implied that the lack of fit was not significant (p=0.9152). The model equation of the biomass (Eq. 2) in terms of coded factors is given below;

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

From Figure 4b it can be concluded that an increase in the OP concentration in the linear mixture OP+AP 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 was 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, sugar cane bagasse are used in solid state fermentations. [34-36] Hence, 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 polygalacturonases from a wide variety of fungal strains and agro-industrial wastes under optimized conditions. The maximum PG activity in this study was nearly 9 times higher than the activity obtained by Anuradha et al. (2010) (16 U ml^{-1}) using orange peel. [24] Moreover, Mohamed et al. (2009) obtained a maximum PG activity of 10 U ml^{-1} with *Trichoderma harzianum* grown on mandarin *Citrus reticulate* peel as culture medium, levels lower than the maximum enzyme activity obtained in the current study. [23] On the other hand, Pedrolli et al. (2008) focused on the production of PG from *Aspergillus giganteus* by submerged fermentation using agro-industrial wastes like wheat bran, lemon peel, sugar beet, apple, and orange bagasse. [22] In their study, enzyme activity using citrus pectin as sole carbon source, the highest extracellular 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. (2006) reviewed some polygalacturonase activities by submerged fermentation with different microorganisms using various substrates. [36] Fontana and Silveira (2012) performed submerged fermentation by using non-hydrolyzed and partially hydrolyzed pectin as C source for the cultivation of *Aspergillus oryzae* in stirred tank bioreactor and found 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 as 51.82 U ml^{-1} in submerged fermentation by *Aspergillus niger* ATCC 9642 using pectin as C source in the study performed by Gomes et al. (2011). [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 mixture of orange peel and apple pomace as substrate in order to obtain optimum polygalacturonase production conditions. Data obtained in this study showed us that the apple pomace and orange peel combination was superior to these agro-industrial residues with respect to PG production.

4. Conclusion

The potential of important agro-industrial wastes; apple pomace and orange peel as C sources, using an industrially important microorganism *Aspergillus sojae* were used in the maximization of the PG production. In the screening experiments, it was observed that maximum PG activity was achieved at a combination of non-hydrolyzed-AP+OP and ammonium sulphate at the end of 8 days of incubation. 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 into 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 the waste accumulation in the food industry with economical returns.

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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 D.K, Attri C, Gopal K, Devi M, Gupta G, Bhalla TC. Bioethanol production from apple pomace left after juice extraction, Internet J. Microbiol. 2008; 5:2.

[7] Cao X, Wang C, Pei H, Sun B. Separation and identification of polyphenols in apple pomace by high-speed counter-current 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] Sathishkumara P, Palvannana T, Murugesan K and 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, and 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 and 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. 144 (2008) 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, Bioresource 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. Energ. Combust. 2008; 34:551-573.

[28] Sanchez OJ, Cardona CA. Trends in biotechnological production of fuel ethanol from different feedstocks, Bioresource Technol. 2008; 99:5270-5295.

[29] Margeot A, Hahn-Hagerdal B,. Edlund M, Slade R, Monot F. New improvements for lignocellulosic ethanol, Curr. Opin. Biotech. 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, Bioresource 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, Bioresource 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.

Tables

Table 1. Factors and levels of screening experiments

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

AP (hyd) : Apple pomace (hydrolyzed)

AP (hyd)+OP : Apple pomace (hydrolyzed)+Orange peel

AP (nonhyd) : Apple pomace (nonhydrolyzed)

AP (nonhyd)+OP : Apple pomace (nonhydrolyzed)+Orange peel

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

	Factor 1	Factor 2	Response 1	Response 2
Run	**A:Ammonium sulphate**	**B:Orange peel+Apple pomace**	**PG Activity (U ml^{-1})**	**Biomass (mg ml^{-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

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

	Component 1	Component 2	Factor 3	Response 1	Response 2
Run	**A:Orange peel**	**B:Apple pomace**	**C:Ammonium sulphate**	**PG Activity (U ml^{-1})**	**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

1 **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 $m^{-1}l$)**	**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

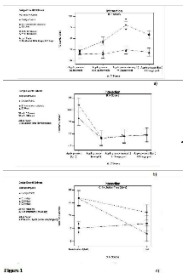
Figure Captions

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

Figure 2 (a) Effect of OP+AP mixing ratio (B) and b) interaction of ammonium sulphate amount and OP+AP mixing ratio (AB) on PG production, c) effect of OP+AP mixing ratio (B) and d) 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) 4.5g l^{-1}, c) 8g l^{-1}.

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



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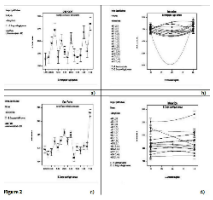

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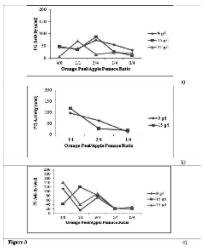

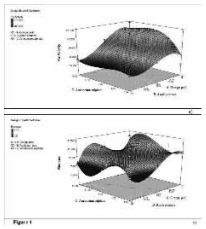

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