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Cloning and Expression of the *XPR2* Gene from *Yarrowia lipolytica* in *Pichia pastoris*

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Yarrowia lipolytica is a dimorphic yeast able to secrete different types of proteases depending on the pH of the environment. At neutral pH, the production of an extracellular alkaline protease (AEP) is induced. This protease could be useful in the leather, detergent, or food industries. The *XPR2* gene, coding for AEP, was extracted from the pINA154 vector and cloned into the pHIL-D2 vector to obtain a new protease-producing recombinant *Pichia pastoris* strain. The gene was efficiently integrated in the *P. pastoris* genome and expressed from the AOX1 promoter actively induced by methanol. Finally, the protease was successfully secreted by *P. pastoris* GS115.

KEYWORDS: Cloning; expression; protease; *XPR2*; *Yarrowia lipolytica*; *Pichia pastoris*

INTRODUCTION

Proteases are enzymes that catalyze the breakdown of the peptide bonds in proteins and are involved in both normal and abnormal biochemical pathways, conferring them the interesting aspect of being potential targets for the development of new therapeutic agents. Additionally, proteases are one of the three main enzyme groups used industrially. Although these enzymes are widely distributed in nature, in both animals and higher plants, microbial proteases represent an interesting source with clear industrial projection because of the rapid and profitable growth of the microorganisms and their amenability to genetic modification, this allowing the construction of new superproducing strains (*1*−*3*).

Yarrowia lipolytica is a dimorphic yeast able to secrete a variety of enzymes including proteases. The type of protease secreted depends on the pH of the environment. Thus, at acidic pH, an acidic protease is produced (AXP), whereas neutral pH induces the formation of an alkaline one (AEP) (*4*, *5*). AEP has been characterized as a serine protease, in particular, a member of the subfamily of subtilisins with a molecular mass of 30 kDa and an optimum pH of activity of 9−10 (*6*, *7*). Its corresponding gene (*XPR2*) has been cloned and sequenced, resulting in a mature peptide encoded from an 890 bp sequence preceded by an extensive pro-region including a signal peptide (*8*). Alkaline proteases have been widely used for the detergents industry, the best candidates being those who show broad substrate specificity to facilitate the removal of a large variety of proteinaceous substances. Also, both their stability at high pH and their compatibility with other chelating and oxidizing agents added to detergents are of great interest (*3*). Alkaline proteases are also used in the leather industry, where extremely alkaline conditions are often used for soaking, dehairing, or bating. In addition, fungal proteases are often used in the food industry to lower the bitter taste of protein hydrolyzates (*3*).

Pichia pastoris has routinely been used as an excellent host for heterologous expression. In addition, the *Pichia* expression systems allow the generation of stable recombinants microorganisms where the DNA is permanently integrated in the chromosomes (*9*), this being important for preventing gene spreading (*10*). As an expression tool, *P. pastoris* has many advantages, such as its economy of growth, the possibility of making complex post-translational modifications, or the availability of a variety of plasmids as cloning vehicles (*11*, *12*). In addition, *P. pastoris* secretes low levels of native proteins, which can ease the purification steps of the recombinant product. This methylotrophic yeast is able to secrete larger than 30 kDa heterologous proteins to a better extent than *Saccharomyces cerevisiae*, where the cloned product is often hyperglycosylated and hence may even lose its biological activity (*5*). The present paper describes the cloning and expression in *P. pastoris* of an alkaline protease (AEP) from *Y. lipolytica*.

MATERIALS AND METHODS

Strain, Plasmids, and Culture Media. The strains employed were *P. pastoris* GS115 (*his4* genotype, *Pichia* Expression Kit, Invitrogen, Carlsbad, CA) and *E. coli* TOP10 (Zero Blunt Topo PCR Cloning Kit, Invitrogen, Carlsbad, CA).

