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Utilisation of whole wheat flour for the production of extracellular pectinases by some fungal strains

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Abstract

The possibility of producing pectinases by *Rhizopus stolonifer* and *Aspergillus awamori*, using cereal raw materials as substrate, was investigated. The whole wheat flour acted as a good nutrient source for the cultivation of the microorganisms and exo- and endo-polygalacturonases (PG) were produced in submerged culture. In this respect, it was possible to obtain polygalacturonase activities at an acceptable yield, in comparison with a typical defined medium described in the literature for pectinase production. The synthesis of both enzymes occurred in both strains in the absence of pectin, demonstrating the constitutive nature of these enzymes; nevertheless, production was increased by the addition of a small amount of pectin to the flour.

Keywords: Pectinases; *Rhizopus stolonifer*; *Aspergillus awamori*; Submerged culture; Whole wheat flour; Cereal raw material

1. Introduction

Traditionally, the main objectives of agriculture have been to produce more and better food. However, increasingly nowadays, agriculture has to focus not only on food production, but also on the production of crops as alternative raw materials for energy and chemicals production. Efforts to develop cereal-based non-food products are now increasing, especially in those countries where there is sufficiency in cereal production.

Generally, cereal grains are composed mainly of starch plus other carbohydrates, protein, oil and fat. More importantly, they contain all the essential macro- and micronutrients, such as minerals and vitamins, to sustain microbial growth. This nutritional characteristic makes them potentially ideal as alternative, renewable, raw materials for chemical production [1,2]. The fermentation industries that use glucose as a carbon source rely on hydrolysis of purified starch from cereals. However, the direct use of whole-wheat flour as a fermentation feed stock would remove the need to purify the starch to produce glucose, and would have the benefit of providing a more complete supply of nutrients. Hence, overall production costs can be reduced. Moreover, the use of cereal grains as a starting material for the production of fine chemicals through fermentation would offer potentially cleaner and more environmentally friendly processes [3].

Pectinase enzymes are extensively used in the industrial clarification of wine and fruit juices, owing to their degradative action on pectic substances that are present in fruits [4]. The quality of such products can be improved by treating fruit pulps with pectolytic enzymes, which reduce the viscosity, and can increase the yield of the juice [5]. Other applications include removal of citrus fruit peels and increasing the firmness of some fruits [6]. Recent applications have emerged such as the treatment and degumming of natural fibres [7].

The ability to synthesise pectinase enzymes is widespread among all microbial groups, but for industrial purposes moulds are preferred because as much as 90% of the enzyme can be excreted into the culture medium

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[8]. Specifically, the fungal genera *Aspergillus*, *Rhizopus* and *Trichoderma* are the chief sources of pectinolytic enzymes. The synthesis of these enzymes has been reported to be highly influenced by the components of the growth medium, particularly by the carbon source [9–13]. Another important factor influencing the synthesis of pectic enzymes is the presence of inducers in the cultivation medium [14,15]. In this respect, the production of extracellular pectinases is induced by pectin and its derivatives [16,17]. Typical substrates are agroindustrial wastes such as citrus fruit peels [18–20], sugar beet [10] or extract of wheat bran [21–23]. However, no work has been done in testing whole-wheat flour as the sole nutrient for the production of pectolytic enzymes. In this respect, this direction of research may be of interest in the innovative use of agricultural residues, as fermentation media, for the manufacture of high value products. The advantage of such a feedstock, in comparison with all the other agro-industrial wastes (citrus fruit peels, sugar beet or extract of wheat bran) is that, in principle, it contains all the necessary nutrients for many different fermentations. All the others, by contrast, must be combined with an appropriate nitrogen or carbon source and other minor nutrients, making the economic advantage of the whole wheat flour even greater.

The main objectives of this work were, therefore, to explore the feasibility of using a wheat flour solution as the sole nutrient source to grow some moulds, and at the same time, to test the capability of these strains to produce pectinases.

2. Materials and methods

2.1. Microorganisms

Two types of fungi, *Rhizopus stolonifer* and *Aspergillus awamori*, were used. The strain of *Rhizopus stolonifer* was purchased from the Type Culture Collection in Valencia, Spain. The strain of *Aspergillus awamori* 2B.361 U2/1, which according to the Commonwealth Mycological Institute is classified in the *Aspergillus niger* complex, produces a range of hydrolytic enzymes and has been used with whole wheat flour to produce a generic fermentation medium [3]. Both microorganisms were propagated and stored on PDA (potato-dextrose-agar) slants at 4 °C.

