JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 11471–11477 **11471**

Apple Juice Clarification by Immobilized Pectolytic Enzymes in Packed or Fluidized Bed Reactors

NADIA DIANO,[†,‡,§] TIZIANA GRIMALDI,[‡] MARIANGELA BIANCO,[‡] SERGIO ROSSI,[§] KATYA GABROVSKA,[||] GALYA YORDANOVA,[||] TZONKA GODJEVARGOVA,[||] VALENTINA GRANO,[‡,§] CARLA NICOLUCCI,[‡] LUIGI MITA,[‡] UMBERTO BENCIVENGA,[§] PAOLO CANCIGLIA,[||] AND DAMIANO G. MITA*[,†,‡,§]

Department of Experimental Medicine, Second University of Naples, Via S. M. di Costantinopoli, 16, 80138 Naples, Italy, National Institute of Biosystems and Biostructures (INBB), Viale Medaglie d'Oro, 305, 00136 Rome, Italy, Institute of Genetic and Biophysics "ABT", Via P. Castellino, 111, 80131 Naples, Italy, Department of Biotechnology, University "Prof. Dr. A. Zlatarov", Bourgas 8010, Bulgaria, and Department of Pharmacobiology, University of Messina, Via SS Annunziata, 90168, Messina, Italy

The catalytic behavior of a mixture of pectic enzymes, covalently immobilized on different supports (glass microspheres, nylon 6/6 pellets, and PAN beads), was analyzed with a pectin aqueous solution that simulates apple juice. The following parameters were investigated: the rate constant at which pectin hydrolysis is conducted, the time (τ_{50}) in which the reduction of 50% of the initial viscosity is reached, and the time ($\tau_{comp,dep}$) required to obtain complete depectinization. The best catalytic system was proven to be PAN beads, and their pH and temperature behavior were determined. The yields of two bed reactors, packed or fluidized, using the catalytic PAN beads, were compared to the circulation flow rate of real apple juice. The experimental conditions were as follows: pH 4.0, $T = 50$ °C, and beads volume = 20 cm^3. The initial pectin concentration was the one that was present in our apple juice sample. No differences were observed at low circulation rates, while at higher recirculation rates, the time required to obtain complete pectin hydrolysis into the fluidized reactor was found to be 0.25 times smaller than in the packed bed reactor: 131 min for the packed reactors and 41 min for the fluidized reactors.

KEYWORDS: Pectin hydrolysis; bed reactors; apple juice; PAN beads; nylon pellets; glass microspheres; pectic enzymes

INTRODUCTION

The raw juices obtained after pressing apples are cloudy, dark in color, extremely viscous, and tend to settle during storage. For this reason, raw apple juices need to be clarified. Turbidity is caused by the presence of colloids, which essentially consist of pectic substances and starch. The amount of pectic substances in raw apple juice ranges from 1 to 5 g/L (*1*). Pectic substances are natural polysaccharides, present in vegetable tissues, and are essentially composed of linear polymers of α-D-galacturonic acid units joined in α-D-glucosidic linkage (polygalacturonic acid) (*2*−*4*). Because of their fiber-like structure, pectins are responsible for the viscosity of the juice, thus affecting the ultrafiltration processes usually employed in fruit juice clarification (*5*−*11*). To overcome this problem and to degrade pectins, pectinases are employed. In general, pectinases are classified into de-esterification and depolymerizing enzymes based on the pectin degradation mechanism (*12*−*19*). Several different pectic enzymes and their reaction mechanisms, which depend upon the substrate nature, are illustrated in **Figure 1**. In particular, pectinlyase (EC 4.2.2.10) and polygalacturonase (EC 3.2.1.15) act on pectin and polygalacturonic acid, respectively. Pectinlyase (or transeliminases) breaks the glycosidic linkages at C-4 and simultaneously eliminates H from C-5, producing a 4.5-unsatured galacturonic acid group. Polygalacturonase catalyzes the hydrolytic cleavage of the polygalacturonic acid chair with the introduction of water across the oxygen bridge. Pectinesterase (EC 3.1.1.11) is responsible for pectin de-esterification, thus producing a polysaccharide that can be subsequently hydrolyzed by a polygalacturonase. As a result of these specific enzymatic activities, it is possible to find commercial preparations of a mixture of these enzymes. These preparations are

* To whom correspondence should be addressed: Institute of Genetic and Biophysic "ABT" of CNR, Via P. Castellino, 111, 80131 Naples, Italy. Telephone/Fax: +39-081-6132608. E-mail: mita@igb.cnr.it.

[†] Second University of Naples.

[‡] National Institute of Biosystems and Biostructures (INBB).

[§] Institute of Genetic and Biophysics "ABT".

[||] University "Prof. Dr. A. Zlatarov".

[⊥] University of Messina.