Plasmid pINA154 containing the *XPR2* gene from *Y. lipolytica* was obtained from Dr. Gaillardin (Collection de Levures d'Intérêt Biotechnologique, UMR INA-PG-INRA-CNRS Microbiologie Génétique Moléculaire, INA-PG, Thiverval-Grignon, France) (*7*). Plasmid pCR-Blunt II-TOPO, containing the kanamycin resistance gene (Invitrogen, Carlsbad, CA), was employed to clone blunt-ends PCR products and plasmid pHIL-D2 (Invitrogen, Carlsbad, CA) to express the *XPR2* gene. The pHIL-D2 vector is an integrative vector often used for intracellular

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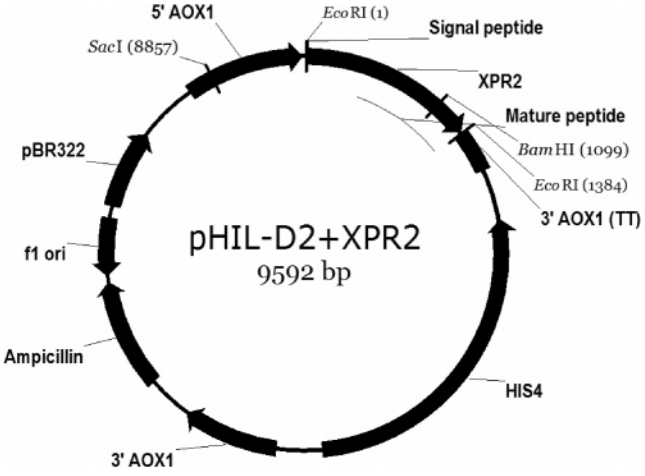

Figure 1. Map of the plasmidic construction of pHIL-D2 vector containing the cloned *XPR2* gene.

expression in *P. pastoris* that contains the *AOX1* promoter induced by methanol, two regions of the *AOX1* gene obtained from the *P. pastoris* genome, an *E. coli* origin of replication, and the ampicillin resistance gene and the *HIS4* gene for selection of transformants in *E. coli* and in *P. pastoris* GS115, respectively (see **Figure 1**). The selection of transforming bacteria was performed on LB medium (tryptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 5 g/L) supplemented with the appropriate antibiotic (ampicillin 100 μg/mL or kanamycin 50 μg/mL) and incubated overnight at 37 °C. Growth of yeast was done in YEPD medium (yeast extract, 10 g/L; peptone, 10 g/L; and glucose, 20 g/L) at 30 °C and 200 rpm. Selection of the transforming *P. pastoris* strains was done in SD medium (YNB w/o aminoacids, 7 g/L; and glucose, 20 g/L) supplemented with all aminoacids and bases except histidine, according to Sherman et al. (*13*). Heterologous expression in *P. pastoris* GS115 was accomplished at 30 °C and 300 rpm by first growing the strain in BMGY medium (yeast extract, 10 g/L; peptone, 20 g/L; 0.1 M potassium phosphate buffer pH 6.0, YNB, 14 g/L; biotine, 4×10^{-4} g/L; and glycerol, 10 g/L) and then induced for, at least, 2 days in BMMY medium, this containing the same composition as the previous medium but replacing glycerol by methanol. Proteolytic activity of the recombinant clones was visualized on Petri dishes using milk medium (beef extract, 3 g/L; bactotryptone, 5 g/L; glucose, 1 g/L; and skimmed powdered milk, 24 g/L) as described by Phaff et al. (*14*). Some studies on the cloned gene were carried out using milk medium but increasing the glucose or glycerol concentrations from 1% to 2%. Likewise, 1% methanol was added to the milk medium to study its effect over protease production. In this case, 100 μL of 100% methanol was regularly added to the plates every 24 h during 6 days. When necessary, solid media plates were made up adding 2% agar.

Enzyme Preparation. Cultures of yeast were centrifuged at 1500*g* for 15 min at 4 °C, and the supernatants were filtered through 0.22 μm membranes and ultrafiltered with Amicon (Millipore, Billerica, MA) devices using PM 10 membranes to obtain 20-fold concentrates. The samples were dialyzed overnight against 0.05 M Tris-HCl, pH 7.5, with gentle stirring at 4 °C.