2.2. Spore production

Spores, which were stored on PDA slants, were washed in 5 ml of 0.01% (v/v) Tween 80 solution. A total of 0.5 ml of the spore solution was spread on the surface of 100 ml PDA medium in 500 ml Erlenmeyer flasks. The inoculated Erlenmeyer flasks were incubated at 30 °C for 7 days. After the incubation period, 40 ml of 0.01% (v/v) Tween 80 solution were added and spores were suspended by gentle shaking of the flasks, then counted in an Improved Neubauer Counting Chamber (Assistent-Germany; BDH). The spore suspension was adjusted to a final concentration in the culture medium of 1.0×10^6 spores/ml.

2.3. Culture media

Several different fermentation media were prepared during this study. To all of these, before sterilisation, some drops of silicone antifoam were added to prevent foaming. Sterilisation was carried out at 121 °C and 15 psi for 20 min.

To check that both microorganisms were able to grow and produce pectinases on whole wheat flour, media containing nothing but hammer milled whole soft wheat flour (5% w/v) were prepared. The initial pH in all these experiments was 6.5.

A combined medium, containing 5% (w/v) hammer milled whole soft wheat flour plus 1% (w/v) citrus pectin (Sigma Chemical Co., USA), was also prepared in order to assess the effect of combining different carbon sources on the production of pectinases. The initial pH was 6.5.

An appropriate defined medium [24] was chosen to compare the results obtained, in terms of enzyme production, with those from the flour based media. The medium composition was 3% (w/v) pectin from citrus fruit (Sigma Chemical Co., USA), 0.66% (w/v) $(NH_4)SO_4$ (BDH-England), 0.35% (w/v) KH_2PO_4 (FSA-England), 0.015% (w/v) $FeSO_4$ (BDH-England) and 0.01% (w/v) $MgSO_4 \cdot 7H_2O$ (FSA-England). Medium pH was adjusted to 6.5 with 0.5 M NaOH.

2.4. Fermentation experiments

Submerged cultures were performed in 500 ml Erlenmeyer flasks with a working volume of 200 ml. The flasks were incubated at 33 °C on a rotary shaker at 220 rpm for 7 days.

2.5. Sample preparation

During the fermentations, samples of 10 ml were collected from the bulk liquor at regular intervals of 48 h (days 1, 3, 5 and 7) for further analysis. Samples were centrifuged at 7000 rpm for 15 min (WIFUG-2000S, England) and the supernatant was used for measuring the pH, exo-polygalacturonase and endo-polygalacturonase activities, and reducing sugars. Solids were used for measuring the cell dry weight.

2.6. Assay for pectinolytic activity

The pectinolytic activity was determined at 45 °C by viscometry for endo-polygalacturonase (endo-PG) and by the release of reducing sugars, expressed as galacturonic acid, for exo-polygalacturonase (exo-PG). For endo-PG, 2.5 ml of sample was mixed with 7.5 ml of 1% (w/v) pectin in 0.1 M acetate buffer, pH 4.5. Reduction in viscosity was followed with an Ostwald viscometer (Technico, BS/U, England). One endo-PG unit (U) was defined as the amount of enzyme that reduces the viscosity of the solution by 50% per minute under the conditions mentioned above. For exo-PG, 0.25 ml of sample was added to a solution containing 0.25 ml of 1% pectin in 0.1 M acetate buffer, pH 4.5. Samples were incubated at 45 °C for 30 min and reducing sugars were determined by the dinitrosalicylic acid (DNS) method [25]. One exo-PG unit (U) was defined as the amount of enzyme that liberates one micromole of galacturonic acid per minute under the conditions mentioned above. All measurements were made in triplicate.

2.7. Dry matter

Dry weight measurements were based on samples of the bulk liquor. After removing the supernatant from the centrifuged samples, solids were washed twice in 10 ml of distilled water and then filtered through pre-dried and pre-weighed filter papers (Whatman No. 1). The filter papers were dried at 105 °C for about 18 h, until a constant weight was obtained.

