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Expression and Partial Characterization of Alkaline Pectate Lyase A from *Paenibacillus barcinonensis* in a Glycosylation-Deficient Strain of *Saccharomyces cerevisiae*

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Abstract

The alkaline pectate lyase A from *Paenibacillus barcinonensis*, encoded by *pelA* (GenBank accession no. CAB40884), is an enzyme with high activity on pectin and potential application in sustainable industrial biotechnology. In this study, *pelA* was expressed in *Saccharomyces cerevisiae* by using different domains of the cell wall protein Pir4 as translational fusion partners. Given the presence of five potential N-glycosylation sites in the amino acid sequence coded by *pelA*, and two of them in conserved regions of class III pectate lyases, the effect of glycosylation on the enzymatic activity of the recombinant enzyme was investigated by expressing the recombinant fusion proteins in both standard and glycosylation deficient strains of *S. cerevisiae*. The correct targeting of the recombinant fusion proteins was confirmed by Western blot analysis using Pir-specific antibodies, whilst enzymatic activity on polygalacturonic acid was demonstrated on both plate assays and colorimetric assays. Maximum activities were over two and a half times higher when the enzyme was expressed in the glycosylation deficient strain, suggesting a better adaptation of this strain to the secretion of the functional enzyme.

Keywords: alkaline pectate lyase; *Paenibacillus barcinonensis*; *Saccharomyces cerevisiae*; Pir4 cell wall protein; secretion of recombinant proteins; sustainable bioprocessing

1. Introduction

Pectinases, naturally produced by plants and microorganisms, are enzymes capable of degrading pectin. Based on the degradation mechanism they can be classified in three groups: deesterifying enzymes, depolymerizing enzymes, and protopectinases [1]. Deesterifying enzymes (pectinesterases) catalyze the deesterification of the methyl groups in pectin, producing polygalacturonic acid or pectate. The depolymerizing enzymes or depolymerases can break the glycosidic bonds between the monomers of polygalacturonic acid by hydrolysis (hydrolases) or transelimination (lyases). Finally, the protopectinases are enzymes capable of solubilizing protopectin.

Within this group of heterogeneous enzymes, pectate lyases (EC 4.2.2.2.), depolymerizing enzymes capable of cleaving the α-1,4 glycosidic bonds linking monomers of galacturonic acid in pectin by transelimination, are especially important [2–4]. Based on their mode of action, they can be classified as endo- or exo-pectate lyases. Endo-pectate lyases randomly degrade links in the polygalacturonic acid chains, while exo-pectate lyases

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release sugars progressively from the ends of the chain [5]. Their most common substrate is polygalacturonic acid but can also degrade pectins with a low degree of methylation, making them indistinguishable from pectin lyases, except by the fact that pectate lyases require calcium for their activity [6,7].

Enzyme production constitutes a significant sector within industrial biotechnology, and pectinase production is estimated to represent up to 10% of the global enzyme market [8–11]. The industrial use of pectinases in the food industry dates back to 1930, initially in wine and fruit juice production, and continues today in more sustainable biotechnological processes [12–15], with most commercial preparations still applied in fruit juice production, specifically in the clarification step to reduce the turbidity caused by the presence of insoluble pectin in suspension. Furthermore, pectinases are used to decrease the viscosity of pulps, enhancing the performance of juice extraction, and to prevent gelling in the preparation of fruit concentrates [5]. These enzymes remain central in various food and beverage processes. In winemaking, pectinases can be added at the stage of pressing, facilitating the extraction of the must, or at the final stages after fermentation, aiding the clarification process, increasing the filtration rates, enhancing sensory properties, and reducing energy consumption and processing time, thereby improving the overall winemaking process and lowering costs [16–18]. Similarly, pectinases, together with other cell wall-degrading enzymes, can be used in vegetable oil production, reducing the need for chemical solvents [19]. Finally, pectinases play a very important role in the processing and production of coffee and tea. In tea production, the enzymatic treatment accelerates the fermentation process, while in coffee, the use of pectinolytic microorganisms during fermentation facilitates the removal of the mucilaginous layer surrounding the beans, enhancing flavour and aroma [20–22]. These applications illustrate the long-standing industrial relevance of pectinases and their growing potential for sustainable biotechnological processes.

Textile industries are a highly promising field of application for pectate lyases. Alkaline pectate lyases are increasingly used in textile industrial processing and degumming to facilitate the release of fibres from jute, flax, ramie, and other plants, offering an environmentally friendly alternative to conventional retting with harsh chemicals [23–26]. Degumming is normally performed by treatment with hot NaOH solutions in a process that is energy-intensive and highly polluting, whereas enzymatic degumming with pectate lyases offers milder conditions, limited fibre damage, and reduced environmental impact [27–32]. However, enzymatic degumming requires an alkaline environment at a moderate temperature to be effective, since the gum-like materials released by the process are soluble under these conditions [23,25], explaining the growing interest in alkaline pectate lyases [24,25,31–36].

Paenibacillus barcinonensis was isolated after enrichment culture in rice straw for two months from samples taken in a rice field in the Ebro River in Spain [37], and was found to be able to use xylan as the only carbon source [38], with XynA, an alkali stable xylanase, being the main xylanolytic enzyme. *P. barcinonensis* is characterized by the secretion of multiple polysaccharide depolymerizing enzymes with characteristics that make them good candidates for industrial use [39].

