



Orange Solid Waste Valorization: Optimization of Pectinase Extraction and Enzymatic Treatment of Orange Press Liquor

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During orange juice production only approximately a half of fresh oranges weight is transformed into juice while the other half is considered as production waste. Orange solid wastes can be pressed with lime to obtain a press liquor that is currently concentrated up to 65-70°Brix by multiple effect evaporation. A prior preconcentration step by reverse osmosis (RO) reduces significantly energy consumption during evaporation. However, RO treatment is not easily performed due to the high viscosity of the press liquor, attributed to the presence of pectic substances.

The aims of this work are to study the extraction of pectinase enzymes from orange peels, as well as to assess the effect of the enzyme extract on pectic content and viscosity of press liquor. Pectinmethylesterase enzymes were characterized in terms of the typical enzyme kinetic parameters.

The optimum extracting conditions were pH=5.5 and NaCl=1.7 M. The characteristic kinetic parameters of the pectinmethylesterase were $K_M=0.5\%$ and $V_{max}=2.3$ mg/min·mL. The application of the enzymatic extract to real orange press liquor reduced considerably its pectin content (77.72%) and viscosity (48.30%), suggesting that enzyme extraction could be included as a step in the integrated valorization of orange wastes.

1. Introduction

Oranges are around the 10% of the world fruit production (FAOSTAT). During orange juice production only approximately the half of fresh oranges weight is transformed into juice (Braddock, 1999) while the other half is considered as production waste. These wastes are, in most cases, spread on soil areas adjacent to the production locations, for a final use as raw material for cattle feed, or burned (Martin et al., 2010). This way of waste handling produces highly polluted wastewater in terms of chemical and biological oxygen values (COD and BOD) that can negatively affect the soil and the ground and superficial water.

An alternative to improve the management of orange solid wastes is their treatment by addition of lime and a latter pressing, obtaining a press cake and a press liquor rich in sugars with a total concentration of around 10°Brix. Other components present in the press liquor are citric acid, protein, pectin and ethanol (Braddock, 1999). Currently, the orange press liquor (OPL) is concentrated up to 65-70°Brix by multiple effect evaporation to obtain molasses that are used in the production of beverage alcohol (Bampidis and Robinson, 2006), and as cattle feed (Wing et al., 1988). The evaporative concentration implies very high energy consumptions when compared with non-thermal membrane operations, as

reported in the preconcentration of sucrose solutions by reverse osmosis (RO) (Garcia-Castello et al., 2006).

Some attempts of OPL preconcentration by RO and forward osmosis (FO) have been done. In both cases, the presence of pectin in synthetic press liquor made very difficult its treatment mainly due to its high viscosity (Garcia-Castello et al., 2011; Garcia-Castello and McCutcheon, 2011). Hence, a depectinization step is strongly recommended for membrane preconcentration of press liquor.

To our knowledge there are no studies in literature aimed to remove pectin from OPL. However, there are many works of juice depectinization to obtain a clarified product. The most extended procedure is based on the addition of commercial enzymes such as pectinmethylesterase (EC 3.1.1.11) or polygalacturonase (EC 3.2.1.15). Pectinmethylesterase (PME) and polygalacturonase enzymes are naturally present in the peel of several fruits such as oranges and apples (Liu et al., 2011).

The aims of this work were to study the extraction of PME enzymes from orange peels, identifying the optimum extracting conditions, as well as to assess the effect of the enzyme extract on pectin content and viscosity of orange press liquor.

2. Material and Methods

2.1 Raw materials

"Valencia Late" oranges (*Citrus sinensis* L.) were purchased from a local market in Valencia, Spain. Oranges were carefully washed with tap water and stored at 5°C until use.

2.2 Press liquor

Around 20 kg of whole oranges were squeezed in a full-scale industrial juice extractor (mod. FOMEX, Fomesa, S.A., Spain) obtaining separately orange juice (10 kg aprox.) and solid waste (peel and pulp). Lime (Ca(OH)₂ 95% purity, Sigma) was added to the solid waste in a ratio of 0.3% of the waste weight. Mixture was stirred for 20 minutes and afterwards was pressed in a hydraulic press for 10 minutes. The obtained OPL was collected and kept apart. The pressing process was repeated and the resulting OPL was added to the first liquor. The total weight of OPL was around 2 kg and was stored at -20°C.