DW measurements on defined medium allowed the direct estimation of the amount of microbial biomass and its increase with time, i.e. growth rate. However, as mycelial cells became intricately interwoven with the suspended solids in the flour based media and, hence, separation of the fungal biomass from other solids was not possible, DW measurements in representative samples indicated the result of a balance between solids consumption and fungus growth.

2.8. Reducing groups in the culture media

The reducing groups in the culture media were determined using a DNS method [25] and expressed, via a calibration curve, as galacturonic acid.

3. Results and discussion

As stated previously, the basic aim of this work was to examine the feasibility and the efficiency of using cereal raw materials for the production of pectinases. So, as a first approach, hammer milled whole soft wheat flour was used in aqueous solution as the sole nutrient. This medium was later supplemented with pectin to investigate its effect on pectinase production. A parallel fermentation, using a defined medium, was carried out with the aim of comparing the results obtained.

3.1. Microorganism growth on whole-wheat flour versus defined medium

As can be seen in Fig. 1, it was possible to grow both *A. awamori* and *R. stolonifer* cells using 5% w/v whole wheat flour as the sole nutrient source in the culture medium. Therefore, this substrate contains all the necessary nutrients for the regular growth of both moulds.

When *A. awamori* was grown on wheat flour medium, a reduction in DW occurred from the 1st to the 3rd day, followed by an increase. This trend can be explained considering both the rate of suspended solids consumption and the rate of microorganism growth. Moreover, it is necessary to consider that DW measurements from the bulk liquor include all solids present in

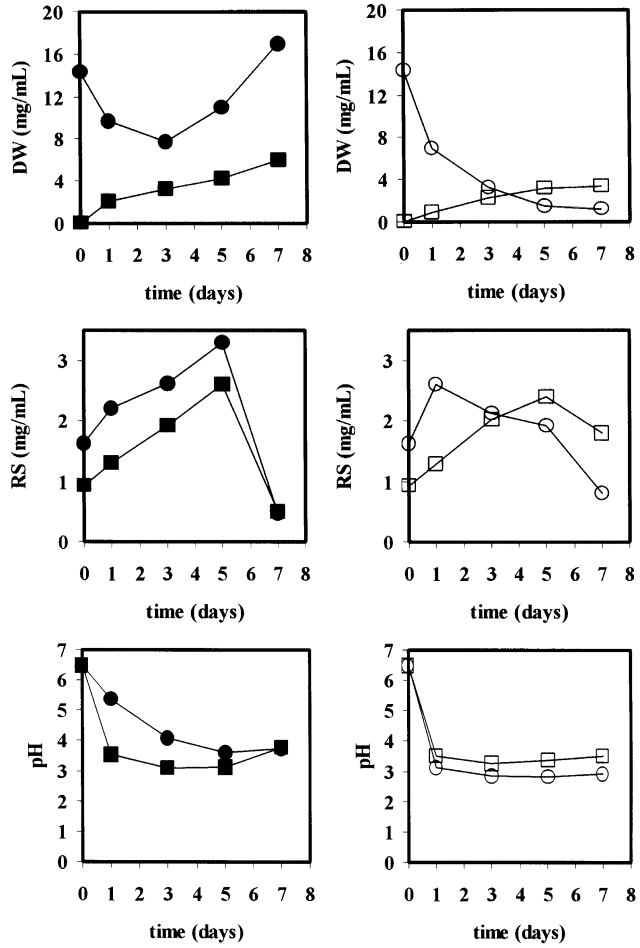

Fig. 1. Dry weight, reducing sugars concentration and pH time course for *A. awamori* (●, ■) and *R. stolonifer* fermentation (○, □) on 5% (w/v) hammer milled whole soft wheat flour medium (●, ○) and defined medium (■, □).

the fermentation broth. In this respect, no-suitable method was found for the effective separation of fungal hyphae from wheat bran solids. Probably, during the initial period, the rate of destruction of digestible solids exceeded the contribution of cell growth to total dry weight, resulting in a continuous reduction in DW. In this respect, in previous studies *A. awamori* demonstrated not only the ability to hydrolyse wheat starch into glucose, but also the capability of disintegrating the aleurone layer in wheat bran. The complete destruction of aleurone layer lasts for at least 30 h, starting at about 15 h and proceeding until 45 h or beyond [3]. Therefore, the subsequent increase in DW is a more direct indicator of the increase in the cell concentration. It is highly predictable that the comprehensive destruction of all digestible solids would lead to an increase in total dry weight owing to the uninterrupted microorganism growth before nutrient exhaustion. In contrast, as the defined medium has no suspended solids, its DW values are always lower than the corresponding values for the wheat flour medium and represent directly the fungus growth.

