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Expression of the bovine papillomavirus type 1, 2 and 4 L1 genes in the yeast *Pichia pastoris*

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ABSTRACT. Papillomaviruses are known to cause benign or malignant lesions in various animals. In cattle, bovine papillomavirus (BPV) is the etiologic agent of papillomatosis and neoplasia of the upper gastrointestinal tract and urinary bladder. Currently, there are no standard diagnostic tests or prophylactic vaccines. Protection against papillomavirus infection is conferred by neutralizing antibodies directed towards the major structural protein L1. These antibodies can be efficiently induced by immunization with virus-like particles that are formed spontaneously after L1 gene expression in recombinant systems. The yeast *Pichia pastoris* is known to provide an efficient system for expression of proteins due to reduced cost and high levels of protein production. We evaluated *P. pastoris* for expression of the L1 gene from BPV1, BPV2 and BPV4. After methanol induction, the recombinants were able to produce L1 proteins of the three different BPV types. To increase heterologous L1 protein levels, a codon optimization strategy was used for production under bioreactor conditions. The BPV1 L1

protein was identified by monoclonal antibody anti-6xHis. This is the first report of BPV L1 expression in yeast.

Key words: Bovine papillomavirus; *Pichia pastoris*; L1 gene

INTRODUCTION

Papillomaviruses are a heterogeneous group of viruses associated with specific epithelial lesions. They are small, double-stranded DNA viruses related to a wide variety of proliferative lesions of epithelial origin that are associated with different oncogenic processes in humans and other vertebrates (zur Hausen, 2002). Papillomavirus infection begins in basal cells that are accessed through microabrasions resulting from various forms of physical trauma (Campo, 2002).

In cattle, bovine papillomavirus (BPV) is the etiologic agent of cutaneous papillomatosis and neoplasia of the upper gastrointestinal tract and urinary bladder. BPV has been studied as an infectious agent, as an animal model to investigate the relationship between papillomavirus and its natural hosts, and as a model for human papillomavirus vaccination studies (Campo, 2006). Eleven BPV types have been characterized associated with specific lesions with various histopathological characteristics. The bovine papillomatosis is an important disease leading to economic depreciation of animals and the deterioration of the appearance of the animal and animal leather. The lesions may progress to cancer due to the synergistic action of genetic or environmental co-factors (Leal et al., 2003; Borzacchiello and Roperto, 2008). Although papillomaviruses are considered strictly species specific, BPV1 and, less frequently, BPV2 are recognized as the most important etiological agents in the development of fibroblastic skin tumors, or equine sarcoids, which affect horses, donkeys, and mules (Chambers et al., 2003; Bogaert et al., 2008; Nasir and Campo, 2008).

The main strategies to develop vaccines directed to papillomaviruses are related to the virus capsid proteins L1 and L2. The L1 protein comprises 90% of the capsid structure and is organized in pentamers associated with L2 protein (Campo, 2006). When expressed in eukaryotic and some prokaryotic systems, L1 can self-assemble into a structure similar to that of the viral capsid. These virus-like particles (VLPs) (Kirnbauer et al., 1992; Campo, 2006) are morphologically indistinguishable from native virions and preserve the necessary conformational epitopes to induce high titers of neutralizing antibodies (Dupuy et al., 1999; Palker et al., 2001). Because the VLPs do not contain the viral genome, they are not infectious or oncogenic and thus represent an excellent target for the development of prophylactic vaccines against papillomavirus. The expression of papillomavirus capsid protein has been reported in several heterologous expression systems such as bacteria, yeast, baculovirus-infected insect cells, transgenic plants, and mammalian cells (Liu et al., 2005; Aires et al., 2006; Park et al., 2008). BPV VLPs produced in insect cells have been described as providing potent prophylactic vaccines against BPV4 infection (Kirnbauer et al., 1996).

During the last several decades, a *Pichia pastoris* expression system has been used successfully to produce various recombinant proteins. This system offers many advantages, such as tight regulation of the promoter of the alcohol oxidase 1 gene (*AOX1*) by methanol, easy growth at high cell densities, high levels of protein production at the intra- or extracellular level, and the opportunity to perform posttranslational modifications typically associated

with higher eukaryotes (Macauley-Patrick et al., 2005). The aim of this work was to evaluate a *P. pastoris* expression system for BPV1, BPV2, and BPV4 L1 gene expression and consequent protein production.