Proteolytic Assays. To test the proteolytic activity of the recombinant *P. pastoris* strains, enzyme preparations were subjected to the cup-plate assay described by Poza et al. (*15*) or the strains grown in milk medium where haloes surrounding the colonies were evaluated. The proteolytic activity of recombinant *P. pastoris* strains was quantified according to the method described by Kunitz (*16*), in which casein is diluted in a potassium phosphate buffer 1 M, pH 7.5, and employed as substrate. Standard reaction mixtures were incubated for varying times at 37 °C. Readings at 280 nm were referred to a tyrosine standard, defining 1 enzymatic unit as the amount of enzyme that releases 1 mmol of tyrosine at 37 °C in 1 h. To analyze the optimum pH of action for the recombinant enzyme activity, the substrate (casein) was diluted using potassium phosphate buffer 1 M at different pH values (ranging from 6 to 10), and the reactions were maintained 1 h at 37 °C. Protein concentration was determined by the method described by Lowry (*17*).

Molecular Exclusion Chromatography. Concentrated enzymatic samples (5 mL) were loaded onto a Sephacryl S-200 column (80 × 3.5 cm) equilibrated with 0.05 M Tris-HCl, pH 7.5. Fractions of 3.5 mL were collected at a flow rate of 0.54 mL/min. Fractions were monitorized at 280 nm. Aldolase (158 kDa), bovine serum albumin (67 kDa), albumin from egg (45 kDa), chymotrypsinogen A (25 kDa), and lysozyme (14 kDa) were used as molecular weight standards. The molecular weight of the recombinant protease was estimated by the method described by Whitaker (*18*).

Polyacrylamide Gel Electrophoresis (PAGE). Sodium dodecyl sulfate-PAGE (SDS-PAGE) was employed to check protein purity and to evaluate molecular weights. The stacking and separation gels contained 5% and 12% polyacrylamide, respectively, and were performed as described by Ausubel et al. (*19*). Proteins were visualized by staining with Coomassie blue G-250 (BIO-RAD, Hercules, CA).

Nucleic Acid Extraction and Purification. Plasmidic DNA was purified with the Qiaprep Spin Miniprep Kit (Qiagen, Valencia, CA). Nucleic acid extraction from agarose gels was accomplished by using the Wizard PCR Preps Purification System (Promega, Madison, WI), and genomic DNA from *P. pastoris* was extracted by using the Wizard genomic DNA Purification Kit (Promega, Madison, WI). The RNeasy Mini Kit (Qiagen, Valencia, CA) was used for RNA extraction from *P. pastoris*. In all cases, the manufacturer's instructions were followed.

DNA Amplification. Amplification of the *XPR2* gene from the pINA154 construction was done by PCR, employing the oligonucleotides YCOD, 5′-ATG AAG CTC GCT ACC GCC TT-3′, and YCOD reverse, 5′-CTA AAT GCC AAC ACC GTT GTA-3′, synthesized from the first codon of the peptide signal to the stop codon of the mature peptide of the *XPR2* gene according to the nucleotide sequence corresponding to the AEP preprotein of *Y. lipolytica* (Genbank accession code; M17741) (*8*). The reaction mixture contained 5 ng of plasmid DNA, 0.5 μL of a dNTP mixture (Bioline, Randolph, MA), 5 μL of Cl_2Mg 25 mM (Bioline, Randolph, MA), 5 μL of Taq polymerase buffer (Bioline, Randolph, MA), 1 μL of a Taq (Bioline, Randolph, MA) and Pfu (Stratagene, La Jolla, CA) polymerases mixture (5 U/μL each) at a ratio of 7:3, and 200 ng of each oligonucleotide and sterile ultrapure water at a final volume of 50 μL. The cycling program was: 1 cycle at 94 °C for 5 min and 30 cycles of 94 °C for 1 min; 60−64 °C for 1 min and 72 °C for 2 min. The process was completed with a final cycle of 72 °C for 5 min.

