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review

Production of Hydrolytic Depolymerising Pectinases

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Summary

Polygalacturonases (PGases) or hydrolytic depolymerases are enzymes involved in the degradation of pectic substances. They have a wide range of applications in food and textile processing, degumming of plant rough fibres and treatment of pectic wastewaters. Bacteria, yeasts and fungi under both submerged (SmF) and solid-state fermentation (SSF) conditions produce these enzymes. Bacteria produce mainly alkaline and thermostable PGases, whilst fungi are the major producers of acidic PGases. In order to obtain high yields of pectinase production, strain improvement and optimization of culture conditions should be considered. This review is an overview of the microorganisms, substrates, and type of culture used for PGase production. It also provides a description about the strategies used to enhance the production of PGases.

Key words: pectinases, polygalacturonases, alkaline pectinases, thermostable pectinases

Introduction

Pectin is a structural polysaccharide found in primary cell wall and middle lamella of fruits and vegetables. The predominant structure of pectin is homopolymeric, made of a partially methylated poly-α-(1,4)-galacturonic acid. Sections of α-(1,2)-L-rhamnosyl-α-(1,4)-D-galacturonosyl containing branch-points with L-arabinose and D-galactose can be incorporated in the main polymeric chain. Pectin may also contain residues of D-glucuronic acid, D-apiose, D-xylose and L-fucose attached to poly-α-(1,4)-D-galacturonic acid sections (*1,2*).

On the other hand, pectinases are a group of related enzymes involved in the breakdown of pectin from a variety of plants. These enzymes are classified based on their preferred substrate (pectin, pectic acid or oligo-D-galacturonate), the degradation mechanism (transelimination or hydrolysis) and the type of cleavage (random [endo-] or terminal [exo-]) (*3*).

Pectinases are extensively used in fruit juice processing (extraction and clarification), vegetable oil extraction, processing of alcoholic beverages and a variety of applications in food industries. Actual and potential industrial applications of pectinases were reviewed (*3–6*). The classification of pectinolytic enzymes, as well as some aspects related to their assay methods, purification, and physicochemical and biological properties were reviewed (*7–9*).

Due to the potential and wide applications of pectinases, there is a need to highlight recent developments on several aspects related to their production. Microbial production of pectic transeliminases was reviewed (*10*); however, aspects regarding the most common microorganisms and processes for hydrolytic depolymerising pectinase (PGase) production have not been considered until now. The aim of this review is to present an overview of the PGase activity values obtained by different microbial strains in submerged and solid-state fermentations, as well as the strategies used to obtain higher activities with both types of cultures.

Endo- and Exopolygalacturonases

PGases are hydrolytic depolymerases with endo and exo activities. Endo-PGases (E.C. 3.2.1.15) are important enzymes involved in fruit ripening and in fungal and bac-

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terial attack on plants, and are commonly used in the treatment of certain vegetables like tubers, apples, *etc*. Their enzymatic reaction involves random hydrolysis of *O*-glycosyl bonds in 1,4-α-D-galactosyluronic linkages in homogalacturonans. On the other hand, galacturan 1,4-α-galacturonidases (E.C. 3.2.1.67), or exo-PGases, are enzymes that degrade polygalacturonan by hydrolysis of the glycosidic bonds from the nonreducing ends yielding the corresponding 1,4-α-D-galacturonide and galacturonic acid. Depending on the type of substrate (*i.e.* pectin, polygalacturonate or polymethylgalacturonate) and the mode of action (endo or exo activity), PGase activity can be quantified, and therefore expressed in different units, whether by the reduction of viscosity in the reaction mixture or by the release of reducing groups during the enzymatic reaction under established conditions.

Polygalacturonase Production

Microbial sources of polygalacturonases

More than 30 different genera of bacteria, yeasts and moulds have been used for the production of PGases (*8*). However, *Erwinia*, *Bacillus*, *Saccharomyces*, *Kluyveromyces*, *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus* have been the genera most frequently studied in the last 15 years, with strains of *Aspergillus*, *Penicillium* and *Erwinia* mainly used for enzyme production studies.

Selection of the microbial source for PGase production depends on several features, such as the type of culture (solid-state or submerged fermentation), number and type of the produced pectinases (esterases, hydrolytic depolymerases and eliminative depolymerases), pH and thermal stability of the enzymes, and genotypic characteristic of the strain (wild type, mutagenized strain, homologous or heterologous recombination).

