CAIO ROBERTO SOARES BRAGANÇA

CONSTRUÇÃO DE UMA LINHAGEM RECOMBINANTE DE *Kluyveromyces marxianus* UFV-3 PARA EXPRESSÃO DA PROTEÍNA NÃO ESTRUTURAL (NS1) DO VÍRUS DA DENGUE-1

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae.*

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APROVADA: 15 de março de 2013.

Sérgio Oliveira de Paula
(Coorientador)

Silvia Almeida Cardoso

Wendel Batista da Silveira
(Presidente da Banca)

"Enquanto eu descanso em Deus Ele me dá tudo o que eu preciso
É descansar, confiar, entregar, todo o meu cuidado a Deus!"
(Diante do Trono)

A Deus, minha essência. Aos meus amados pais, Osvaldo e Marlene, que com carinho, amizade e compreensão me encorajaram e me incentivaram em todas as situações ao longo do mestrado. À minha irmã Sabrina pelo imenso apoio. À minha sobrinha Lana, pelo seu sorriso inesquecível. À minha vó e à memória de meu avô pelo amor incondicional. Aos meus tios e primos pela confiança. À minha família de viçosa (PIBV) pela lealdade de uma amizade genuína. Aos amigos LABFIS, pelo companheirismo e ensinamentos compartilhados, e à minha orientadora Flávia e coorientadores por ter acreditado e confiado em mim.

Dedico.

BIOGRAFIA

Caio Roberto Soares Bragança, filho de Osvaldo Santana Bragança e Marlene Soares Bragança, nasceu em 29 de julho de 1987, na cidade de Ipatinga, Minas Gerais. Iniciou a graduação em fevereiro de 2006, no Centro Universitário do Leste de Minas Gerais (UNILESTE-MG), no qual se graduou Bacharel em Ciências Biológicas em 08 de dezembro de 2008. Em março de 2011, ingressou no curso de Mestrado em Microbiologia Agrícola da Universidade Federal de Viçosa, submetendo-se a defesa de dissertação em 15 de março de 2013.

RESUMO

BRAGANÇA, Caio Roberto Soares, M.Sc., Universidade Federal de Viçosa, março de 2013. **Construção de uma linhagem recombinante de *Kluyveromyces marxianus* UFV-3 para expressão da proteína não estrutural (NS1) do vírus da dengue-1**. Orientadora: Flávia Maria Lopes Passos. Coorientadores: Wendel Batista da Silveira e Sérgio Oliveira de Paula.

A levedura *Kluyveromyces marxianus* tem sido considerada uma candidata hospedeira para a síntese industrial de biomoléculas. Apesar de seu potencial, são poucos os estudos que relatam a expressão de proteínas heterólogas utilizando esta levedura. Neste trabalho, foi relatado pela primeira vez, a expressão da proteína do vírus da dengue em *K. marxianus*. O gene que codifica a proteína não estrutural (NS1) do vírus da dengue-1 foi integrado no genoma da levedura *K. marxianus* UFV-3 no *locus LAC4*, utilizando um vetor integrativo adaptado projetado para expressão de proteínas recombinantes em *Kluyveromyces lactis*. O gene de dengue-1 NS1 foi otimizado utilizando os códons preferenciais para aumentar os níveis de expressão de proteínas em leveduras. O gene sintético foi clonado "*in frame*" com o peptídeo sinal (*mating-α-factor*) de *K. lactis* e o plasmídeo recombinante obtido foi utilizado para transformar *K. marxianus* UFV-3 por eletroporação. As células transformantes selecionadas em YPD (yeast extract peptone dextrose) contendo 200 ug mL^{-1} de geneticina foram mitoticamente estáveis. A análise das linhagens recombinantes por meio de RT-PCR e a detecção da proteína utilizando Dot-blot confirmou a transcrição e a expressão dos peptídeos extracelulares. Após a indução com galactose, a proteína NS1 foi analisada por SDS-PAGE e Western blot. A produção da proteína foi investigada sob duas condições: com pulso de galactose e biotina com intervalos de 24 horas durante 96 horas após a indução e sem pulso de galactose e biotina. A atividade proteolítica não foi detectada no sobrenadante das culturas. Nossos resultados indicam que células recombinantes de *K. marxianus* podem ser consideradas boas hospedeiras para a produção de proteínas do vírus de dengue, que têm um potencial para aplicações em diagnósticos.

