Article

Not peer-reviewed version

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

María Mormeneo , Ismaïl Moukadiri , Jesús Zueco *

Posted Date: 2 April 2026

doi: 10.20944/preprints202604.0070.v1

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

 doi:10.20944/preprints202604.0070.v1

Article

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

María Mormeneo [1], Ismaïl Moukadiri [2] and Jesús Zueco [1,*]

[1] Unidad de Microbiología, Facultad de Farmacia, Universidad de Valencia Avda. Vicente Andrés Estellés s/n, 46100 Burjassot (Valencia), Spain

[2] Engineering School in Biomedical and Biotechnology, Euromed University of Fes, Eco-Campus UEMF, Route de Meknes (RN6, Rond-Point Bensouda), 30070 Fez, Morocco

* Correspondence: jesus.zueco@uv.es

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*, 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*. 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. Hyper- glycosylation of the enzyme when expressed in the standard strain of *S. cerevisiae* did not occur, however 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. Notably, pectate lyase activity was approximately fourfold higher when the *pelA* gene was expressed in this yeast strain compared to its expression in a prokaryotic host such as *Bacillus subtilis* or *Escherichia coli*.

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 are able to 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

 doi:10.20944/preprints202604.0070.v1

classified as endo- or exo-pectate lyases. Endo-pectate lyases randomly degrade links in the polygalacturonic acid chains, while exo-pectate lyases release sugars progressively from the ends of the chain [5]. Their most common substrate is polygalacturonic acid but can also degrade pectins with 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 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 [24,25,31,32,35,36]

In this work, we describe the expression of *pelA* (GenBank accession no. CAB40884) from *Paenibacillus barcinonensis*, an alkaline pectate lyase with activity on both polygalacturonic acid and highly methylated pectin [2], in a standard and a glycosylation- deficient [37] strain of *Saccharomyces cerevisiae*.

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 100μg 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, 35mg/l; methionine, 20mg/l; histidine, 80mg/l; leucine, 20mg/l).

 doi:10.20944/preprints202604.0070.v1

Cultivation was carried out on an orbital shaker at 28ºC and 180 revolutions per minute for 24-48 hours.

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-BglII)	This study
P2-BY4741	MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, (YEplac195-PIR4/pelA-SalI)	This study
P3-BY4741	MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, (YEplac195-PIR4/pelA-BglII-SalI)	This study
P1-mnn9	MATα, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1, ypl050c::kanMX4, (YEplac195-PIR4/pelA-BglII)	This study
P2-mnn9	MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, ypl050c::kanMX4, (YEplac195-PIR4/pelA-SalI)	This study
P3-mnn9	MATα, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1, ypl050c::kanMX4, (YEplac195-PIR4/pelA-BglII-SalI)	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 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* was performed as described by Sambrook *et al*. 1989 [38]. Yeast transformation was carried out following the lithium acetate method [39,40]. Plasmid DNA from *E. coli* was prepared using the Quantum Prep ® Plasmid Miniprep Kit (Bio-Rad Laboratories) and DNA fragments were purified from agarose gels using the Agarose Gel DNA Extraction Kit (Roche).

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 pel*A (GenBank accession no. CAB40884) gene (Soriano et al. 2000), minus the 5´region coding leader peptide, in the *Bgl*II and *Sal*I sites of *PIR4* (ORF YJL158C; see www.yeastgenome.org for complete sequence). For this, a 591-bp fragment of *pel*A was amplified using oligonucleotides PBGL5-PBGL3 and PSAL5-PSAL3 (Table 2), and plasmid pBR322pelA as template. The oligonucleotides included the restriction sites for the enzymes *Bgl*II and *Xho*I, which leaves overhangs compatible with the overhangs left by *Sal*I, and had been designed so that the *pel*A fragment was inserted in-frame in *PIR4* in construction pIA1 [41]. The PCR fragments amplified using Expand High Fidelity DNA Polymerase (Roche) were subcloned in the *Hinc*II site of pUC18; digested out with *Bgl*II or *Sal*I and inserted in pIA1 previously digested with *Bgl*II or *Sal*I. The correct orientation of the inserts was monitored by PCR performed directly on the colonies of transformants using

 doi:10.20944/preprints202604.0070.v1

oligonucleotides PIR5′ and the corresponding 3′oligonucleotides used in the amplification of *pel*A (Table 2).

Construction P3 involved the substitution of a fragment of *PIR4* by the coding sequence of *pel*A. In this construction, the 591-bp fragment of *pel*A was amplified using oligonucleotides PBGL5 and PSAL3 (Table 2) and plasmid pBR322*pel*A as template, subcloned in the *Hinc*II site of pUC18; digested out with *Bgl*II and *Sal*I and subcloned in pIA1 previously digested with enzymes *Bgl*II and *Sal*I with the loss of 365 bp of the 5′region of the *PIR4* ORF.

Table 2. Primers used to amplify the coding sequence of the pelA gene minus the region coding the leader peptide (PB5-3, PS5-3) and for confirmation of the orientation of inserts (PIR5-3). Restriction sites used in subcloning are underlined.