Systems for polygalacturonase production

Submerged (SmF) and solid-state fermentation (SSF) processes have been widely used for PGase production by different types of microorganisms. SSF is considered more suitable for fungi than for bacteria and yeast growth (*11*,*12*). This is justified in terms of the natural habitat conditions for fungi (mainly soil), in contrast with those for bacteria or yeast, which are present in soils or liquid environments. The capability of fungi to grow under culture conditions with low water activity makes them more interesting for their use in SSF processes. Moreover, one of the major characteristics of this type of process is its low water content, used to avoid bacterial contamination by improving the fungal metabolism.

Several studies have shown that the production of PGases by SSF is higher than that obtained by SmF. One of the pioneer works on pectinase production by SSF demonstrated that PGase production by *A. niger* was about 11 times higher in SSF than in SmF (*13*). The use of pectin as sole carbon source also allowed demonstrating that PGase production by *A. niger* was 6 times higher in SSF than in SmF, and required a shorter time for enzyme production (*14*). Addition of sucrose in SmF acted as a catabolite repressor for PGase activity, while in SSF, an enhancement of activity (more than 10 times) was observed after the addition of sucrose to the culture medium (*15*). Solis-Pereira *et al*. (*13*) also observed this lack of catabolite repression when the culture medium was supplemented with glucose. Later, a comparative study of alkaline PGases from *Bacillus* sp. MG-cp-2 demonstrated that the enzyme production was more than 60 times higher per gram of dry matter in SSF than per mL of culture medium in SmF. The enzyme production under both types of cultures was improved by the addition of surfactants, amino acids and vitamins; however, whilst Tween 60, DL-serine and folic acid enhanced PGase activity in SmF, Tween 80, pyridoxine and DL-ornithine monohydrochloride were the most stimulating agents in SSF. These results suggest differences in the regulation of PGase biosynthesis in *Bacillus* sp. MG-cp-2 as a function of the type of culture (*16*). Endo-PGase production by *Paecilomyces clavisporus* was 28 times higher per gram of solid substrate in SSF than per mL of culture medium in SmF (*17*). Production of PGase by the fungus *Fusarium moniliforme* grown on a solid medium containing wheat bran and orange pulp resulted in a threefold increase in comparison with the production obtained in SmF. This increased PGase production in SSF, as compared to that in SmF, was partially attributed to the expression of a second PGase (PG II), biochemically different from the one produced in SmF (*18*). More recently, Kuhad *et al*. (*19*) improved pectinase production by the alkalophilic strain of *Streptomyces* sp. RCK-SC using an immobilized cell system and SSF in comparison with SmF system.

Differences in enzyme production by SmF and SSF can be mainly attributed to: (*i*) the lack of catabolic repression in SSF as compared to that observed in SmF, (*ii*) lower diffusion of substrate to the microorganism in SSF, or (*iii*) higher oxygen levels in SSF at the solid-air interface that, in turn, support faster growth (*13*,*15*).

The advantages of SSF over SmF and the economic feasibilities of adopting SSF technology in the commercial production of industrial enzymes have recently been evaluated (*20*). In terms of economic feasibility of the production step, it is required to design processes using inexpensive raw materials that would enable obtaining high enzymatic concentrations and productivities.

Thermostable polygalacturonases

Thermal stability and activity of pectinases are of great significance in biotechnological processes. In case of yeasts, the optimal temperatures of PGases produced range from 40 to 50 °C. However, some yeasts (*Tephrosia candida* and *Kluyveromyces fragilis*) produce PGases with maximal activities at temperatures up to 60 °C (*21*). Pectinases produced by fungi are generally acidic and have optimum activities at temperatures up to 50 °C. Nevertheless, some strains of *Thermoascus aurianticus* (*22*), *Aspergillus aculeatus* (*23*) and *Fusarium oxysporum* (*24*) produce PGases with optimal activity at temperatures from 60 to 65 °C. Regarding bacterial pectinases, the optimal temperature for a PGase from *Streptomyces* sp. QG-11-3 is 60 °C and the hyperthermophilic bacterium *Thermotoga maritima* produces an enzyme with optimum activity at 80 °C (*25*).