2.3 Extraction of pectin enzymes from orange peels. Experimental design

Fruits were cut into halves and the juice was extracted using a domestic squeezer. Immediately after juice extraction peels were treated to extract pectin enzymes.

A central composite design (CCD) with two variables (NaCl concentration and pH) and five replicates of the center point was used. Real and coded values for each variable are listed in Table 1.

Response variables in every experiment were total pectinase (TP) activity and pectinmethylesterase (PME) activity in the extract.

A sample of around 25 g of orange peels was suspended into 100 mL of extracting aqueous solution at different NaCl concentrations. The mixture was homogenized in an electrical blender. The pH of the homogenate was adjusted to different values according to the experimental design (Table 1) using NaOH and HCl solutions. Homogenates were stirred in an orbital shaker at 175 rpm and at constant temperature of 4°C. The extraction time was fixed following the procedure explained below. After the extraction, samples were vacuum filtered. The crude extract was centrifuged at 13000 rpm for 30 minutes at 4°C. The supernatant (enzymatic extract) was kept at -20°C until analysis.

In order to fix the time of extraction, extraction kinetics were followed at different times (0.33, 0.83, 1.5 and 2 hours) and at experimental conditions of pH 5.5 and NaCl concentration of 1.25 M. Extracts were analyzed in terms of PME activity. Extraction kinetics was made in duplicate.

2.4 Determination of enzymatic activity

The enzymatic activity of extracts was determined by measuring the PME and TP activities. The PME activity of the extracts was measured by using the experimental method used by several authors (Hou et al., 1997; Vivar-Vera et al., 2007). One unit of PME activity was defined as 1 µequivalent of carboxyl groups released per minute and millilitre of enzymatic extract (U/mL).

TP activity was calculated from the viscosity decrease (Alkorta et al., 1998; Zhou et al., 2000; Rai et al., 2004) of a standard citrus pectin solution (1%, 20 mL) before and after adding a known volume (1 mL) of the enzymatic extract, leaving the system to react during 5 minutes at 25±2°C. The viscosity

determinations were done using an Ostwald capillary viscosimeter. The TP activity was expressed as the percentage of viscosity decrease of the 1% pectin solution.

Table 1. Experimental design for enzyme extraction. Independent variables: pH and NaCl concentration. Response variables: Pectinmethylesterase and pectinase activities (Agitation speed=175 rpm; Temperature=4°C; Extraction time=90 min).

Experiment	Coded values		Real values		Response variables	
	X ₁	X ₂	pH	NaCl (M)	PME activity (U/mL)	TP activity (%)
1	-1	-1	4.0	0.50	1.05	12.74
2	-1	+1	4.0	2.00	5.10	10.30
3	+1	-1	7.0	0.50	0.76	20.48
4	+1	+1	7.0	2.00	4.55	13.89
5	0	0	5.5	1.25	6.26	22.89
6	0	0	5.5	1.25	5.27	22.63
7	0	0	5.5	1.25	5.77	22.12
8	0	0	5.5	1.25	6.44	24.27
9	0	0	5.5	1.25	5.48	26.86
10	+1.414	0	7.6	1.25	5.16	35.88
11	-1.414	0	3.4	1.25	5.24	33.71
12	0	+1.414	5.5	2.31	5.88	16.88
13	0	-1.414	5.5	0.19	0.08	2.77

2.5 Enzymatic characterization

The enzymatic characterization was made by the calculation of the typical enzyme kinetic parameters in the Michaelis-Menten equation (1) that describes the rate of the enzymatic reactions by relating the reaction rate, V , to the substrate concentration, C_S .

$$V = \frac{V_{max} \cdot C_S}{K_M + C_S} \quad (1)$$

where V_{max} is the maximum reaction rate and K_M is the Michaelis constant (substrate concentration at which the reaction rate is at half-maximum). V_{max} and K_M were obtained by using the Lineweaver-Burk (or double reciprocal) plot widely used by several authors (Vivar-Vera et al., 2007). It was measured the release of galacturonic acid per mL of enzymatic extract as a function of reaction time in pectin solutions at different concentrations (0.25, 0.5, 1.0, 1.5 and 2.0% (w/v)). The specific reaction rate was expressed as mg of galacturonic acid released per minute and mL of enzymatic extract.