MATERIAL AND METHODS

Construction of expression vectors

Escherichia coli TOP10 (Invitrogen, São Paulo, Brazil) was used as a host strain for plasmid cloning experiments. For expression in *P. pastoris*, the wild-type X-33 strain (Mut$^+$, His$^+$) and pPICZA expression vector were purchased from Invitrogen as part of an EasySelect® *Pichia* Expression kit. DNA manipulations were performed according to standard protocols recommended by Sambrook et al. (1989).

The L1 genes were amplified with polymerase chain reaction (PCR) from the complete viral genomes of BPVs 1, 2, and 4 with Platinum *Taq* DNA polymerase High Fidelity (Invitrogen). Specific primers were designed based on complete BPV sequences deposited in GenBank. The forward primers contained the yeast Kozak consensus sequence and a restriction site for *Eco*RI in the 5' end. The reverse primers had a restriction site for *Xho*I in the 5' end. The amplified L1 fragments were initially cloned into a pGEM-T Easy vector (Promega, Wisconsin, USA). After digestion with *Eco*RI and *Xho*I enzymes (New England Biolabs, Massachusetts, USA), the inserts were cloned in frame into the pPICZA expression vector. Then, *E. coli* TOP10 were transformed and plated onto low-salt Luria-Bertani medium (0.5% yeast extract, 0.5% NaCl, 1% tryptone, pH 7.5) supplemented with 25 µg/mL Zeocin® (Invitrogen). The presence and orientation of the insert were analyzed with PCR, enzyme digestion, and sequencing (ABI3100 sequencer, Applied Biosystems). The L1 proteins were expressed fused to a 6xHis tag at the C-terminus in the pPICZA vector.

In addition, the BPV1 L1 gene was synthesized with codons optimized for expression in *P. pastoris* yeast. Codon usage analyses of the heterologous gene and the host organism were performed using the online program Graphical Codon Usage Analyzer (http://gcua.schoedl.de). We also analyzed the GC content of the heterologous gene using Generunner for Windows version 3.05 (Hasting Software). The codon-optimized L1 gene was cloned into the pPICZA expression vector, and the presence of the insert was confirmed with PCR, enzyme digestion, and sequencing.

Transformation of *P. pastoris* and selection of recombinants

P. pastoris X-33 was transformed through electroporation with the expression plasmids previously linearized with the *Sac*I enzyme (New England Biolabs) using a Multiporator system (Eppendorf, Hamburg, Germany). The X-33 strain transformed with the empty pPICZA plasmid was used as a negative control. Immediately after the pulse, cold 1 M sorbitol was added and the cells were incubated for 2 h at 30°C. The transformants were selected on plates with YPDS medium (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol) containing 100 µg/mL Zeocin® and incubated for 2-3 days at 30°C. The presence of the integrated expression plasmids in the transformants was confirmed using colony PCR according to manufacturer recommendations with primers complementary to the 5' and 3' region of *AOX1*.

Expression of L1 genes in *P. pastoris* in shake flasks

The positive recombinants for colony PCR were grown in minimal glycerol medium (MGY; 1% glycerol, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.002% biotin) in baffled flasks at 28°C for 24 h under agitation. For induction of L1 gene expression, the cells were centrifuged, transferred to minimal methanol medium (the same components as those of MGY with glycerol replaced by methanol 0.5%), and the culture was kept at 28°C under vigorous agitation for 96 h. To maintain the induction of recombinant protein, methanol was added to the culture every 24 h to a final concentration of 2%.

Expression of L1 genes in *P. pastoris* in a bioreactor

Cultivations were performed in a 1.3-L bioreactor (BioFlo 110, New Brunswick Scientific, USA) at 28°C and an agitation of 350 rpm. Pre-cultures were inoculated into in 50 mL MGY medium in shake flasks. The cells were grown to an optical density at 600 nm of 3 before adding the inoculum of 50 mL to the bioreactor to reach a working volume of 500 mL MGY medium. After 24 h of biomass generation and total consumption of glycerol in the medium, methanol was added every 24 h to a final concentration of 1% (v/v) to induce protein expression for 48 h. The glycerol and methanol feed batches during bioreactor cultivation were carried out according to the *Pichia* fermentation protocol (Invitrogen). Aeration was kept constant at 1.0 vvm, and pure oxygen was supplied as needed. The medium pH was adjusted and controlled at 6.0 with the addition of 28% (v/v) ammonium hydroxide. Foam formation was suppressed by the addition of antifoam reagent (Antifoam C; Sigma-Aldrich, São Paulo, Brazil).