ABSTRACT

BRAGANÇA, Caio Roberto Soares, M.Sc., Universidade Federal de Viçosa, March, 2013. **Construction of recombinant *Kluyveromyces marxianus* UFV-3 to express dengue virus type 1 nonstructural protein 1 (NS1)**. Adviser: Flávia Maria Lopes Passos. Co-advisers: Wendel Batista da Silveira and Sérgio Oliveira de Paula.

The yeast *Kluyveromyces marxianus* has been considered a candidate host for industrial synthesis of biomolecules. Despite its potential, there are few studies reporting the expression of heterologous proteins using this yeast. Here, it was reported for the first time a dengue viral protein expression in K. marxianus. The dengue virus type 1 nonstructural protein 1 (NS1) was integrated into the *K. marxianus* UFV-3 genome at the *LAC4* locus using adapted integrative vector designed for high-level expression of recombinant protein in *Kluyveromyces lactis*. The gene of dengue-1 NS1 was optimized using preferential codons to increase the levels of proteins expression in yeast. The synthetic gene was cloned in frame with *K. lactis* mating-α-factor signal peptide and the recombinant plasmid obtained was used to transform *K. marxianus* UFV-3 by electroporation. The transformants cells selected in Yeast Extract Peptone Dextrose (YPD) containing 200 µg mL-1 Geneticin were mitotically stable. The analysis of recombinant strains by RT-PCR technique and the protein detection using blot analysis have confirmed both transcription and expression of the extracellular peptides. After induction with galactose, the NS1 protein was analyzed by SDS-PAGE and immunogenic detection. The protein production was investigated under two conditions: with galactose and biotin pulse at 24 hours intervals during 96 hours of induction and without galactose and biotin pulse. Protease activity was not detected into the medium. Our results indicate that the constructed recombinant *K. marxianus* can be considered good host for the production of dengue virus proteins, which have a potential for diagnostic applications.

SUMÁRIO

Construction of recombinant *Kluyveromyces marxianus* UFV-3 to express dengue virus type 1 nonstructural protein 1 (NS1)[1]

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[1] Formatação realizada conforme as instruções da revista *Applied Microbiology and Biotechnology.*

Construction of recombinant *Kluyveromyces marxianus* UFV-3 to express dengue virus type 1 nonstructural protein 1 (NS1)

ABSTRACT

The yeast *Kluyveromyces marxianus* has been considered a candidate host for industrial synthesis of biomolecules. Despite its potential, there are few studies reporting the expression of heterologous proteins using this yeast. Here, it was reported for the first time a dengue viral protein expression in *K. marxianus*. The dengue virus type 1 nonstructural protein 1 (NS1) was integrated into the *K. marxianus* UFV-3 genome at the *LAC4* locus using adapted integrative vector designed for high-level expression of recombinant protein in *Kluyveromyces lactis*. The gene of dengue-1 NS1 was optimized using preferential codons to increase the levels of proteins expression in yeast. The synthetic gene was cloned in frame with *K. lactis* mating-α-factor signal peptide and the recombinant plasmid obtained was used to transform *K. marxianus* UFV-3 by electroporation. The transformants cells selected in Yeast Extract Peptone Dextrose (YPD) containing 200 μg mL^{-1} Geneticin were mitotically stable. The analysis of recombinant strains by RT-PCR technique and the protein detection using blot analysis have confirmed both transcription and expression of the extracellular peptides. After induction with galactose, the NS1 protein was analyzed by SDS-PAGE and immunogenic detection. The protein production was investigated under two conditions: with galactose and biotin pulse at 24 hours intervals during 96 hours of induction and without galactose and biotin pulse. Protease activity was not detected into the medium. Our results indicate that the constructed recombinant *K. marxianus* can be considered good host for the production of dengue virus proteins, which have a potential for diagnostic applications.

Keywords: *Kluyveromyces marxianus* · Heterologous expression · dengue virus · NS1 protein

1. Introduction

The yeast *Kluyveromyces marxianus* is phylogenetically related to the common species of *Saccharomyces cerevisiae* and it is closer to *Kluyveromyces lactis*, but it has particular characteristics which are differential for its biotechnological applications. The most common feature among *K. lactis* and *K. marxianus* is the ability to assimilate lactose and use this carbohydrate as sole carbon and energy source. Because of this feature, which is absent in *Saccharomyces cerevisiae*, these yeasts are common in dairy sources such as fermented milks, cheeses and yogurts (Lane and Morrissey 2010) which gives this yeast the status of Generally Recognized as Safe (GRAS) allowing its use at pharmaceutical and food industry. This yeast has been also isolated from several other environments, which explain its high metabolic diversity. As a consequence of this, several biotechnological applications have been investigated in this yeast, as for example the production of aromatic compounds and bioingredientes from cheese whey (Fonseca et al. 2008), ethanol formation (Dos Santos et al. 2013; Silveira et al. 2005) and most recently as a host for heterologous protein synthesis (Rocha et al. 2010; Rocha et al. 2011). In addition the potential of *K. marxianus* for industrial purposes has been highlighted be some characteristics such as thermotolerance, high growth rate, and a broad substrate spectrum (Fonseca et al. 2008).