2.6 Pectin content in press liquor

The pectin content in raw and treated press liquor was determined as described by Untiveros (2003). Pectin content was expressed as mg/mL.

2.7 Statistical analysis

All analytical determinations were conducted in triplicate. Data were expressed as the means of these values. Experimental data from the CCD were analyzed using the response surface methodology (RSM) and fitted to Equation (2).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2 \quad (2)$$

Fitting of eq. (2), analysis of variance (ANOVA), surface plots and the optimized value of the two independent variables for the best response were determined using the statistical software Statgraphics 5.1 (Statpoint Technologies Inc.). Evaluations were based on a significant level of $p \leq 0.05$.

3. Results and Discussion

3.1 Extraction kinetics

It was found a clear asymptotic trend in the enzymatic extraction kinetics (experimental data were satisfactorily adjusted to a third order polynomial, $R^2 = 0.99$, data do not shown). At the beginning of the extraction process PME activity increases fastly, at 60 min the increase is less accentuated and after 90 min PME activity remains practically constant. Then, the optimum extraction time was selected at 90 minutes and was used in the following extraction experiments. This time is similar to that used by Spagna et al. (2003) in the PME extraction from different parts of orange.

3.2 Enzyme extraction

Extraction experiments were performed at the process conditions shown in Table 1. In that table are also shown experimental results in terms of PME and TP activities in the extracting media. Data of PME activity was fitted to Eq. 2 resulting in Equation 3.

$$PMEact(U/mL) = -10.72 + 10.71 \cdot NaCl + 3.06 \cdot pH - 3.09 \cdot NaCl^2 - 0.28 \cdot pH^2 - 0.06 \cdot NaCl \cdot pH \quad (3)$$

The overall quadratic model was significant with an R^2 value of 0.93. Statistical analysis indicated that first-order effect of NaCl concentration (X_2) and second-order effect of NaCl concentration ($X_2 \cdot X_2$) were significant at 95% confidence level.

Figure 1(a) shows the response surface obtained from eq. (3). As observed, PME activity is clearly influenced by NaCl concentration, showing a maximum value approximately at 1.7 M. pH of the extracting liquid shows a slight effect (not significant as indicated in the ANOVA results), giving a maximum value around pH= 5.5. These pH and NaCl values are in the range of the optimum PME extraction conditions for fruits and vegetables, as reported by several authors (Hou et al., 1997; Pires and Finardi-Filho, 2005).

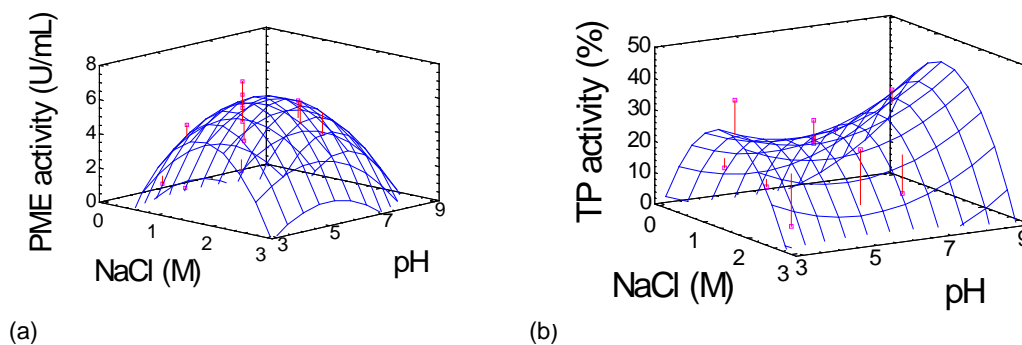


Figure 1: Effect of NaCl concentration and pH on: (a) pectinmethylesterase activity of orange waste aqueous extracts; (b) total pectinase activity of orange waste aqueous extracts.

The strong influence of NaCl on citrus PME extraction suggests that the enzyme is associated with the cell wall by ionic interaction. The decrease in PME activity with NaCl concentrations higher than 1.7 M might indicate a “salting-out” effect (Vivar-Vera et al., 2007). In the salting-out process, salt removes water molecules that hydrate protein and allows hydrophobic-hydrophobic interactions between different proteins. This effect leads to higher molecular weight aggregates that precipitate. In case of enzymes, the precipitation provokes their activity lost (Tokunaga et al., 2006).

