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GENERATION OF MULTICOPY PICHIA CLONES FROM GAP VECTOR.

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ABSTRACT

The methylotrophic yeast *Pichia pastoris* was used as a host to generate multicopy clones using in-vitro multimerization and GAP vector approaches. The latter approach relied on the selection of spontaneously occurring multiple integrants based on zeocin resistance. Higher levels of heterologous protein could result using these approaches.

KEYWORD: In vitro multimerization, GAP vector, Zeocin, *Pichia*

INTRODUCTION

The methylotrophic yeast, *Pichia pastoris* has emerged as a powerful and inexpensive heterologous system for the production of high levels of functionally active recombinant proteins. It is also the only system that offers the benefits of *E.coli* (high-level expression, easy scale-up, and inexpensive growth) combined with the advantages of expression in a eukaryotic system (protein processing, folding, post-translational modifications) (Higgins and Cregg, 1998).

The generation of recombinant strains with multiple copies of the expression plasmid integrated into the genome has been shown to result in an increase in heterologous protein production via a gene dosage effect for a number of different heterologous genes (Clare et al, 1991 (a) Clare et al 1991 (b) Romanos, 1991; Romanos et al, 1995; Scorer et al, 1994). In addition to screening for relatively rare spontaneous events, there are several methods to generate recombinant strains containing multiple plasmid copies. Among such methods are: invitro multimerization and G418 hyper resistance screening of His + transformants (Scorer et al. 1994).

Zeocin belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from Streptomyces. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and antitumour drugs. They show strong toxicity against bacteria, yeast, plants, and mammalian cells (Berdy, 1980). Zeocin hyper resistant clones arise from, multiple copies of the Zeocin resistance gene, which in turn corresponds to multiple plasmid copies and thus multiple copies of the heterologous gene being expressed. If expression levels of the heterologous gene product respond to a gene dosage effect, higher levels of heterologous protein can result. The use of the Zeocin marker has the added benefit that zeocin hyper resistant transformants can be selected directly. The selection of zeocin resistant clones generates an enrichment in recombinant strains with multiple copies of the integrated vector. Also, the product of the zeocin resistance gene functions stoichiometrically, not enzymatically, as does the kanamycin gene product, which may lead to a more efficient copy number correlation with a zeocin hyper resistant phenotype (Higgins and Cregg, 1998). In this study the multipcopy clones were achieved as shown in Fig. I using invitro multimerization and Glyceraldehyde phosphate (GAP) vector approaches.

MATERIALS AND METHOD

Host Strain and Plasmid Vectors

The *P.pastoris* host strain used in this study was the histidine requiring auxotroph, G115(his 4). The *Pichia* vectors P AO815 and PGAP were used. Selection of these vectors in *P. pastoris* was based on a single small dominant selectable marker that confers resistance to the drug zeocin. Both GS115 (his 4) host strain as well as the vectors PAO815 and PGAP were from International Centre for Genetic Engineering and Biotechnology, India.

Preparation of PAOX-HBsAg-TT Fragment

The 2kb PAOX-HBsAg-TT Fragment was prepared by digesting PAO815+ Ic-HBsAg plasmid with Bam HI and Bgl II.

In vitro multimerization

A self-ligation reaction using purified HBsAg expression cassette (EC) DNA fragment was set up using 2kb HBsAg EC fragment, Ligase buffer, and T4 DNA Ligase. They were mixed, spun down for a few seconds and incubated at 16°C over night. The ligation reaction was heat-inactivated at 65°C for 20 minutes. The BamHI/Bgl II double digestion was also set up to eliminate unwanted products.

The ligated products were analyzed on a 0.8% agarose gel with 1kb DNA marker ladder.

Zeocin Screening of GAP clones

The GAP vector approach relies on the selection of spontaneously occurring multiple integrants based on zeocin resistance. Four Yeast extracat peptone Dextrose (YPDS) plates containing 0.1, 0.5, 1.0 and 2.0mg zeocin/ml were marked. The plates contained a uniform grid with the individual boxes numbered identically.

