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MiniReview

Production of pectic enzymes in yeasts

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Abstract

When grown in the appropriate medium, several yeast species produce pectinases able to degrade pectic substances. It is mainly exocellular endopolygalacturonases that break pectins or pectate down by hydrolysis of α-1,4-glycosidic linkages in a random way. Biochemical characterisation of these enzymes has shown that they have an optimal pH in the acidic region and an optimal temperature between 40 and 55°C. Their production by yeasts is a constitutive feature and is repressed by the glucose concentration and aeration. Pectic substances and their hydrolysis products are used as carbon sources by a limited number of yeasts and hence these enzymes must be involved in the colonisation of different parts of plants, including fruits. The first yeast pectic enzyme (encoded by the *PSE3* gene) was cloned from *Tichosporon penicillatum*. Recently, a polygalacturonase-encoding gene from *Saccharomyces cerevisiae* has been cloned and overexpressed in several strains and the gene for an extracellualar endopolygalacturonase from *Kluyveromyces marxianus* has also been described. Taking all the results together, the idea is now emerging that this type of yeast enzyme could offer an alternative to fungal enzymes for industrial applications.

Keywords: Pectinase; Pectic enzyme; Polygalacturonase; Yeast; *Saccharomyces cerevisiae*

1. Introduction

Pectic substances are complex structural polysaccharides that occur mainly in the middle lamella and primary cell wall of higher plants. They consist of a main backbone containing a variable but normally large proportion of partially methyl-esterified galacturonic acid subunits linked by α-1,4 glycosidic linkages. This compound is known as pectin, while the demethylated compound is known as pectic acid or polygalacturonic acid. Several L-rhamnopyranosyl residues can be linked through their C-1 and C-2 atoms in the main chain. Moreover, galacturonate residues may be acetylated at positions C-2 and C-3 and side chains of neutral sugar residues can be linked to galacturonic acid or to C-4 of the rhamnose residues in the main chain [1].

The enzymes that hydrolyse pectic substances are known as pectic enzymes, pectinases, or pectinolytic enzymes. They are classified in two main groups, namely pectinesterases (PE) (able to de-esterify pectin by removal of methoxyl residues) and depolymerases (which readily split the main chain). The depolymerising enzymes are divided into polygalac-

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Table 1
Classification of pectic enzymes

PEs (EC 3.1.1.11) de-esterify pectin by the removal of methoxyl residues. Depolymerases split the main backbone
1. By hydrolysis of α-(1,4) linkages
1.1. PGs, acting on pectate
Exo-PG (EC 3.2.1.67)
Endo-PG (EC 3.2.1.15)
1.2. Polymethylgalacturonases (PMG) (EC 3.2.1.15), acting on pectin
2. By β-elimination
2.1. PL, acting on pectate
Exo-PL (EC 4.2.2.9)
Endo-PL (EC 4.2.2.2)
2.2. Pectin methyl-lyase (PML) (EC 4.2.2.10), acting on pectin

turonases (PG), enzymes that cleave the glycosidic bonds by hydrolysis, and lyases (PL), which break the glycosidic bonds by β-elimination (Table 1). In addition, the latter two types of enzymes are classified on the basis of whether they exhibit a preferential hydrolytic power against pectin, pectic acid or oligogalacturonate as the substrate and whether the pattern of action is random (endo-) or terminal (exo-) [2].

The production of pectic enzymes has been widely reported and thoroughly studied in bacteria and filamentous fungi because they play an essential role in the phytopathogenesis. In addition, fungal pectinases are important in the food industry, especially in the extraction and clarification of fruit juices. An assumed 'natural' role in plants includes fruit maturation, growth, abscission and pollen development. The pectinase production in yeasts has received less attention and a few yeast species show this ability. In this sense, the biochemical characterisation of some PG from yeasts has been reported and heterologous genes have been successfully expressed in *Saccharomyces cerevisiae* [3,4]. However, to the best of our knowledge, little has been published about the genetic determination of these enzymes in yeast.

This review covers yeast pectic enzyme production and the biochemical characterisation of these enzymes and includes current knowledgement of the genetics and molecular bases of the pectolytic enzyme production in this kind of eukaryotic microorganisms. Finally, the role and applications of yeast pectinases, as well as possible hints for their use in the future are discussed.