Despite the potential for biotechnological application, there is no commercial cloning and expression system available for *K. marxianus* and little knowledge has accumulated about its genetic manipulation. An interesting feature of *K. marxianus* strains as a host system is that vectors that replicate extrachromosomally (episomal vectors) or that integrate into the genome (integrative vectors) can be used to transform cells. Some studies have shown that the use of episomal vectors showed low copy number and low stability in nonselective medium (Fonseca et al. 2008). Therefore, the option is to use the commercial available integrative expression vector, pKLAC2 (*New England Biolab*®) which was designed for high-level expression of recombinant protein in *K. lactis* yeast cells. Yeast transformants with pKACL2 vector can be selected using the acetamidase selectable marker (*amdS*), which is expressed from the yeast *ADH1* promoter. Acetamidase expressed from pKLAC2 permits transformed cells to utilize acetamide as a sole nitrogen source on defined medium. When we try this system, we found a problem on the selection of recombinant *K. marxianus* UFV-3. The wild type strains were able to utilize acetamide a sole nitrogen source. Therefore, to resolve this problem, the acetamidase selectable marker need to be replaced by a LoxP-KanMX-LoxP cassete that would confers resistance to the geneticin for correct selection of transformants cells. Thus we expect to have *K. marxianus* be usefull for recombinant protein expression and secretion. In order to evaluate the potential of *K marxianus* as a host for recombinant protein synthesis, a protein with immunogenic or vaccinal function was tested.

Dengue is a tropical mosquito-borned viral disease caused by infection with 1 of 4 serotypes of dengue virus (Coller et al. 2011), for which there is no preventive vaccine or effective treatment available currently (Swaminathan and Khanna 2009). Dengue virus contains a single positive-stranded RNA genome of about 11 kb that encodes three structural proteins and seven nonstructural proteins (Guzman et al. 2010). Dengue nonstructural protein (NS1) is a glycoprotein of approximately 40 to 46 KDa, with two N-linked glycosylation sites, that may form homodimers and is detected in serum of infected individuals and also in vitro infected cells (Noisakran et al. 2007). The NS1 is known to be protective antigen (Athmaram et al. 2012), and several studies conducted revealed the importance of dengue NS1 antigen as a biomarker, because can be detected before the formation of antibodies (Alcon et al. 2002; Singh et al. 2010). The use of NS1 antigen has been suggested for early diagnosis of dengue infection after the onset of fever (Chaiyaratana et al. 2009; McBride 2009; Ramirez et al. 2009).

There are several reports on the use of heterologous expression systems like vaccinia virus, cell lines, *Pichia pastoris* and *Escherichia coli* for expressing NS1 protein (Athmaram et al. 2012; Noisakran et al. 2007; Zhao et al. 1987; Zhou et al. 2006). However, no systematic studies have been carried out using *Kluyveromyces* yeasts for protein synthesis from synthetic genes encoding viral proteins. The ideal is to establish a system for large scale synthesis of the rNS1 protein performed in standard yeast medium, which does not require the explosion-proof fermentation equipment necessary for large-scale growth of methylotrophic yeasts such as *P. pastoris* and to drive secretion of the protein into the culture supernatant, facilitating downstream operations. With this background, the current study was focused on construction of *K. marxianus* UFV-3 strains to produce recombinant NS1 (rNS1) protein, which has an important application for diagnostic test.

2. Material and Methods

2.1 Microorganism and maintenance

The yeast strain used in this work is designated as *Kluyveromyces marxianus* UFV-3. It was isolated from Brazilian southeast regional dairy industry, and taxonomically identified by the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands) as *Candida kefir* (Beijerinck) Van Uden & Buckey [non-ascospore forming state of *Kluyveromyces marxianus* (Hansen) Van der Walt]. The yeast strain was maintained on YPD plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar]. For storage of the long-term, cells were maintained frozen at -80 °C with 20% (v/v) glycerol. All *K. marxianus* UFV-3 strains (wild type and selected mutants) were precultured in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose]. YPD agar containing 200 μg mL^{-1} of geneticin (G418, SIGMA®) was used to select transformants. *Escherichia coli* strain DH5α^{TM} was used for routine recombinant plasmid manipulations. The wild type and recombinant *E. coli* cells were sub-cultured regularly in Luria-Bertani (LB) medium at 37 °C, supplemented with ampicillin (50 μg mL^{-1}) as appropriate.