The *XPR2* gene integration in the *P. pastoris* genome was investigated by PCR using genomic DNA extracted from *P. pastoris* by means of the Lyse-N-Go PCR Reagent (Pierce Biotechnology, Rockford, IL). The cells of one *P. pastoris* colony were thoroughly rinsed with 0.01 M Tris-HCl, pH 8.0, and resuspended in 10 μL of the reagent. After they had been spun down at 800*g* for 10 min and resuspended again in 10 μL of the same reagent, they were subjected to the following conditions to ensure cellular breakage: 65 °C, 30 s; 8 °C, 30 s; 65 °C, 90 s; 97 °C, 180 s; 8 °C, 60 s; 65 °C, 180 s; 97 °C, 60 s; 65 °C, 60 s; and 80 °C until the next step. Finally, PCR was carried out under the aforementioned conditions, but 40 cycles were used instead of 30, only Taq polymerase was added, 1 μL of the cellular suspension treated as described above was used as the DNA template, and the oligonucleotides AOX1, 5′-GAC TGG TTC CAA TTG ACA AGC-3′, and AOX1 reverse, 5′-GCA AAT GGC ATT CTG ACA TCC-3′, were used.

Cloning of the *XPR2* Gene into pCR-Blunt-II-TOPO and pHIL-D2 Vectors. The PCR product was cloned in the pCR-Blunt-II-TOPO vector by use of a Zero Blunt TOPO PCR cloning kit (Invitrogene, Carlsbad, CA) following the manufacturer's instructions. Next, cohesive ends insert was obtained from an *Eco*RI-digestion of the pCR-Blunt-II-TOPO+*XPR2* construction. To clone the insert in the pHIL-D2 vector, the plasmid was also *Eco*RI-digested and dephosphorylated using shrimp alkaline phosphatase (all enzymes from Promega, Madison, WI). Ligation was carried out with T4DNA Ligase (Promega, Madison, WI). *Sac*I and *Bam*HI enzymes (Promega, Madison, WI) were used for investigating the insert orientation in the pHIL-D2 vector, and *Sac*I was also used to linearize the construction pHIL-D2+*XPR2* before the *P. pastoris* transformation. For all reactions, the manufacturer's instructions were followed.

Transformation of *E. coli* and *P. pastoris*. Transformation of *E. coli* TOP10 was accomplished as described in the Zero Blunt TOPO

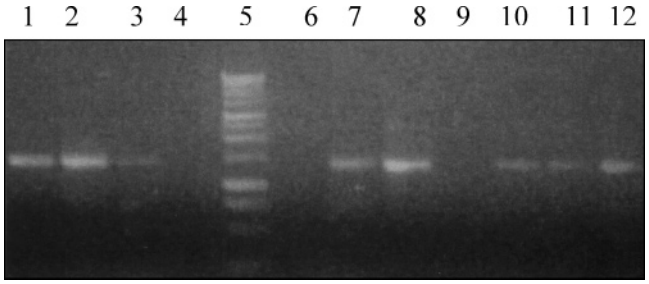

Figure 2. PCR-amplification of the *XPR2* gene (band of ca. 1.4 Kbp) from 8 pretreated fresh colonies of recombinant *P. pastoris*: lanes 1, 2, 3, 7, 8, 10, 11, and 12. On lane 5: 1 Kbp ladder (Promega).

PCR Cloning Kit (Invitrogen, Carlsbad, CA) when pCR-Blunt-II-TOPO was used, or the protocol described by Sambrook et al. (*20*) was followed for the transformation with the pHIL-D2 vector. The yeast was transformed using LiCl, following the method described by Geitz and Schiestl (*21*).