10.1021/jf8019437 CCC: $40.75
Published on Web 11/06/2008

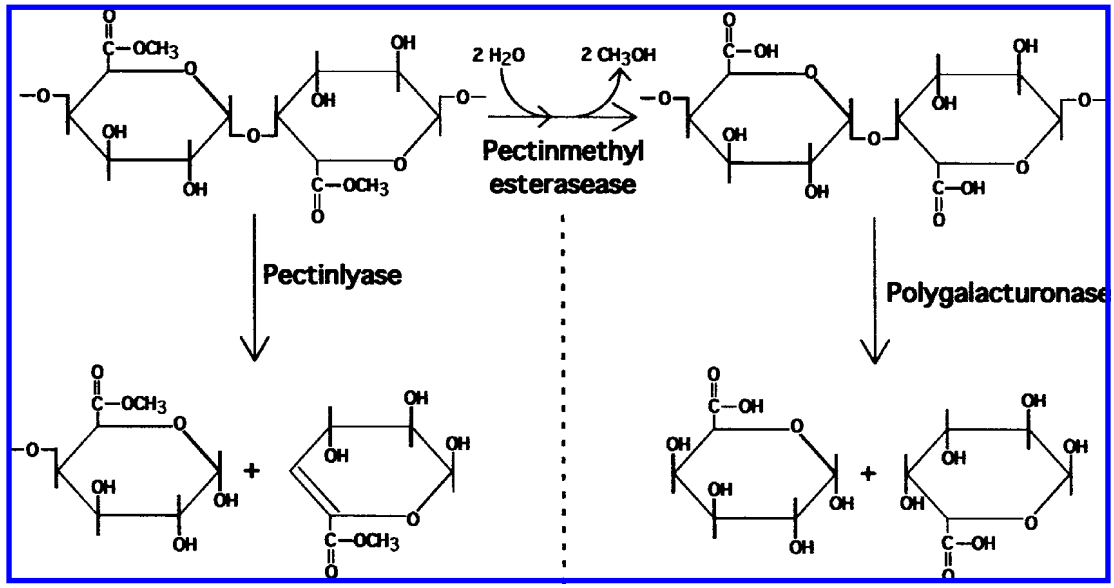

Figure 1. Different types of pectinases and their action mechanisms on pectic substances.

widely used in the food industry to improve the extraction yield or to decrease the viscosity of fruit juice. As for all enzymes employed in industrial processes, this enzyme mixture can also be used in the soluble form or can be immobilized. Several methods have been developed for the preparation of immobilized pectinases on various supports, including nylon (*20*−*23*), Eupergit C (*23*−*25*), glass (*26*−*28*), ion-exchange resin (*29, 30*), and chitosan (*31*). A survey of the main data on immobilization of pectolytic enzymes has been reported in Dinnella et al. (*32*). In this paper, we will discuss the catalytic yields of a commercial mixture of pectinase (Macer8 FJ) immobilized on three different spherical beads: glass, nylon, and polyacrylonitrile (PAN). The latter was found to be best and was employed in bed reactors packed or fluidized. The rate of change in apple juice viscosity will be discussed as a function of different operative parameters of the system.

MATERIALS AND METHODS

Materials. An industrial preparation of pectinases from *Aspergillus* sp. (Macer8 FJ) with a protein content of 80 mg/mL was used as a catalyst. Macer8 FJ, produced by Biocatalyst Limited (Wales, U.K.), contains a balanced mixture of pectinlyase (PL, EC 4.2.2.10) and polygalacturonase (PG, EC 3.2.1.15) designed to provide controlled depectinization of fruit. The low levels of pectin esterase (PE, EC 3.1.1.11) ensure that only minimal concentrations of methanol are produced during pectin hydrolysis.

Pectin from apple (Sigma-Aldrich, Milano, Italy), with a degree of esterification between 70 and 75%, was used as the substrate.

Glass microspheres, nylon pellets, or PAN beads were used separately as solid supports for enzyme immobilization. GL-0271 aluminoborosilicate, alkali-free, glass spheres (MS-302E), 2.5−3.5 mm in diameter, were purchased from MO-SCI Specialty Products, LLC (Rolla, MO). Nylon 6/6 pellets (5 mm in diameter) were purchased from Sigma-Aldrich. Porous PAN beads were made in our own laboratories using PAN powder (Look Oil Co., Bourgas, Bulgaria).

Owing to different diameters of the different bead types, we have always used the same volume, 20 cm^3.

All other chemicals were purchased from Sigma-Aldrich and used without further purification.

Methods. *Support Preparation and Activation. Glass Microspheres.* A total of 20 cm^3 (30 g) of glass microspheres was cleaned with concentrated nitric acid for 48 h. After several washes in double-distilled water, the microspheres were immersed for 3 h at 60 °C in a 10% (v/v) 3-aminopropyltriethoxysilane (APTS) aqueous solution (15 mL). APTS was used to modify the surface of the glass support to enable enzyme immobilization (*33*). The pH of the silane solution was adjusted to pH 4 by means of 6 N HCl. After this step, the glass microspheres were washed with double-distilled water and dried overnight in an oven at 80 °C.

Nylon Pellets. Because a few end groups are available on the nylon surface for covalent enzyme immobilization, pellets were pretreated to generate potentially reactive centers. The reactive center production involves an O-alkylation process of the amide bonds, creating imidoester groups on the support surface (*33, 34*).

A total of 20 cm^3 (14 g) of nylon pellets was immersed for 4 min at 100 °C in 15 mL of dimethyl sulfate reagent. After the O-alkylated nylon pellets were washed with ice-cold methanol, they were immersed in a 10% (v/v) hexamethylenediamine (HMDA) aqueous solution (15 mL) for 90 min at room temperature. HMDA was used as a spacer. The pellets were then washed with double-distilled water.

PAN Beads. PAN powder (18 g), $LiNO_3$ (1 g), and glycerin (3 g) were dissolved in 78 mL of dimethylformamide. The homogenized mixture was pipetted and precipitated in water. The obtained beads were washed with distilled water and immersed for 24 h in a 30% (v/v) glycerin aqueous solution. After this step, the beads were dried at 70 °C to a constant weight.

