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# Production of L1 protein from different types of HPV in *Pichia pastoris* using an integrative vector

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## Abstract

Human papillomavirus (HPV) infection is the most common sexually transmitted disease in the world and is related to the etiology of cervical cancer. The most common high-risk HPV types are 16 and 18; however, the second most prevalent type in the Midwestern region of Brazil is HPV-33. New vaccine strategies against HPV have shown that virus-like particles (VLP) of the major capsid protein (L1) induce efficient production of antibodies, which confer protection against the same viral type. The methylotrophic yeast *Pichia pastoris* is an efficient and inexpensive expression system for the production of high levels of heterologous proteins stably using a wild-type gene in combination with an integrative vector. It was recently demonstrated that *P. pastoris* can produce the HPV-16 L1 protein by using an episomal vector associated with the optimized L1 gene. However, the use of an episomal vector is not appropriate for protein production on an industrial scale. In the present study, the vectors were integrated into the *Pichia* genome and the results were positive for L1 gene transcription and protein production, both intracellularly and in the extracellular environment. Despite the great potential for expression by the *P. pastoris* system, our results suggest a low yield of L1 recombinant protein, which, however, does not make this system unworkable. The achievement of stable clones containing the expression cassettes integrated in the genome may permit optimizations that could enable the establishment of a platform for the production of VLP-based vaccines.

Key words: Human papillomavirus; Cervical cancer; *Pichia pastoris*; L1

## Introduction

Human papillomavirus (HPV) infection is the most common sexually transmitted disease in the world and is related to the etiology of cervical cancer. Approximately 500,000 new cases of cancer are diagnosed each year, with a mortality rate of 50% (1). Latin America, Sub-Saharan Africa and Asia (South and Southeast) are the regions with the highest incidence (2). Among more than 120 types, 16 and 18 are the most prevalent HPV types responsible for 70% of cervical cancer cases (3). However, the prevalence of HPV types found in cervical cancer shows geographical variations worldwide (4). In Brazil, HPV type 16 is also the most frequent, but there are regional variations regarding the second most frequent type. In the Northeast and Midwest, HPV types 31 and 33 have the higher incidence (5), while in other regions such as the Southeast, HPV-18 is the second most frequent type among cervical cancer cases (6).

Studies indicate that vaccines based on virus-like particles (VLP), built with the L1 capsid protein, are more effective against infection because they induce high neutralizing antibody titers directed at conformational epitopes of this protein, which composes 90% of the capsid structure (7). VLP can be obtained through the production of HPV L1 protein in heterologous expression systems using mammalian cells (8), plants (9), bacteria (10), insect cells (11), and yeast (12).

The methylotrophic yeast *Pichia pastoris* is an efficient and inexpensive expression system used to produce high levels of heterologous proteins. More than 550 proteins

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have been produced in this yeast (13), including those used as subunit vaccines against viruses, such as the surface antigen of hepatitis B and glycoprotein D of bovine herpes virus (14). *P. pastoris* is the second most used system for heterologous expression after the bacteria *Escherichia coli* (15). Several factors contribute to this fact: the system uses the inducible *AOX1* promoter, which encodes the alcohol oxidase 1 gene. The promoter is repressed by glucose and strongly induced by methanol. The possibility to improve post-translational modifications typically associated with higher eukaryotes and required for the biological activity of proteins (16) as well as the easy adaptation to large-scale fermentation, reaching levels of expression that are not often obtained with other cell types, are remarkable advantages of this system (17).

The viability of *P. pastoris* for HPV-16 L1 protein production using an episomal vector associated with the optimized L1 gene has been recently demonstrated (18). However, the use of an episomal vector is not appropriate for large-scale industrial protein production. Since the expression system based on an episomal vector is not ideal for industrial-scale production (19,20) and the expression system based on *P. pastoris* yeast cells has been used successfully to express different heterologous genes using an integrative vector, and without codon optimization (16), the aim of the present study was to evaluate the viability of expression of the wild-type L1 gene from different human papillomavirus types (types 16, 18, and 33) in *P. pastoris* cells using an integrative vector.