Northern Blotting. The probe was obtained from the *XPR2* gene amplified by PCR using the oligonucleotides YCOD and YCOD reverse. A digoxigenine-labeled probe was produced using the Digoxigenine High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH, Mannheim, Germany). RNA was transferred from gel to Hybond-N^+ nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) using a Vacuum Blotting System (Amersham Pharmacia Biotech, Piscataway, NJ) and was cross-linked by means of UV. Northern blotting protocol was performed as described in the instructions manual of the Dig Northern Starter Kit for transcription-labeling of RNA with digoxigenin and SP6/T7/T3 RNA polymerases and chemiluminescent detection with CDP-Star, ready-to-use (Roche Diagnostics GmbH, Mannheim, Germany).

DNA Sequencing and Analysis. DNA sequencing of the *XPR2* gene cloned into the pHIL-D2 vector was carried out in an external DNA sequencing service using AOX1 and AOX1 reverse primers. DNA sequences obtained were compared to databases using the Blast 2.0 application of the Web Site of the National Center for Biotechnology Information.

RESULTS

Cloning of the *XPR2* Gene into Plasmid PCR-Blunt II-TOPO. The *XPR2* gene was PCR-amplified from the pINA154 construction (*7*), and the ca. 1.4 Kb fragment obtained was extracted from the agarose gel and ligated into the pCR-Blunt II-TOPO vector. The construction obtained was used to transform *E. coli* TOP10 strain, and transformants were selected on LB agar plates supplemented with kanamycin. The rescued plasmids were purified and digested with *Eco*RI for later cloning into the pHIL-D2 vector.

Cloning of the *XPR2* Gene into the pHIL-D2 Vector. *Eco*RI-digested and dephosphorylated pHIL-D2 plasmid was successfully ligated to the insert generated above, at a molar ratio (vector:insert) of 1:5. Transforming *E. coli* TOP10 cells containing the pIHL-D2+*XPR2* construction (**Figure 1**) were obtained. Insert orientation was further investigated, and the cloned insert of 1366 bp was sequenced and probed to be the *XPR2* gene from *Y. lipolytica* present in databases.

Transformation of *P. pastoris*. Appropriate recombinant constructions were linearized before transforming *P. pastoris* GS115 to stimulate DNA recombination. *P. pastoris* transformants were obtained on SD medium lacking histidine. The presence of the *XPR2* gene in the *P. pastoris* genome was PCR-investigated using fresh colonies as template DNA, revealing that a high number of colonies carried the gene integrated (**Figure 2**).

Expression Analysis of the *XPR2* Gene in *P. pastoris*. The recombinant clones of *P. pastoris* GS115 were first grown and after induced, and, as expected, the supernatants obtained showed strong proteolytic activity when evaluated by the cup-plate technique (**Figure 3a**). The activity of those proteolytic samples was also quantified as shown in **Figure 3b**. The protease was actively secreted into the extracellular medium where it accumulated for at least 20 h, as deduced from the increase in its specific activity. This ability was restricted to transformants, whereas the original strain did not secrete protease activity under these conditions. The recombinant clones of *P. pastoris* GS115 were also checked on plates containing milk medium where 25% of the colonies constitutively secreted protease activity. As shown in **Figure 4**, when these colonies were picked and regrown on milk plates, they formed haloes regardless of the presence or the absence of methanol.