A total of 20 cm^3 (12 g) of PAN beads was activated at 50 °C for 60 min by treatment with 15% (w/v) NaOH aqueous solution. After thorough washing with distilled water, the beads were treated with a 10% (v/v) aqueous solution of 1,2-diaminoethane (15 mL) for 60 min at room temperature. The beads were then thoroughly washed once more with distilled water.

Enzyme Immobilization. Each activated support type was treated separately for 1 h at room temperature with a 2.5% (v/v) glutaraldehyde (GA) aqueous solution (15 mL). GA was used as coupling agent. After the supports were washed at room temperature with double-distilled water, they were incubated for 16 h at 4 °C with the Macer8 FJ solution diluted at 1:4 in 0.1 M sodium acetate buffer solution at pH 4. Afterward, the supports were washed with the 0.1 M sodium acetate buffer solution at pH 4 to remove the unbound enzyme and were stored at 4 °C.

The amount of immobilized enzymes was calculated by subtracting the amount of pectinases recovered in the solution at the end of the immobilization process and in the washing solutions from the amount of pectinases initially used for the immobilization. The pectinase concentration was measured using the Lowry method (*35*). Under the experimental conditions reported above, the amount of immobilized pectinases was 15.23 ± 0.46 mg on glass microspheres, 24 ± 0.62 mg on nylon pellets, and 29.12 ± 0.87 mg on PAN beads, respectively.

Determination of Catalytic Activity. The determination of the catalytic activity was carried out by using the viscosity assay (*36, 37*).

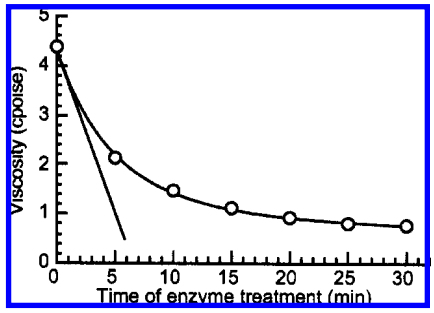

Figure 2. Viscosity of a 5 mg/mL pectin aqueous solution as a function of the time of the enzyme treatment.

The catalytic activity of the immobilized pectic mixture was assessed by following the viscosity reduction of pectin solution at regular time intervals using an Ostwald viscosimeter (water flow rate of 75−100 sec, Vetrotecnica s.r.l., Naples, Italy) immersed in a constant temperature bath (30 °C). The measurements were carried out in triplicate, starting with 10 mL of 0.5% (w/v) pectin in 0.1 M sodium acetate buffer solution at pH 4 containing 10 mM of Ca^{2+}. Ca^{2+} ions were added because pectinlyase needs this ion as a cofactor (*15, 18, 38*).

No operational problems were experienced while conducting viscosity measurements with immobilized pectinase, because the measurements were performed by removing the solution from the batch reactor and putting them into the viscosimeter. At the end of the measuring process, the solution was put back into the batch reactor. For the free pectinases, we used the viscosimeter as a reactor and the viscosity measurements were made at regular time intervals. Taking into account the fact that the measurements were taken during the enzyme reaction, the time of the measurement was established by adding the half-time of the measurement duration to the time from the beginning of the experiment.

Because the time flow is proportional to the viscosity, it is possible to report a decrease in the substrate solution viscosity as a function of the time of the enzyme treatment (**Figure 2**). Enzyme activity, in particular, initial enzyme activity, is defined as the limit for $\Delta t \rightarrow 0$ ($d\eta/dt$), i.e., the slope of the tangent in the initial part of **Figure 2**. Similarly, we can define the relative enzyme activity as the ratio between the maximum enzyme activity, at a determined pH or temperature, and the values of the enzyme activity at different pH or temperature.

All of the experimental points represent the average value of three independent experiments carried out under the same conditions.

Apple Juice Production. The following procedure was used to obtain the raw apple juice to be treated in our reactors with our catalytic supports. A total of 600 g of "Annurca" apple (*39−42*) was cut into small cubes (6 mm each side) and mixed with a 0.1 M sodium acetate buffer solution at pH 4 containing 100 μL of Macer8 FJ, diluted 1:10. After 2 h of maceration at room temperature and constant mixing, the apple pulp was pressed using a small fruit press (mod. PM20, Agrolmacchine, Taurianova, Italy), obtaining 300 mL of raw juice. After filtration on grade 3 Whatman Paper, the raw juice was pasteurized to denature any residual enzymes. To do this, the juice was circulated by means of a peristaltic pump for 5 min in a capillary pirex pipe (inner diameter of 2 mm) immersed in a thermostatic bath set at 90 °C.

The apple juice thus obtained was used in the bed reactors for the subsequent depectinization. The amount of pectin in raw apple juice was determined by Eureco, a srl company, and estimated to be 4.6 ± 0.7 mg/mL. We have used a more simple method using the alcohol precipitation test (*37*). A total of 1 mL of 90% ethanol was added to 0.5 mL of apple juice, causing the gelification of the pectic polymers. The same assay was used to verify pectin removal after enzyme treatment.

Apparatuses. *Batch Reactor.* The activity of free or immobilized enzymes was tested in a batch reactor. The batch reactor was realized using a vessel filled with the solution to be treated and the enzyme system. In the free case, 0.1 mL of Macer8 FJ enzyme solution was mixed with 10 mL of buffered substrate solution and shaken in a water bath. When immobilized enzyme was used, a volume of 20 cm^3 of catalytic beads was put into 10 mL of buffered substrate solution and shaken in a water bath.