2.2 Isolation and manipulation of nucleic acids and dengue NS1 nucleotide sequences

Maps of the plasmids used on this study are shown on Figure 1. The vector pKLAC2 (*New England Biolab®*) was taken as a base. The construction pKLJC/GME (ROSA, 2011) was used to obtain the LoxP-KanMX-LoxP cassete that confers resistance to the aminoglycosid antibiotic G418 (geneticin) and Kanamycin, and was subcloned into *BsrG*I and *Xma*I sites by replace of acetamidase selectable marker (amdS) from pKLAC2 vector, now designated as pKMCL. The construction pTZ57R/T+NS1-Denv1 was used as template for recombinant NSI/Denv1 (rNS1/Denv1) fragment amplification with the primers ns1/denv1-F and ns1/denv1-R (Table 1). The rNS1 fragments were obtained in amplification cycle: 30 seconds 98 °C, 10 seconds 98°C, 1 minute 70 °C, 1 minute 72 °C (35 cycles) and were subcloned into pGEM T Easy Vector (Promega®) and further transferred to pKMCL generating the plasmid pKMCL/NS1-Denv1. All ligation reactions were performed with T4 DNA ligase Kit from Invitrogen®.

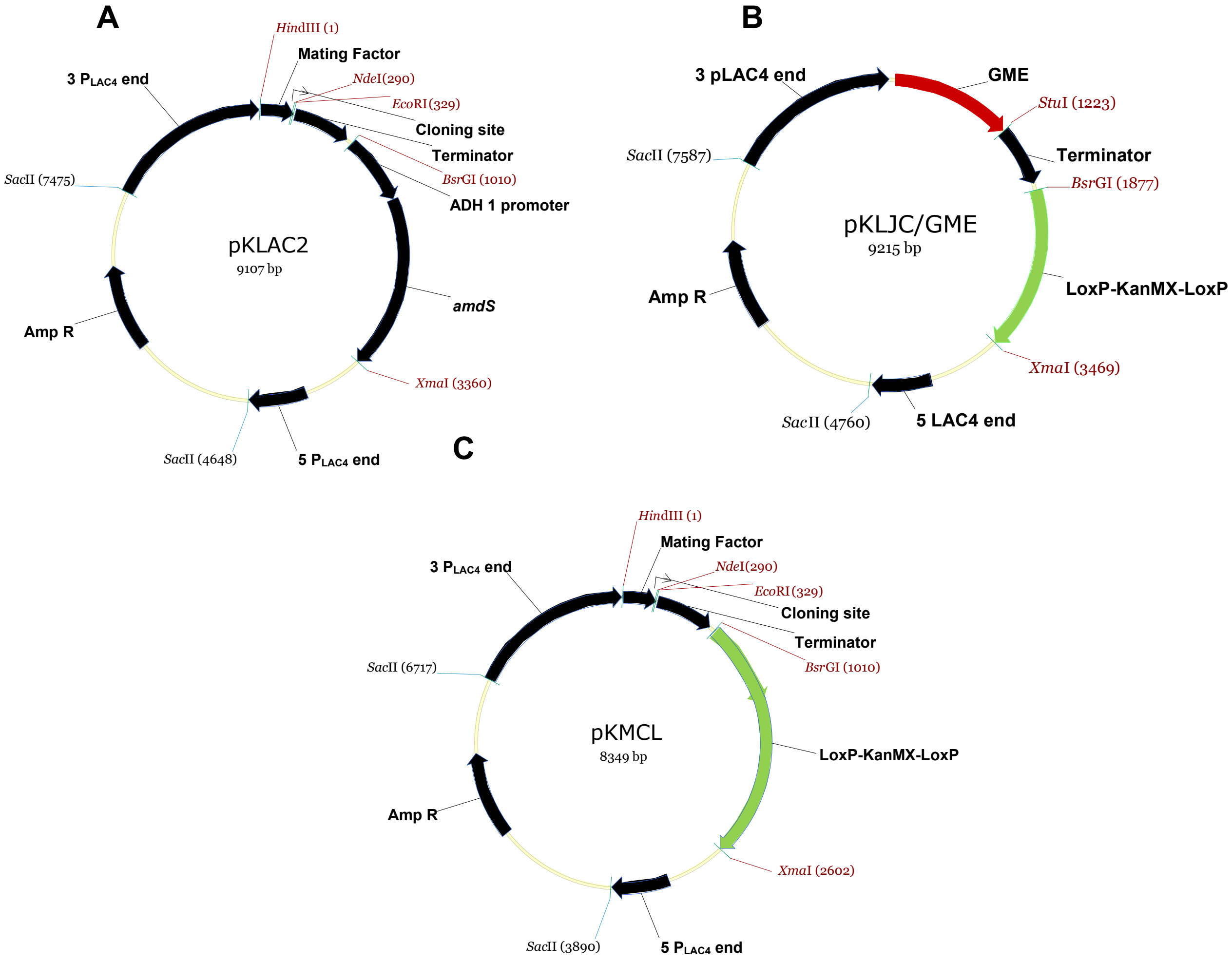

Figure 1. Maps of the plasmid vectors used for rNS1 gene expression. pKLAC2 (A). pKlJC/GME (B) and pKMCL (C) vectors are derived from pKLAC1 and pKLAC2 respectively. pKMCL was modified with the cassette LoxP-KanMX-LosP, between the restriction sites *BsrG*I e *Xma*I. The cassette LoxP-KanMX-LosP confers resistance to geneticin.

Table 1: Primers sequences used in this study.

Name	***Sequence***	***Restriction site***
NSI-DENV 1 F	5'TTCATATGGACTCAGGTTGCGTCATA3'	Nde I
NS1-DENV 1 R	5'TGAATTCTTAATGATGATGATGTGCGGATACCATACTCTTTA 3'	EcoR I
Primer 1	5'ACACACGTAAACGCGCTCGGT3'	
Primer 2	5'ATCATCCTTGTCAGCGAAAGC3'	