2. The occurrence of pectic enzymes in yeasts

The presence of enzymes exhibiting this biochemical property has been reported in several yeast species. The first authors to describe endopolygalacturonase (endo-PG) production by yeasts were Luh and Phaff [5] in 1951, in *Saccharomyces fragilis*. In 1954, those authors [6] published a study on the properties of these enzymes in comparison to those reported for fungi. In 1953 [7], Roelofsen reported the ability of certain yeasts to attack cell wall pectin, indicating that they contain true PG. These yeasts belonged to the genera *Candida, Pichia*, *Saccharomyces* and *Zygosaccharomyces* and included species of *S. fragilis* and its imperfect form *Candida pseudotropicalis*. Many of the strains were isolated from fermenting cocoa from Java and West Africa. Bell and Etchells [8] described the ability of certain salt tolerant yeasts to hydrolyse pectin. These yeasts were possibly involved in the softening of cucumbers in brine fermentations and included strains of *S. fragilis* and *S. cerevisiae*. Vaughn et al. [9] found that the production of pectin methyl esterase and PG enzymes by *Rhodotorula* sp. was associated with softening in olives. Wimborne and Rickard [10] characterised a pectic enzyme produced by a strain of *S. fragilis* as a PG and Lim et al. [11] reported the presence of multiple forms of endo-PGs in this yeast. Since 1980 and up to the present, the ability to produce pectic enzymes has been described in several other yeast species. These include *Cryptococcus albidus* [12], which has been reported to produce an inducible endo-PG that is involved, together with some filamentous fungi, in the spoilage of preserved fruit, *Fabospora macedoniensis* [13], several species of *Candida* [14–16], *Saccharomycopsis fibuligera* [17], *Saccharomycopsis chevalieri* [15] and several species of the genus *Saccharomyces* including *S. cerevisiae* [18] and *S. pastorianus* [19]. In addition, pectinases have been found in *Kluyveromyces marxianus*, which produces an endo-PG assumed to be important in the breakdown of the pectinaceous layer surrounding coffee and cocoa beans during processing [20,21], in *K. fragilis* [22], *K. lactis* [23] and *Geotrichum lactis* [24].

Regarding the presence of pectic enzymes in *S. cerevisiae*, as mentioned above, the first authors to raise the matter were Luh and Phaff [5] who analysed as many as 140 different strains belonging to this