PRIMERS	SEQUENCES
PIR5	TGCATTCCATACGATTTCCACGGG
PIR3	GTGTATATTAAAGGCTGCATGTGG
PB5	TATATAAGATCTTAGCGCCAACCGTCGTCAATTC
PB3	TATAGGAGATCTTATACTGTGTATTTCCGGACTG
PS5	AAGCCTCTCGAGGCGCCAACCGTCGTCAATTC
PS3	GATCCCCTCGAGATACTGTGTATTTCCGGACTGG

2.5. Isolation of 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 10mM, pH 7.4, 1mM 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 seconds, with 1-minute intervals, in a CO_2-cooled MSK homogenizer (Braun Melsungen, Germany). Breakage was confirmed by phase contrast microscopy, and the walls were washed six to eight times in buffer A. Removal of non-covalently bound proteins was achieved by boiling the walls in buffer A containing 2% SDS (10ml per gram of walls, wet weight) for 10 minutes, followed by six to eight washes in buffer A. The purified cell walls were finally resuspended in 10mM ammonium acetate buffer, pH 6.3, containing 2% (v/v) β-mercaptoethanol (5 ml per gram of walls, wet weight) and incubated for 3 hours at 30ºC 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 [42] in 10% (w/v) Polyacrylamide gels. The proteins separated by SDS-PAGE were transferred onto Hybond-C nitrocellulose membranes as described by Burnette [43]. 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 [44] at a dilution of 1: 5,000. 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 plates containing 1.25% polygalacturonic acid (Sigma-Aldrich) according to the method described by McKay [45]. A stock of 2.5% polygalacturonic acid, adjusted with potassium hydroxide to pH 5.5, was prepared and medium in the plates contained 1%

 doi:10.20944/preprints202604.0070.v1

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 [46].

After 72 h incubation at 28 °C, the plates were washed with distilled water to remove the colonies and stained with Ruthenium Red (Sigma-Aldrich) for 5 min., a purple halo appearing in areas where the polygalacturonate had been degraded by the enzyme activity.

Quantification of pectate lyase activity was carried out by evaluating the formation of unsaturated products from polygalacturonic acid at 232 nm [2,47].

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, 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.

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. Pir4 belongs to the family of PIR cell wall proteins 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 [44,48]. 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 *pelA* gene, lacking the leader peptide, to achieve secretion of pectate lyase A to the growth medium (Figure 1). This combination of strategies has been successfully applied previously to achieve expression of many different recombinant proteins previously [49–54]. Moreover, recent structural and functional studies of the Pir protein family have renewed interest in PIR-based fusion partners as efficient anchors for yeast cell wall display of heterologous proteins [55].

 doi:10.20944/preprints202604.0070.v1

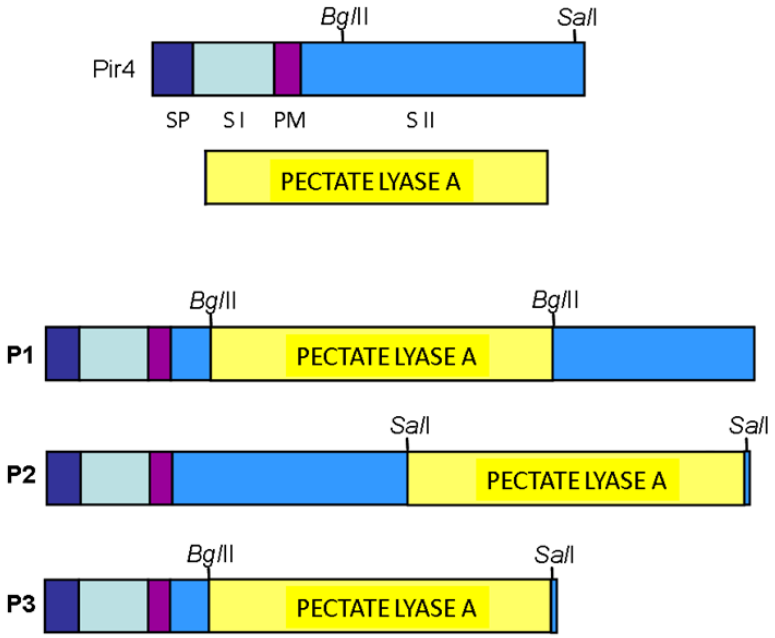

Figure 1. Schematic representation of the PelA and PIR4 genes together with the P1, P2 and P3 gene fusions. SP, signal peptide; SI, subunit I; PM, PIR motive; SII, subunit II; LP, leader peptide. Mature Pir4 corresponds to subunit II after removal of subunit I by Kex2 protease at the Golgi.

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 YEplac112, 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 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.

 doi:10.20944/preprints202604.0070.v1

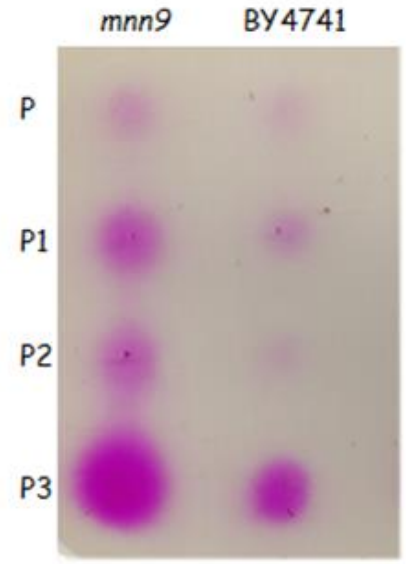

Figure 2. Pectinase plate assay of the different strains harbouring constructs P1 to P3 on plates containing 1.25% polygalacturonic acid as substrate. Colonies were grown for 72 hours, washed from the plates with distilled water and the plates were stained with Ruthenium Red; purple halos around the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