## Material and Methods

### Cloning of the L1 gene of HPV-16, -18 and -33 in expression vectors

The pBR322H16 and pBR322H18 plasmids containing the complete genome of HPV-16 and -18, respectively, were used for PCR of the L1 gene. The HPV-33 L1 gene was amplified from a clinical sample. The primers were constructed on the basis of the known gene sequences of L1 of HPV-16, -18 and -33, deposited in databases (GenBank accession Nos. gi: 27752860, gi: 194272988 and gi: 333049, respectively). The consensus sequence for translation in yeast (Kozak sequence) was added to the forward primer sequences as well as the restriction sites in the 5' end for insertion into the pPICZA vector (*Kpn*I for the primer sequence of HPV-16 and *Bst*BI for the primer sequences of HPV-18 and -33) and pPICZαA vector (*Kpn*I for the primer sequence of HPV-16). In the forward primer for insertion of the HPV-16 L1 gene in the pPICZαA vector it was not necessary to add the Kozak sequence because of the presence of the secretion factor in this vector. The expression vectors were obtained with the EasySelect™ *P. pastoris* expression kit, Invitrogen (USA). The reverse primers included a restriction site for *Sal*I (HPV-16 primer sequence) and *Xho*I (HPV-18 and -33 primer sequences).

**Table 1.** Primers for amplification of the L1 gene of HPV-16, -18 and -33.

| Primers | Sequences* |
|---|---|
| H16Fw | 5'-GGTACCTC**ACAATAATGTCT**CTTTGGCTGC-3' |
| H16Rev | 5'-GTCGACGAGCTTACGTTTTTTGC-3' |
| H18Fw | 5'-GATTCGAA**AAAAATGTG**CCACGGGTCC-3' |
| H18Rev | 5'-GTCCCTCGAGTTCCTGGCACGTACACG-3' |
| H33Fw | 5'-GATTCGAA**AAAATGTCC**GTGTGGCGGCCTAG-3' |
| H33Rev | 5'-TCCCTCGAGTTTTTAACCTTTTTGCG-3' |
| H16αFw | 5'-GGTACCCTTTGGCTGCCT-3' |
| H16αRev | 5'-GTCGACCAGCTTACGTTTTTTGCG-3' |

*Primer-based sequences are deposited in databases: GenBank accession Nos. gi: 27752860 (L1 of HPV-16), gi: 194272988 (L1 of HPV-18) and gi: 333049 (L1 of HPV-33). Underlined: sites for restriction enzymes: *Kpn*I and *Sal*I for H16Fw and H16Rev, respectively; *Bst*BI and *Xho*I for H18Fw and H18Rev, respectively; *Bst*BI and *Xho*I for H33Fw and H33Rev, respectively; *Kpn*I and *Sal*I for H16αFw and H16αRev, respectively. Bold: the Kozak consensus sequence for initiation of translation in yeast (21).

The stop codon of the L1 gene was removed in order to promote the link of the poly-histidine (6xHis) tag present in the vectors (Table 1).

The clones in *E. coli* TOP 10 were obtained by transformation with $CaCl_2$ (22) and selected on low salt LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 2% agar) with Zeocin™ (due to the presence of the *Sh ble* gene in the vectors that confers resistance to this antibiotic). Recombinants were confirmed by PCR, restriction enzyme digestion and sequencing.

### Transformation of *P. pastoris*

The pPICZL1H16, pPICZL1H18, pPICZL1H33, and pPICZαL1H16 constructs were linearized with the *Sac*I enzyme to transform the *P. pastoris* X-33 strain by electroporation (according to the Eppendorf multiporator protocol). *P. pastoris* X-33 was also transformed with empty vectors (pPICZA and pPICZαA) for negative control tests. Aliquots of 50, 100, and 200 mL were spread on YPDS plates (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, 2% agar) containing 100 µg/mL Zeocin for incubation at 30°C for 2-3 days. Clones were detected by colony PCR using the conditions and primers provided in the EasySelect™ *P. pastoris* expression kit. The primers used for detection anneal in the flanking regions of the insert (AOX1 and α-factor) and are reported in Table 2.