Data of TP activity was fitted to Eq. (2) resulting in Equation (4). The overall quadratic model was significant with an R^2 value of 0.75. Statistical analysis indicated that only second-order effect of NaCl concentration ($X_2 \cdot X_2$) was significant at 95% confidence level.

The response surface shown in Figure 1(b) is based on the model described in Eq. 4. It can be observed that NaCl concentration had the most significant effect on TP activity. From Eq. (4) it was

determined the NaCl concentration and pH values that would give maximum TP activity: pH=5.1 and NaCl=1.3 M.

$$TPact(\%) = 31.14 + 46.69 \cdot NaCl - 14.91 \cdot pH - 15.92 \cdot NaCl^2 + 1.57 \cdot pH^2 - 0.92 \cdot NaCl \cdot pH \quad (4)$$

There was not found a significant correlation between these two enzymatic activities ($R^2=0.31$). This lack of correlation is explained considering that TP activity is the sum of the activities of three types of enzymes, pectinmethylesterases (de-esterifying enzymes), depolymerising enzymes (pectin-hydrolases and pectin-lyases enzymes) and protopectinases (Alkorta et al., 1998). In orange peels there are large amounts of PME but there are also present low natural amounts of polygalacturonase (pectin-hydrolase) (Braddock, 1998; Liu et al., 2011).

The optimum extraction conditions in terms of pH and NaCl concentration were selected according to the conditions for the maximum PME activity since R^2 value was higher than the R^2 obtained for the TP activity ($0.93 > 0.75$).

3.3 Pectinmethylesterase kinetic characterization

The enzyme extract used for the enzyme characterization was obtained under the optimum conditions (pH=5.5 and NaCl=1.7 M). K_M and V_{max} values (see Eq. 1) were calculated from the intercepts on x and y axis of the Lineweaver-Burk plot (figure not shown). The specific maximum reaction rate, V_{max} , of galacturonic acid release was 2.3 mg/min·mL of enzymatic extract. The K_M value indicates the affinity of the enzyme for its natural substrate. The K_M value obtained was 0.50% (w/v) (5.0 mg/mL), a value close to others reported for PME from several plant sources (Vivar-Vera et al., 2007).

3.4 Effect of enzymatic extract on orange press liquor

As commented in 3.3, enzymatic extract obtained under the optimum extraction conditions (NaCl=1.7 M; pH=5.5) was used for the depectination of the press liquor. The calcic pectate content in the raw press liquor was 3.86 mg/mL and after the application of the enzymatic extract it was found a pectin reduction of 77.7 %.

Viscosity reduction in the press liquor was determined using the same procedure for the determination of TP activity, but OPL was used as substrate. It was found a viscosity reduction of 48.3%. Sarioglu et al. (2001) reported that using commercial pectinase from *Aspergillus aculeatus* on pectin aqueous solutions it was obtained a viscosity reduction of approx. 83% using a 3.5% pectin solution. In that case, they used commercial enzymes while we used enzymatic extracts. In addition, the incubation time for our experiments was 5 min while the incubation time reported by those authors was 20 min. It can be considered that the viscosity decrease effect of the enzymatic extract obtained in this work is quite good when compared with other literature works.

The percentage in viscosity reduction obtained is lower than the percentage in calcic pectate reduction. This fact can be explained by the nonlinear dependence of viscosity with pectin concentration (Guimaraes et al., 2009). Furthermore, OPL is composed by other substances such as sugars, proteins and citric acid that promote viscosity too. The products of the enzyme activity (sugars, short chain carbohydrates) may additionally contribute to the viscosity of the hydrolyzed press liquor.

4. Conclusions

Pectinase enzymes from orange peel have been successfully extracted by using aqueous NaCl solutions at controlled pH. The optimum conditions for the enzyme extraction were pH=5.5 and NaCl=1.7 M. The typical parameters for the enzyme kinetic characterization of the pectinmethylesterase resulted in a $V_{max}=2.3$ mg/min·mL and a $K_M=0.50\%$ (w/v).

The application of the enzymatic extract to orange press liquor reduces considerably its pectin content and viscosity. Then, it can be finally concluded that pectinase enzyme extraction from orange peels could be an interesting step to be included in an integrated orange waste management process, where the enzymatic extract could be used to depectinize the orange press liquor for its later preconcentration by membrane technology.

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