In this work, we describe the expression of *pelA* (GenBank accession no. CAB40884) from *P. barcinonensis*, an alkaline pectate lyase with activity on both polygalacturonic acid and highly methylated pectin [2], in a standard and a glycosylation-deficient [40] strain of *Saccharomyces cerevisiae*. Expression was achieved by fusing the *pelA* gene to *Pir4*, a gene in *S. cerevisiae* that codes Pir4, a cell wall protein of the Pir cell wall protein family of *S. cerevisiae* (Pir-CWPs), all of which share the presence of a signal peptide and a pro-peptide (Subunit I) that is processed at the Golgi by the Kex2p protease. The mature protein (Subunit II) includes a 19 amino acid repetitive domain and a conserved carboxy-terminus that contains

four cysteine residues at fixed positions [41,42]. Gene fusion with *Pir4* has been successfully applied to achieve the expression of many different recombinant proteins including an endoglucanase from *P. barcinonensis* [43]. The additional use of a yeast glycosylation-deficient strain was decided based on the presence of five potential N-glycosylation sites in the amino acid sequence coded by *pelA*, two of them in conserved regions of class III pectate lyases, since the potential glycosylation in any of these sites might affect the enzymatic activity of the recombinant enzyme. The questions we were trying to answer with the present work were twofold: Will the presence of specific N-glycosylation sites in *pelA* affect glycosylation, and will glycosylation affect stability, secretion, and enzymatic activity.

2. Materials and Methods

2.1. Strains and Media

Escherichia coli DH5α was used as the cloning host; it was cultivated in Luria Bertani broth supplemented with 100 µg of Ampicillin per millilitre when necessary. The standard *Saccharomyces cerevisiae* strains BY4741 (MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1) and *mnn9* (MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, ypl050c::kanMX4) used in this study were obtained from the EUROSCARF collection (Heidelberg, Germany). Table 1 presents a summary of the strains used in this study. Yeast strains were cultivated in YPD (1%yeast extract, 2% Bacto peptone, 2% glucose, pH 6.5), or synthetic minimal medium YNB: 0.7% yeast nitrogen base without amino acids, 2% glucose, pH 6.5 with amino acids added as required (uracil, 35 mg/L; methionine, 20 mg/L; histidine, 80 mg/L; leucine, 20 mg/L). For the growth of strains transformed with YEpLac195-based constructions, YNB supplemented with methionine, histidine, and leucine, but not uracil, was used. Cultivation was carried out on an orbital shaker at 28 °C and 180 revolutions per minute for 24–48 h.

Table 1. List of strains used in this study.

***S. cerevisiae* Strains**	**Genotype**	**Origin**
BY4741	MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1	EUROSCARF
mnn9	MATα, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1, ypl050c::kanMX4	EUROSCARF
P1-BY4741	MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, (YEplac195-*PIR4*/*pelA-Bgl*II)	This study
P2-BY4741	MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, (YEplac195-*PIR4/pelA-Sal*I)	This study
P3-BY4741	MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, (YEplac195-*PIR4/pelA-Bgl*II-*Sal*I)	This study
P1-*mnn9*	MATα, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1, ypl050c::kanMX4, (YEplac195-*PIR4/pelA-Bgl*II)	This study
P2-*mnn9*	MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, ypl050c::kanMX4, (*YEplac195-PIR4/pelA-Sal*I)	This study
P3-*mnn9*	MATα, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1, ypl050c::kanMX4, (YEplac195-*PIR4/pelA-Bgl*II-*Sal*I)	This study

2.2. Reagents

Agar, yeast extract, peptone, and yeast nitrogen base were purchased from Pronadisa (Madrid, Spain); DNA restriction and modification enzymes were from Thermo Fisher

Scientific. Phenylmethylsulphoyl fluoride (PMSF), Tris-base, HCl, and other buffer reagents were purchased from Sigma-Aldrich (Burlington, MA, USA) and from Panreac (Barcelona, Spain). Electrophoresis reagents were from Bio-Rad Laboratories. Nitrocellulose membranes, the chemiluminescence ECL reagents for developing Western immunoblots, and the goat anti-rabbit IgG-peroxidase were from Sigma-Aldrich. Polygalacturonic acid, ruthenium red, and all reagents for activity and assays were also purchased from Sigma-Aldrich.

2.3. Transformation of Strains and DNA Isolation

Basic DNA manipulation and transformation in *E. coli* were performed as described by Sambrook et al. 1989 [44]. Yeast transformation was carried out following the lithium acetate method [45,46]. Plasmid DNA from *E. coli* was prepared using the Quantum Prep ® Plasmid Miniprep Kit (Bio-Rad Laboratories, Hercules, CA, USA) and DNA fragments were purified from agarose gels using the Agarose Gel DNA Extraction Kit (Roche, Basel, Swithzerland).

2.4. Construction of the Gene Fusion Between Pir4 and pelA

Constructions P1 and P2 consisted in the insertion of the coding sequence of *Paenibacillus barcinonensis pelA* (GenBank accession no. CAB40884) gene, minus the 5′ region coding leader peptide, in the BglII and SalI sites of *Pir4* (ORF YJL158C; see www.yeastgenome.org for complete sequence (accessed on 4 May 2026)). For this, a 591 bp fragment of *pelA* was amplified from plasmid pBR322*pelA* using oligonucleotides including the restriction sites for the enzymes *Bgl*II and *Xho*I, designed so that the *pelA* fragment was inserted in-frame in *Pir4* in construction pIA1 [47], digested with *Bgl*II or *Sal*I, or both. Constructions P1 and P2 involved the in-frame insertion of *pelA* in *Pir4* whilst construction P3 involved the substitution of a fragment of *Pir4* by the coding sequence of *pelA*. Primers used to amplify *pelA* and confirm the constructions can be found in the Supplementary Material, Supplementary Table S1.