Unlike the results for *A. awamori*, Fig. 1 shows an apparent reduction in DW throughout the fermentation with *R. stolonifer*. This is due to the morphology of *R. stolonifer* during its growth in shaken flasks. Under these conditions, the fungus formed a single large aggregate—a solid cake—from the 2nd day of incubation. In this way, *R. stolonifer* acted as a sack, that was able to include all suspended solids from the medium. As a consequence, by the end of the fermentation period, the liquid in the flask had a clear, bright yellow colour, without a trace of any of the insoluble flour components or the fungal mycelium, which by this time had been completely incorporated into the aggregate. As the DW measurements presented are based on samples of the liquid phase only, an apparent continuous decrease in total dry weight was measured. Clearly the conditions of aeration and agitation affect aggregation and the use of higher agitation speeds could possibly have prevented the solid cake formation. On the other hand, when the defined medium was employed, no such aggregate formation occurred and, therefore, measured DW increased throughout the fermentation period as a result of the microorganism growth.

In relation to the time course for reducing sugars using *A. awamori* (Fig. 1), the same trend was observed for both culture media. In the first stage, a continuous increase in reducing sugars concentration was observed, obtaining on the 5th day the highest value. In a second stage, a significant decrease was seen. As has been shown previously [3], *A. awamori* demonstrated the ability to disintegrate the aleurone layer in wheat bran and to assimilate the nutrient components, existing in the cells. When *A. awamori* is cultivated on flour based medium it secretes amylolytic enzymes, which hydrolyse the carbon source (starch). So, during the first stage, owing to the presence of starch, and low cell concentrations, the rate of glucose production is higher than the rate of glucose consumption, resulting in a continuous increase in reducing sugars concentration. In the second stage, owing to the exhaustion of starch, and the further increase in cell concentration, the rate of sugar utilisation surpasses the rate of sugar production, leading to a sharp decrease in reducing sugars concentration. For the defined medium, the increase in reducing sugars concentration occurs due to the production of pectolytic enzymes, which degrade the carbon source (pectin), releasing sugars into the fermentation liquid.

In the case of *R. stolonifer*, the evolution of reducing sugars was dependent on the medium employed (Fig. 1). As for *A. awamori* cells, two stages were clearly observed when *R. stolonifer* was grown on whole-wheat flour medium. However, in this case, the major reducing sugars concentration was measured 24 h after inoculation, and, after reaching this maximum, the reducing sugars concentration started to decline in a uniform way. Therefore, as for *A. awamori* cells, when *R. stolonifer* is grown on whole-wheat flour medium it secretes starch degrading enzymes which hydrolyse the carbon source. However, the different trends followed by reducing sugars concentration suggest that the rate of secretion of starch degrading enzymes by *R. stolonifer* during the first stage is higher than for *A. awamori* cells, while the rate of sugars consumption by *A. awamori* cells during the second stage is higher than for *R. stolonifer* cells. In contrast, for the defined medium, with pectin as the carbon source, the reducing sugars evolution was the same as for the *A. awamori* cells.

From Fig. 1, it can be observed that the pH profile in both culture media using *R. stolonifer* or *A. awamori* were very similar: pH sharply decreased during the first 24 h of incubation from 6.5 to about 3.5 and then continued to decrease much more slowly.

3.2. Pectinase production on whole-wheat flour versus defined medium

Endo-PG and exo-PG activities produced by *R. stolonifer* and *A. awamori* cells on different media are presented in Fig. 2. Endo-PG activities appeared, in all cases, after 24 h of culture, reaching the maximum value by the 5th or 7th day of fermentation. Similar trends were followed by endo-PG activity produced by *A. awamori* on both culture media. The enzymic activity increased during the first 3 days of incubation, decreased by the 5th day, and reached the maximum value on the 7th day of culture. Moreover, equal maximum values of endo-PG activity were measured on both culture media. Unlike *A. awamori*, in the case of *R. stolonifer*, the endo-PG activity increased continu-