### Induction of L1 gene expression in *P. pastoris* cells

The selected clones were induced together with the positive control strains of the intracellular (GS115/β-galactosidase) and extracellular (GS115/albumin) expression provided by the EasySelect™ *P. pastoris* expression kit. The colonies were inoculated in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate,

pH 6.0, 1.34% yeast nitrogen base (YNB), 4 x $10^{-5}$% biotin, and 1% glycerol) to obtain biomass over a period of 16-24 h at 28-30°C. After growth reached an absorbance at 600 nm of 10-20, medium with glycerol was removed by centrifugation and BMMY was added (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x $10^{-5}$% biotin, and 0.5% methanol). During the subsequent 24 h (until 96 h) methanol (inducer of the *AOX1* promoter) was added to achieve a final concentration of 1-2%. Forty clones with the pPICZαL1H16 construction and the pPICZαA only (used as a negative control) were induced in a deep-well plate with 1 mL BMMY medium for 72 h for analysis of extracellular expression. The clones of intracellular expression (approximately 90), as well as the negative control (with the pPICZA only), were induced under the conditions described earlier in 500-mL flasks containing 50 mL medium. For subsequent analysis of L1 gene expression, samples were taken from cultures every 24 h, including aliquots of supernatants and pellets stored at -80°C.

### Transcriptional analysis of the L1 gene

Total RNA was extracted from recombinant clones (X33/pPICZL1H16, X33/pPICZL1H18, X33/pPICZL1H33, and X33/pPICZαL1H16) by the acid-phenol method (24). The cDNA of the three types of HPVs was synthesized using the IMPROM-II™ Reverse Transcription System from Promega (USA). The presence of heterologous gene mRNA in *Pichia* was detected by RT-PCR with primers that anneal in the internal sequence of the L1 genes (Table 3), amplifying a region of 500 bp.

### Detection of L1 protein

L1 protein production was determined by Coomassie-stained SDS-PAGE (12.5%) and by dot blot immunoassay. The intracellular proteins were obtained by lysis with glass beads and the supernatant for analysis of extracellular expression was obtained by centrifugation of the induced culture. The samples were quantified by the Bradford method (25) and subjected to a dot blot protocol that consisted of protein ligation (0.9 µg) to a nitrocellulose membrane for 1 h, wash with 1X PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) for 5 min, blocking with 2.5% milk for 40 min addition of alkalane phosphatase-conjugated anti-6xHis antibody (Sigma, USA) diluted 1:10,000 in 5% milk for incubation at 37°C for 1 h, three washes with 1X PBS-0.05% Tween (10 min each), and development with NTB/BCIP (Sigma).

## Results

### Cloning of the L1 gene for expression in *P. pastoris*

The identity of pPICZL1H16, pPICZL1H18, pPICZL1H33, and pPICZαL1H16 constructs was confirmed by PCR (Figure 1). The integration of these expression cassettes into the *Pichia* genome was confirmed with primers, which anneal to flanking regions of the cloning site in the vector. The results show that constructs were successfully inserted in the expression system and that the expression cassettes were integrated into the *Pichia* genome (Figure 2A). The pattern of amplification shows that clones with the $Mut^S$ and $Mut^+$ phenotypes were obtained due to double-crossover events (replacements) and single-crossover events (insertions) that can occur in gene insertions at the *AOX1* locus, respectively.

### L1 gene expression in *P. pastoris* cells

*Transcription analysis by RT-PCR*. After induction of L1 gene expression in all clones tested, total RNA was extracted for cDNA synthesis and subsequent amplification by RT-PCR. The result was the expected fragment of 500 bp (Figure 2B), confirming the transcription of the L1 gene of the three HPV types in *P. pastoris* cells.