2.5. Isolation of Yeast Cell Wall Mannoproteins

Cell walls were purified and extracted with β-mercaptoethanol as follows: cells in the early logarithmic phase were harvested and washed twice in buffer A (Tris-HCl 10 mM, pH 7.4, 1 mM PMSF). The harvested biomass was resuspended in buffer A in a proportion of 2 mL per gram wet cell weight. Glass beads (0.45 mm in diameter) were added up to 50% of the final volume, and the cells were broken by shaking four times for 30 s, with 1 min intervals, in a CO_2-cooled MSK homogenizer (Braun Melsungen, Melsungen, Germany). Breakage was confirmed by phase contrast microscopy, and the walls were washed six to eight times in buffer A. The removal of non-covalently bound proteins was achieved by boiling the walls in buffer A containing 2% SDS (10 mL per gram of walls, wet weight) for 10 min, followed by six to eight washes in buffer A. The purified cell walls were finally resuspended in 10 mM ammonium acetate buffer, pH 6.3, containing 2% (*v*/*v*) β-mercaptoethanol (5 mL per gram of walls, wet weight), and incubated for 3 h at 30 °C in an orbital incubator at 200 revolutions per minute. The β-mercaptoethanol cell wall extract was separated from the cell walls by centrifugation and concentrated by lyophilisation.

2.6. SDS-Polyacrylamide Gels and Western Blot Analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [48] in 10% (*w*/*v*) polyacrylamide gels. The proteins separated by SDS-PAGE were transferred onto Hybond-C nitrocellulose membranes as described by Burnette [49]. Membranes were blocked overnight in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% non-fat milk. The blocked membranes were washed three times in TBST and incubated for 1 h in TBST containing an antibody that

reacts with Pir cell wall proteins [41] at a dilution of 1:5000. After another three washes in TBST, membranes were incubated for 20 min in TBST containing goat anti-rabbit IgG-peroxidase at a dilution of 1:12,000 and washed again in TBST. Finally, antibody binding was visualized on X-ray film by using the ECL method (Amersham/Sigma-Aldrich).

2.7. Determination of Pectate Lyase Activity

Pectate lyase activity was detected on 9 cm plates containing 1.25% polygalacturonic acid (Sigma-Aldrich), according to the method described by McKay [50]. A stock of 2.5% polygalacturonic acid, adjusted with potassium hydroxide to pH 5.5, was prepared and the medium in the plates contained 1% glucose, 1.25% polygalacturonic acid, 50 mM potassium phosphate (pH 5.5), yeast nitrogen base (6.7 g/L), the necessary amino acids, and 0.5% agar [51].

10 µL of a 1/10 dilution of overnight cultures adjusted to an OD of 1.5 were spotted on the plates with a separation of 1.5 cm. After 72 h incubation at 28 °C, the plates were washed with distilled water to remove the colonies and stained with 0.05% Ruthenium Red (Sigma-Aldrich) for 5 min, a purple halo appearing in areas where the polygalacturonate had been degraded by the enzyme activity.

The quantification of pectate lyase activity was carried out by evaluating the formation of unsaturated products from polygalacturonic acid at 232 nm [2,52].

The assay mixture contained 0.2% polygalacturonic acid in a final volume of 2.5 mL of 50 mM glycine buffer pH 10 containing either 2 mM $CaCl_2$ in the assays of strains based on BY4741, or 3 mM for the assays of those strains based on *mnn9*. After adding 500 µL of the test sample, generally the culture medium supernatant of cultures adjusted at an OD of 1.5, and the increase experienced by the absorbance at 232 nm was measured. One unit of enzyme activity was defined as the amount of enzyme which produced 1 µmol of unsaturated product per minute under assay conditions. The determinations were repeated three times, and each sample was determined in duplicate for each separate experiment.

2.8. Statistical Analysis

Means and standard deviation were determined. Mean comparisons were performed by using the Student's *t*-test when two independent sets of data were compared, and a post hoc Bonferroni test when more than two independent sets of data were analyzed. Statistical significance thresholds were $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, from lower to higher significance. All analyses were performed with the software SPSS version 28.0.1.1 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Pir4/pelA Gene Fusion Strategies and Expression in Saccharomyces cerevisiae

For expression in *Saccharomyces cerevisiae*, three different gene fusion strategies were used to achieve the targeting of the pectate lyase A from *Paenibacillus barcinonensis* either to the cell wall or to the growth medium. The first two fusion strategies consisted of inserting the coding sequence of the *pelA* gene, minus the 5′ fragment coding the leader peptide, in the naturally occurring *Bgl*II site close to the amino-terminus of subunit II of *Pir4*, or in the also naturally occurring *Sal*I site close to the carboxy-terminus of subunit II to achieve cell wall retention (Figure 1). In the third case, the *Bgl*II-*Sal*I region of *Pir4* was substituted by the coding sequence of the *pelA* gene, lacking the leader peptide, to achieve the secretion of pectate lyase A to the growth medium (Figure 1).

The three constructs, named P1 (*Bgl*II, cell wall targeting), P2 (*Sal*I, cell wall targeting) and P3 (*Bgl*II-*Sal*I, secretion to the growth medium), based in YEplac195, were transformed into the parental BY4741 and the *mnn9* glycosylation-deficient strains of *S. cerevisiae*. The

resulting recombinant strains were assayed for pectate lyase activity on polygalacturonic acid plates (Figure 2). As can be deduced from the halos formed around the colonies, most constructs conferred pectate lyase activity to both the standard and the glycosylation deficient strains. However, the highest detectable activity was observed in the strains carrying the P3 construct, designed to secrete the recombinant enzyme to the growth medium, with the *mnn9* strain showing the highest signal.