Underlined are represented the restriction sites.

Nucleotide sequences encoding the viral protein were designed from the primary sequence of the NS1/Denv1 peptide considering the preferential yeast codons (Figure 2). The two N-linked glycosylation sites were maintained. This sequence constitutes the ORF (*open reading frame*) of 1056 base pairs encoding the NS1 protein from dengue virus type 1 and was cloned into the pTZ57 R / T vector. The construction pTZ57R/T+NS1-Denv1 was gently ceded by Dr. Eduardo Rezende Honda.

Protein Alignment (Optimized Region)

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Optimized    1    DSGCVINWKGRELKCGSGIFVTNEVHTWTEQYKFQADSPKRLSAAIGKAWEEGVCGIRSA
Original     1    DSGCVINWKGRELKCGSGIFVTNEVHTWTEQYKFQADSPKRLSAAIGKAWEEGVCGIRSA
Optimized   61    TRLENIMWKQISNELNHILLENDMKFTVVVGDVSGILAQGKKMIRPQPMEHKYSWKSWGK
Original    61    TRLENIMWKQISNELNHILLENDMKFTVVVGDVSGILAQGKKMIRPQPMEHKYSWKSWGK
Optimized  121    AKIIGADVQNTTFIIDGPNTPECPDNQRAWNIWEVEDYGFGIFTTNIWLKLRDSYTQVCD
Original   121    AKIIGADVQNTTFIIDGPNTPECPDNQRAWNIWEVEDYGFGIFTTNIWLKLRDSYTQVCD
Optimized  181    HRLMSAAIKDSKAVHADMGYWIESEKNETWKLARASFIEVKTCIWPKSHTLWSNGVLESE
Original   181    HRLMSAAIKDSKAVHADMGYWIESEKNETWKLARASFIEVKTCIWPKSHTLWSNGVLESE
Optimized  241    MIIPKIYGGPISQHNYRPGYFTQTAGPWHLGKLELDFDLCEGTTVVVDEHCGNRGPSLRT
Original   241    MIIPKIYGGPISQHNYRPGYFTQTAGPWHLGKLELDFDLCEGTTVVVDEHCGNRGPSLRT
Optimized  301    TTVTGKTIHEWCCRSCTLPPLRFKGEDGCWYGMEIRPVKEKEENLVKSMVSA
Original   301    TTVTGKTIHEWCCRSCTLPPLRFKGEDGCWYGMEIRPVKEKEENLVKSMVSA
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Figure 2. Protein alignment encoding the NS1/Denv1 peptide. The sequences were designed considering the preferential yeasts codons