Alkalophilic and acidic polygalacturonase production

Fungi and yeasts produce mainly acidic PGases, whilst alkaline pectinases are mainly produced by bacteria. The highest reported values for PGase production (from 3600 to 23 076 IU/g) were obtained by alkalophilic strains of *Bacillus* sp. and *Streptomyces* sp. under SSF conditions (Table 1), whilst the production by the same strains under SmF was considerably lower (with a maximum value of 360 IU/mL). The PGases produced by *Streptomyces* sp. were optimally active at 60 °C with the half-life of the activity of 3 h at 70 °C. These alkalophilic enzymes can be used in both, degumming of rough fibres or fibre crops and treatment of alkaline pectic wastewater from vegetable and fruit processing industries (*3,6,19*).

Table 1. Production of alkalophilic polygalacturonases

Microorganism	pH	PGase activity*		Reference
Bacillus sp.	10	23076.0	IU/g	(*16*)
Bacillus sp.	8	8050.0	IU/g	(*26*)
Streptomyces sp.	8	4857.0	IU/g	(*19*)
Bacillus gibsonii	10.5	3600.0	IU/g	(*27*)
Bacillus sp.	10	360.0	IU/mL	(*16*)
Streptomyces sp.	8	76.0	IU/mL	(*19*)
Bacillus pumilus	10.5	20.5	IU/mL	(*28*)
Bacillus sp.	10	6.0	IU/mL	(*29*)

*International units expressed as the amount of enzyme required for liberating one μmol of galacturonic acid per minute. Activity units are expressed as IU/g and IU/mL for SSF and SmF, respectively

Besides acidic PGases produced by fungi and yeast, some bacteria strains produce PGases with optimum activities at low pH values. Beg *et al.* (*30*) found a pectinase produced by *Streptomyces* sp. QG-11-3 optimally active at pH=3 and 60 °C. Since this enzyme was stable in the pH range of 2 to 9, it could be used for reactions under acidic or alkaline conditions. In addition, a PGase produced by *Aspergillus aculeatus* was found to be optimally active at pH=3 and 60 °C (*23*). However, most of the studies on acidic PGase production usually determine their activity at temperatures ranging from 40 to 45 °C and pH values from 4.0 to 4.5.

Independently of the type of culture (SSF or SmF), the highest titres of acidic PGase have been obtained with strains of *Aspergillus*, *Penicillium* and *Candida* (Tables 2 and 3). Enzymatic activities between 153 and 480 IU/g were obtained in SSF with *Aspergillus* strains using wheat bran or wheat bran supplemented with soy bran as sole source of nutrients (Table 2), whilst activities from 162 to 500 IU/mL were obtained with several microbial strains in SmF (Table 3) with different carbon and inducer sources (glucose and pectin (*43*), apple pomace (*44*), citrus pectin or cellulose (*45*)) and aqueous extract of wheat bran supplemented with citrus pectin (*38*). Such high activities were obtained by using genetic strategies or the optimization of culture media and physicochemical parameters.

Strain improvement for polygalacturonase production

Antier *et al.* (*62*) isolated pectinase UV hyperproducing mutants of *Aspergillus niger* by looking for a deoxyglucose resistance phenotype. Some of these mutants were less sensitive to catabolite repression than the wild type.

Table 2. Polygalacturonase production by solid-state fermentation

Microorganism	Substrate	PGase activity* IU/g	Reference
Aspergillus carbonarius	Wheat bran	480.0	(*31*)
Aspergillus carbonarius	Wheat bran	400.0	(*32*)
Aspergillus niger	Wheat bran and glucose	370.0	(*33*)
Aspergillus niger	Pectin	357.5**	(*15*)
Aspergillus niger	Wheat and soy bran	152.5**	(*34*)
Fusarium moniliforme	Wheat bran and orange pulp	81.0	(*18*)
Penicillium viridicatum	Orange bagasse and wheat bran	71.2	(*35*)
Thermoascus auriantacus	Wheat bran or orange bagasse	43.0	(*22*)
Aspergillus niger	Sugar cane bagasse and pectin	38.0	(*13*)
Aspergillus niger	Wheat bran and dextrose	36.3	(*36*)
Lentinus edodes	Strawberry pomace	29.4	(*37*)
Aspergillus niger	Wheat bran and pectin	27.5	(*38*)
Moniliella sp.	Sugar cane bagasse and wheat bran	26.0	(*39*)
Aspergillus awamori	Grape pomace	25.0	(*40*)
Chaetomium sp.	Palm oil mill fibre	18.9	(*41*)
Penicillium sp.	Sugar cane bagasse and wheat bran	12.0	(*39*)
Aspergillus awamori	Wheat grains	9.6	(*42*)
Aspergillus niger	Sugar cane bagasse and pectin	3.8**	(*14*)