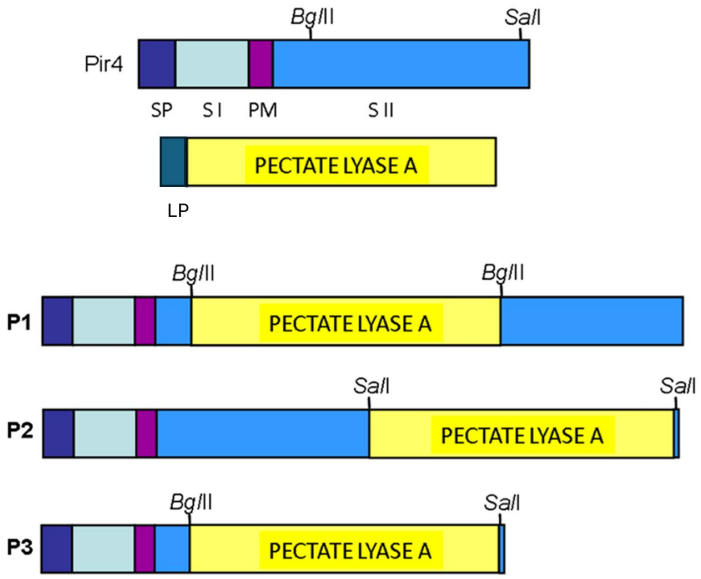


Figure 1. Schematic representation of the *PelA* and *Pir4* genes together with the P1, P2, and P3 gene fusions. SP, signal peptide; SI, subunit I; PM, Pir motive; SII, subunit II; LP, leader peptide. Mature Pir4 corresponds to subunit II after the removal of subunit I by Kex2 protease at the Golgi.

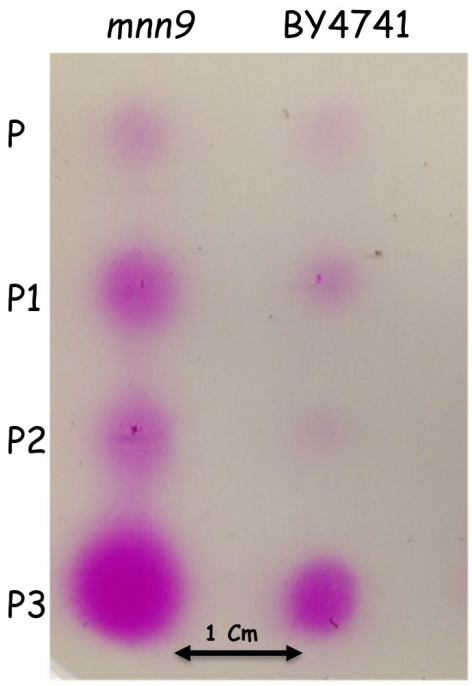


Figure 2. Pectinase plate assay of the different strains harbouring constructs P1 to P3 on plates containing 1.25% polygalacturonic acid as substrate. Colonies were grown for 72 h, washed from plates with distilled water, and plates were stained with Ruthenium Red; purple halos around colonies represent areas where polygalacturonic acid had been degraded by enzyme activity. Strains based on *mnn9* and BY4741. P corresponds to the untransformed parental strains.

3.2. A Study of the Localization of the Recombinant Pir4–pelA Fusion Proteins by Western Blot Analysis

To confirm that the Pir4–pelA fusion proteins derived from constructs P1, P2, and P3 were being correctly targeted to the cell wall or the growth medium, β-mercaptoethanol extracts of purified cell walls of the different strains, and concentrated samples of growth medium, were probed by Western blot using an antibody that reacts with Pir-CWPs of *S. cerevisiae* [41]. In the standard *S. cerevisiae* BY4741 strain (Figure 3A), specific bands of approximately 70–80 kDa were detected in the β-mercaptoethanol extracts from purified cell walls of the P1 and P2 transformed strains. These bands are absent in the extracts of either the parental or the P3 transformed strain, indicating the correct targeting of the Pir4–pelA fusions derived from constructs P1 and P2. The apparent molecular weight was slightly higher than the expected size, corresponding to the observed size of Pir4 (around 40–45 kDa) plus the 20 kDa of pelA, but not sufficiently increased to suggest N-glycosylation. Analysis of the growth medium supernatants revealed the presence of a single band of approximately 55 kDa exclusively in the P3- transformed strain (Figure 3B), consistent with secretion of the Pir4–pelA fusion protein. This band was not present in the growth medium of the parental strain or the P1 or P2 transformed strains (Figure 3B), indicating the correct expression and targeting of Pir4–pelA fusion protein to the growth medium. Comparable analysis of the β-mercaptoethanol extracts from purified cell walls in the P1 and P2 transformed glycosylation-deficient *mnn9* strain revealed the presence of bands of similar size to those detected in the BY4741 strain transformed with same constructs, that is, in the 70–80 kDa range (Figure 4A), suggesting the absence of N-glycosylation. The size of the Pir4–pelA fusion protein detected in the growth medium of the P3-transformed *mnn9* strain was also similar to that detected in the P3-transformed BY4741 strain (Figure 4B), further suggesting the absence of N-glycosylation.