2.3 Yeast transformation

K. marxianus UFV-3 transformation was carried out according to Sanchez et al. 1993, with some modifications. Fresh *K. marxianus* UFV-3 cells were plated on YPD agar medium and incubated overnight at 37°C. An isolated colony was innoculated in 50 mL YED medium [1% (w/v) yeast extract, 1% (w/v) glucose] culture at 30°C, 200 rpm overnight. 50 mL YED were inoculated with this pre-cultured cells to start $O.D_{600}$ 0,0025 per mL (0,1 O.D.). When $O.D_{600}$ reached approximately 0,8, the cells were harvested at 3000 *g* for 5 minutes at 4°C and washed with 20 mL sterile distilled water ice-cold. A volume of 20 mL of pretreatment buffer (YED, 25 mM DTT and 20 mM HEPES-Tris pH 8.0) were added and further incubated at 30°C for 30 minutes and 100 rpm. Cells were collected at 3000 *g* for 5 minutes at 4°C and resuspended in 0,3 mL electroporation buffer EB (10mM Tris-HCl, pH 7.5, 270 mM sucrose and 1mM $MgCl_2$) and splitted on 60 µL aliquots of competent cells in Eppendorf tube on ice. On each aliquot were added 50 µg SS-DNA (*Salmon Sperm DNA*) plus 2 µg transforming DNA and kept on ice for 15 minutes. The mixture was transferred to chilled electroporation cuvette (2 mm) on ice and eletroporated at 1KV, 25 µF and 400 Ohm. Immediately, 1 mL YED ice-cold was added and incubated on ice for 15 minutes and at 37°C for 1 hour, 200 rpm. The cells were plated on YPD agar plates containing 200 µg mL^{-1} of geneticin and kept at 37°C for 2 days.

2.4 Total DNA extraction and yeast transformants screening

K. marxianus UFV-3 cells were grown in 5 mL YPD containing selectable marker at 37°C to saturation. The cell mass were collected by centrifugation, resuspended in 0,2 mL lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH8, 1 mM EDTA) and transferred to 2 mL screwcap tube. Afterwards, was added 0,2 mL PCI [phenol pH 6.7- chloroform-isoamylalcohol (25:24:1)] and 0,3 g glass beads. The cells were broken at fastprep machine, speed 6 for 20 seconds followed by centrifugation at 10000 *g* for 10 minutes. The supernatant were transferred to new tube, 0,5 mL ethanol was added and kept on -20°C at least 20 minutes. The total DNA was pelleted by centrifugation, 14000 *g*, 10 minutes and washed with 70% ethanol and dried at room temperature. The DNA samples were dissolved in 20 µL nuclease-free water and kept on -20°C. A aliquot of 1 µL from total DNA extracted was used as template for 50 µL PCR reaction. The specific primers used to detect the single cassette insertion into the *LAC4* promoter locus are shown on table 1. The amplification cycles comprised 5 minutes 98°C, 10 seconds 98°C, 30 seconds 70°C, 1 minute 72°C (35 cycles) and 5 minutes 72°C.

2.5 Total RNA extraction from yeast and RT-PCR

The transformants yeast cells were grown in 20 mL YPD medium containing selectable marker at 37°C, 200 rpm overnight. The cells were pelleted by centrifugation at 9000 *g* for 5 minutes at 4°C and the supernatant was discarded. The total RNA from recombinant *K. marxianus* UFV-3 yeast cells was extracted using the hot acid phenol method as described by Collart and Oliviero (2001). The cDNA synthesis from the total RNA extracted was achieved using the *Reverse Transcription System* from Promega®. A 2 µL cDNA aliquot from each sample were used in a 50 µL PCR reaction in order to qualitatively detect mRNA expression of the rNS1 gene inserted into *K. marxianus* UFV-3 genome. The RT-PCR was achieved using the same primers and amplification cycles used for viral gene amplification.

2.6 Evaluating the genetic stability of the recombinant strains

Recombinant strains were transferred to YPD agar and incubated at 37°C until the appearance of isolated colonies. Next, five colonies of each transformant were successively transferred to nonselective complete medium, YPD, in a total of five replating. Each replating was incubated at 37°C for 48 hours. At the end of the fifth replating, the colonies were transferred to selective medium (YPD agar plates containing 200 µg mL^{-1} of geneticin) and incubated at 37 ° C for over 48ºC. The transformants cells were subjected to an induction phase test.