*International units expressed as the amount of enzyme required for liberating one μmol of galacturonic acid per minute
**Estimated from the activity value reported as IU/mL

Table 3. Polygalacturonase production by submerged fermentation

Microorganism	Substrate	PGase activity* IU/mL	Reference
Aspergillus japonicus	Pectin and glucose	500.0	(*43*)
Candida utilis	Apple pomace	239.0	(*44*)
Penicillium occitanis	Citrus pectin	221.0	(*45*)
Streptomyces sp.	Pectin	162.0	(*46*)
Aspergillus oryzae	Wheat bran and pectin	54.0	(*47*)
Streptomyces sp.	Pectin	46.0	(*30*)
Aspergillus niger	Wheat bran extract and pectin	34.0	(*48*)
Alkalophilic bacteria	Citrus pectin	29.1	(*49*)
Penicillium dierckxii	Sugar beet pectin	32.6	(*50*)
Aspergillus niger	Sucrose	20.0	(*51*)
Aspergillus niger	Pectin	14.5	(*52*)
Penicillium frequentans	Pectin	3.0	(*53*)
Aspergillus niger	Wheat bran and pectin	2.4**	(*38*)
Aspergillus niger	Corn meal and glucose	1.9	(*54*)
Polyporus squamosus	Pectin	1.5	(*55*)
Aspergillus niger	Corn and glucose	1.3	(*56*)
Trichoderma reesei	Sugar beet pulp	1.1	(*57*)
Penicillium griseoroseum	Pectin	0.7	(*58*)
Sporotrichum thermophile	Citrus peel	0.5	(*59*)
Mucor flavus	Sugar beet pulp and citrus pectin	0.4	(*60*)
Fusarium moniliforme	Citrus pectin and glucose	0.3	(*18*)
Aspergillus niger	Pectin	0.055	(*14*)
Aspergillus awamori	Pectin	0.046	(*61*)
Rhizopus stolonifer	Pectin	0.04	(*61*)

*International units expressed as the amount of enzyme required for liberating one μmol of galacturonic acid per minute
**Estimated from the activity value reported (140 U per 5 grams of wheat bran)

The use of culture media with different levels of ethylene glycol, a water activity depressant, helped to isolate mutants that were suited to produce pectinases on solid substrates with low water activity, such as coffee pulp (*63*). Loera and Viniegra-González (*64*) and Larralde-Corona *et al.* (*65*) applied a mathematical model of mycelia growth in order to evaluate, in Petri dishes, the phenotypes of those mutants in relation to the growth rate and pectinase activity, using image analysis techniques. Loera *et al.* (*66*) showed that some parasexual diploids of *Aspergillus niger* hyperproducing mutants doubled the production of the parental strains. Thus, selection of deoxyglucose resistant mutants and parasexual recombination, together with automated image analysis of colonies, seems to be an interesting way to produce superior mutants for pectinase production.

Genetic improvement for pectinase production by using protocols of mutagenesis or protoplast fusion has been successful. Cao *et al.* (*29*) used the P/C value (ratio of the diameter of clearance zones [P] to the diameter of colonies [C]) on plates in order to identify hyperproducing mutants of pectinases. The use of rifampin to isolate spontaneous mutants of the alkalophilic *Bacillus* sp. NTT33 allowed the obtaining of two mutants resistant to catabolic repression that produce PGase activities up to 82.4 % higher than the maximum achieved for the wild type. The use of mutants resistant to catabolic repression (deregulated mutants) would allow obtaining higher titres of enzymatic activity at lower production costs. Hadj-Taieb *et al.* (*45*) used this strategy to isolate, after mutagenesis with nitrous acid, a constitutive mutant producing 15 times more endo- and exo-PGase than the wild type strain of *Penicillium occitanis*. This wild type strain had previously been subjected to an improvement program (*67*). After mutagenesis with *N*-methyl-*N*--nitro-*N*-nitrosoguanidine, ethyl methane sulphonate and UV light, a catabolic repression resistant mutant, Po16, was isolated. This mutant presented improved cellulose and pectinase production as compared to the wild type strain.