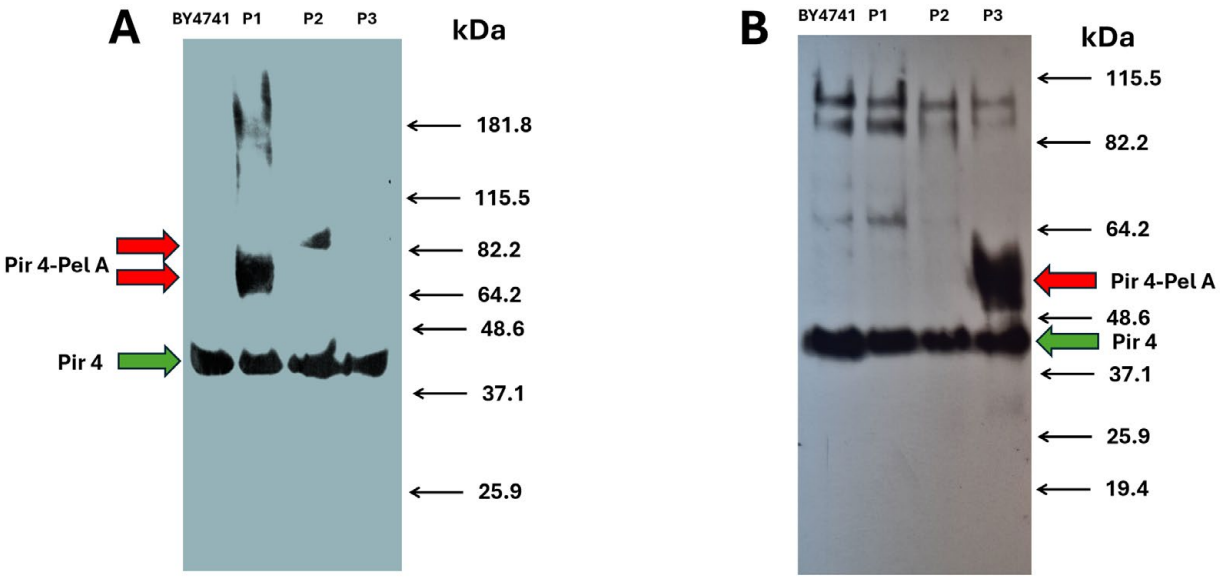


Figure 3. Western immunoblot, developed with a polyclonal antibody that reacts with Pir4 protein, of the material extracted with β-mercaptoethanol from purified cell walls (**A**), and of the supernatant of the concentrated culture medium (**B**), of the BY4741 strain transformed with constructions P1–P3. Lane 1 corresponds to the untransformed BY4741parental strain. Red arrows point to Pir4–pelA fusion proteins. Native Pir4 is indicated with a green arrow.

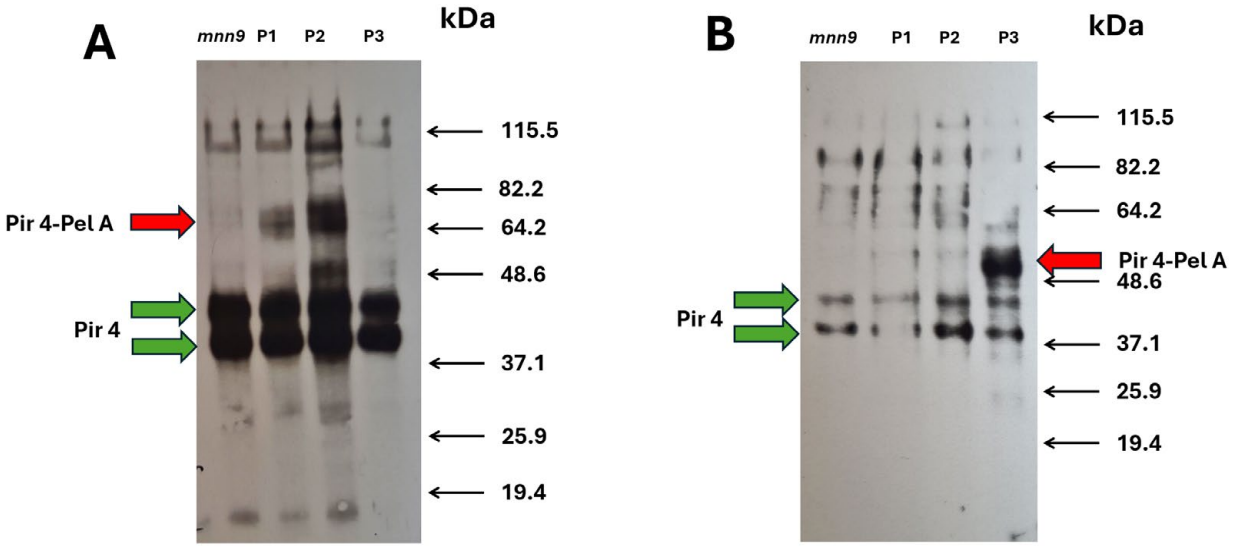


Figure 4. Western blot, developed with a polyclonal antibody that reacts with Pir4 protein, of the material extracted with β-mercaptoethanol from purified cell walls (**A**), and of the supernatant of the concentrated culture medium (**B**) of the *mnn9* strain transformed with constructions P1–P3. Lane 1 corresponds to the untransformed *mnn9* parental strain. Red arrows point to Pir4–pelA fusion proteins. Native Pir4 (green arrows) appears as a double band due to incomplete processing by Kex2 in this strain.