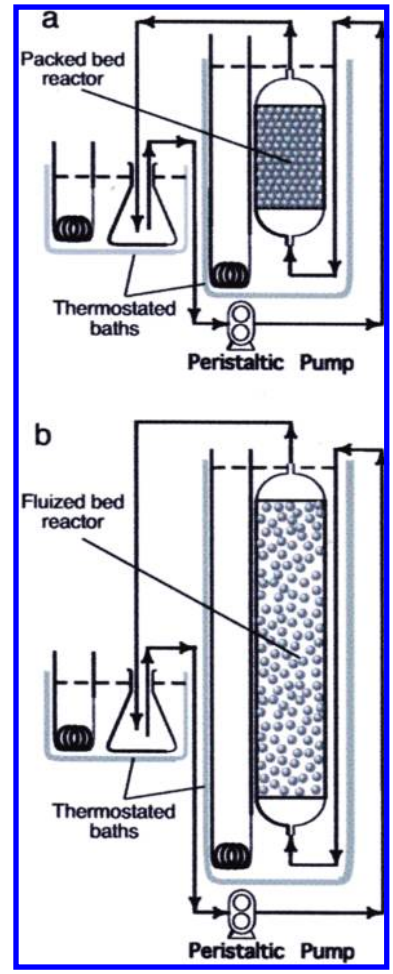

Figure 3. (a) Packed and (b) fluidized bed reactor filled with Macer8 FJ/PAN beads. In both reactors, the ratio of pellet volume/apple juice volume is 20:50.

Bed Reactors. A polystyrene pipe with an inner diameter of 1.3 cm and a length of 15 cm was used as a bed reactor, in which 12 g (20 cm^3) of the catalytic PAN beads was packed (see **Figure 3a**). A total of 50 mL of raw apple juice, which was thermostatted at 50 °C, was recirculated into the reactor using a peristaltic pump.

The same configuration was used for the fluidized bed reactor (**Figure 3b**). The only difference was the length of the pipe (30 cm). We maintained the same operative parameters used for the packed reactor, i.e., the ratio beads/solution volume (20 cm^3 of PAN beads/50 mL of apple juice). The temperature and circulation rate were also the same.

RESULTS AND DISCUSSION

To select the best catalytic bead system to be used in the clarification of apple juice, we first simulated apple juice using a 0.1 M sodium acetate buffer at pH 4.0 enriched with pectin (5 mg/mL).

When immobilized enzymes were put into the batch reactor, a decrease was observed over time in the viscosity from the initial value of 4.4 cpoise (the value measured at 5 mg/mL of pectin) to the final value of 0.8 cpoise. The rate constant of this decrease was found to be a function of the amount of immobilized enzymes and the nature of the carrier, which introduces some "limitations" to the substrate diffusion toward the catalytic site. In **Figure 4**, the relative viscosity of the solution is reported as a function of the time of enzymatic treatment. **Figure 4a** refers to the catalytic

Table 1. Kinetic Parameters Characterizing Our Catalytic Systems

catalytic system	K_{init} (min^{-1})	K_{fin} (min^{-1})	τ_{50} (initial) (min)	τ_{50} (stabilized) (min)	$\tau_{comp,dep}$ (min)	$\tau_{comp,dep}/mg_{enzymes}$ (min mg^{-1})
glass microspheres	0.115	0.040	8.76	24.23	90	6.5
nylon pellets	0.080	0.058	11.86	16.72	60	2.5
PAN beads	0.790	0.247	1.22	4.0	15	0.5

glass microspheres. **Figure 4b** refers to the catalytic nylon pellets. **Figure 4c** refers to the catalytic PAN beads. In each figure, we reported the initial behavior, the behavior after three reuses, and the stabilized behavior after eight reuses. After eight reuses, the catalytic power of each catalytic system remained stable for about 40 successive reuses. In the present case, relative viscosity is defined as the ratio $\eta_r = \eta(t)/\eta(0) \propto \tau(t)/\tau(0)$, i.e., the ratio between the flow time of the pectin solution after t minutes of enzyme treatment and the initial flow time in the absence of enzyme treatment. Obviously, the relative viscosity during the enzyme treatment varies from 1 to 0.2 with a time constant that is dependent upon the previously outlined parameters. In this case too, the experimental points were fitted well by an exponential curve of the type $\eta_r = \eta(t)/\eta(0) = 0.2 + 0.8\exp(-Kt)$, where K is a time constant measured in min^{-1}.

In **Table 1**, we have listed, for each catalytic system, the K values from the equations interpolating the initial and stabilized curves, K_{init} and K_{stab}, respectively; the τ_{50}, i.e., the time necessary to obtain a 50% of reduction in viscosity; and the $\tau_{comp,dep}$, i.e., the time required to obtain the complete depectinization using the stabilized systems. In **Figure 5**, the K constants of each system are reported as a function of the respective τ_{50}, for the initial and stabilized catalytic systems. **Figure 5a** refers to the absolute value of K, while **Figure 5b** refers to the K values for milligrams of immobilized enzyme mixture. The data in parts **a** and **b** of **Figure 5** clearly indicate that the more efficient catalytic support was that constituted by PAN beads. The same result emerges from the analysis of the times for the complete depectinization of the pectin solution, when these times are normalized for the amount of immobilized enzymes ($\tau_{comp,dep}/mg_{enzymes}$). Indeed, this ratio amounts to 6.5 for the glass microspheres, 2.5 for the nylon pellets, and about 0.5 for the PAN beads.