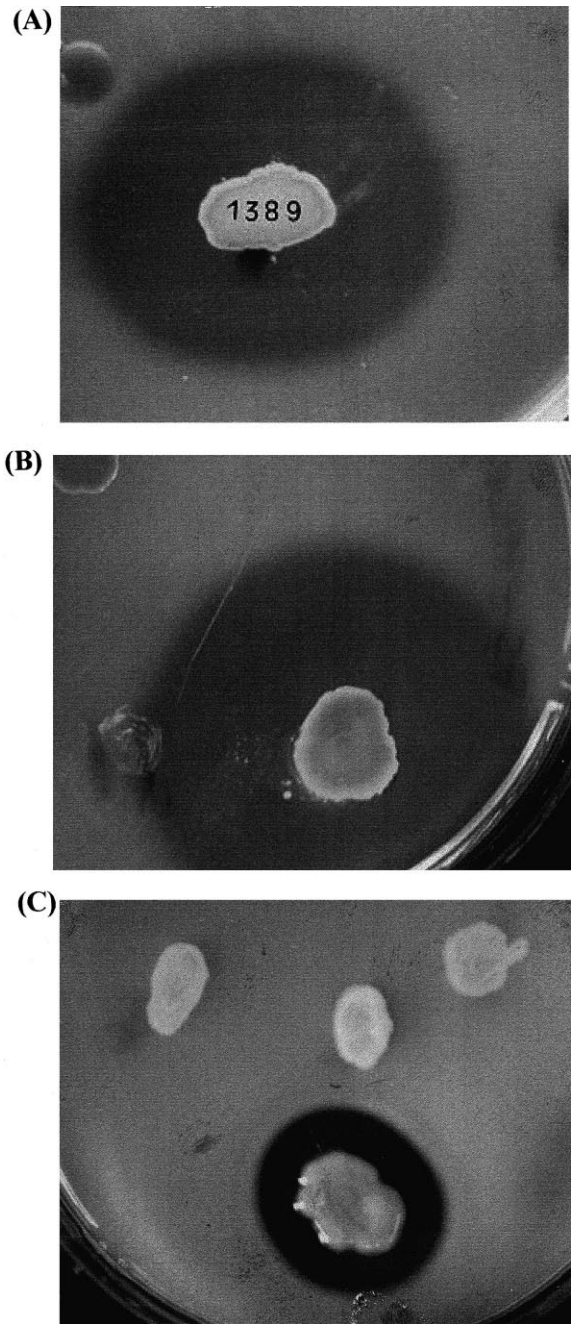


Fig. 1. Hydrolysis haloes on plates with polygalacturonic acid originated by (A) *Saccharomyces cerevisiae* 1389, (B) *Cryptococcus* sp. and (C) by the bacteria *Xanthomonas campestris*.

species and reported that none of them had this ability. However, in 1956 Bell and Etchell [8] described that some strains of *S. cerevisiae* did show some pectolytic activity. A few studies concerning this subject attracted much attention until the one published by McKay [25], who reported the degradation of polygalacturonic acid (PGA) by two strains of *S. cerevisiae*. Also, in 1994, Gainvors et al. [26] found PG, PE and PL activity in strain SCPP 2180 of this species and Blanco et al. [18] characterised the endo-PG from the wild-type strain 1389, as well as the differences between this enzyme and the one synthesised by the genetic strain IM1-8b [27]. The apparent contradiction between the results found by Luh and Phaff [5] and those of other authors in more recent years might be due to the detection method employed in each case. The first authors measured the ability of yeasts to clarify a pectin solution whereas more recently, methods have relied on the production of hydrolysis haloes on plates (Fig. 1).

3. Factors affecting the pectinase production by yeast

Pectinase production is a constitutive capacity in yeasts because pectin, PGA and galacturonic acid are not required to induce the synthesis of these enzymes. However, the pectolytic capacity of a few species, such as *C. albidus* [12], *G. lactis* [24] or *K. fragilis* [28], has been described as inducible and the PG from *S. fragilis* [11] is partially constitutive, the PG activity being higher after growth in 2% glucose+1.8% pectin than when the cells are grown in 1% glucose alone. In *K. marxianus*, pectin does not induce any increase in pectolytic activity [29] and the same is true for the PG from *S. cerevisiae* [18,27]. However, galacturonic acid, which is not the end product of the enzymic reaction, has been described as an inducer of pectolytic activity in several strains as *S. cerevisiae* [18] and *C. albidus* [12].

Another common feature among pectic enzymes is their glucose repressible synthesis. This effect has been reported in *S. cerevisiae* [18,27] and *C. albidus* [12]. Schwan and Rose [29] found that the repressive effect due to glucose on PG from *K. marxianus* was quite evident when the sugar concentration in the media was circa 10%. The effect of sucrose was similar whereas other carbon sources, such as galactose

or fructose, retarded the PG secretion. By contrast, in *S. cerevisiae* strain 1389, galactose is the best carbon source for PG production [18].

The PG activity in yeasts is also related to the amount of dissolved oxygen in the medium. Several strains, such as *K. marxianus* [20,29], *K. fragilis* [28] and *K. lactis* [23], exhibit pectolytic ability when they are grown without shaking and even under anaerobic conditions and no activity is found at high aeration rates. Wimborne and Rickard [10] demonstrated that in *S. fragilis* the PG activity is completely repressed when the dissolved oxygen tension (DOT) is 60%, however, they found a normal activity when the DOT was near 0% after oxygen had been replaced by CO_2 or N_2. In *Saccharomycopsis fibuligera* [17], a reduction in the concentration of dissolved O_2 in the culture medium causes an increase in the rate of pectin decomposition. The PG production in *S. cerevisiae* is also affected by the amount of dissolved oxygen, depending on the strain. For example, *S. cerevisiae* 1389 (wild-type strain) showed a pectolytic activity when it was grown with moderate shaking whereas the genetic strain IM1-8b displayed a lower activity under these conditions. Both showed activity when they were grown without aeration while the enzymic activity was fully repressed with a high aeration. However, in other strains such as NCY 365 and NCY 373, the pectolytic activity is not repressed by the amount of oxygen [25]. The effect of oxygen in other species, such as *C. albidus* [12] and *G. lactis* [24], does not seem to be as crucial. Further investigation is required to determine the effect of oxygen on the synthesis of pectic enzymes in yeast as well as which regulatory mechanisms are involved in this kind of behaviour. In addition, it is possible that early screenings may have been carried out under suboptimal conditions, affording negative results when they should have been positive.