3.3. Quantification of Pectate Lyase Activity Associated with the Different Strains

Pectate lyase activity was quantified by determining the formation of unsaturated products from polygalacturonic acid. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of unsaturated product per minute under the assay conditions. Although the enzyme had been previously characterized as to its optimum pH and Ca^{2+} requirements [2], we evaluated whether fusion to Pir4 and the heterologous expression in *S. cerevisiae* affected these properties. As expected, the levels of activity in the growth medium of the strains harbouring the P1 and P2 constructions were low, consistent with the targeting of the enzyme to the cell wall. As for the Ca^{2+} requirements (Figure 5A,B), the case of the *mnn9* strain harbouring the P3 construction (2 mM) was clearly different from the Ca^{2+} requirements originally described for the enzyme (0.5 mM). These differences were statistically significant between the 2 mM and all the rest of the concentrations tested ($p < 0.001$). However, in the case of the BY4741 harbouring the P3 construction, although the mean maximum of activity was reached at 3 mM, the differences with the rest of the $CaCl_2$ concentrations was not statistically significant ($p > 0.05$) Regarding pH dependence, following a preliminary screening, enzymatic activity was monitored at pH 7 and pH 10. Relevant activity was detected exclusively at pH 10 (Figure 6A,B), with statistically significant differences ($p = 0.015$ for BY4741 and $p = 0.006$ for *mnn9*), in agreement with the optimal pH previously described for this enzyme [2]. Finally, pectate lyase activity levels measured under the optimized conditions are shown in Figure 7. As anticipated, the levels of activity in the growth medium of the strains transformed with constructions P1 and P2 were relatively low. In contrast, strains expressing construct P3, which directs the secretion of the enzyme into the culture medium, exhibited substantially higher activity levels, with up to a threefold increase observed in the *mnn9* strain. This difference in the activity expressed in the two strains harbouring the P3 construction was statistically significant ($p = 0.019$).

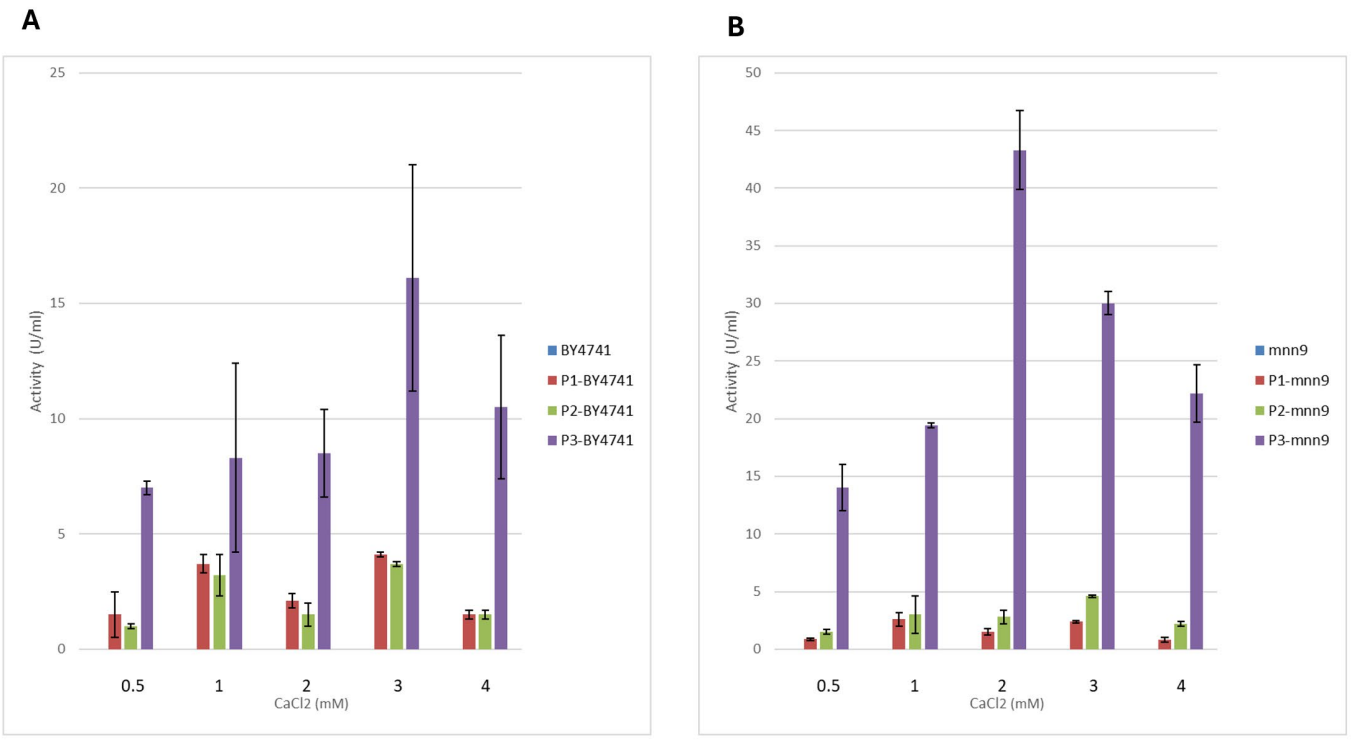


Figure 5. Influence of the concentration of added calcium to the pectate lyase activity of the BY4741-based strains (**A**) and *mnn9*-based strains (**B**) at pH 10. Bars represent means and standard deviations. A post hoc Bonferroni test was used for statistical analysis. Differences in activity were statistically significant in the case of the *mnn9* strain harbouring the P3 construction between the values obtained at 2 mM $CaCl_2$ and all the remaining concentrations tested ($p < 0.001$). Differences in activity were not statistically significant in the case of the BY4741 strain harbouring the P3 construction between the values obtained at 3 mM $CaCl_2$ and all the remaining concentrations tested ($p > 0.05$).

