A SINGLE-STEP SCREENING PROCEDURE FOR Pichia pastoris CLONES, BY PCR

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ABSTRACT

The screening of *Pichia pastoris* transformants is always necessary to avoid any possible selection of "false" transformants resulting from a gene conversion event between the transforming vector his4 gene and the mutant *his4* locus in the *Pichia* genome. True transformants can be identified by conventional Southern blot or by polymerase chain reaction (PCR) analysis of purified genomic DNA, although both methods have the disadvantage of being time-consuming and the number of clones that can be simultaneously screened is limited. In this report, we describe a faster method for the screening of *P. pastoris* clones, using a single-step PCR procedure from fresh-intact colonies.

Key words: PCR, *Pichia pastoris*, methylotrophic yeast, AOX1

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RESUMEN

El análisis de transformantes de *Pichia pastoris* es siempre necesario para eliminar la selección de "falsos" positivos resultantes del evento de conversión génica entre el gen *his4* del vector de transformación y el locus *his4* en el genoma de *Pichia*. Los transformantes positivos pueden ser identificados por técnicas convencionales como Southern blot o reacción en cadena de la polimerasa (RCP), a partir de ADN genómico purificado. No obstante, ambos métodos tienen la desventaja de ser procedimientos de larga duración y de permitir el análisis de un número limitado de clones simultáneamente. En este reporte describimos un método de pesquisaje en un solo paso de clones de *P. pastoris* mediante RCP de colonias frescas intactas.

Palabras claves: RCP, *Pichia pastoris*, levadura metilotrófica, AOX1

Introduction

The methylotrophic yeast *Pichia pastoris* has been widely used as a suitable host for the expression of foreign genes. In the last few years, a highly efficient expression system (available as a kit from Invitrogen; San Diego, USA) was designed using the methanol-inducible *P. pastoris* alcohol oxidase1 (*AOX1*) promoter and vectors which, carrying a cloned sequence of interest, are inserted by recombination into the *Pichia* genome with replacement (or not) of the resident *AOX1* locus (1). When the *AOX1* locus is not affected by recombination, the clones are able to use methanol efficiently as a carbon source (Mut$^+$ phenotype). On the contrary, when the *AOX1* locus is replaced, only the *AOX2* gene remains active and methanol is then used very slowly (Mut^s phenotype).

Since the commercially available *P. pastoris* strains are mutated at the *his4* locus and the vectors used carry a functional *his4* gene, after transformation the clone selection is currently accomplished by growing in a histidine-deficient media. However, about 10 % to 20 % of the transformants are the result of a gene conversion event between the *his4* gene of the transforming vector and the mutant *his4* locus in the *Pichia* genome, making a subsequent screening step of true transformants necessary. True transformants can be identified by conventional Southern blot or by polymerase chain reaction (PCR) analysis of purified genomic DNA, though both methods have the disadvantage of being time-consuming and the number of clones that can be screened simultaneously is limited. Recently, Linder *et al.* (2) proposed a method for the PCR screening of *P. pastoris* clones from colonies previously treated with lyticase. In this report, we describe a faster, single-step PCR screening procedure that uses intact yeast colonies, being the only limiting factor the thermal-cycler space.

Materials and Methods

The *P. pastoris* strain GS115 (Invitrogen; San Diego, USA) was used in our experiments after transforming it with a pPIC-3K vector (3) carrying a 2.13 kb DNA fragment coding for the toxic region of the Cry1Ab protein of *Bacillus thuringiensis* var. *kurstaki* HD-1. The resulting clones were called PPK19. Fresh colonies were taken from YNB (Difco, USA) glucose plates and used as a template in the PCR reaction mixture. PCR of PPK19 clones was

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performed in a HYBAID thermal-cycler (UK) using *AOX1* primers (Table 1).

Results and Discussion

The protocol for the single-step PCR screening procedure is depicted in Table 1. The present method was designed for both the detection of *Pichia* integrants and the determination of the clone Mut phenotype by PCR using 5′ and 3′ *AOX1* primers. For the appropriate interpretation of the PCR results when pHIL and pPIC plasmids are used, the distances in base pairs between the *AOX1* primers should be added to the size of the cloned insert (*e.g.*: pHIL-D2: + 0.159 kb, pPIC-3K: + 0.228 kb). From Mut^+ clones two bands were generated and detected, one corresponding to the insert (2.35 kb) and the other to the *AOX1* gene (approx. 2.2 kb), while for Mut^S clones only the band corresponding to the insert (2.35 kb) was detected (Figure 1).

In an attempt to obtain a higher PCR product yield, we tried the incubation of yeast cells in a common microwave oven or alternatively in a water bath at 100 °C for disruption of the cell wall and membranes prior to amplification. However, no improvement was attained (data not shown).