3.2. 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* [44]. 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 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 PelA–Pir4 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 the PelA-Pir4 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 KDda 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.

 doi:10.20944/preprints202604.0070.v1

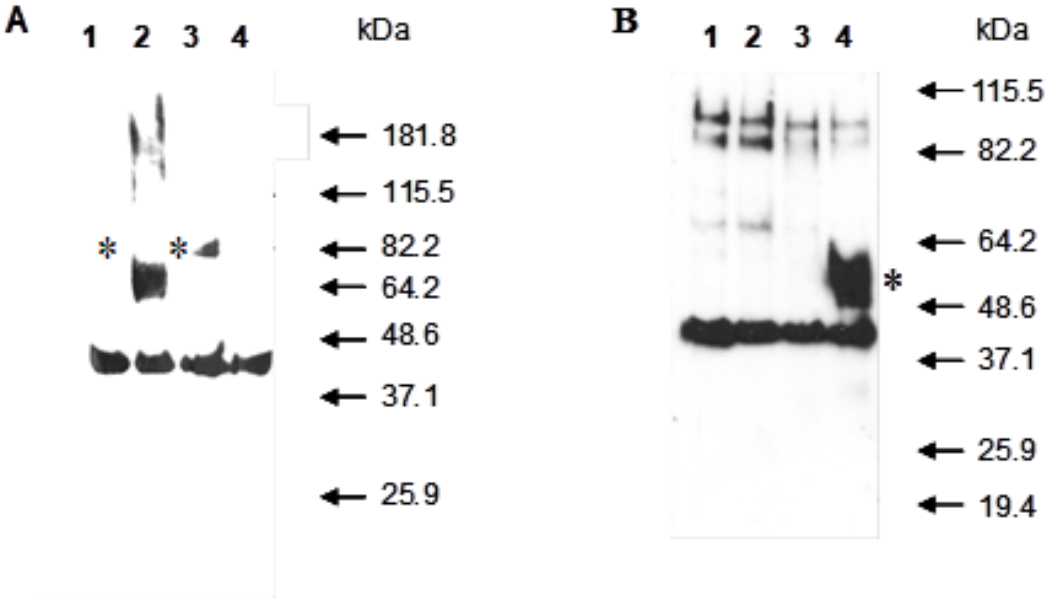

Figure 3. Western immunoblot, developed with a polyclonal antibody that reacts with Pir4 protein, of the material extracted with β-mercaptoethanol from purified cell walls (A), and of the supernatant of the concentrated culture medium (B), of the BY4741 strain transformed with constructions P1-P3 (lanes 2-4). Lane 1 corresponds to the untransformed BY4741parental strain. * Pir4-pelA fusion protein.

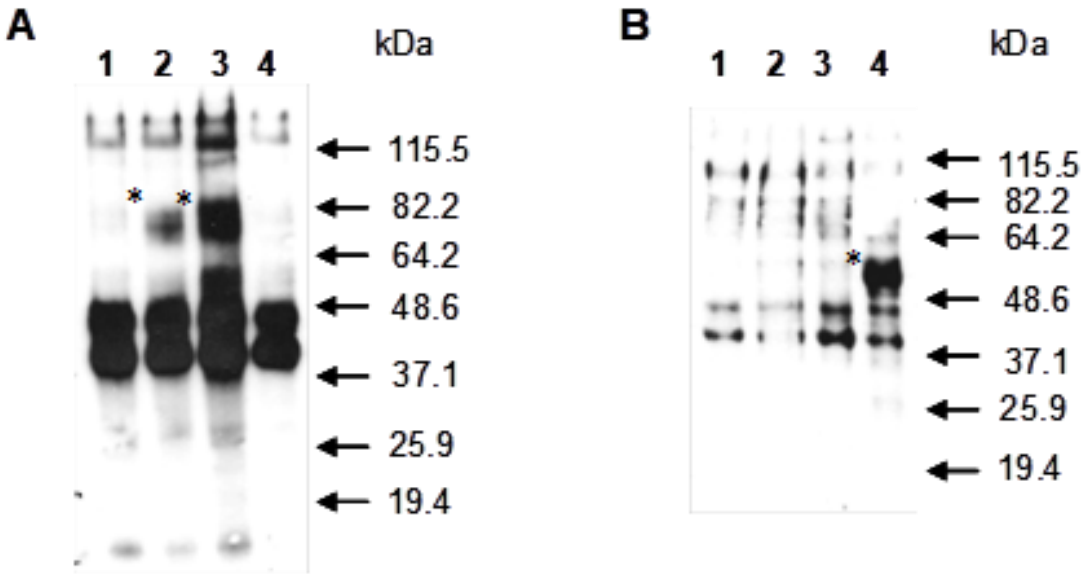

Figure 4. Western blot, developed with a polyclonal antibody that reacts with Pir4 protein, of the material extracted with β-mercaptoethanol from purified cell walls (A), and of the supernatant of the concentrated culture medium (B), of the mnn9 strain transformed with constructions P1-P3 (lanes 2-4). Lane 1 corresponds to the untransformed mnn9 parental strain. * Pir4-pelA fusion protein.