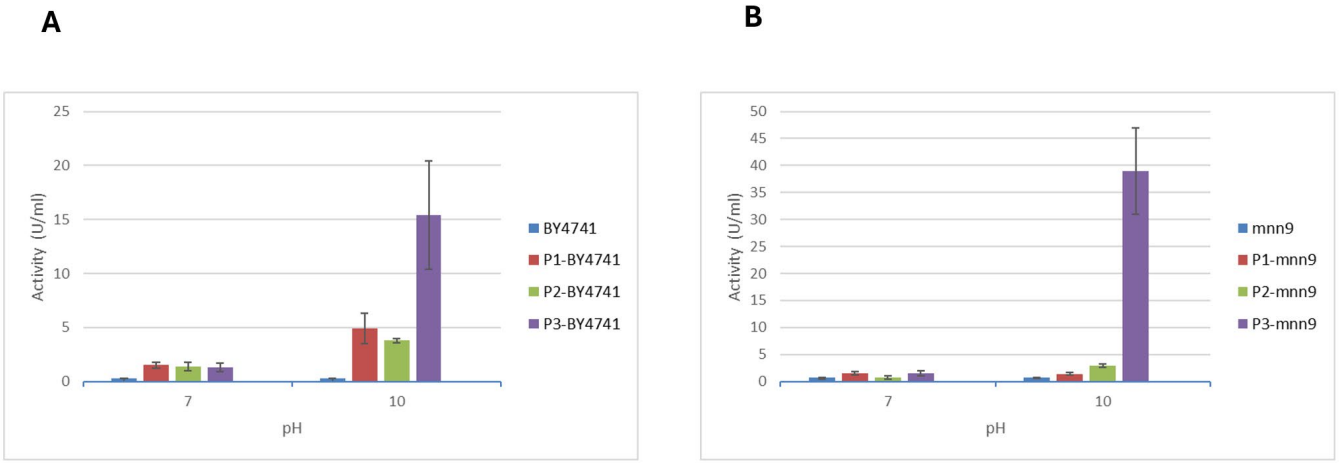


Figure 6. Effect of pH on pectate lyase activity of BY4741-based strains (**A**) and *mnn9*-based strains (**B**). Bars represent means and standard deviations. Student's *t* test was used for statistical analysis. Differences in activity according to pH were statistically significant ($p = 0.015$ for BY4741 and $p = 0.006$ for mnn9).

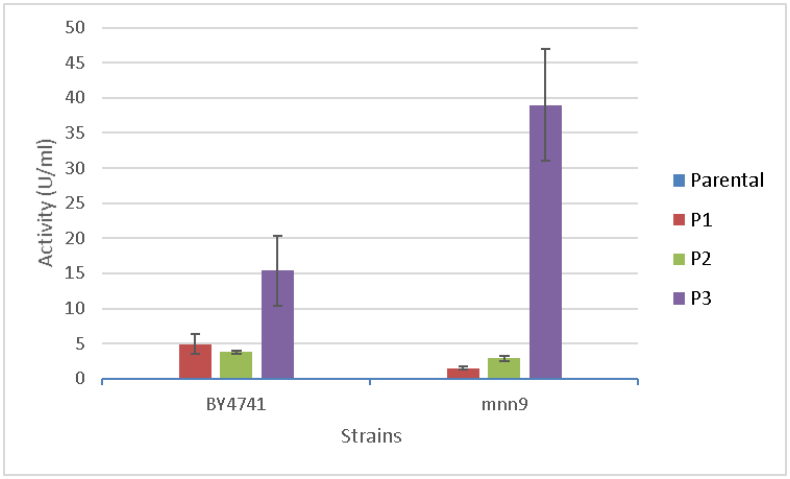


Figure 7. The pectate lyase activity of the growth culture supernatants from the BY4741- and *mnn9*-based strains. Bars represent means and standard deviations. The Student's *t*-test was used for statistical analysis. The difference in the activity expressed in the two strains harbouring the P3 construction was statistically significant ($p = 0.019$).

4. Discussion

Pectinolytic enzymes, including pectate lyases, have been traditionally used in the production of fruit juice and wine but, because of their versatility, these enzymes have found application not only in the food industry [17,22,28] but also in the paper and textile industries [35].

Previous studies have allowed the characterization of pectate lyase A from *Paenibacillus barcinonensis*, a Class III alkaline pectate lyase with activity on highly methylated pectin, and its expression in *Escherichia coli* [2,53]. The aim of the present work was the expression of the enzyme in *S. cerevisiae*, to study the possible effect of glycosylation and the characteristics of the enzyme when expressed in a standard and a glycosylation deficient strain of *S. cerevisiae*, and to compare the level of expression with that achieved in a prokaryotic host. The yeast expression of bacterial alkaline pectate lyases is increasingly attracting attention for its potential application in ramie degumming, as yeast does not secrete cellulases that would damage the vegetal fibres and it is possible to increase the production of the enzyme in comparison with the original host [22,23,35]. In this context, *S. cerevisiae* represents an attractive alternative host due to its genetic tractability, robustness, and industrial relevance.