It should be mentioned that inconsistent PCR results were obtained after keeping plates stored at 4 °C for several weeks. The reasons accounting for this observation can not be formulated. A possible explanation could be that the strength of the cell wall of certain methylotrophic yeasts increases with age (4), making the rupture inefficient by this published method. Given this, it follows that the efficient am-

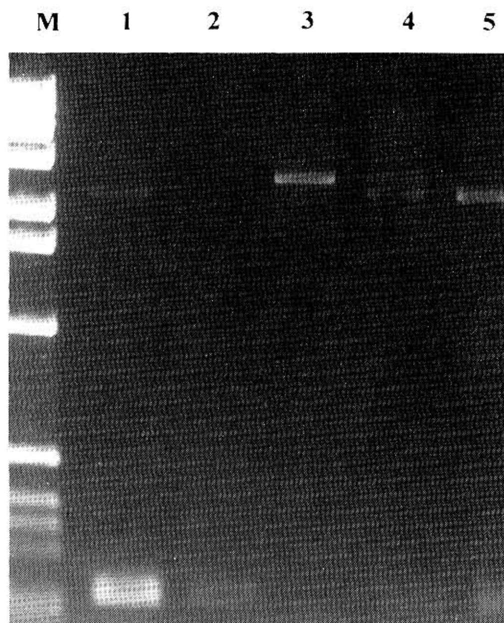

Figure 1. Analysis of PCR products obtained from intact *P. pastoris* colonies. Volumes of 10 μL of the PCR reactions were run on a 1.2 % agarose gel and stained with ethidium bromide. Lane 1: a Mut^+ clone carrying the pPIC-3K vector without insert (size of the resulting bands: 2.2 kb and 0.228 kb); lane 2: no yeast colony as a negative control; lane 3: a Mut^S PPK19 clone (size of the resulting band: 2.35 kb); lane 4: a Mut^+ PPK19 clone (size of the resulting bands: 2.2 kb and 2.35 kb); lane 5: wild-type yeast strain showing the characteristic band of the *AOX1* gene (2.2 kb) and lane M: DNA ladder (1 kb ladder, BRL).

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Table 1. Protocol for a single-tube PCR screening procedure.

1. Pick a pinpoint-size portion of a yeast colony from the fresh agar plate and resuspend the cells in a microcentrifuge tube containing 5 μL of 10x Taq DNA polymerase reaction buffer (500 mM KCl; 100 mM Tris-HCl pH = 9.0 at 25 °C; 25 mM $MgCl_2$; 1 % v/v Triton X-100) and overlay with 20 μL of mineral oil.
2. Prepare a cocktail mixture with the following reagents:
   - deoxyribonucleoside triphosphate mixture (2,5 mM each): **5** μL
   - AOX1 5′ primer, 5′-GACTGGTTCCAATTGACAAGC-3′ (20 pmol/μL): **1** μL
   - AOX1 3′ primer, 5′-GCAAATGGCATTCTGACATCC-3′ (20 pmol/μL): **1** μL
   - *Taq* DNA polymerase: 2 U (*EnziBiot*, Cuba)

   complete with H_2O to a final volume of **45** μL.
3. Add 45 μL of the cocktail mixture to each sample and spin tubes briefly.
4. Put the samples into the cycler and heat at 95 °C for 5 min.
5. Cycle 30 times at the following conditions:
   - denaturation step: 95 °C, 1 min
   - annealing step: 60 °C, 1 min
   - extension step: 72 °C, 2 min

   Add a final step of a 10 min extension at 72 °C to the end of the cycling reaction.
6. Run 10 μL of the reaction samples on agarose gels of an appropriate percentage.

The values in bold letters correspond to the quantities per sample.

plification of any genomic sequence by our PCR procedure should necessarily require the use of intact fresh yeast cells (preferably not older than two weeks) as a source of template. In addition, a proper design of the primer set and optimization of PCR conditions should be accomplished in order to obtain a reliable DNA amplification. For *Pichia* clone screening we recommend an extension step of at least 2 min to ensure amplification of the 2.2 kb *AOX1* locus in Mut⁺ clones.

In summary, this new screening method has the advantages that it is time-saving and cost-effective since it does not require expensive reagents (cell-wall degradative enzymes) or specialized equipment for cell disruption. Consequently, it is feasible to obtain a ready-to-amplify sample by just introducing a pinpoint-size portion of a fresh yeast colony directly into the reaction tube, thus avoiding other rather complicated steps and reducing handling. Finally, misclassification of Mut^+ clones as Mut^s was not observed.

This procedure, together with our previous report (5) on the genetic transformation of *P. pastoris* via colony electroporation would greatly facilitate routine genetic manipulation of yeast and speed up future work on this topic.

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