A sterile P-200 tip was used to pick a colony from the plate of zeocin-resistant clones and patched sequentially on to four plates, making sure that it was

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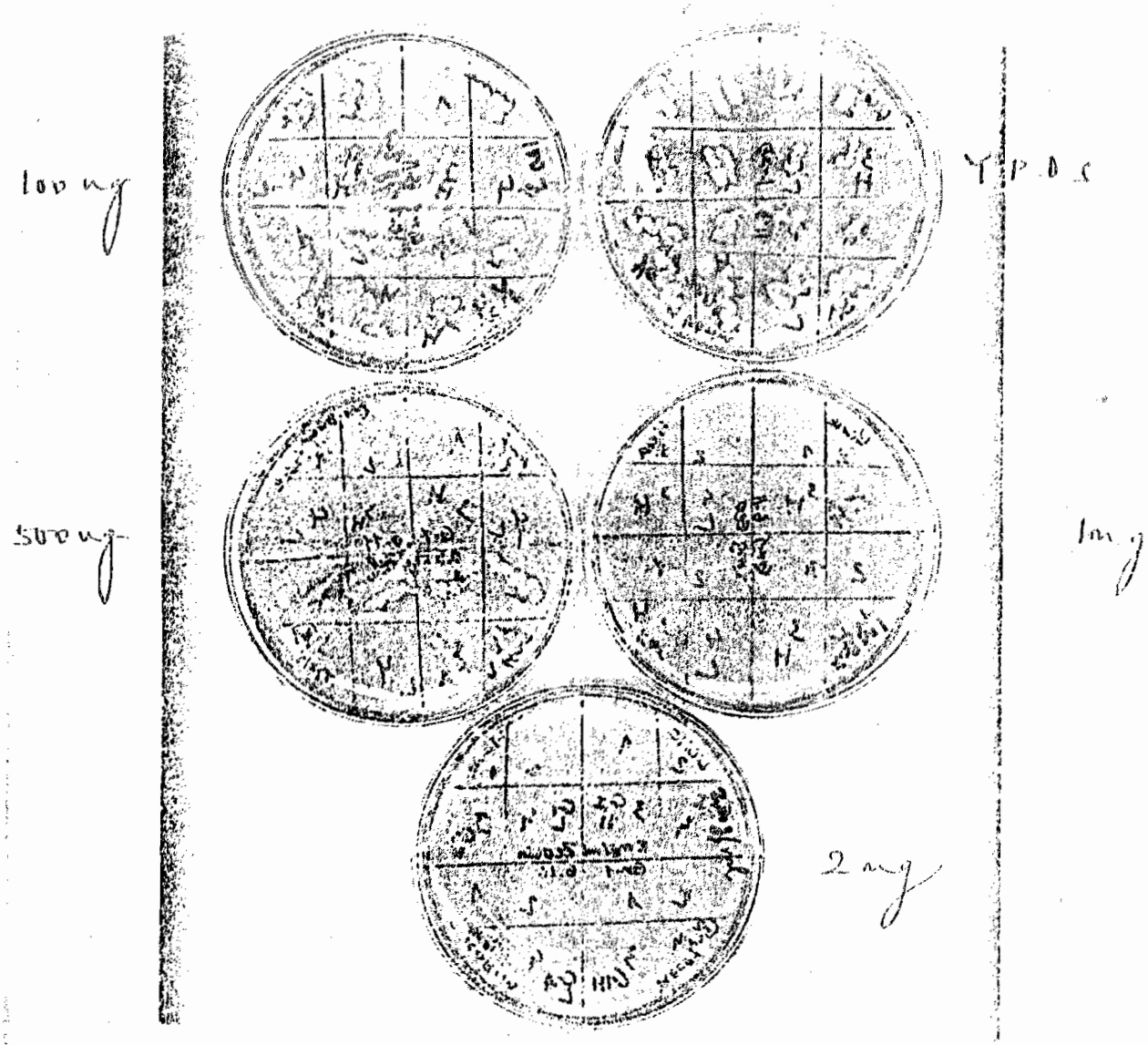

Fig. 1: Zeocin Screening of GAP clones

patched on its designated slot in each plate. The plates were incubated at 30°C for 2-3 days and the ability of each clone to grow in the presence of increasing zeocin concentrations was evaluated. (Higgins and Cregg, 1998).

RESULT

In the PAOX-HBsAg-TT fragment, the Bgl II and Bam HI over hangs are present at the 5^1 and 3^1 ends respectively. Ligation of the expression cassette generated head- to-tail, head-to head, and tail-to-tail multimers because the Bgl II and Bam HI over hangs are compatible with each other. Creation of head-to-tail multimers by the ligation of Bam HI over hang at 3^1 end of one HBsAg-EC to the Bgl II over hang at the 5^1 end of a second HBsAg-EC destroyed both sites between the adjacent HBsAg-ECs. Digestion of the multimers in the ligated reaction with Bam HI and Bgl eliminated those multimers with tail to tail and head-to-head orientation.

Multicopy *Pichia* clones were generated from *Pichia* vectors as snown in Figs. II and III. The first lane in Fig. II showed the ladder. In Fig. II the multi copies were shown for each lane. In Fig III, apart from showing the multicopies, the uncut lanes were also shown. Plate 1 showed the different plates containing different zeocin concentration. It showed that zeocin-resistant transformant is hyperresistant to zeocin and is likely to represent multicopy events.