In this work, we employed an expression system based on creating gene fusions with the *Pir4* gene, coding a cell wall protein of *S. cerevisiae*, which allows the specific targeting of the recombinant protein either to the cell wall or to the culture medium [43,54,55]. A total of three gene fusions were created, two of them (P1 and P2) intended to target the recombinant protein to the cell surface, and a third (P3) designed to determine secretion. The three constructions were then transformed into both a standard (BY4741) and a glycosylation-deficient (*mnn9*) strain of *S. cerevisiae*.

Analysis of the concentrated culture medium of the different strains confirmed that only those strains transformed with construction P3 secreted the recombinant protein, as identified by the specific antibody, with a size compatible with that expected after removing 365 bp from the sequence of *Pir4* and replacing it with 591 bp of the coding sequence of the pectate lyase A, with a final size slightly higher than that of Pir4. The observed size, slightly larger than native Pir4, was consistent with the predicted fusion protein. Importantly, no differences in size between the fusion proteins expressed in the parental and the glycosylation-deficient strains were observed, suggesting that, despite the presence of five potential N-glycosylation sites in the amino acid sequence of pectate

lyase A, the recombinant protein does not seem to be glycosylated when expressed in *S. cerevisiae*. If this was the case, it would suggest the limited accessibility of the N-X-S/T motifs or rapid folding of the bacterial enzyme within the yeast secretory pathway.

Although our primary interest was the secretion of the enzyme, the aim of the constructions that included the cell wall anchoring domain was to explore the possibility of immobilizing the enzyme, a strategy that in some cases allows the separation of the enzyme from the reaction mixture and its reuse, and to study the effect of the immobilization on the enzyme itself. Correct targeting of the recombinant protein to the cell surface was confirmed by studying the β-mercaptoethanol extracts from the purified walls of the standard (BY4741) and the glycosylation-deficient (*mnn9*) strain of *S. cerevisiae*, containing the different constructions. As expected, those strains transformed with constructions P1 and P2, both BY4741 and *mnn9*, showed specific bands that reacted with the antibody which were not present in the corresponding extracts of neither the parental strains, nor the strains transformed with the P3 construction. These bands had a size compatible with that expected of the fusion protein, although there appeared to be a slight difference in size between the bands detected in the strains based on the BY4741 and those based on the glycosylation-deficient *mnn9* strain. However, this difference opposes expectations of the N-glycosylation, and may be attributable to incomplete processing by the Kex2p protease of subunit I of Pir4 in this strain [41], which causes the fusion protein, also including subunit I, to be slightly larger in size. Again, these results suggest that none of the potential sites of N-glycosylation in *pelA* are glycosylated.

Having located the fusion protein, we proceeded to evaluate the pectate lyase activity shown by the different strains. Plate assays showed a marked difference between the levels of activity shown by the *mnn9*- and BY4741-based strains, with the *mnn9*-based strains showing higher levels. This could speculatively be attributed to increased cell wall permeability in the *mnn9* mutant, which may facilitate enzyme diffusion.

Before quantifying the enzymatic activity associated with each of the strains, we proceeded to test whether the expression in *S. cerevisiae* modified the Ca^{2+} ion requirements or the pH optimum of the enzyme which had previously been characterized in *E. coli*. Assays were performed both on *mnn9*- and BY4741-based strains. While the optimum pH was unchanged, we found a change in the requirements of the Ca^{2+} ion, as the maximum activity on polygalacturonic acid was obtained at a 3 mM Ca^{2+} concentration in BY4741 strains and at 2 mM in those based on the *mnn9* strain. This is in contrast with the 0.5 mM optimum Ca^{2+} concentration in *E. coli*. Such differences likely reflect host-dependent effects on enzyme conformation or microenvironment. In any case, the maximum activity obtained was always higher in the *mnn9*-based strains, with the P3 transformed strain giving the highest activity.

The determination of activity in the cell free concentrated culture medium of the different strains showed the absence of detectable activity in the untransformed parental strains. Strains transformed with either P1 and P2 constructions, both BY4741- and *mnn9*-based, showed low levels of activity, surely a consequence of the recombinant protein being localized mainly in the cell wall and the determination being performed in the culture supernatant, whereas those strains transformed with the P3 construction obtained the highest levels of activity, reaching 39 UIA/mL culture in the *mnn9*-based strain and 15.5 UIA/mL culture in the BY4741-based strain. These results emphasize the difference in enzymatic activity between the *mnn9*-based P3 transformed strain and the BY4741-based P3 transformed strain. The 2.5 times higher activity detected in the *mnn9*-based strain probably reflects an increased concentration of the enzyme in the medium, related to the increased permeability of the *mnn9* cell wall to the passage of proteins [56]. In absolute

terms, the activity results obtained suggest that the expression system is quite effective, even using a selective medium such as YNB where cell densities remain relatively low.

While the recombinant expression of pectinases with potential biotechnological applications has attracted considerable interest [2,23,24,33–35,53,57], pectate lyase expression in *S. cerevisiae* is much less common and usually involves the design of complex expression and secretion cassettes in which promoters and signal sequences of different bacteria and yeasts are combined [17,58]. In the specific case of alkaline pectate lyases, expression has been successful in *Pichia pastoris* [22,25] but, to our knowledge, this is the first report describing the expression of an alkaline pectate lyase with biotechnological potential in *S. cerevisiae* using a simple and well-established Pir-based system.

In conclusion, this study demonstrates for the first time the expression, secretion, and/or immobilization of alkaline pectate lyase A from *P. barcinonensis* in *S. cerevisiae* and provides a partial functional characterization of the enzyme in this novel host, highlighting the potential of Pir-mediated secretion or cell wall anchoring strategies for industrial enzyme production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres17060106/s1, Table S1: Primers used in this study.

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