*Analysis of L1 protein expression*. The total lysate of the clones induced after 96 h for intracellular expression and the supernatants of the clones for extracellular expression were subjected to SDS-PAGE (approximately 40 µg) to determine the integrity of the proteins. It was not possible to identify the L1 heterologous protein in the protein profile of the two extracts. The efficiency of induction of the expression system was confirmed by the use of *Pichia* control strains that express heterologous proteins such as

**Table 2.** Primers for the detection of recombinant *Pichia* clones.

| Primers | Sequences* |
|---|---|
| *AOX1*Fw | 5'-GACTGGTTCCAATTGACAAGC-3' |
| *AOX1*Rev | 5'-GCAAATGGCATTCTGACATCC-3' |
| α-factorFw | 5'-TACTATTGCCAGCATTGCTGC-3' |

*The sequences of primers are available on EasySelect *Pichia* Expression Kit Manual (23).

**Table 3.** Primers for RT-PCR.

| Primers | Sequences* | Nucleotide position |
|---|---|---|
| H16Fw_int | 5'-GGTCCATTAGGTGTGGG-3' | 634-659 |
| H16Rev_int | 5'-AGCTGTCGCCATATGGTTCTG-3' | 1111-1133 |
| H18Fw_int | 5'-ATGGTAGATACTGGATATGGTGC-3' | 781-804 |
| H18Rev_int | 5'-CTGCTATACTGCTTAAATTTGGTAG-3' | 1255-1280 |
| H33Fw_int | 5'-GGAACATTGGGGTAAAGGTGTTGC-3' | 500-524 |
| H33Rev_int | 5'-CCACAGTAACAAATACCTGATTGCC-3' | 975-1000 |

*Primer-based sequences are deposited in databases: GenBank accession Nos. gi: 27752860 (L1 of HPV-16), gi: 194272988 (L1 of HPV-18) and gi: 333049 (L1 of HPV-33).

the beta-galactosidase (intracellular) and the human serum albumin (HSA) (extracellular).

L1 protein production in *P. pastoris* was detected by dot blot assay using an antibody against the poly-His tag. The lysates of the intracellular expression clones showed specific reactions due to the presence of L1, as shown in Figure 3A.

The dot blot used to evaluate the extracellular expression of the HPV-16 L1 protein was based on the screening of many induced clones in a deep-well plate for detection of the more efficient. After pre-selection, two clones (15 and 20) that showed intense reactions, as well as the strain used as negative control, were induced in shake flasks. The culture supernatants quantified by the Bradford method

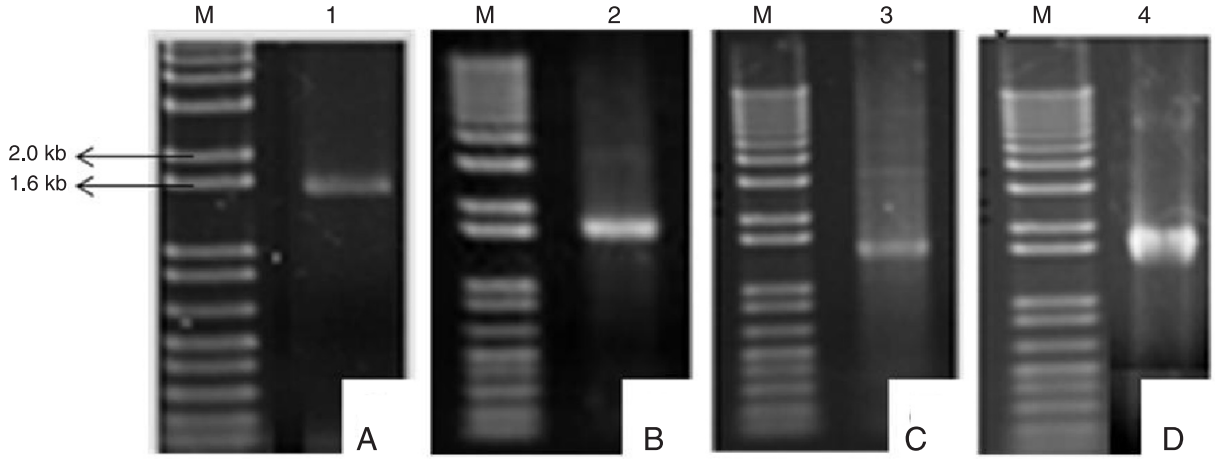


**Figure 1.** Confirmation of the intra- and extracellular constructs for L1 expression by PCR. *A*, Amplification of the HPV-16 L1 gene (1.5 kb) cloned in the pPICZα vector. *B*, *C*, and *D*, HPV-16 (1.5 kb), HPV-18 (1.7 kb), and HPV-33 (1.5 kb) L1 genes, respectively, cloned in the pPICZ vector. On all gels, we used the molecular weight marker 1 kb plus (lane M; Invitrogen).