Having established, for the purposes of possible industrial applications, that PAN beads are the best of those used in this research, we then proceeded to study the pH and temperature behavior of the PAN beads. **Figure 6** shows the pH and temperature dependence of the Macer8 FJ mixture immobilized on the PAN beads, expressed as relative activity (%). **Figure 6a** refers to pH, while **Figure 6b** refers to temperature. For the sake of comparison, we have also reported in parts **a** and **b** of **Figure 6** the relative activities of the soluble Macer8 FJ. Inspection of the results in **Figure 6a** shows that (1) the optimum pH is shifted from the values of 5.6 (for the soluble enzymes) to the value of 6.2 (for the immobilized enzymes) and (2) the immobilization procedure increases the pH dependence of the enzyme mixture, insomuch as a restricted optimum pH range is exhibited in this case. When the optimum pH range is defined as the range where the relative activity is higher than 90%, it is

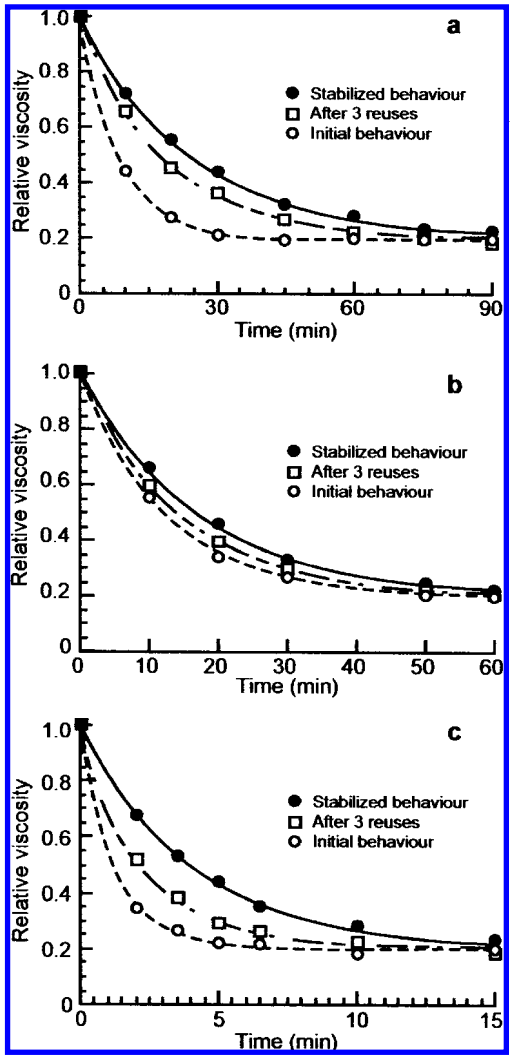

Figure 4. Relative viscosity of a pectin (5 mg/mL) aqueous solution as a function of the enzyme treatment in the case of Macer8 FJ immobilized on (a) glass microspheres, (b) nylon pellets, and (c) PAN beads.

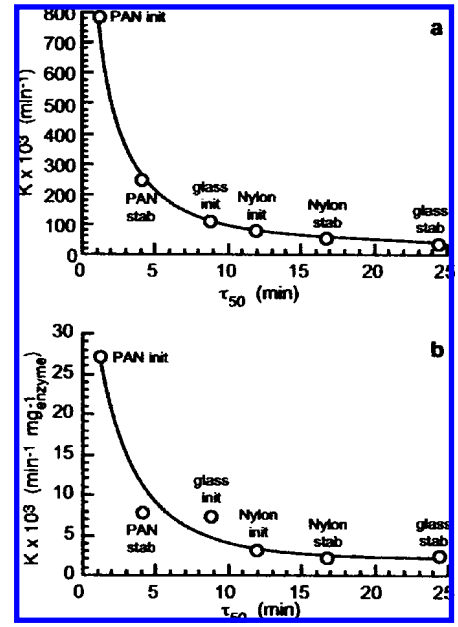

Figure 5. (a) Absolute and (b) normalized values of the constant time K as a function of τ_{50}. Subscripts "init" and "stab" stay for values calculated from the initial and stabilized curves in **Figure 4**, respectively.

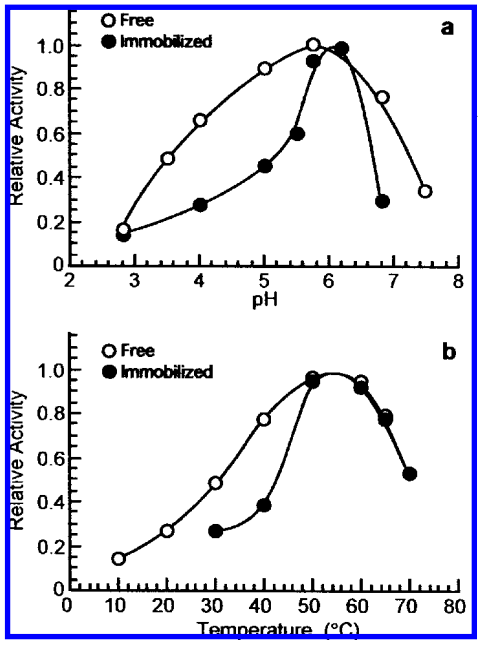

Figure 6. Relative activity of free and immobilized Macer8 FJ as a function of (a) pH and (b) temperature.

possible to appreciate how this range spans from pH 5.6 to 6.4 for the immobilized enzymes and from pH 5.1 to 6.2 for soluble enzymes.