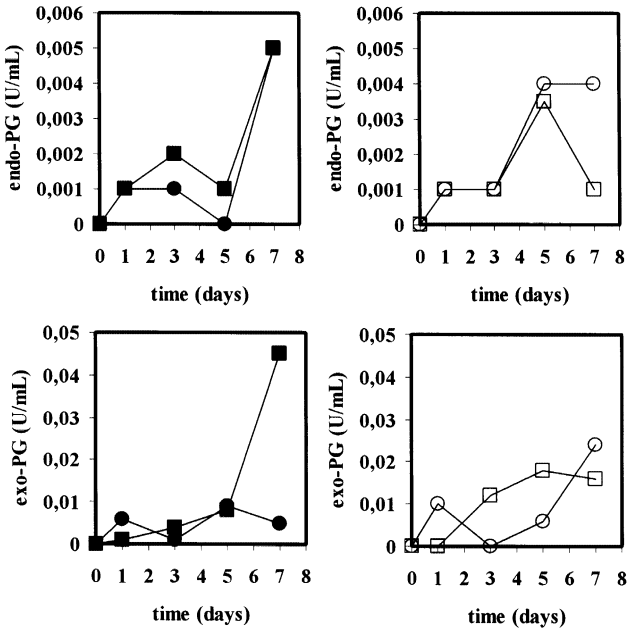

Fig. 2. Endo-polygalacturonase and exo-polygalacturonase activities produced by *A. awamori* (●, ■) and *R. stolonifer* (○, □) on 5% (w/v) hammer milled whole soft wheat flour medium (●, ○) and defined medium (■, □).

ously during the first 5 days of culture. However, on the 7th day, the enzymic activity on the flour based medium remained constant but decreased for the defined medium.

The maximum endo-PG activity was registered for both fungal strains on the 5th or 7th day of fermentation, when the pH was in the range 3.0–3.8. Similar behaviour has been reported by Aguilar et al. [26], who specified that endopectinase produced by *Aspergillus* sp. is strongly induced at pH 3.5, but, virtually absent at pH values above 4.2, using pectin with a low degree of esterification, as carbon source [26]. This suggests the importance of pH in the induction of endo-PG production. The presence of pectin, however, appears to be less important with synthesis of the endo-PG occurring in both strains growing in the absence of peptic substances (whole-wheat flour is devoid of pectin), thus supporting the views that this enzyme is constitutive [27,28].

The time course of exo-PG activities, produced by *A. awamori* and *R. stolonifer* on both culture media, are also represented in Fig. 2. As for the endo-PG activity, the synthesis of the exo-PG occurs in both strains in the absence of pectic substances, indicating the constitutive nature of this enzyme too.

As can be seen in Fig. 2, exo-PG production depends on the fungal strain and the culture medium. Therefore, different trends can be observed. When *A. awamori* was grown on the flour based medium, the production of exo-PG oscillated during the period of fermentation, and peaks of maximum activity occurred on the 1st and 5th day. However, when defined medium was employed, exo-PG activity increased continuously from the 1st day of fermentation, reaching a maximum on the 7th day. For *R. stolonifer* fermentation in defined medium, exo-PG activity increased continuously from the 1st day of incubation, reaching a maximum value on the 5th day. Practically the same enzymic activity was measured on the 7th day.

Taking into consideration these differences and those in Fig. 1, and considering that exo and endo-PG enzymes are constitutive, it can be concluded that *A. awamori* and *R. stolonifer* may be metabolically different, with different pathways leading to the enzyme production in each case.

3.3. Effect of pectin addition on pectinase production

The maximum endo-PG and exo-PG activities produced by *A. awamori* and *R. stolonifer* on the different culture media tested are shown in Fig. 3. As can be seen in this figure, flour appears to be as good or better than the defined medium (except for exo-PG activity by *A. awamori*) for the production of polygalacturonase activity. Therefore, the direct use of whole-wheat flour as the sole nutrient for the microorganism growth allows the production of pectolytic enzymes at an acceptable yield in comparison with a typical defined medium.