P. pastoris cell lysis

Yeast cells were harvested by centrifugation, washed, and resuspended in breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol). An equal volume of 0.5-mm acid-washed glass beads (Sigma-Aldrich) was added to the tubes, and a total of 10 cycles of vortexing and incubation on ice for 30 s each were performed. The samples were centrifuged, and the supernatant was transferred to a new tube.

Protein purification

After induction with methanol, the yeast cells were recovered, and the protein extract was obtained. The purification of the recombinant protein was performed on a nickel column with a ProBond® Purification System kit (Invitrogen) following manufacturer recommendations.

RT-PCR

Yeast cells were recovered, and total RNA was extracted from the samples using an SV Total RNA Isolation System kit (Promega) following manufacturer recommendations. The L1 messenger RNA (mRNA) was detected using RT-PCR with an Improm-II Reverse Transcription System (Promega). The amplification was performed with specific primers that amplify a 500-bp internal fragment from BPV1, 2, and 4 L1 genes.

Dot blot assay

For dot blot analysis, protein adsorption was carried out by spotting approximately 10 µL protein extract on a nitrocellulose membrane. The membrane was dried and then blocked with 2.5% nonfat dry milk and incubated with antibody anti-6xHis alkaline phosphatase conjugated (Sigma-Aldrich) at a dilution of 1:20,000. After 3 washes with PBS 0.05% Tween 20, the membranes were revealed with nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (NTB/BCIP®) substrate (Sigma-Aldrich).

SDS-PAGE and Western blot assay

The proteins were boiled in SDS-loading buffer (10% 2-mercaptoethanol, 4% SDS, 0.004% bromophenol blue, 20% glycerol, 0.125 M Tris-HCl, pH 6.8) for 10 min and separated using 12.5% polyacrylamide gel electrophoresis (PAGE). The gel was stained with Coomassie brilliant blue. For the Western blot, proteins were transferred onto PVDF membranes (Millipore, São Paulo, Brazil). The membranes were blocked in 5% nonfat dry milk for 1 h and incubated overnight at 4°C with antibody anti-6xHis alkaline phosphatase conjugated at a dilution of 1:1000. The membranes were then washed 3 times with PBS 0.05% Tween 20 for 10 min. The L1 protein detection was performed using the VISIGLO® chemiluminescent substrate (Amresco, Ohio, USA).

RESULTS

Expression of BPV1, 2, and 4 L1 genes in *P. pastoris*

BPV1, 2, and 4 L1 genes were cloned into the pPICZA expression vector under the control of an inducible *AOX1* promoter. *P. pastoris* transformed with these constructs were grown in medium with methanol and analyzed for L1 gene expression and protein production. After 72 h of methanol induction, recombinant *P. pastoris* were analyzed for BPV1, 2, and 4 L1 gene expression using RT-PCR. A 500-bp fragment of the L1 gene was detected (Figure 1), indicating L1 transcription. As a genomic DNA contamination control, a reverse transcription reaction without reverse transcriptase enzyme was performed for the samples analyzed (see Figure 1).

For analysis of L1 protein production in recombinant *P. pastoris*, the protein extract was analyzed using SDS-PAGE. Although the protein profile showed no signal of degradation, the band related to the L1 protein (~55 kDa) could not be visualized (Figure 2). The production of L1 protein was confirmed by dot blot assay using anti-6xHis antibody in the protein extract of the same recombinants positive for BPV1, 2, and 4 L1 gene transcription (Figure 3). The L1 protein was detected in protein extracts of the clones in the amounts of protein tested (0.3, 0.6, and 1.2 µg). The X-33/pPICZL1B4 clone displayed a weak signal at the levels evaluated; however, a new dot blot using larger amounts of protein extract (1.7, 3.4, and 6.8 µg) demonstrated better results, confirming the production of BPV4 L1 protein in the protein extract of *P. pastoris* clones (see Figure 3). Several Western blot assays using anti-6xHis antibody were then performed, with no satisfactory results.