3.3. Quantification of Pectate Lyase Activity Associated to 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

 doi:10.20944/preprints202604.0070.v1

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. The Ca^{2+} requirements in the case of the strains harbouring the P3 construction were slightly different in the case of the BY4741 (3 mM) and *mnn9* (2 mM) strains (Figure 5A and B), and both differed from the Ca^{2+} requirements originally described for the enzyme (0.5 mM). Regarding pH dependence, following a preliminary screening, enzymatic activity was monitored at pH 7 and pH10. Significant activity was detected exclusively at pH10 (Figure 6A and B), 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 7A and B. 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 secretion of the enzyme into the culture medium, exhibited substantially higher activity levels, with up to a threefold increase observed in the *mnn9* strain. Notably, these activity levels exceeded those previously reported for expression of the enzyme in *Escherichia coli* or *Bacillus subtilis* [56].

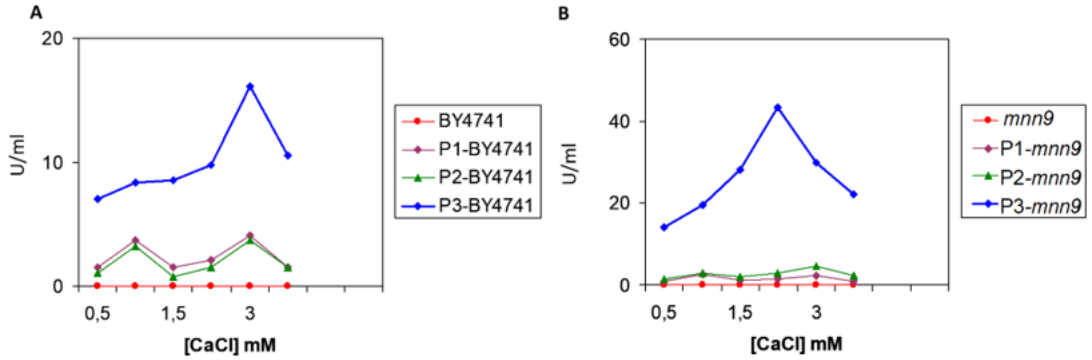

Figure 5. Influence of the concentration of added calcium to the pectate lyase activity of the BY4741 based strains (A) and mnn9 based strains (B) at pH 10.

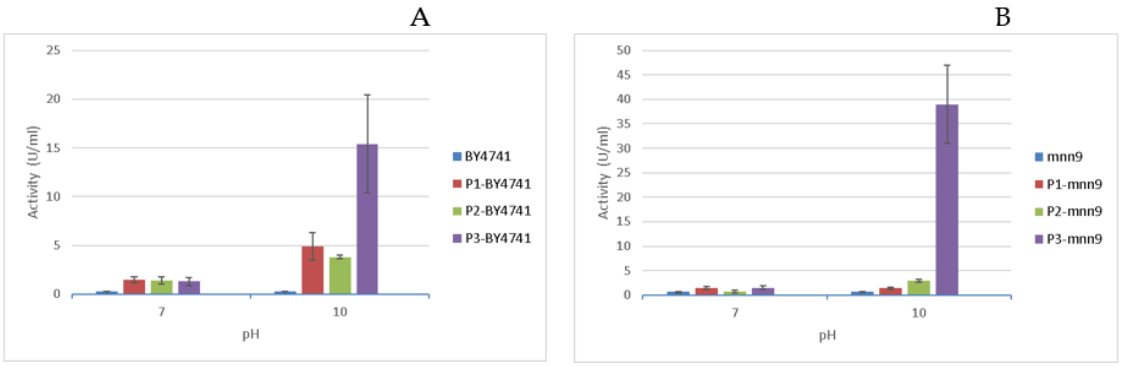

Figure 6. Effect of pH on pectate lyase activity of the BY4741based strains (A) and mnn9 based strains (B).

 doi:10.20944/preprints202604.0070.v1

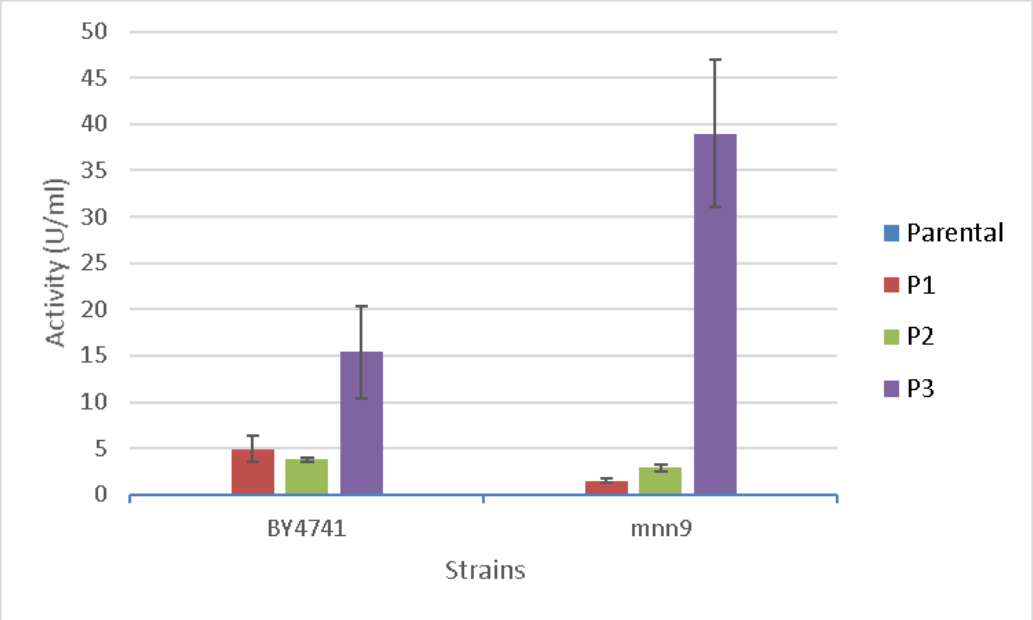

Figure 7. Pectate lyase activity of the growth culture supernatants from the BY4741 and mnn9 based strains.