The influence of other parameters such as the pH of the medium, inoculum size, incubation time or the addition of nitrogen sources has received less attention. Murad and Foda [23] found that the highest value of enzymic activity can be obtained in a medium with an initial pH of 5.0, the inoculum size being circa 4% (v/v). The effect of the nitrogen source on the pectolytic activity was studied in only two cases. Murad and Foda [23] reported that in *K. lactis* the activity increased when the media were supplied with an organic nitrogen source such as yeast extract or peptone but that when an inorganic nitrogen source was added, the enzymic activity remained unaffected. In these studies, the cultures were usually grown for 3 days [12,18,23] and the pectolytic activity decreased with a longer period of culture. Despite this, in *K. marxianus* [21], the PG secretion started earlier in the exponential phase and was completed after 24 h.

4. Biochemical characterisation

Yeast pectinases are usually exocellular enzymes of a varying molecular mass (Table 2) and are of glycoproteinaceous nature [11,20]. In some species, the presence of several isoenzymes has been demonstrated, this is the case in *S. fragilis* [11] and *K. marxianus* [20]. All these enzymes exhibit an optimum pH in the acidic region between 3.5 and 5.5 and their optimum temperature lies between 40 and 55°C, except in the cases of those described for endo-PG from *S. chevalieri* [15] and *C. albidus* [12], with optimum temperatures of 25 and 37°C, respectively. Because these enzymes are unstable when challenged by changes in pH, pI values have been determined in only a few cases, precisely when the pI values are neither too high or too low (5.7–6.3). The pI of the endo-PG from *C. albidus* [12] is 8.1 and the putative pI for the PG from *S. cerevisiae* is 8.5, in clear agreement with the results obtained by cation exchange chromatography [30].

The K_m values for different pectic substances lie between 14.08 mg ml^{-1} for an endo-PG from *S. cerevisiae* 1389 and 0.09 mg ml^{-1} for exo-PG from *G. lactis* [24,30], indicating different affinities for these substrates, although they usually fall in the 0.1–0.6 mg ml^{-1} range (Table 2).

Pectic enzymes from yeasts are mainly endo-PG, that is, enzymes which randomly degrade the main chain of the pectic backbone, mainly via hydrolysis of α-1,4-glycosidic linkages. Only in a few cases has a different pectic activity been reported. In this sense, Gainvors et al. [26] found a strain of *S. cerevisiae* (SCPP 2180) showing PG, PE and PL activities. However, these enzymes have not yet been characterised. Vaughn et al. [9] described PE activity in *Rhodotorula* sp. and Wimborne and Rickard [10] re-

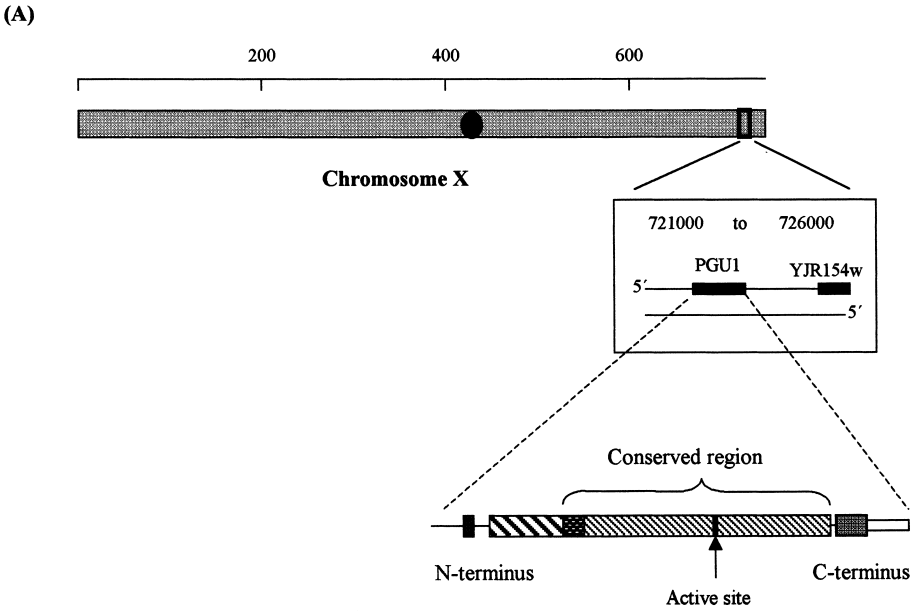
Table 2
Summary of some biochemical properties of yeast pectic enzymes