2.7 Induction of protein expression

The recombinant yeast cells were precultured overnight in 50 mL YPD, 200 rpm at 37°C for obtaining cell mass. The culture was washed with peptone water 0,01% (w/v) and used to inoculate 1 L Yeast Nitrogen Base with amino acids and 5 $g{\cdot}L^{-1}$ ammonium sulfate (YNB Sigma, St. Louis, USA) containing 4 $g{\cdot}L^{-1}$ of galactose (inducer), yeast extract 0,5 % (w/v) supplemented with biotin 4 x 10^{-5}% (w/v). The cells were cultured at 37°C (optimum temperature determined for growth), 250 rpm, pH was maintained at 5.0 by adding of either 10% (w/v) HCl or 10% (w/v) NaOH as and when required in the 2,5 L - Bioflo® & Celligen® 310 – Fermentor/Bioreactor (New Brunswick). Samples were collected every 24 hours to specific protease activity, SDS-PAGE and rNS1 detection. An induction experiment was carried out in parallel, in which every 24 hours of culture was added a pulse of 4 $g \cdot L^{-1}$ galactose solution and 4 x 10^{-5}% (w/v) biotin. Foaming was prevented by addition of sterile antifoam solution (Sigma Chemicals) as required. Shake-flask experiments were performed under the same conditions described above for comparing.

Total proteins content was measured according to Bradford (1975), using bovine serum albumin (BSA) as standard. The specific protease activity was carried out according to Ray et al. 1992 with some modifications, and was assayed with cell-free culture supernatants, using azocasein as a substrate at a concentration of 1% (w/v), pH 7.5. Enzymatic hydrolysis of azocasein produces stable dye-labelled peptides and aminoacids into the reaction mixture which can be measured easily. Azocasein protease activity was measured by incubating 100 µL of culture supernatant and 100 µL of 0.5% (w/v) azocasein for 12 hours at 30ºC. The reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid and allowed to stand for 15 minutes at room temperature. The mixture was centrifuged at 10000 *g* for 10 minutes to remove a yellow precipitate. The absorbance of the supernatant was measured at 440 nm using a spectrophotometer. The activity of the protease was expressed in arbitrary units, where 1 unit of activity was defined as the absorbance obtained divided by the total protein concentration in $mg \cdot L^{-1}$ per time unit.

2.8 Fractionation of cultures for rNS1 SDS-page analysis and immunogenic detection using positive human serum for dengue virus

Both cells and culture supernatants were routinely assayed for rNS1 detection. The fractionation of cultures into supernatant, cell wall (retained inside the periplasmic space) and cell-bound protein were separated according to the method described by Rouwenhorst et al. 1988. Total protein content were collected during induction time and precipitated with trichloroacetic acid and acetone, resuspended in SDS sample buffer [150mM Tris-HCL pH 7.0, 200mM DTT, 12% (w/v) Sodium dodecyl sulfate (SDS), 30% glycerol, 0,05% (w/v) Coomassie Blue G-250 and 8M urea (instead of water) and store at -20°C. Samples were heated to 100°C for 5 minutes and proteins were separated by SDS-polyacrylamide gel electrophoresis. Electrophoresed proteins were transferred to nitrocellulose membrane and positive human serum for dengue virus was used to detect the rNS1 in culture supernatant by western blot analysis. In brief, for the western blot analysis, the proteins along with prestained protein markers on adjacent lanes were transferred electrophoretically to nitrocellulose membrane. The membrane was blocked overnight with 5% (w/v) bovine serum albumin in TBS buffer (10 mM Tris-HCl, pH 7.6, 1,4 mM NaCl) at 4°C and then washed with TBS buffer plus 0,1% (w/v) Tween 20 (TBS-T) for 20 minutes. Serum samples were diluted 1:10.000 with blocking buffer and incubated with membranes for two hours at room temperature. After incubation, membranes were washed with TBS-T and incubated for 1 hour with human anti-IgG and anti-IgM (Sigma Chemicals). The protein bands were visualized by incubating in substrate 3,3 diaminobenzidin (Sigma Chemicals).

2.9 rNS1 purification

The culture supernatant containing rNS1 protein was rapidly adjusted to pH 8.0 using 5M NaOH, to minimize precipitation of rNS1 (pI: 5.7) and was freshly diluted with an equal volume of binding buffer pH 8.0 (20 mM Tris-HCl). Subsequently was clarified by filtration through a 0.45μm membrane, and then submitted to ion chromatography (IC). The clarified supernatant was load onto a 1 mL HisTrap Capto Q column, previously equilibrated with binding buffer and attached to an AKTA purifier system (GE Healthcare Life Sciences, Uppsala, Sweden). Ten-column volume of binding buffer was passed through the column to remove non-specifically bound proteins, before the elution with 10, 25, 50 100% of elution buffer (20 mM Tris-HCl, 500 mM NaCl), Peak fractions were collected and dialyzed against PBS pH 8.0 using centrifugal filtration devices with a 10,000-molecular weight cut-off (Millipore), for the protein estimation using was determined with a BCA kit (Pierce Chemical Co., Rockford, USA). For the characterization the purified proteins were subjected to 15% SDS-PAGE.