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* [2,57]. 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. Yeast expression of bacterial alkaline pectate lyases is increasingly attracting attention for its potential application in ramie degumming as yeast do 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 in 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 [49–54]. 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 in 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 is not glycosylated when expressed in *S. cerevisiae*. This lack of

 doi:10.20944/preprints202604.0070.v1

glycosylation may reflect limited accessibility of the N-X-S/T motifs or rapid folding of the bacterial enzyme within the yeast secretory pathway.

Correct targeting of the recombinant protein to the cell surface was confirmed by studying the β-mercaptoethanol extracts from 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 for the fusion protein, although there appeared to be a slight difference in size between the bands detected in the strains based on the BY4741 in comparison to those based on the glycosylation-deficient *mnn9* strain. However, this difference goes in the opposite sense of that expected in case of N-glycosylation and may be attributable to incomplete processing by the Kex2p protease of subunit I of Pir4 in this strain [44], 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 *pel*A is 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. Since there not seem to be a difference in glycosylation levels, this difference could tentatively be attributed to the presence of the 43 amino acids of the Pir4 subunit I at the amino-terminus of the fusion protein in the *mnn9* based strains, which may contribute to the stability of the enzyme. Alternatively, increased cell wall permeability in the *mnn9* mutant may facilitate enzyme release or substrate accessibility.

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 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 0.5mM 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, probably a consequence of the recombinant protein being localized mainly in the cell wall, 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 reflecting slight changes in the conformation of the enzyme or the increased permeability of the *mnn9* cell wall to the passage of proteins [58].

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. By comparison, the expression of pectate lyase A in *B. subtilis* MW15 / pMS-RA-P22 resulted in 11.37 UIA / ml (Soriano, 2004), a lower level than in the P3 transformed *S. cerevisiae* BY4741 strain and around 3.5 times lower that in the P3 transformed *S. cerevisiae mnn9* strain. Taken together, these results position *S. cerevisiae*, and particularly the *mnn9* strain, as a competitive host for alkaline pectate lyase production.

While recombinant expression of pectinases with potential biotechnological applications has attracted considerable interest [2,23,24,33–35,57,59], pectate lyase expression in *S. cerevisiae* is much

 doi:10.20944/preprints202604.0070.v1

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 [17,60]. 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 anchoring strategies for industrial enzyme production.

Author Contributions: "Conceptualization, JZ and IM.; methodology, MM.; validation, MM and JZ; formal analysis, MM, IM and JZ; investigation, MM.; resources, JZ; data curation, JZ.; writing—original draft preparation, JZ.; writing—review and editing, MM, IM and JZ.; visualization JZ and IM.; supervision, JZ.; project administration, JZ.; funding acquisition, JZ and IM. All authors have read and agreed to the published version of the manuscript.

Funding: María Mormeneo was a recipient of a pre-doctoral grant from the Programa Nacional de Formación de Profesorado Universitario del Ministerio de Ciencia, Innovación y Universidades of Spain. I.M. was funded by UEMF.

Institutional Review Board Statement: Not Applicable.

Informed Consent Statement: Not Applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

Acknowledgments: The authors wish to thank the support provided by the Servicio Central de Soporte a la Investigación Experimental (SCSIE) Universitat de Vlència.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Gummadi SN, Panda T**.** Purification and biochemical properties of microbial pectinases: a review. Process Biochem. 2003. 38 (7): 987-996. https://doi.org/10.1016/S0032-9592(02)00203-0
2. Soriano M, Blanco A, Díaz P, Pastor FIJ. An unusual pectate lyase from a Bacillus sp. with high activity on pectin: cloning and characterization. Microbiology (Reading). 2000 Jan;146 (Pt 1):89-95. https://doi.org/10.1099/00221287-146-1-89
3. Gummadi SN, Kumar DS. Microbial pectic transeliminases. Biotechnol Lett. 2005 Apr;27(7):451-8. https://doi.org/10.1007/s10529-005-2197-8
4. Jayani RS, Saxena S, Gupta R. Microbial pectinolytic enzymes: a review. Process Biochem. 2005. 40 (9): 2931–2944. https://doi.org/10.1016/j.procbio.2005.03.026
5. Alkorta I, Garbisu C, Llama MJ, Serra JL. Industrial applications of pectic enzymes: a review. Process Biochem. 1998. 33 (1): 21-28. https://doi.org/10.1016/S0032-9592(97)00046-0
6. Sakai T, Sakamoto T, Hallaert J, Vandamme EJ. Pectin, pectinase and protopectinase: production, properties, and applications. Adv Appl Microbiol. 1993;39:213-94. https://doi.org/10.1016/s0065-2164(08)70597-5
7. Zheng Y, Huang CH, Liu W, Ko TP, Xue Y, Zhou C, Guo RT, Ma Y. Crystal structure and substrate-binding mode of a novel pectate lyase from alkaliphilic Bacillus sp. N16-5. Biochem Biophys Res Commun. 2012 Apr 6;420(2):269-74. https://doi.org/10.1016/j.bbrc.2012.02.148
8. Thakur, P, Singh, A. K, Singh, M, Mukherjee, G. 2022. Extracellular alkaline pectinases production: a review. Journal of Microbiology, Biotechnology and Food Sciences, 11(5), e3745. https://doi.org/10.55251/jmbfs.3745