Strain	Molecular weight (Da)	Optimum pH and/or pH activity range	Optimum temperature and/or activity range (°C)	K_m (mg ml^{-1})	Type of pectic enzyme	pI	Reference
S. fragilis		4.4 (3.5–5.5)	50		PG		[6]
Rhodotorula sp.		6.0 (4.9–9.0)	40 (10–50)		PE, PG		[9]
S. fragilis (*K. fragilis*)	I-46000	4.0–5.0 (3.5–6.0)	50	I-0.13	Endo-PG (three isoenzymes)	I-6.10	[11]
	II-50000			II-0.22		II-6.10	
	III-30000			III-0.5		III-5.80	
F. macedoniensis U-480	28000	5.0–5.2 (3.5–6.0)	45–50		PG	6.3	[13]
K. fragilis IFO 0288	33000	5.0 (4–7)	55 (30–55)		Endo-PG (four isoenzymes)	5.6	[22]
S. chevalieri		5.0	25	0.54	Endo-PG		[15]
T. candida		5.0	60	0.20	Endo-PG		
C. norvegensis		5.0	50	0.64	Endo-PG		
K. fragilis		5.0	50–60	0.13	Endo-PG		
C. macedoniensis		4.5	50–53		Endo-PG		[14]
C. albidus	41000	3.75 (4.0–8.0)	37	0.57	Endo-PG	8.10 ± 0.1	[12]
K. marxianus NCYC587	I-29600				Endo-PG (four isoenzymes)	I-6.3	[20]
	II-29800					II-6.0	
	III-36000					III-6.3	
	IV-21900					IV-5.7	
G. lactis	53000	5.0 (3.5–7.0)	40	0.09	Exo-PG		[24]
S. cerevisiae 1389	I-22000	5.0	45	1.8	Endo-PG		[30]
	II-31000	4.5	45	14.08	Endo-PG		
S. cerevisiae IM1-8b	36000	4.5 (3.5–6.5)	45 (20–50)	0.59	Endo-PG		[27,30]
S. pastorianus	43000	4.2	50	0.62	PG	5.4	[19]

ported PG activity in *K. fragilis*. The main enzymes produced, however, were endo-PG, capable of strongly reducing the viscosity of pectin or a pectate solution during the first minutes of the reaction. The percentage of reduction depends on both the type of substrate and the strain used. The end product of such enzymic reactions are always oligosaccharides with a varying number of galacturonic residues, although no free galacturonic acid, which is the end product of exo-PG, has been found. Moreover, all these enzymes preferentially attack pectate over pectin and their activities decrease as the degree of methylation increases. Barnby et al. [20] found that the activity of endo-PG from *K. marxianus* with 37.8% esterified pectin is about 95% and with 61% pectin esterification, the activity decreases to 25%. Likewise, in *S. cerevisiae* 1389 and IM1-8b, when 30% esterified pectin was used, the activity decreased by about 30% and when the methylation was 70%, it decreased by circa 60% [31].