The results in **Figure 6b** also show a higher dependence of immobilized enzyme mixture on the temperature. In this case, the optimum temperature range spans from 48 to 65 °C for the immobilized enzymes and from 43 to 65 °C for the soluble form. The definition of the optimum temperature range is similar to that of the optimum pH range. The optimum temperature for the soluble and immobilized Macer8 FJ is practically coincident (~55 °C).

Having characterized our PAN beads with the model aqueous solutions of pectin, we focused our attention on the real case, i.e., the removal of pectin from real apple juices. For this purpose, we used the apple juice samples prepared according to the procedure of maceration and centrifugation described in the Materials and Methods. Because the raw apple juice obtained after pressing apple is cloudy, as a result of the presence of suspended insoluble particles, which can block the capillary of the viscosimeter, we followed the viscosity changes in pectin concentration by means of the qualitative test based on the use of the ethanol and described in the Materials and Methods (see Apple Juice Production). The runs were carried out in a packed bed reactor. At regular time intervals, samples of enzymatically treated apple juice were taken from the reservoir, filtered on Whatman grade 3 Filter Paper, and mixed with a double volume of ethanol. Depectinization efficiency was qualitatively estimated by means of the measurement of the complete depectinization time, $\tau_{comp,dep}$, i.e., the time required for the disappearance of any form of gel. This was estimated at 66 ± 4 min.

At this point, we studied how the depectinization rate depended upon the rate of circulation of the apple juice in the reactor. The same packed reactor was used. Only the flow rate of the peristaltic pump was changed. In **Figure 7**, we report the time of complete depectinization ($\tau_{comp,dep}$) as a function of the rate of apple juice circulation. The results in **Figure 7** display sigmoidal behavior with two ranges of flow rate, where the complete depectinization times are constants: smaller (~50 min) at low circulation rates (up to 40 mL/min) and higher (~130 min) at a high rate above 85 mL/min, with a sigmoidal trend in

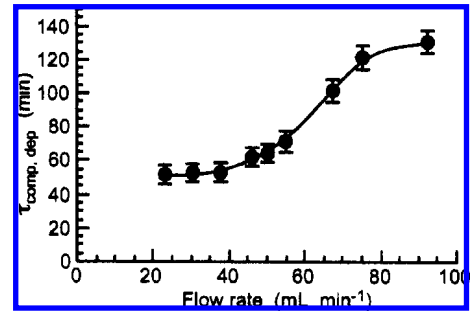

Figure 7. Time of complete depectinization, $\tau_{comp,dep}$, as a function of the apple juice circulation flow rate in the packed bed reactor.

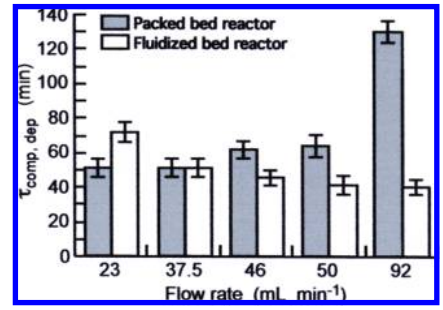

Figure 8. Comparison of the time of complete depectinization in the two types of bed reactors at various apple juice flow rates.

Table 2. Comparison between the Clear Apple Juice Produced in Our Laboratory and Some Apple Juices Available on the Italian Market

	raw apple juice	our apple juice	Zipperle[a]	Pago[b]
pH	3.66–4.03	3.73–3.93	3.4	3.45
°Brix	13.5–15.3	11.6–13	11.3	11.2
color (420 mm)	nd	0.261–0.290	0.3	0.41
turbidity (560 nm)	nd	0.008–0.01	0.008	0.02
viscosity (cpoise)	2	1.4–1.5	1.5	1.4
polyphenols (mg/L)	1200–1334	233–280	828	263
proteins (g/100 g)	0.28	0.05–0.09	0.07	0.1
sugars (g/100 g)	9.15–11.5	9.9–10.2	10.5	10.5

[a] Hans Zipperle SpA, Merano, Italy. [b] Pago Italia Srl, Dosson di Casier, Italy.

between. This means that at a slow circulation rate the pectin diffusion toward the catalytic site is effective and a good depectinization yield occurs, while at a high circulation rate, the substrate diffusion is disturbed and the depectinization rate is low. What is surprising is the apparent difference in times to achieve complete depectinization: 15 min (see **Table 1**) in batch and 50 min in the bed reactor. This discrepancy is only apparent because it is known that the rate of a catalytic process (and, in this case, the time required to obtain complete depectinization) is proportional to the ratio between the amount of catalytic beads (and hence of the enzyme amount, A_{beads}) and the solution volume (V) to be treated.

In our case, the time required for complete depectinization in the batch apparatus was $\tau_{comp,batch} \propto (A_{beads}/V)_{batch} = A_{beads}/10$, while in the packed bed reactor, $\tau_{comp,bed} \propto (A_{beads}/V)_{bed} = A_{beads}/50$. Because the amount of catalytic beads is the same, it follows that $\tau_{comp,batch}/\tau_{comp,bed} \propto V_{bed}/V_{batch}$. The first ratio is 15:50 = 0.3, while the second ratio is 10:50 = 0.2. The fact that the two times are similar is considered to be a good result, taking into account the approximations used in this comparison and, in particular, the fact that the batch reactor operated with an aqueous solution and the bed reactor operated with apple juice, whose viscosity is lower than the viscosity of the pectin buffer solution.