 doi:10.20944/preprints202604.0070.v1

9. Bera S, Rajan E, Shakya S, Perera I, Wijewarna S, Semini M (2023) Revisiting microbial pectinases: an Understanding between struc-ture-functional relationship in the arena of genetic engineering. J Appl Biology Biotechnol. https://doi.org/10.7324/JABB.2023. 27206
10. Satpathy A, Mukherjee K, Nigam VK (2025) Improved production of thermostable pectinase from novel Bacillus siamensis (BIOS-MNF45) using computational-conventional approach. Sci Rep 15(1):11489. https://doi.org/10.1038/s41598-024-77167-2
11. Kaissar FZ, Benine ML, Saadi SA, Khaldi A, Missouri A, Laraki M, Barberini S, Emiliani G, Lebouachera SEI. Alkaline adapted pectinase from polyextremotolerant Bacillus amyloliquefaciens via agro-wastes valorization: dual biocatalyst and multifunctional bioinoculant. Int Microbiol. 2025 Dec;28(8):3243-3257. https://doi.org/10.1007/s10123-025-00761-5
12. Strauss ML, Jolly NP, Lambrechts MG, van Rensburg P. Screening for the production of extracellular hydrolytic enzymes by non-Saccharomyces wine yeasts. J Appl Microbiol. 2001 Jul;91(1):182-90. https://doi.org/10.1046/j.1365-2672.2001.01379.x
13. Busto MD, García-Tramontín KE, Ortega N, Perez-Mateos M. Preparation and properties of an immobilized pectinlyase for the treatment of fruit juices. Bioresour Technol. 2006 Sep;97(13):1477-83. https://doi.org/10.1016/j.biortech.2005.06.013
14. Van Rensburg P, Strauss ML, Lambrechts MG, Cordero Otero RR, Pretorius IS. The heterologous expression of polysaccharidase-encoding genes with oenological relevance in Saccharomyces cerevisiae. J Appl Microbiol. 2007 Dec;103(6):2248-57. https://doi.org/10.1111/j.1365-2672.2007.03474.x
15. Kaul K, Rajauria G, Singh R (2024) Valorization of agro-industrial waste for pectinase production and its influence on circular econ¬omy. Food Bioprod Process 148:141–153. https://doi.org/10.1016/j.fbp.2024.09.008.
16. Haile S, Ayele A (2022) Pectinase from microorganisms and its industrial applications. ScientificWorldJournal 1881305. https://doi.org/10.1155/2022/1881305.
17. Gazaloğlu M, Camarasa C, Nevoigt E. Exploring pectinolytic yeast diversity: toward effective polygalacturonase producers for applications in wine-making. FEMS Yeast Res. 2025 Jan 30;25:foae033. https://doi.org/10.1093/femsyr/foae033
18. Zhao M, Chen J, Pan X, Zabed HM, Arsalan A, Qi X. Advances in Pectinase Engineering for Food Bioprocessing: Novel Sources, Mechanisms, and Optimization Strategies. J Agric Food Chem. 2025 Sep 17;73(37):23078-23097. doi: https://doi.org/10.1021/acs.jafc.5c06547
19. Kashyap DR, Vohra PK, Chopra S, Tewari R. Applications of pectinases in the commercial sector: a review. Bioresour Technol. 2001 May;77(3):215-27. https://doi.org/10.1016/s0960-8524(00)00118-8
20. Angayarkanni J, Palaniswamy M, Murugesan S, Swaminathan K. Improvement of tea leaves fermentation with *Aspergillus spp*. pectinase. J Biosci Bioeng. 2002;94(4):299-303. https://doi.org/10.1263/jbb.94.299
21. Masoud W, Jespersen L. Pectin degrading enzymes in yeasts involved in fermentation of Coffea arabica in East Africa. Int J Food Microbiol. 2006 Aug 1;110(3):291-6. https://doi.org/10.1016/j.ijfoodmicro.2006.04.030
22. Silva, M. E. d. S., Oliveira, R. L. d., Moraes, M. M. d., Camara, C. A. G. d., Silva, S. P. d., & Porto, T. S. (2025). Application of Commercial Pectinase as a Biocatalyst During Self-Induced Anaerobic Fermentation of Coffee (Coffea arabica L. var. Typica). Fermentation, 11(7), 361. https://doi.org/10.3390/fermentation11070361
23. Zhang C, Yao J, Zhou C, Mao L, Zhang G, Ma Y. The alkaline pectate lyase PEL168 of Bacillus subtilis heterologously expressed in Pichia pastoris is more stable and efficient for degumming ramie fiber. BMC Biotechnol. 2013 Mar 19;13:26. https://doi.org/10.1186/1472-6750-13-26
24. Li, X., Zhou, Y., & Zheng, F. 2014. Alkaline pectate lyases for textile degumming: Advances and applications. Applied Microbiology and Biotechnology, 98(20), 8725–8737. https://doi.org/10.1007/s00253-014-5872-9
25. Zheng X, Zhang Y, Liu X, Li C, Lin Y, Liang S. High-Level Expression and Biochemical Properties of A Thermo-Alkaline Pectate Lyase From Bacillus sp. RN1 in Pichia pastoris With Potential in Ramie Degumming. Front Bioeng Biotechnol. 2020 Jul 24;8:850. https://doi.org/10.3389/fbioe.2020.00850