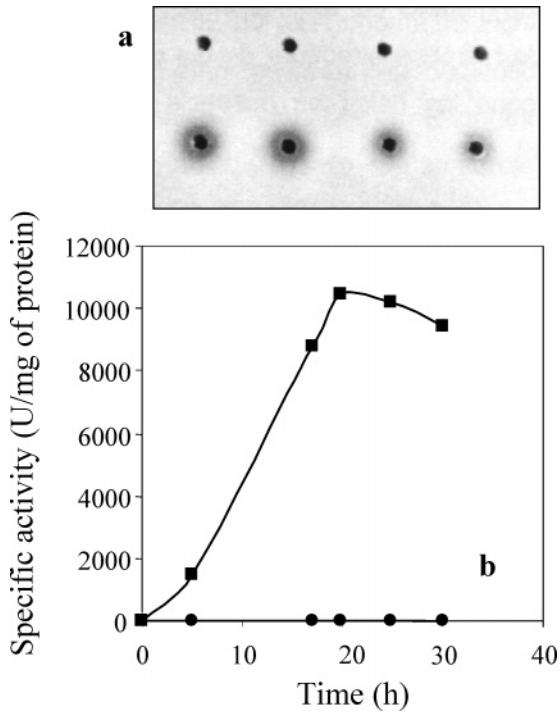

Figure 3. (**a**) Cup-plate proteolytic assay showing haloes generated by milk proteolysis produced by the supernatant obtained from recombinant *P. pastoris* strains. Negative controls (supernatant from a non-recombinant strain) are shown in the upper lane. (**b**) Proteolytic activity of the supernatant obtained from one recombinant *P. pastoris* strain (■) and the non-recombinant *P. pastoris* GS115 strain (●), both induced during 30 h in BMMY medium.

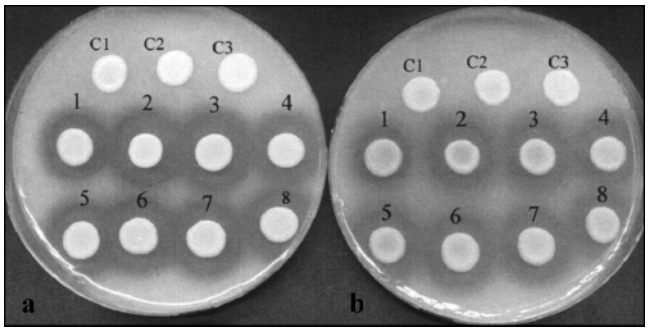

Figure 4. Proteolytic haloes produced by 8 colonies of recombinant *P. pastoris* strains (1–8) and negative controls of non-recombinant *P. pastoris* GS115 strain (C1, C2, and C3) grown in milk medium plates with methanol (**a**) and without methanol (**b**) added.

Northern Blotting Analysis. Transcription of the *XPR2* gene was investigated by Northern analysis. mRNA from both

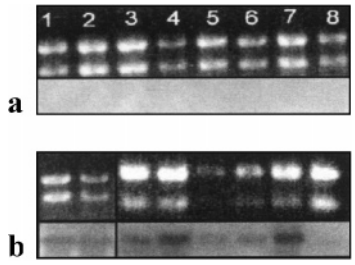

Figure 5. Northern analysis made with *XPR2* probe of 8 recombinant colonies of *P. pastoris* (1–8) grown in BMGY (**a**) and in BMMY (**b**) medium.

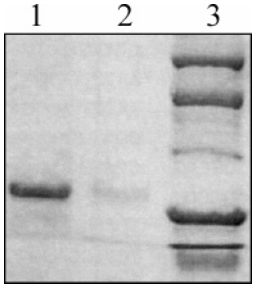

Figure 6. SDS-PAGE. Lanes 1 (non-diluted sample) and 2 (diluted sample) show a band of ca. 31 kDa, which corresponds to the proteolytic sample obtained from gel exclusion chromatography of the supernatant from a recombinant *P. pastoris* strain containing the *XPR2* gene. On lane 3: molecular weight standard (Broad Range, BIO-RAD).

methanol-containing (BMMY) and methanol-free (BMGY) media were extracted and probed with *XPR2*. As expected, positive signals were only detected in samples from clones grown in the presence of methanol (**Figure 5**).

Partial Characterization of the Recombinant Protein. The protease expressed in the *P. pastoris* background was characterized for optimum pH, which was 8. The enzyme was partially purified by Sephacryl S-200 chromatography where it eluted as a single peak with an estimated molecular mass of 31 kDa. This value was also obtained with SDS-PAGE (**Figure 6**). Upon analyzing the expression of the *XPR2* gene in milk medium, the assays revealed that, when 1−2% glycerol was used instead of glucose, the expression from AOX1 promoter was abolished, and the same situation occurred when glucose concentration was increased to 2%.