The final experiment concerned the comparison between the depectinization power of the packed bed reactor (**Figure 3a**) and that of a fluidized bed reactor (**Figure 3b**). To make this comparison, we used a fluidized reactor, in which we maintained the same ratio A_{beads}/V, i.e., the same amount of catalytic beads (20 cm^3) and the same amount of apple juice (50 mL). The results of this study are reported in **Figure 8**, where it is possible to appreciate how, at a low flow rate, the depectinization power of the two types of bed reactors (fluidized or packed) is extremely similar, while at higher circulation rates, the fluidized bed reactor offers an advantage with respect to the packed bed reactor, because the time for complete depectinization in the fluidized reactor is 0.25 times smaller than that in the packed bed reactor.

When the depectinized apple juice is centrifuged (at 5000 rpm and room temperature), we obtained a clear apple juice, whose characteristics are reported in **Table 2**, together with those of raw apple juice and those of other apple juices available on the Italian market. Samples were analyzed by Eureco s.r.l. (Contrada La Fagianeria, Piana di Monte Verna, CE, Italy). Inspection of **Table 2** shows that our clear apple juice displays characteristics that are similar or better, i.e., for example, higher pH values, than those available on the Italian market.

Overall, these results provide a good indication for constructing a pilot plan for the production of clear apple juice using PAN beads.

LITERATURE CITED

(1) Álvarez, S.; Riera, F. A.; Álvarez, R.; Coca, J.; Cuperus, F. P.; Bouwer, S. Th.; Boswinkel, G.; van Gemert, R. W.; Veldsink, J. W.; Giorno, L.; Donato, L.; Todisco, S.; Drioli, E.; Olsson, J.; Traagaardh, G.; Gaeta, S. N.; Panyor, L. A new integrated membrane process for producing clarified apple juice and apple juice aroma concentrate. *J. Food Eng.* **2000**, *46*, 109–125.

(2) BeMiller, J. N. An introduction to pectins: Structure and properties. In *Chemistry and Functions of Pectins*; Fishman, M. L., Jen, J. J., Eds.; American Chemical Society: Washington, D.C., 1986; pp 2−21.

(3) Rombouts, F. M.; Pilnik, W. Pectolytic enzymes. In *Microbial Enzymes and Bioconversions*; Rose, A. H., Ed.; Academic Press: London, U.K., 1980; pp 227−282.

(4) Baumann, J. W. Application of enzymes in fruit juice technology. In *Enzymes and Food Processing*; Birch, G. G., Blakebrough, N., Parker, K. J., Eds.; Applied Science Publisher: London, U.K., 1981; pp 129−147.

(5) Heatherbell, D. A. Cause and prevention of haze and sediment formation in fruit juice and concentrates. Oregon State Tech. Publ. 6234, 1981.

(6) Alvarez, S.; Alvarez, R.; Riera, F. A.; Coca, J. Influence of depectinization on apple juice ultrafiltration. *Colloids Surf., A* **1998**, *138*, 377–382.

(7) Chamchong, M.; Noohorm, A. Effect of pH and enzymatic treatment on microfiltration and ultrafiltration of tangerine juice. *J. Food Process Eng.* **1991**, *14*, 21–34.

(8) Girard, B.; Fukumoto, L. R. Membrane processing of fruit juices and beverages: A review. *Crit. Rev. Food Sci. Nutr.* **2000**, *40*, 91–157.

(9) Hart, F. L.; Fisher, H. J. *Modern Food Analysis*; Springer-Verlag: New York, 1971; Vol. 13.

(10) Sahin, S.; Bayindirli, L. The effect of depectinization and clarification on the filtration of sour cherry juice. *J. Food Eng.* **1993**, *19*, 237–245.

(11) Sheu, M. J.; Wiley, R. C.; Schlimme, D. V. Solute and enzyme recoveries in apple juice juice clarification using ultrafiltration. *J. Food Sci.* **1987**, *52*, 732–736.

(12) Lanzarini, G.; Pifferi, P. G. Enzymes in the fruit juice industry. In *Biotechnology Applications in Beverage Production*; Cantarelli, C., Lanzarini, G., Eds.; Elsevier Applied Science: London, U.K., 1989; p 189.

(13) Sakai, T.; Sakamoto, T.; Hallaert, J.; Vandamme, E. J. Pectin, pectinase and protopectinase: Production, properties and applications. *Adv. Appl. Microbiol.* **1993**, *39*, 231–294.

(14) Alkorta, I.; Garbisu, C.; Llama, M. J.; Serra, J. L. Industrial applications of pectic enzymes—A review. *Process Biochem.* **1998**, *33*, 21–28.

(15) Withaker, J. R. Microbial pectolytic enzymes. In *Microbial Enzymes and Biotechnology*; Fogart, W. F., Kelly, C. T., Eds.; Elsevier Applied Science: London, U.K., 1992; p 133.

(16) Beveridge, T.; Franz, K.; Harrison, J. E. Clarified natural apple juice: Production and storage stability of juice and concentrate. *J. Food Sci.* **1986**, *51*, 411–414.

(17) Rexova-Benkova, L.; Markovic, O. Pectic enzymes. *Adv. Carbohydr. Chem.* **1976**, *33*, 323–385.

(18) Ranveer, S. J.; Shivalika, S.; Reena, G. Microbial pectinolytic enzymes: A review. *Process Biochem.* **2005**, *40*, 2931–2944.