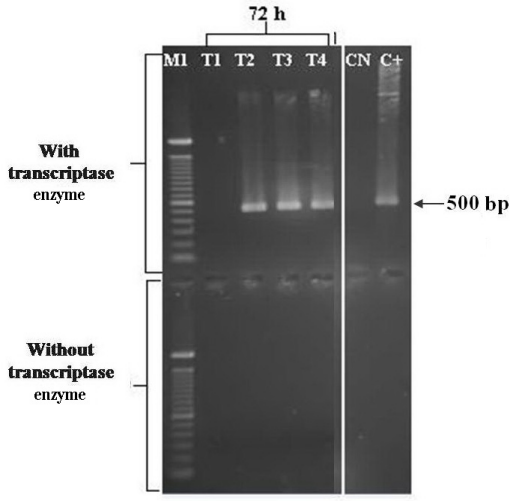

Figure 1. RT-PCR of recombinant *Pichia pastoris* after 72 h of methanol induction. The mRNAs of BPV1, 2 and 4 L1 genes were detected using specific primers that amplify a 500-bp fragment on the central portion of the nucleotide sequence, encoding L1 protein. *Lane M1* = 100-bp DNA ladder (Promega); *lane T1* = X33/pPICZA clone (empty vector, negative control of L1 expression); *lanes T2*, *T3* and *T4* = X33/pPICZAL1B1, X33/pPICZAL1B2 and X33/pPICZAL1B4 clones, respectively; *lane CN* = control reaction (without DNA); *lane C+* = plasmid used as positive control reaction. The upper side corresponds to the RT-PCR experimental test. The lower side corresponds to the control experiment of genomic DNA contamination, in which the reverse transcription reaction was performed without the reverse transcriptase enzyme.

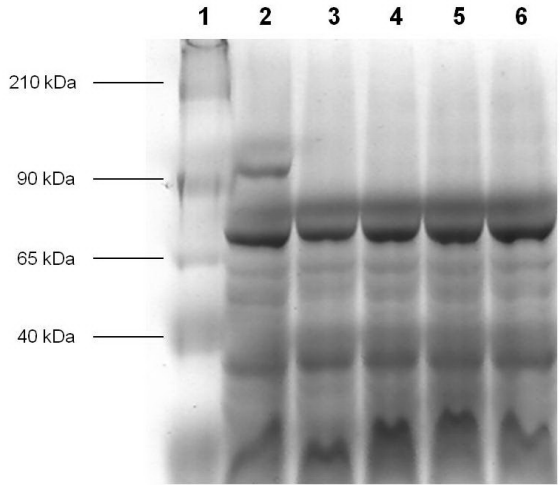

Figure 2. SDS-PAGE of recombinant *Pichia pastoris* after 72 h of methanol induction. The yeast cells were lysed after cultivation in medium with methanol and the soluble protein extract was fractionated on 12.5% SDS-PAGE (around 40 µg/lane). *Lane 1* = Color Burst Electrophoresis Marker (Sigma); *lane 2* = protein extract of recombinant *P. pastoris* GS115/pPICZ*LacZ*, positive control of intracellular expression in *P. pastoris* provided by the EasySelect® *Pichia* Expression Kit (Invitrogen); *lanes 3*, *4* and *5* = protein extract of recombinant *P. pastoris* X33/pPICZL1B1, X33/pPICZL1B2 and X33/pPICZL1B4, respectively; *lane 6* = protein extract of recombinant *P. pastoris* X33/pPICZA, empty vector, negative control of L1 expression. The figure shows that protein degradation did not occur, but the L1 protein band (~55 kDa) could not be verified. Instead, it was possible to detect the band corresponding to β-galactosidase protein (119 kDa). The gel was stained with Coomassie brilliant blue.

