Isolation of genomic DNA from Pichia pastoris *without hydrolases*

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ABSTRACT

Pichia pastoris has become a widely used system for the expression of heterologous genes, and many laboratories around the world are using it to obtain active proteins and to produce them at large scale. The purpose of this paper is to minimize the time and cost in the isolation of *P. pastoris'* genomic DNA for PCR screening of recombinant clones. The proposed method is simple and will speed up the work with this expression system. In contrast with most procedures for DNA isolation, our protocol does not require the use of hydrolytic enzymes for cell wall lysis or other special procedures.

Keywords: gDNA isolation, *Pichia pastoris*, PCR

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RESUMEN

Aislamiento de DNA genómico de *Pichia pastoris* sin el uso de hidrolasas. *Pichia pastoris* se ha convertido en un sistema ampliamente usado para la expresión de genes heterólogos y muchos laboratorios del mundo están usando este sistema para obtener proteínas activas y producirlas a gran escala. El objetivo del presente trabajo fue minimizar el tiempo y costo en el aislamiento de ADN genómico de *P. pastoris*, para la selección de clones recombinantes por PCR. El método propuesto es fácil de realizar y acelerará el trabajo de quienes usan este sistema expresión. A diferencia de la mayoría de los procedimientos para aislamiento de ADN, nuestro protocolo no requiere el uso de enzimas hidrolíticas para la lisis de la pared de celular u otros procedimientos especiales.

Palabras claves: aislamiento de ADN genómico, *Pichia pastoris*, PCR

*I*ntroduction

Pichia pastoris has become a widely used system for the expression of hetorologous genes. The foreign protein expression in *P. pastoris* is based on the use of the alcohol oxidase gene promoter, *AOX1* [1]. Transcription of heterologous genes is tightly regulated by the *AOX1* promoter, with the *AOX1* coding sequence replaced by the gene of interest. Genes under the control of the *AOX1* promoter are rapidly transcribed on adding methanol, the alcohol oxidase substrate. Hence, the selection of transformed cells that contain the gene of interest at the target genome region is necessary.

DNA amplification by polymerase chain reaction (PCR) is now the most commonly used procedure for screening, more so than Southern blot. Both methods require DNA purification. Current procedures for *P. pastoris* genomic DNA isolation are time-consuming and costly because they employ Zymolyase [2] or Lyticase [3] for cell lyses. We have adapted the previously reported method for DNA isolation from fungal mycelia and spores [4] to *P. pastoris.* This method is simple and avoids the use of enzymes and procedures such as the immersion of samples in liquid nitrogen.

*M*aterials and Methods

The competent *P. pastoris* GS115 cells (Invitrogen, San Diego, CA) were electroporated in the presence of the pPIC9K vector [5] carrying a 1.35 Kbp DNA fragment coding for the Fc domain of the tetanus toxin, linearized with *Bgl*II. This linearization with *Bgl*II allowed the endogenous *AOX1* gene in the GS115 genome to be replaced with the Fc and plasmid-encoded histidinol dehydrogenase *(HIS4)* genes on homologous recombination. After selecting *his*$^+$ transformants, the clones were grown in a liquid medium and DNA was purified using the following *DNA isolation* protocol:

1. Inoculate 3 mL the YPD medium (1% yeast extract, 2% peptone, 2% dextrose) with a single colony of *P. pastoris* transformants. Incubate overnight at 30 ºC, with vigorous shaking (250 rpm).
2. Pellet 2 mL of an overnight culture of *P. pastoris* by microcentrifugation at 10,000 x*g* for 1 min at room temperature and remove the medium.
3. Add 400 mL of the lysis buffer (50 mM Tris-HCl pH 7.2, 50 mM EDTA, 3% SDS, 1% β-Mercaptoethanol) and vortex until the mixture is homogeneous. If the mixture is too viscous, add more lysis buffer (up to 700 mL). Incubate at 65 °C for 1 h.
4. Add an equal volume of phenol [50mM Tris-HCl (pH 8.0) saturated]:chloroform (1:1) and vortex for 20 s. Microcentrifuge at 10,000 x*g* for 15 min at room temperature or until aqueous phase is clear.
5. Remove 300 to 350 mL of the aqueous phase containing the DNA to a new tube. Be careful not to take any cellular debris from the interface.

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6. Add 0.1 volume of 3 M sodium acetate buffer, pH 5.2, to the aqueous phase followed by a 0.6 volume of isopropanol. Mix gently by inversion for 2 min. Microcentrifuge at 10,000 x*g* for 15 min. Pour off the supernatant. Rinse the pellet once with 70% ethanol and centrifuge again under the same conditions as above.
7. Dry the pellet at room temperature or in a vacuum oven at 50 ºC for 15 min. Resuspend the pellet in 50 mL water with 50 mg/mL RNase A. The final concentration of total DNA should be in the range of 1 to 10 mg/mL.
8. For PCR, 1 mL of the DNA sample should be diluted in water to a final concentration of 10 ng/mL.