 doi:10.20944/preprints202604.0070.v1

26. Mushtaq B, Nawab Y, Ahmad F, Ahmad S. An enzymatic modification of sisal fiber macromolecules (cellulose/lignin) for spinnable fibers used in value-added textile applications. Int J Biol Macromol. 2025 Sep;323(Pt 1):147046. https://doi.org/10.1016/j.ijbiomac.2025.147046
27. Tzanov T, Paar A, Gudelj M, Gübitz GM, Cavaco-Paulo A. Immobilization of catalases from Bacillus SF on alumina for the treatment of textile bleaching effluents. Enzyme Microb Technol. 2001 Jun 7;28(9-10):815-819. https://doi.org/10.1016/s0141-0229(01)00335-0
28. Hoondal GS, Tiwari RP, Tewari R, Dahiya N, Beg QK. Microbial alkaline pectinases and their industrial applications: a review. Appl Microbiol Biotechnol. 2002 Aug;59(4-5):409-18. https://doi.org/10.1007/s00253-002-1061-1
29. Lenting HBM, Warmoeskerken MMCG. 2004. A fast, continuous enzyme-based pretreatment process concept for cotton containing textiles. Biocatal Biotransformation. 2004. 22(5-6):361–368. https://doi.org/10.1080/10242420400024557
30. Zou M, Li X, Zhao J, Qu Y. Characteristics of polygalacturonate lyase C from Bacillus subtilis 7-3-3 and its synergistic action with PelA in enzymatic degumming. PLoS One. 2013 Nov 13;8(11):e79357. https://doi.org/10.1371/journal.pone.0079357
31. Zhou C, Ye J, Xue Y, Ma Y. Directed Evolution and Structural Analysis of Alkaline Pectate Lyase from the Alkaliphilic Bacterium *Bacillus* sp. Strain N16-5 To Improve Its Thermostability for Efficient Ramie Degumming. Appl Environ Microbiol. 2015 Sep 1;81(17):5714-23. https://doi.org/10.1128/AEM.01017-15
32. Kumar D, Bhardwaj R, Jassal S, Goyal T, Khullar A, Gupta N. Application of enzymes for an eco-friendly approach to textile processing. Environ Sci Pollut Res Int. 2023 Jun;30(28):71838-71848. https://doi.org/10.1007/s11356-021-16764-4
33. Zhou C, Xue Y, Ma Y. Cloning, evaluation, and high-level expression of a thermo-alkaline pectate lyase from alkaliphilic Bacillus clausii with potential in ramie degumming. Appl Microbiol Biotechnol. 2017 May;101(9):3663-3676. https://doi.org/10.1007/s00253-017-8110-2
34. Shi A, Hu H, Zheng F, Long L, Ding S. Biochemical characteristics of an alkaline pectate lyase PelA from Volvariella volvacea: roles of the highly conserved N-glycosylation site in its secretion and activity. Appl Microbiol Biotechnol. 2015 Apr;99(8):3447-58. https://doi.org/10.1007/s00253-014-6146-0
35. Wu P, Yang S, Zhan Z, Zhang G. Origins and features of pectate lyases and their applications in industry. Appl Microbiol Biotechnol. 2020 Sep;104(17):7247-7260. https://doi.org/10.1007/s00253-020-10769-8
36. Han Y, Peng XB, Wei SY, Chen QG, Yang JK. Molecular Characterization of a Novel Alkaline Endo-Pectate Lyase from *Paenibacillus borealis* and Over-Production in Bioreactor Realized by Constructing the Tandem Expression Cassettes in Host Genome. Molecules. 2025 Sep 4;30(17):3612. https://doi.org/10.3390/molecules30173612
37. Hernández LM, Ballou L, Alvarado E, Gillece-Castro BL, Burlingame AL, Ballou CE. A new *Saccharomyces cerevisiae mnn* mutant N-linked oligosaccharide structure. J Biol Chem. 1989 Jul 15;264(20):11849-56. PMID: 2663842.
38. Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory. Cold Spring Harbor N. Y.
39. Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. J Bacteriol. 1983 Jan;153(1):163-8. https://doi.org/10.1128/JB.153.1.163-168.1983
40. Gietz RD, Sugino A. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene. 1988 Dec 30;74(2):527-34. https://doi.org/10.1016/0378-1119(88)90185-0
41. Andrés I, Zueco J, Parascandola P. Immobilization of *Saccharomyces cerevisiae* cells to protein G-Sepharose by cell wall engineering. J Mol Microbiol Biotechnol. 2003;5(3):161-6. https://doi.org/10.1159/000070266
42. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5. https://doi.org/10.1038/227680a0
43. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem. 1981 Apr;112(2):195-203. https://doi.org/10.1016/0003-2697(81)90281-5