5. Genetic determination of pectic enzymes in yeasts

It is quite surprising that despite the importance of pectic enzymes in several industrial processes, our basic knowledge of the genetics and of the control over such enzymes in the major industrial microorganism *S. cerevisiae*, as well as in other yeast species, has remained so scanty. The first approach to this issue was reported by Blanco et al. [30], who analysed the genetic determination of PG production in a wild-type and a laboratory strain of *S. cerevisiae*. Using classical genetic techniques, these authors demonstrated that the pectolytic capacity was determined by at least two structural genes in the wild-type strain 1389 whereas the genetic strain IM1-8b was monogenic. These observations are in good agreement with the finding of two different enzymes in the first strain and only one in the second. Kenichi Iguchi et al. [32] reported the cloning of a protopectinase-encoding gene (*PSE3*) from *Trichosporon*



(B)

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1    MISANSLLIS TLCAPAIATP LSKRDSCTLT GSSLSSLSTV KKCSSIVIKD
51   LTVPAGQTLD LTGLSSGTTV TFEGTTTFQY KEWSGPLISI SGSKISVVGA
101  SGHTIDGQGA KWWDGLGDSG KVKPKFVKLA LTGTSKVTGL NIKNAPHQVF
151  SINKCSDLTI SDITIDIRDG DSAGGHNTDG FDVGSSSNVL IQGCTVYNQD
201  DCIAVNSGST IKFMNNYCYN GHGISVGSVG GRSDNTVNGF WAENNHVINS
251  DNGLRIKTVE GATGTVTNVN FISNKISGIK SYGIVIEGDY LNSKTTGTAT
301  GGVPISNLVM KDITGSVNST AKRVKILVKN ATNWQWSGVS ITGGSSYSGC
351  SGIPSGSGAS C
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Fig. 2. (A) The location of ORF YJR153w (*PGU1* gene) on Chromosome X and the domain organisation of the protein. (B) The predicted amino acid sequence of the protein encoded by *PGU1*: light-grey box indicates the signal peptide, the putative active site is shown in a black box and underlined and the two potential glycosylation points are in bold and double underlined.

penicillatum. The *PSE3* gene contains an ORF encoding a 367 amino acid protein. The deduced amino acid sequence for this gene shows a high homology (65.4%) with the polygalacturonases from *Aspergillus oryzae* and other filamentous fungi. In addition, the existence of an ORF on *S. cerevisiae* chromosome X (ORF YJR153w) (Fig. 2A) that also shows a strong homology with PGs of fungal origin and with the *PSE3* product (57%) has recently been reported. The domain organisation is shown in Fig. 2A. The C-terminal region where the active site is located is common for PGs regardless of their origin. This ORF encodes a protein of 361 amino acids (37 287 Da) with a signal peptide between residues 1 and 18 (Fig. 2B). The active site is a histidine at position 222 flanked by two glycines thus forming the three amino acid active site GHG, well conserved throughout the evolution of PG genes. The protein contains two

potential glycosylation sites at residues 318 and 330. Such an ORF has recently been cloned and named *PGU1* [33]. The activity in transformed strains was enhanced up to 200 times, although the final activity depended on the genetic background of each strain and the plasmid used. It was also shown that all *S. cerevisiae* yeast strains carry the *PGU1* gene, regardless of their pectolytic phenotype. Other workers [34] have also cloned the above mentioned ORF, obtaining similar results. The regulation of this ability as well as the study of different factors, which could enhance or repress the enzymic activity, need to be further investigated.

In addition to the above genes, the sequence of an endopolygalacturonase from *K. marxianus* has been submitted by Siestele et al. to the GenBank (accession number 2597957). This sequence comprises a 361 amino acid protein that shows a high homology (72%) with that of *S. cerevisiae* polygalacturonase.

6. The role of pectic enzymes in yeasts

The function of these enzymes in yeasts is largely unknown and it is noteworthy that two different types of yeasts may be discerned. One group comprises those unable to use pectin, pectate or their hydrolysis products (galacturonic acid) as carbon sources [5,29,31] and these strains mainly produce endopolygalacturonases. Accordingly, most authors have attributed an ecological role rather than a trophic one to yeast polygalacturonases. As far as it is known, these enzymes could be involved in substrate colonisation (fruits), causing the breakdown of plant tissues with a concomitant release of sugars from plant cells, which in turn can be utilised for yeast growth, hence causing further spoilage. The other group encompasses the yeasts that, like filamentous fungi, have the ability to grow using pectic substances as the sole carbon source, suggesting a more complex enzymic system in this type of yeast. Such a characteristic has been reported in *C. albidus* [12] and in the methylotrophic yeast *Candida boidinii* [16]. Taking all this together, one could even envisage a different origin for the pectolytic enzymes present in both groups. Deeper insight into these aspects may, in the foreseeable future, afford interesting results on the origin of pectic genes in yeasts.