On the other hand, a combined medium containing 5% (w/v) whole wheat flour plus 1% (w/v) citrus pectin was used in order to assess the effect of combining

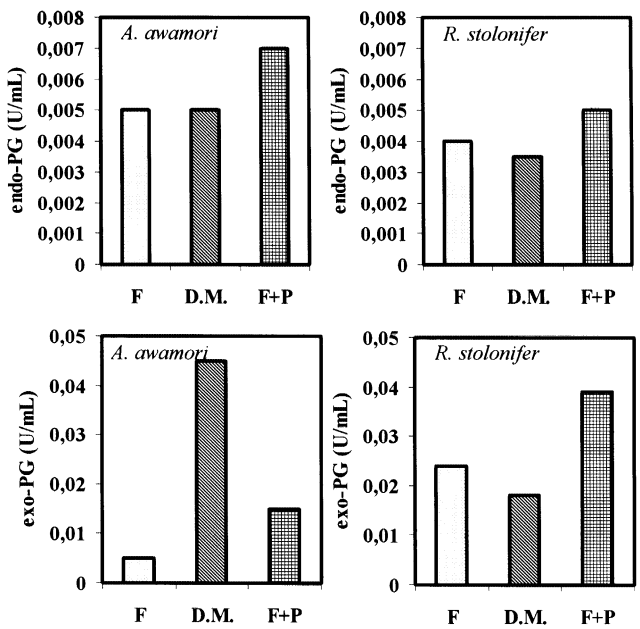

Fig. 3. Endo-PG and exo-PG activities produced by *A. awamori* and *R. stolonifer* on 5% (w/v) hammer milled whole soft wheat flour medium (F), defined medium (D.M.) and 5% (w/v) hammer milled whole soft wheat flour plus 1% (w/v) pectin medium (F + P).

different carbon sources on the production of pectinases. The results obtained are presented in Fig. 3. As can be seen, for both strains, the highest endo-PG activity levels were found when whole-wheat flour plus pectin was used as the fermentation medium. So, the production of endo-PG can be encouraged by the addition of a small amount of pectin to the flour based medium. In the same way, exo-PG synthesis can be increased by supplementing flour with pectin, but in this case, higher activities are obtained when the defined medium is employed for *A. awamori* fermentation.

To summarise, although polygalacturonase enzyme production for both fungi requires no inducer in the culture medium, the production can be enhanced by the addition of small amounts of pectin to the medium. The same kind of behaviour has been reported before by Talboys and Busch [27], who stated that PG activity production in *Verticillium sp*. was constitutive but it was increased by incorporation of pectin to the medium. Ammar et al. [28], on the other hand, found that all sugars failed to induce pectinase production by *Aspergillus niger*, while the highest production was obtained in the presence of pectin in solid state fermentation and in the presence of starch in submerged fermentation.

4. Conclusions

The feasibility of producing fungal pectinases from whole wheat flour of low quality has been demonstrated by the results presented in this work. Batch fermentations with *A. awamori* and *R. stolonifer* showed that satisfactory growth, without the addition of any external sources of nitrogen, carbon, vitamins, or microelements could be achieved. In this way, utilizing whole wheat flour as a cheap and readily available cereal raw material, it was possible to obtain polygalacturonase activities at a very acceptable yield, in comparison with a typical defined medium described in the literature for pectinase production. The synthesis of both endo and exo-PG occurred in both strains in the absence of pectin, demonstrating the constitutive nature of these enzymes; nevertheless, the synthesis was increased by incorporation of a small amount of pectin to the flour.