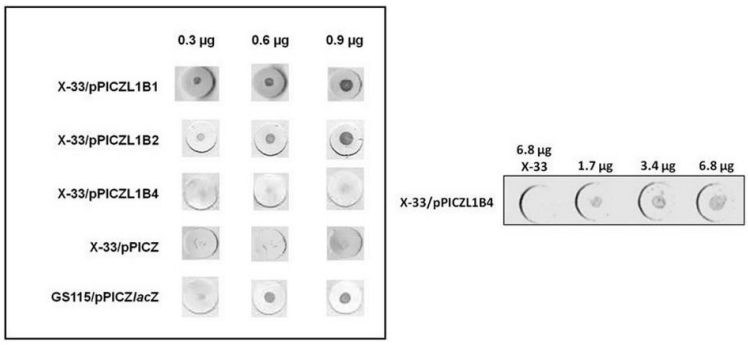

Figure 3. Detection of BPV1, 2 and 4 L1 proteins in recombinant *Pichia pastoris* after 72 h of methanol induction. Dot blots using antibody anti-6xHis alkaline phosphatase conjugated were performed to verify the L1 protein production, which has a C-terminal 6-histidine tag. The assay detected BPV1, 2 and 4 L1 proteins in recombinant *P. pastoris* in the three amounts of proteins tested (0.3, 0.6 and 1.2 µg), while in the X-33/pPICZ clone it was not detected. Although the reaction from the X-33/pPICZL1B4 clone protein extract presented low intensity, the BPV4 L1 protein was observed with greater intensity when tested with larger amounts of protein extract (1.7, 3.4 and 6.8 µg). The assay also was positive in protein extract from *P. pastoris* GS115/pPICZ*LacZ*, provided by the EasySelect® *Pichia* Expression Kit (Invitrogen), which produces β-galactosidase protein fused to 6xHis tag. The samples were diluted and were applied to nitrocellulose membrane. The dot blot was revealed with NBT/BCIP.

Expression of BPV1 L1 gene codon-optimized for *P. pastoris*

After analysis of L1 gene expression in 3 types of BPV, the BPV1 L1 gene was selected for nucleotide sequence modification based on *P. pastoris* preferred codons to increase the expression levels of L1 protein. The codon-optimized gene cloned into the pPICZA vector was successful expressed after cultivation in a bioreactor. Gene transcription was verified using RT-PCR (data not shown) and protein production by Western blot, which detected a 56-kDa band corresponding to the L1 protein (Figure 4). Higher-molecular-weight proteins were also observed. These bands may be of L1 protein dimers.

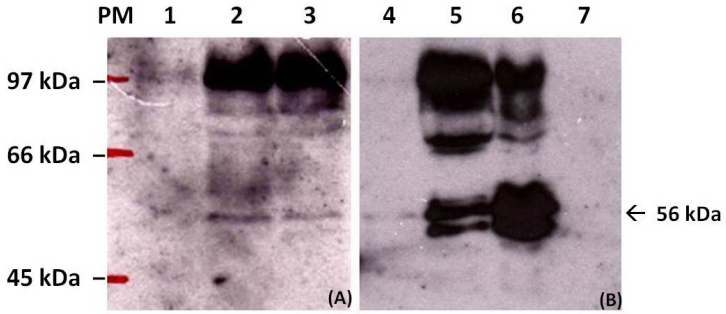

Figure 4. Detection of the recombinant BPV1 L1 protein (56 kDa) in *Pichia pastoris* by Western blot. Proteins were separated by SDS-PAGE and transferred onto PVDF membrane for Western blot assay using antibody anti-6xHis alkaline phosphatase conjugated. **A.** Protein extract was obtained at 0, 24, and 48 h after methanol induction. *Lane PM* = Protein molecular marker LMW (GE Healthcare); *lane 1* = expression of the recombinant L1 protein at 0 h; *lane 2* = expression of the recombinant L1 protein at 24 h; *lane 3* = expression of the recombinant L1 protein at 48 h. **B.** BPV1 L1 protein was purified from protein extract obtained after 48 h of methanol induction using the ProBond® Purification System. *Lane 4* = First elution of the purified recombinant L1 protein; *lane 5* = second elution; *lane 6* = third elution; *lane 7* = fourth elution.

DISCUSSION

BPV is distributed worldwide in cattle herds and is recognized as the etiologic agent associated with various benign and malignant tumors and major economic losses. Currently, no vaccine or effective treatment exists to control these infections. Traditionally, prophylactic vaccines for animal viruses are produced with live attenuated or inactivated viruses. Papillomaviruses cannot be cultured *in vitro* for the production of virions or viral proteins, which would be a source of antigens. The developing papillomavirus vaccines are related to the technology of genetic engineering. This report is the first of heterologous expression of BPV genes in yeast. In this work, BPV1, 2, and 4 L1 genes were expressed in *P. pastoris* under control of the methanol-inducible *AOX1* promoter. After optimization of the BPV1 L1 gene and cultivation of recombinant *P. pastoris* in a bioreactor, we detected the 56-kDa L1 protein with Western blot.