DISCUSSION

P. pastoris is a methylotrophic yeast capable of metabolizing methanol as its sole carbon source. Secretion in *P. pastoris* with pHIL-D2 requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. Therefore, the AXO1 promoter contained in the pHIL-D2 vector is used to drive expression of the gene of interest. Growth of *P. pastoris* in glucose represses transcription, and growth on methanol induces AOX1 expression. The *XPR2* gene was extracted from the pINA154 vector and cloned into other vectors for subsequent expression in the yeast *P. pastoris* GS115. Heterologous protease expression has been often achieved in this microorganism, examples being a keratinolytic metalloprotease from *Microsporum canis* (*12*) or an aspartic protease from *Rhizopus microsporus* (*22*).

The *XPR2* gene from *Y. lipolytica* has been cloned in the pHIL-D2 expression vector, and the encoded protein was successfully secreted by the recombinant *P. pastoris* strain into the culture medium, suggesting that the endogenous peptide signal from the *Y. lipolytica XPR2* gene cloned is functional in the *Pichia* background. When cells pregrown in glucose-containing medium were transferred to induction medium, both expression of the *XPR2* gene from the AOX1 promoter and secretion of protease activity were observed. Thus, during active growth (in BMGY), the yeast used glycerol as the carbon source and accumulates biomass, and later, in BMMY medium, the alcohol activated expression from the AOX1 promoter, as expected. Previously, this system of heterologous expression in *P. pastoris* through use of pHIL-D2 vector and strain GS115 has been used successfully (*9*, *23*). When the recombinant *P. pastoris* colonies were PCR-analyzed, it was found that the *XPR2* gene had been correctly cloned. The *XPR2* gene was successfully integrated in the *P. pastoris* genome. Crossover events may occur between the *AOX1* regions included in the pIHL-D2 vector and the *AOX1* locus in the *P. pastoris* genome, but also these events may take place between the *HIS4* region located in the vector and the *his4* region (mutant) in the *P. pastoris* GS115 genome. By linearizing the recombinant vector pHIL-D2 at a restriction enzyme site (*Sac*I) located in the 5′ *AOX1* regions, His^+ recombinants can be obtained by single crossover events between these regions in the vector and the locus *AOX1* in the *P. pastoris* GS115 genome. Double crossover events (replacements) may occur with less frequency, this leading to the loss of the *AOX1* locus, which in turn makes the strain grow slowly in the presence of methanol (Mut^s phenotype). No Mut^S clones were found in our recombinant strains screening; therefore, we can conclude that in our recombinant strains of *P. pastoris* single crossover events occurred between the 5′*AOX1* region in the vector and the *AOX1* locus in the genome. Gene integration ensures clone stability, on the one hand, and avoids gene spreading, on the other. Northern analysis revealed that recombinant DNA was correctly transcribed in all cases into mRNA. Although *P. pastoris* synthesized and actively secreted this product of the *XPR2* gene, the final protease exhibited some differences in comparison with the native enzyme. One of these was the optimum pH, which was 8 instead of 9, the value originally described for the AEP of *Y. lipolytica* (*4*, *5*). This may be interpreted in terms of different patterns of glycosylation in both yeasts. One striking result was the protease production in milk medium without the need of methanol induction. When glucose concentration in milk medium was increased from 1% to 2% or when glucose was completely replaced by 1−2% glycerol, gene expression was repressed, as expected, suggesting that indeed expression from AOX1 promoter was taking place somehow in normal milk medium (*24*−*26*). All of these data suggest that a casein derivative present in milk medium may somehow mimic the inductive effect of methanol or that a second nearby promoter may also direct AEP synthesis without the direct participation of methanol. In no case was a second protease detected, reinforcing the conclusion that the expression of the *XPR2* gene in the *P. pastoris* background does occur but generates a protease with a different behavior from that of the native enzyme. The recombinant enzyme keeps anyway its alkaline character, which could lead to the application of this new superproducing strain of *P. pastoris* in the leather, detergent, or food industries.

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