References

[1] Webb C. Food feed and fermentation: the future for grain processors. Part I. World Grain 1996: March pp. 18–19.
[2] Webb C. Food feed and fermentation: the future for grain processors. Part II. World Grain 1996: April pp. 31–34.
[3] Webb C, Wang RH. Development of a generic fermentation feedstock from whole-wheat flour. In: Campbell GM, Webb C, McKee SL, editors. Cereals: Novel Uses and Processes. New York: Plenum Press, 1997:205–18.
[4] Uhlig H. Processing of fruit, vegetables, and wine. In: Gerhartz W, editor. Enzymes in Industry; Production and Applications. New York: VCH publishers, 1990:126–8.
[5] Chang TS, Siddiq M, Sinha NK, Cash JN. Plum juice quality affected by enzyme treatment and fining. J Food Sci 1994;59:1065–9.
[6] Baker RA, Wicker L. Current and potential applications of enzyme infusion in the food industry. Trends Food Sci Technol 1996;7:279–84.
[7] Tanner RD, Prokop A, Bajpai RK. Removal of fibre from vines by solid-state fermentation/enzymic degradation: a comparison of flax and kutzu retting. Biotechnol Adv 1993;11:635–43.
[8] Solis S, Flores ME, Huitron C. Isolation of endopolygalacturonase hyperproducing mutants of *Aspergillus* sp. CH-Y-1043. Biotechnol Lett 1990;12:751–6.
[9] Nakadai T, Nasuno S. Culture conditions of *Aspergillus oryzae* for production of enzyme preparation. J Ferment Technol 1988;66:525–33.
[10] Leuchtenberger A, Friese E, Ruttloff H. Variation of polygalacturonase and pectinesterase synthesis by aggregated mycelium of *Aspergillus niger* in dependence on the carbon source. Biotechnol Lett 1989;11:255–8.
[11] Aguilar G, Huitron C. Constitutive exo-pectinase produced by *Aspergillus* sp. CH-Y-1043 on different carbon source. Biotechnol Lett 1990;12:655–60.
[12] Nair SR, Rakshit T, Panda T. Effect of carbon sources on the synthesis of pectinase by *Aspergilli*. Bioproc Engin 1995;13:37–40.
[13] Nair SR, Panda T. Statistical optimation of medium components for improved synthesis of pectinase by *Aspergillus niger*. Bioproc Engin 1997;16:169–73.
[14] Kilara A. Enzymes and their uses in the processed apple industry. A review. Proc Biochem 1982;17:35–41.
[15] Friedrich J, Cimerman A, Steiner W. Production of pectolytic enzymes by *Aspergillus niger*: effect of inoculum size and potassium hexacyanoferrate II-trihydrate. Appl Microbiol Biotechnol 1990;33:377–81.
[16] Solis S, Flores ME, Huitron C. Improvement of pectinase production by interspecific hybrids of *Aspergillus* strains. Lett Appl Microbiol 1997;24:77–81.
[17] Minjares-Carranco A, Trejo-Aguilar BA, Aguilar G, Vinegra-Gonzalez G. Physiological comparison between pectinase-producing mutants of *Aspergillus niger* adapted either to solid-state fermentation or submerged fermentation. Enzyme Microb Technol 1997;21:25–31.
[18] Maldonado MC, Navarro A, Callieri AS. Production of pectinase by *Aspergillus* sp. using differently pretreated lemon peel as the carbon source. Biotechnol Lett 1986;8:501–4.
[19] Fonseca MJV, Said S. The pectinase produced by *Tubercularia vulgaris* in submerged culture using pectin or orange-pulp pellets as inducer. Appl Microbiol Biotechnol 1994;42:32–5.
[20] Ismail AM. Utilization of orange peels for the production of multienzyme complexes by some fungal strains. Proc Biochem 1996;31:645–50.
[21] Lobarzewski J, Fiedurek J, Ginalska G, Wolski T. New matrices for the purification of pectinases by affinity chromatography. Biochem Biophys Res Commun 1985;131:666–74.
[22] Fiedurek J, Ilczuk Z, Lobarzewski J. Influence of the mycelium growth conditions on the production of amylolytic, proteolytic and pectinolytic enzymes by *Aspergillus niger*. Acta Biotechnol 1989;9:355–61.
[23] Taragano V, Sanchez VE, Pilosof AMR. Combined effect of water activity depression and glucose addition on pectinases and protease production by *Aspergillus niger*. Biotechnol Lett 1997;19:233–6.

[24] Solis-Pereira S, Favela-Torres E, Viniegra-Gonzalez G, Gutierrez-Rojas M. Effect of different carbon sources on the synthesis of Pectinase by *Aspergillus niger* in submerged and solid state fermentations. Appl Microbiol Biotechnol 1993;39:36–41.
[25] Miller GL. Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:426–8.
[26] Aguilar G, Trejo BA, Garcia JM, Huitron C. Influence of pH on Endo- and Exo-Pectinase production by *Aspergillus* sp. CH-Y-1043. Can J Microbiol 1991;37:912–7.
[27] Talboys PW, Busch LV. Pectic enzymes produced by *Verticillium* species. Trans Br Mycol Soc 1970;55:367–81.
[28] Ammar MS, Louboudy SS, Azab MS, Afifi MM. A new method for the estimation of fugal pectinases(s) using the pectin clearing zone (BCZ) technique and its application in food industries. Al-Azhar Bull Sci 1995;6:325–39.