(19) Busto, M. D.; García-Tramontín, K. E.; Ortega, N.; Perez-Mateos, M. Preparation and properties of an immobilized pectinlyase for the treatment of fruit juices. *Bioresour. Technol.* **2006**, *97*, 1477–1483.

(20) Lozano, P.; Manjio, A.; Eomojaro, F.; Canovas, M.; Iborraet, J. L. A cross-flow reactor with immobilized pectolytic enzymes for juice clarification. *Biotechnol. Lett.* **1987**, *9*, 875–880.

(21) Lozano, P.; Manjòn, A.; Romajaro, F.; Iborra, J. L. Properties of pectolitic enzymes covalently bound to nylon for apricot juice clarification. *Process Biochem.* **1988**, 75–78.

(22) Alkorta, I.; Garbisu, C.; Llama, M. J.; Serra, J. L. Immobilization of pectin lyase from penicillum italicum by covalent binding to nylon. *Enzyme Microb. Technol.* **1996**, *18*, 141–146.

(23) Spagna, G.; Pifferi, P. G.; Tramontini, M. Immobilization and stabilization of pectinlyase on synthetic polymers for application in the beverage industry. *J. Mol. Catal. A: Chem.* **1995**, *101*, 99–105.

(24) Stratilovà, E.; Capka, M.; Rexova-Benkova, L. Endopolygalacturonase immobilized on epoxide-cointaining supports. *Biotechnol. Lett.* **1987**, *9* (7), 511–516.

(25) Spagna, G.; Pifferi, P. G.; Martino, A. Pectinlyase immobilization on epoxy supports for application in food processing industry. *J. Chem. Technol. Biotechnol.* **1993**, *57*, 379–385.

(26) Weibel, M.; Barrios, R.; Delotto, R.; Humphrey, A. Immobilized enzymes: Pectinesterase covalently coupled to porous particles. *Biotechnol. Bioeng.* **1975**, *17*, 85–98.

(27) Baraniak, A. Covalent methods of bindig polygalacturonase to porous glass carriers. *Acta Alim. Pol.* **1981**, *8*, 219–226.

(28) Rexova-Benkova, L.; Stratilovà, E.; Capka, M. Endopolygalacturonase immobilized on ceramic materials substituited with aminopropyl groups. Syntesis, kinetics and mode of action. *Collect. Czech. Chem. Commun.* **1986**, *51*, 2924–2932.

(29) Kminkova, M.; Kucera, J. Comparison of pectolytic enzymes using different methods of binding. *Enzyme Microb. Technol.* **1983**, *5*, 204–208.

(30) Sartoglu, K.; Demir, N.; Acar, J.; Mutlu, M. The use of commercial pectinase in the fruit juice industry, part 2: Determination of the kinetic behaviour of immobilized commercial pectinase. *J. Food Eng.* **2001**, *47*, 271–274.

(31) Pifferi, P. G.; Tramontini, M.; Malacarne, A. Immobilization of endo-polygalacturonase from *Aspergillus niger* on various types of macromolecular supports. *Biotechnol. Bioeng.* **1989**, *33*, 1258–1266.

(32) Dinella, C.; Stagni, A.; Lanzarini, G. Pectolytic enzymes coimmobilization on γ-alumina spheres via organophosphate compounds. *Process Biochem.* **1997**, *32*, 715–722.

(33) Bickerstaff, G. F. Immobilization of enzymes and cells. In *Methods in Biotechnology*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1997.

(34) Hornby, W. E.; Goldstein, L. Immobilization of enzymes on nylon. *Methods Enzymol.* **1976**, *44*, 118–134.

(35) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
(36) Collmer, A.; Ried, J. L.; Mount, M. S. Assay methods for pectic enzymes. *Methods Enzymol.* **1988**, *161*, 329–35.
(37) Ceci, L.; Lozano, J. Determination of enzymatic activities of commercial pectinases for the clarification of apple juice. *Food Chem.* **1998**, *61*, 237–241.
(38) Margo, P.; Varvaro, L.; Chilosi, G.; Avanzo, C.; Balestra, G. M. Pectinolytic enzymes produced by *Pseudomonas syringae* pv. Glycinea. *FEMS Microbiol. Lett.* **1994**, *117*, 1–6.
(39) Lintas, C.; Paoletti, F.; Cappelloni, M.; Gambelli, L.; Monastra, F.; Ponziani, G. Agronomic, nutritional and texture evaluation of "Annurca" apple clones. *Adv. Hort. Sci.* **1993**, *7*, 165–168.
(40) Floris, M. *Melo e Pero nella Frutticoltura Italiana ed Europea*; Federchimica Agrofarma: Milan, Italy, 1997; Vol. 1, pp 43−48.
(41) Lo Scalzo, R.; Testoni, A.; Genna, A. "Annurca" apple fruit, a southern Italy apple cultivar: Textural properties and aroma composition. *Food Chem.* **1997**, *73*, 333–343.
(42) Lo Scalzo, R.; Forni, E.; D, L.; Giudetti, G.; Testoni, A. Change of pectic composition of "Annurca" apple fruit after storage. *Food Chem.* **2005**, *93*, 521–530.

Received for review June 25, 2008. Revised manuscript received October 1, 2008. Accepted October 2, 2008. This work was partially supported by the Italian Ministry of Universities and Research and by Eureco (Contrada La Fagianeria, Piana di Monte Verna, CE, Italy). The work was performed as part of a scientific collaboration agreement between the "National Institute of Biosystems and Biostructures" (Italy) and the University of Bourgas (Bulgaria).

JF8019437