Protoplast fusion was used to obtain hybrid strains with improved characteristics for PGase production. A prototrophic hybrid (HJ) was obtained after protoplast fusion of auxotrophic mutants of *Aspergillus* sp. CH-Y--1043 (*ade*-) and *A. flavipes* ATCC 16795 (F7 *lys*-). This hybrid presented 1.5-fold endo-PGase production, whilst exo-PGase was similar to that obtained by the wild strain of *Aspergillus* sp. (*68*). Interspecific protoplast fusion of *A. niger* and *A. carbonarius* was carried out to obtain hybrids with both, higher production of pectinases (6-fold more than the wild type strain) and improved growth on SSF with wheat bran as sole source of nutrients (*32*).

Optimization of polygalacturonase production

In addition to strain improvement programs to obtain pectinase hyperproducing mutants, studies on me-

dium composition and culture conditions by using experimental designs have demonstrated that pectinase production might be considerably improved. For instance, PGase production by *Streptomyces* sp. QG-11-3 increased 5.6-fold when grown in a culture medium supplemented with DL-norleucine, L-leucine and DL-isoleucine (*46*). Similarly, addition of amino acids (DL-serine and DL-ornithine) to the culture medium in SmF and SSF enhanced (4.0 and 5.7 times, respectively) the PGase production by *Bacillus* sp. MG-cp-2 (*16*). Kashyap *et al.* (*26*) demonstrated that the addition of a multivitamin additive Neurobion (mixture of B_1, B_6, and B_{12}) to the culture medium increased 1.75-fold the pectinase production by *Bacillus* sp. DT7 in a SSF system. Moreover, some studies about the production of extracellular pectinases by wood-rot fungi demonstrated that pectinase production by *Bjerkandera adusta* 40 was stimulated (127 %) by the addition of $NaNO_3$ as nitrogen source, whilst that of *Coriolus versicolor* 24 was stimulated (154 %) by the addition of $(NH_4)_2SO_4$ (*69*). Even though the evaluation of the effect of individual components added to the culture medium allowed enhancing the pectinase production, this approach did not permit to evaluate interactions between the operating conditions and the media composition.

Sharma and Satyanarayana (*28*) optimized the production of a highly alkaline and thermostable pectinase of *Bacillus pumilus* in SmF by using a Plackett-Burman design, identifying the most relevant variables for pectinase production, with a further optimization using the response surface methodology (RSM) of a central composite design. The use of this statistical approach increased the enzyme production in lab fermentor scale up to 41-fold. Likewise, a central composite rotatable design and the RSM were used to find optimal conditions of pH, temperature and incubation period for the pectinolytic activity of *Kluyveromyces wickerhamii* (*70*). The study revealed that pH and temperature were the most significant factors that influence the enzyme production, finding optimal values of 5 and 32 °C, respectively, which resulted in an increase in PGase production up to 6.67-fold. In another study, a multivariate regression analysis was applied to the five main constituents of the solid culture medium to optimize pectinase production by *A. niger*. A mathematical analysis showed that Tween 80 had a negative effect on pectinase production, and led to the definition of the optimal choice of physicochemical parameters and culture medium composition, increasing the pectinase production up to 36.3 IU/g (*36*). The rotating simplex method for optimization of physicochemical parameters (pH, agitation and aeration rate) was used to find the optimal conditions for extracellular pectinase production by *A. niger* in SmF, obtaining a twofold increase in PGase production, with respect to the lowest activity registered (*56*).

As showed above, strategies of strain improvement by classical methods (mutagenesis) or process optimization by experimental designs have allowed to achieve considerable increases (up to 41-fold) in pectinase production yields. Recombinant DNA technology (RDT) also offers important opportunities in order to improve yields of specific activities. However, since pectinases are conformed by a group of enzymes acting on pectic substances with different composition (*3*), the use of RDT necessarily involves recombinant strains producing, each one, single pectinase molecular species. Nevertheless, post-transcriptional and secretional problems associated with cellular regulations have affected the expression of cloned genes for heterologous single protein production (*71*).

Concluding Remarks

Recent work on pectinase microbial production has been oriented in three main directions: a) comparison between solid-state and submerged fermentation systems, b) genetic improvement of strains and c) optimization of culture conditions. Such work has confirmed earlier findings that solid-state technique provides higher enzyme yields than conventional submerged fermentation. A variety of approaches for mutant selection of superior pectinase producing strains has been developed, oriented mainly on the combined use of selection screens, parasexual recombination and protoplast fusion. Nevertheless, the use of advanced genetic engineering has been limited by problems related to unsolved problems of post-transcriptional modification and excretion of pectinases. The use of advanced statistical designs has helped to identify the optimal choice of cultural conditions.

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