DISCUSSION

The most commonly used vectors for heterologous protein expression in *Pichia pastoris* carry its wild type HIS4 gene and the bacterial ampicilin resitance gene as selectable markers (Cregg, et al, 1885; Romanos et al, 1992: Cregg et al, 1993). H1S4-based vectors are [illegible] typically between 7 and 10kb in size, creating diff[illegible] for routine cloning manipulations, especially when generating constructions with large or multiple insertions.

Generation of significantly smaller vectors has

1 2 3 4 5 6 7 8 9 10 11

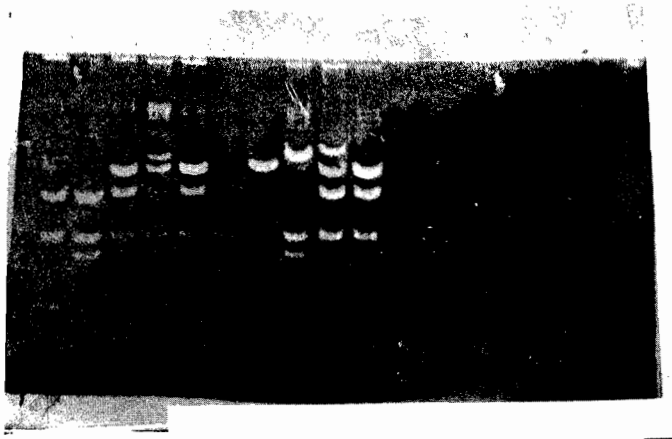

FIG. II Multicopy Pichia clones generated from Vectors

Lane 1, Ladder; Lane 2, Zero copy; Lane 3, 1 copy; Lane 4, 3copy ; Lane 11. 3 copy

1 2 3 4 5 6 7 8

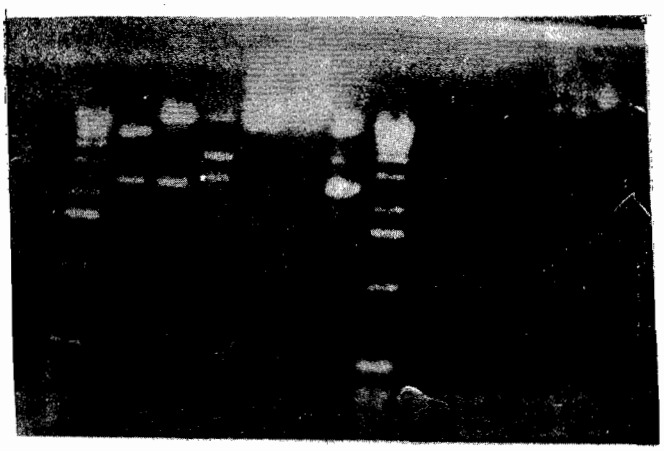

Fig. III. Multicopy Pichia clones and uncut lanes

Lane 1, Ladder; Lane 2, 1 copy, Lane 3, 3 copy , Lane 4 p GAP Lane 5, uncut for 2; Lane 6 uncut for 3; Lane 7 uncut for 4, Lane 8, Ladder.

been achieved by using a single dominant selectable marker that functions both in *E. coli* and *P. pastoris* replacing the H1S4, ampicillin and Kanamycin genes (Higgins and Creggy, 1998). The marker is the sh ble gene from *Streptoalloteichus hindustanus*. The sh ble gene product confers resistance to the drug zeocin in *E.coli*, yeast (including *P. pastoris*) and other eukaryotes (Wenzel et al., 1992). A net reduction in vector size or approximately 3.5kb is achieved because the small zeocin resistance gene substitutes for the H1S4, ampicillin and kanamycin genes.

Furthermore, selection of zeocin resistant transformants at high concentration of zeocin generate an enrichment in recombinant strains with multiple copies of the integrated vector. Direct selection of zeocin hyperresistant transformants can be used routinely to generate a population of multicopy clones that may ultimately result in an increase in the level of heterologous protein production (Higgins and Creggy, 1998).

In vitro multimeration has been used to enhance expression levels using higher gene dosage as reported by Vassileva et al (2001).

In conclusion, muticopy *Pichia pastoris* clones were generated in this work using in vitro multimerization approach, where Hepatitis B. Surface Antigen (HBsAg) was fused in a stepwise fashion to generate multimers in which copy number was predetermined. Generating multicopy clones using the GAP vector which relied on the selection of spontaneously occurring multiple integrante based on zeocin resistance.

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