To ensure that the desired recombinant events took place, PCR was amplified with AOX1 5´ (5´-GACTGGTTCCAATTGACAAGC3´) and AOX1 3´ (5´GCAAATGGCATTCTGACATCC 3´) primers (Invitrogen, San Diego, CA) on the dilutions of the resultant DNA samples. The PCR amplification was made by using 1 mL of each diluted DNA sample in a total volume of 20 mL of reaction mixture that contained 2 mL of the 10x PCR buffer, 2 mL of $MgCl_2$ (25 mM), 0.5 mL of deoxyribonucleoside triphosphate mixture (25 mM each of dATP, dGTP, dCTP and dTTP), 0.5 mL each of 10 mM AOX1 5´ and AOX1 3´ primers, and one unit of *Taq* DNA polymerase (GIBCO BRL, Gaithersburg, MD). The PCR reaction was performed on a Perkin-Elmer 9600 Thermal Cycler (PE Biosystems, Foster City, CA), using the following thermal cycling program: after the initial denaturation at 94 ºC for 3 min, the reaction mixtures underwent 25 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 30 s, and extension at 72 ºC for 2 min, with an additional 7 min extension at 72 ºC.

Results and Discussion

As shown in the gel electrophoretic analysis (Figure 1), the isolation of genomic DNA from transformed clones via this method results in enough *P. pastoris* DNA to perform more than one PCR analysis of the samples. In addition, the quality is suitable for dot and southern blot analysis (data not shown). PCR analysis using the isolated DNA from transformed clone candidates as the template (Figure 2) showed the presence of the gene of interest in certain clones. Unlike the methodologies for direct PCR from plaque colonies [6], that show in our experience non-repetitive results, the PCR analysis performed here does not require a change in thermal parameters, the presence of additional components in the reaction mixture, or the use of special primers to obtain high yields of PCR products, as is frequently the case when samples of low quality DNA are used as template.

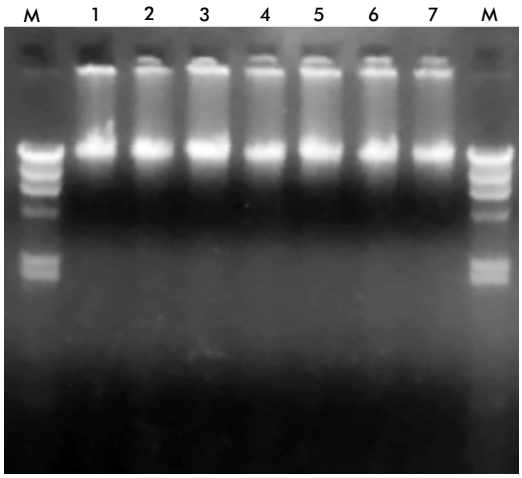

Figure 1. Genomic DNA isolated from *P. pastoris* clones. 1 mL of DNA isolated with the proposed protocol from several clones were run on 0.8% agarose and stained with ethidium bromide. Lanes from 1 to 7 contain DNA from transformed *his*$^+$ clones. Lane M contains lambda phage DNA *Hind*III digests.

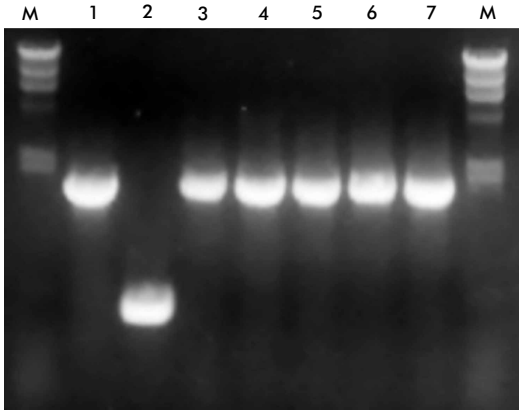

Figure 2. PCR analysis of *P. pastoris* clones. 10 μL of PCR reaction were run on 1% agarose and stained with ethidium bromide. Lane 1: DNA amplification of pPIC9K-Fc plasmid as a positive control for PCR reaction. Lane 2: *his*$^+$ clone carrying the pPIC9K vector without the insert. Lanes 3–7: 1.9 Kbp PCR product from *his*$^+$ transformants corresponding to 1.35 Kbp DNA of fragment Fc + 490 bp of the vector. Lane M contains lambda phage DNA *Hind*III digests.

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