 doi:10.20944/preprints202604.0070.v1

44. Moukadiri I, Jaafar L, Zueco J. Identification of two mannoproteins released from cell walls of a *Saccharomyces cerevisiae mnn1 mnn9* double mutant by reducing agents. J Bacteriol. 1999 Aug;181(16):4741-5. https://doi.org/10.1128/JB.181.16.4741-4745.1999
45. McKay AM. A plate assay method for the detection of fungal polygalacturonase secretion. FEMS Microbiol. Lett. 1988 56(3): 355-358. https://doi.org/10.1111/j.1574-6968.1988.tb03206.x
46. Gognies S, Gainvors A, Aigle M, Belarbi A. Cloning, sequence analysis and overexpression of a *Saccharomyces cerevisiae* endopolygalacturonase-encoding gene (PGL1). Yeast. 1999 Jan 15;15(1):11-22. https://doi.org/10.1002/(SICI)1097-0061(19990115)15:1<11::AID-YEA336>3.0.CO;2-O
47. Collmer A, Ried JL, Mount MS. Assay methods for pectic enzymes. Methods. Enzymol. 1988. 161: 329-335. https://doi.org/10.1016/0076-6879(88)61037-8
48. Moukadiri I, Zueco J. Evidence for the attachment of Hsp150/Pir2 to the cell wall of *Saccharomyces cerevisiae* through disulfide bridges. FEMS Yeast Res. 2001 Dec;1(3):241-5. https://doi.org/10.1111/j.1567-1364.2001.tb00040.x
49. Andrés I, Gallardo O, Parascandola P, Javier Pastor FI, Zueco J. Use of the cell wall protein Pir4 as a fusion partner for the expression of *Bacillus sp.* BP-7 xylanase A in *Saccharomyces cerevisiae*. Biotechnol Bioeng. 2005 Mar 20;89(6):690-7. https://doi.org/10.1002/bit.20375
50. Andrés I, Rodríguez-Díaz J, Buesa J, Zueco J. Yeast expression of the VP8* fragment of the rotavirus spike protein and its use as immunogen in mice. Biotechnol Bioeng. 2006 Jan 5;93(1):89-98. https://doi.org/10.1002/bit.20696
51. Mormeneo M, Andrés I, Bofill C, Díaz P, Zueco J. Efficient secretion of Bacillus subtilis lipase A in *Saccharomyces cerevisiae* by translational fusion to the Pir4 cell wall protein. Appl Microbiol Biotechnol. 2008 Sep;80(3):437-45. https://doi.org/10.1007/s00253-008-1549-4
52. Paciello L, de Alteriis E, Mazzoni C, Palermo V, Zueco J, Parascandola P. Performance of the auxotrophic *Saccharomyces cerevisiae* BY4741 as host for the production of IL-1beta in aerated fed-batch reactor: role of ACA supplementation, strain viability, and maintenance energy. Microb Cell Fact. 2009 Dec 30;8:70. https://doi.org/10.1186/1475-2859-8-70
53. Mormeneo M, Pastor FJ, Zueco J. Efficient expression of a *Paenibacillus barcinonensis* endoglucanase in *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol. 2012 Jan;39(1):115-23. https://doi.org/10.1007/s10295-011-1006-8
54. Yang N, Yu Z, Jia D, Xie Z, Zhang K, Xia Z, Lei L, Qiao M. The contribution of Pir protein family to yeast cell surface display. Appl Microbiol Biotechnol. 2014 Apr;98(7):2897-905. https://doi.org/10.1007/s00253-014-5538-5
55. Martinić Cezar, T., Lozančić, M., Novačić, A., Matičević, A., Matijević, D., Vallée, B., Mrša, V., Teparić, R., & Žunar, B. (2023). *Streamlining N-terminally anchored yeast surface display* via *structural insights into S. cerevisiae Pir proteins*. **Microbial Cell Factories, 22**, Article 174. https://doi.org/10.1186/s12934-023-02183-2
56. Soriano M, Diaz P, Pastor FI. Pectinolytic systems of two aerobic sporogenous bacterial strains with high activity on pectin. Curr Microbiol. 2005 Feb;50(2):114-8. https://doi.org/10.1007/s00284-004-4382-8
57. Soriano M, Diaz P, Pastor FIJ. Pectate lyase C from Bacillus subtilis: a novel endo-cleaving enzyme with activity on highly methylated pectin. Microbiology (Reading). 2006 Mar;152(Pt 3):617-625. https://doi.org/10.1099/mic.0.28562-0
58. de Nobel JG, Klis FM, Priem J, Munnik T, van den Ende H. The glucanase-soluble mannoproteins limit cell wall porosity in *Saccharomyces cerevisiae*. Yeast. 1990 Nov-Dec;6(6):491-9. https://doi.org/10.1002/yea.320060606
59. Sieiro C, Poza M, Vilanova M, Villa TG. Heterologous expression of the *Saccharomyces cerevisiae* PGU1 gene in *Schizosaccharomyces pombe* yields an enzyme with more desirable properties for the food industry. Appl Environ Microbiol. 2003 Mar;69(3):1861-5. https://doi.org/10.1128/aem.69.3.1861-1865.2003
60. Louw C, La Grange D, Pretorius IS, van Rensburg P. The effect of polysaccharide-degrading wine yeast transformants on the efficiency of wine processing and wine flavour. J Biotechnol. 2006 Oct 1;125(4):447-61. https://doi.org/10.1016/j.jbiotec.2006.03.029

 doi:10.20944/preprints202604.0070.v1