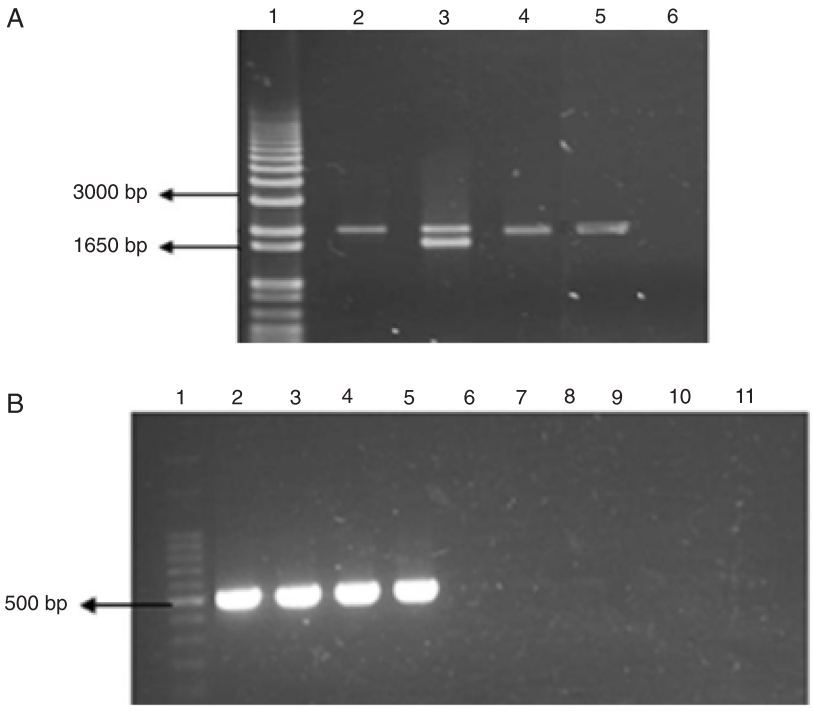


**Figure 2.** PCR detection and RT-PCR of *Pichia* recombinant colonies. *A*, PCR with primers that anneal in the *AOX1* region, amplifying a fragment of 325 bp (in addition to the gene) and in the alpha secretion factor region, amplifying a fragment of 299 bp (in addition to the gene) in pPICZA and pPICZα vectors, respectively. *Lane 1*, 1 kb plus molecular weight marker (Invitrogen); *lane 2*, amplicon obtained from the X33/pPICZL1H18 recombinant (1700 + 325 = 2025 bp); *lane 3*, an amplicon obtained from the X33/pPICZL1H33 recombinant (1500 + 325 = 1825 bp) and an amplicon that refers to the *AOX1* gene (2200 bp) of *P. pastoris* from its $Mut^+$ phenotype; *lane 4*, amplicon obtained from the X33/pPICZL1H16 recombinant (1500 + 325 = 1825 bp); *lane 5*, amplicon obtained from the X33/pPICZαL1H16 recombinant (1500 + 299 = 1799 bp); *lane 6*, negative control (without DNA). *B*, Representative RT-PCR of clones. *Lane 1*, lambda *Hin*dIII molecular weight marker (Invitrogen); *lanes 2* and *3*, X33/pPICZL1H33 and X33/pPICZL1H18 clones, respectively; *lane 4*, X33/pPICZL1H16 clone; *lane 5*, X33/pPICZαL1H16 clone; *lane 6*, X33/pPICZ clone (empty vector, negative control); *lane 7*, negative control (without DNA); *lanes 8*, *9*, *10*, and *11*, controls without reverse transcriptase of all clones.

for standardization of the samples were submitted to a new dot blot, where specific reactions were observed for clones evaluated with anti-6xHis antibody, confirming the presence of the L1 protein in the extracellular environment (Figure 3B).