The methylotrophic yeast *P. pastoris* has become a highly successful system for the production of a variety of heterologous proteins (Daly and Hearn, 2005; Dummer et al., 2009; Yu et al., 2009); however, many proteins have been produced with varying degrees of success for reasons that remain unclear. Some reports have shown that AT-rich regions of heterologous genes can interrupt transcription, which considerably reduces protein production (Romanos, 1995; Boettner et al., 2007). In *Saccharomyces*, a premature termination of transcription in TA-rich sequences such as TTTTTATA, which resembles a sequence in human immunodeficiency virus 1 gp 120, has been verified to cause premature mRNA termination when expressed in *Pichia* (Scorer et al., 1993). In this study, we used RT-PCR to check the transcription of BPV1, 2 and 4 L1 genes from recombinant *P. pastoris*, confirming that the clones were positive for L1 mRNA transcription.

SDS-PAGE was carried out with various protein concentrations to assess whether degradation of protein extract occurred, and although the protein profile indicated that degradation did not occur, the band related to the L1 protein (55-58 kDa) was not visualized. To detect the presence of heterologous protein in the intracellular extract of recombinant yeasts, we initially performed a dot blot assay using an anti-His antibody to identify the 6xHis tag fused to L1 protein. Western blots were performed with positive samples for RT-PCR and dot blot using anti-His antibody, but the heterologous protein signal was not seen, which could be related to low levels of heterologous gene expression.

Enhancing expression levels in *P. pastoris* has required optimization of the heterologous gene through an increase in GC content and the replacement of rare codons with more frequent ones (Bazan et al., 2009; Kotzé et al., 2011). Eukaryotic genes are GC rich and thus more efficiently translated than are viral genes, which are generally rich in AT (Haas et al., 1996). Sinclair and Choy (2002) have shown that heterologous production can have limited success despite high levels of transcription. Thus, the authors compared a codon-optimized construct to a construct with altered GC content and, surprisingly, found that the latter provided higher translation efficiency in *P. pastoris*. Zhou et al. (1999) have observed that the BPV1 L1 gene contains rare translational codons. They therefore replaced the rare codons with codons more commonly used and compared the expression levels of a wild-type and a codon-modified BPV1 L1 gene in transient transfection experiments. Both produced high levels of L1 mRNA in the cytoplasm of cells, but only the codon-modified gene produced detectable levels of L1 protein. In the present study, data obtained from the analysis of BPV1, 2, and

4 L1 sequences revealed a difference of 10-13% between the codon usage of the L1 gene and those of the *P. pastoris* yeast. It is convenient, then, to emphasize that low levels of expression may be associated with the presence of rare codons in relation to the expression host. After codon optimization of BPV1 L1 gene, the codon usage frequency between the wild-type and the codon-optimized gene was reduced from 13 to 8.5%. This small reduction seems to be relevant to the success of heterologous expression in *P. pastoris*.

The levels of L1 protein expression detected in our results may even be related to the conditions adopted in the bioreactor for yeast cultivation or in the induction of the *AOX1* promoter. Although the expression of heterologous proteins in *P. pastoris* can be successfully performed in flasks, production levels are higher when bioreactors are used because parameters such as pH, aeration, and feeding rates of carbon sources can be controlled (Macauley-Patrick et al., 2005; Kotzé et al., 2011). In addition, the metabolism of methanol requires high levels of oxygen; thus, the expression of genes regulated by the *AOX1* promoter is negatively affected by oxygen limitation (Cereghino et al., 2002). Kotzé et al. (2011) have reported a failure to detect the HPV16 L1 gene intracellularly in *P. pastoris* using flasks. Significant levels of expression were detected only when the gene was expressed in a bioreactor. Conversely, Bazan et al. (2009) have expressed a codon-optimized HPV16 L1 gene in *P. pastoris* using flasks; however, they applied a strategy using a non-integrative plasmid expression system. Unlike integrative systems, episomal plasmids have some important disadvantages such as clonal instability, which requires continuous antibiotic selection.

In the Western blotting, we visualized some bands higher than 56 kDa, the expected molecular weight for the L1 protein. These bands probably correspond to dimers of L1 (Kirnbauer et al., 1996; Modis et al., 2002). Interestingly, we observed that after the recombinant protein was purified, we could recover a larger amount of the L1 monomer, possibly owing to the treatment with denaturing buffer from the purification kit.

The present study shows the viability of the *P. pastoris* system for the production of BPV L1 protein, a natural candidate for the development of vaccination strategies against BPV infection. In addition, the technology of BPV VLP production may be useful in the development of diagnostic strategies and vehicles with which to deliver epitopes or genes as well as for the design of interaction studies between papillomaviruses and their hosts.

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