7. Applications of yeast pectinases

The importance of pectolytic enzymes as virulence factors during the microbial pathogenesis has always been well known. In addition, microbial pectinases are of commercial interest in that they are used in many industrial food applications, particularly in fruit and vegetable processing. These applications include extraction and clarification of fruit juices, grape must and apple juice consumed in massive quantities all over the world. Problems in clarification are mainly caused by the presence of pectic substances suspended as insoluble particles. Pectic enzymes are therefore used to depectinise juices in order to remove turbidity and prevent cloud-forming spoilage. Other interesting uses of pectic enzymes are related to the maceration of vegetables as well as oil extraction [1,2]. Also, pectolytic enzymes can be used in complex mixtures with cellulases and other carbohydrases in the formulation of animal feed so as to facilitate nutrient assimilation by the animal.

Until now, the main source of pectic enzymes for industrial use is the mold *A. niger* because it produces fair amounts of these enzymes, in addition to being a GRAS (generally recognised as safe) microorganism. Commercial preparations of fungal origin are in fact lytic broths that contain a complex mixture of different enzymes with pectinolytic activity including endo- and exo-PG, PL and the undesirable PE. Other non-pectic enzymes with no suitable effect (amylases, arabinofuranosidases, etc.) are also present in the mixture.

In this sense, yeast PGs may have some advantages over fungal ones and could offer a good alternative to fungal enzymic preparations. As we have shown, some yeast strains (like *S. cerevisiae*, *K. marxianus* and *G. lactis*) are able to produce only one type of pectolytic enzyme and do not present PE activity. Further, they are GRAS microorganisms and pectic enzymes can be produced inexpensively as by-products during the collection of single cell protein.

However, there are not yet many practical applications for yeast pectolytic enzymes. This is because the total hydrolysis of some substrates often requires the concerted action of several enzymes. Despite this, pectolytic systems from yeasts could be very appropriate for industrial purposes as standardised enzyme mixtures, offering the additional advantage that such mixtures could be prepared with different proportions of each type of enzyme (that is, endo-/exo-PG and PL activities). In this sense, it is interesting to note that in the case of apple juice, complete pectin breakdown can only be ensured if the different types of pectic enzymes are present at the correct proportions [35].

A few efforts have already been made to investigate whether yeast pectinases can be used in certain industrial processes. Thus, Gainvors et al. [36] demonstrated that when added to grape must, a crude enzymic extract from *S. cerevisiae* (SCPP 2180) with PE, PL and PG activities had the same effect on the turbidity as the same quantity of the commercial preparation Endozyme (Pascal Biotech SARL-Paris). Along the same line, Blanco et al. [31] have shown that when wine fermentations are carried out using PG+strains of *S. cerevisiae*, the clarification process is greatly facilitated, the filtration time being reduced up to 50% in some cases.

Probably the main problem in using yeast pectolytic enzymes in industrial processes lies in the low fermentation yield. This, however, may be overcome by cloning and overexpression of the respective structural genes in different genetic backgrounds. Regarding this, it would be interesting to study the regulation of yeast pectic enzymes. The fact that many yeasts have only one gene and therefore a single polypeptide certainly facilitates this task. In relation to the overexpression of cloned genes, those from *S. cerevisiae* and *K. marxianus* are of great interest since they can be expressed in special strains of the same host whose expression systems are largely known. In this sense, the *PGU1* gene has been overexpressed in different *S. cerevisiae* strains [33]. Likewise, the *PSE3* gene from *T. penicillatum* has also been overexpressed in *S. cerevisiae*, with significant increases in yield in comparison with the wild-type [32]. The same approach has been employed for the heterologous cloning of genes from filamentous fungi [3,4]. It would be very interesting to ascertain whether cloning in systems such as those of *Pichia pastoris* or *Yarrowia lipolytica* does in fact increase the fermentation yields.

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