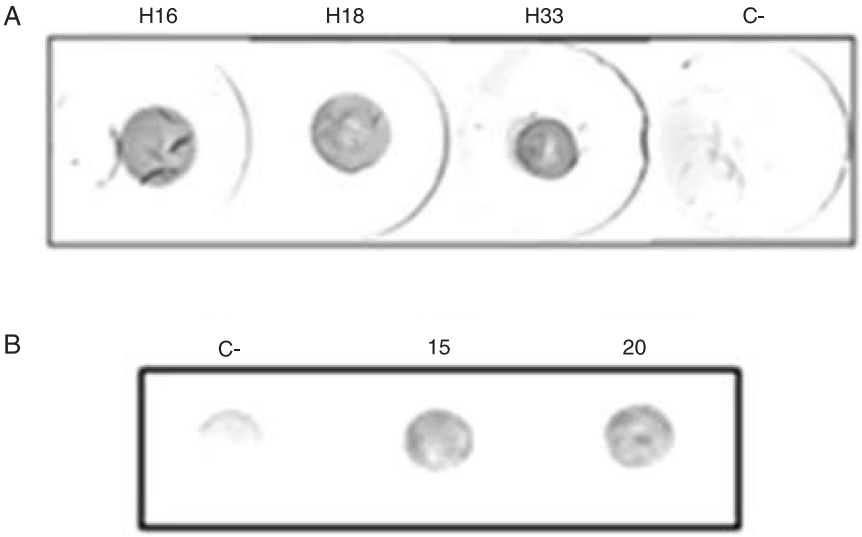


**Figure 3.** Dot blot for the detection of intracellular and extracellular production of the L1 protein. The samples were standardized to an amount of 0.9 µg, which was applied to a nitrocellulose membrane. The specific reactions with the lysates of clones, shown in *A* by H16, H18 and H33 and in *B* by clones 15 and 20, confirm the presence of the L1 protein of the three viral types. In contrast, in the lysate and in the supernatant of the negative controls (C-) there were no reactions.

## Discussion

The aim of this study was to evaluate the possibility of expressing the L1 gene of different HPV types (16, 18, and 33) in *P. pastoris* yeast cells for the production of the L1 major capsid protein using integrative vectors.

Some reports have shown that gene codon optimization can improve the expression levels in *P. pastoris* compared to wild-type gene expression (26,27). However, according to Higgins and Cregg (28), there is a 50-75% probability that the *P. pastoris* system will produce foreign proteins at a reasonable level without the need of optimization. Since 1984 different proteins have been well expressed in *P. pastoris* and several studies have demonstrated the strong and intrinsic *Pichia* ability to express heterologous genes without optimizations (14,29,30).

Bazan et al. (18) demonstrated that, using the episomal vector, the production of the HPV-16 L1 protein in *P. pastoris* was only possible after codon optimization of the L1 gene. It is known that for large-scale production episomal vectors would not be advantageous since the transformed yeast can lose these vectors after successive mitotic divisions because they are not integrated into the genome and thus can be lost in the absence of selection pressure. The need of antibiotics for the maintenance of these clones raises the costs of production and may disagree with the rules imposed by regulatory agencies (31). Thus, to be able to produce the L1 protein on an industrial scale (vaccine production) it is important to obtain stable clones expressing the L1 gene and a manner to do this is to use integrative vectors. In addition, the use of an integrative vector may favor the integration of more than one copy of the heterologous gene, which in many cases increases protein production (32).

The present data allow us to conclude that, although preliminary, the *P. pastoris* expression system is suitable for the L1 protein production from different HPV types. The use of integrative vectors and the consequent stability of clones make this heterologous expression system a potential tool that can be subjected to optimization parameters (use of a bioreactor and/or codon-optimization of the L1 gene) in order to achieve higher levels of the L1 protein and thus establishing a platform for the production of VLP-based vaccine.

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