3. Results

3.1 Construction of expression vector with kanamicin marker and harboring the *NS1* gene to function in *Kluyveromyces* UFV-3

In order to replace the acetamidase selectable marker (*amdS*) from the pKLAC2, the LoxP-KanMX-LoxP cassete of 1592 base pairs that confers resistance to the geneticin from the vector pKLJC/GME was obtained by cleavage with BsrG I and Xma I restriction endonucleases. The DNA fragments were recovered from the agarose gel and subcloned in pKLAC2 vector now designed as pKMCL (Figure 3).

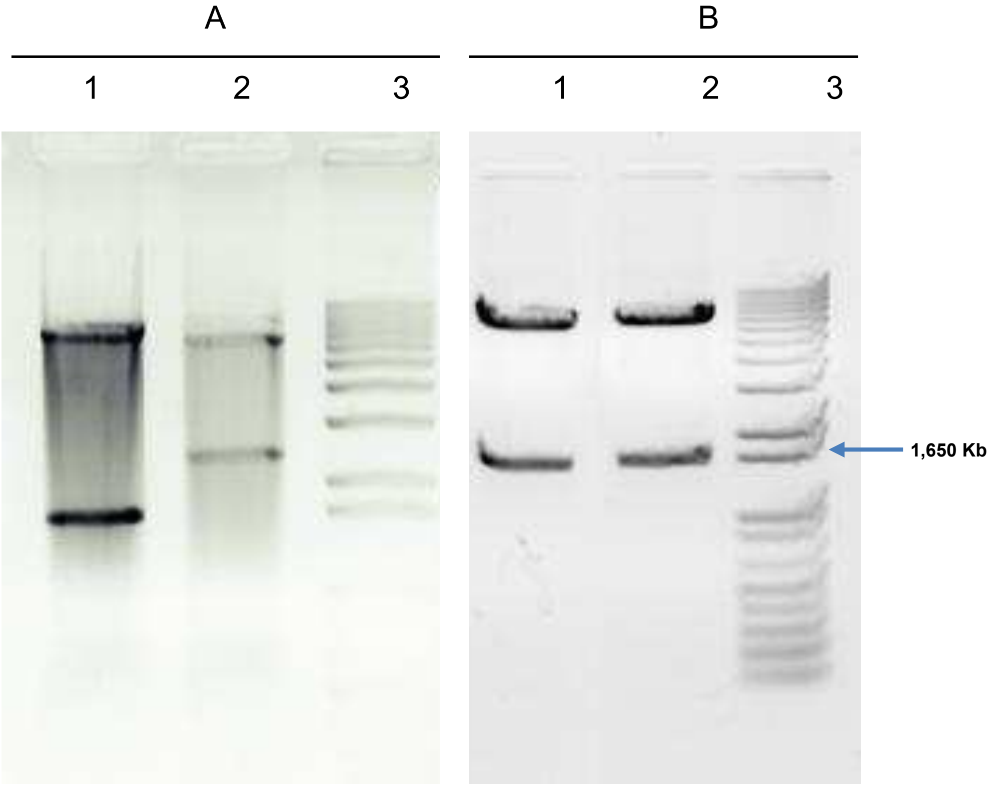


Figure 3. Construction of expression vector. (A) Agarose gel electrophoresis 1,2% (w/v) of double digestion restriction fragments (BsrG I and Xma I) of the pKLJC/GME and pKLAC2 vectors (1,2) respectively; (3) 1 Kb DNA ladder plus (Invitrogen). (B) Agarose gel electrophoresis 1,2% (w/v) of subcloning confirmation by cleavage of pKMCL vector with BsrG I and Xma I restriction endonucleases (1); (2) pKLJC/GME cleaved with the same enzymes (positive control); (3) 1 Kb DNA ladder plus (Invitrogen).

After confirming the pKMCL vector construction, the nucleotide sequence designed from the primary amino acid sequence of the NS1/Denv1 peptide was obtained by PCR amplification. The amplification resulted in fragment about of 1056 bp approximately, consistent with the expected size for the gene of NS1. This DNA fragment obtained by PCR was used to the construction of expression vector pKMCL-rNS1/Denv1. In the pKMCL-rNS1/Denv1 construct, the NS1 region from DENV-1 was cloned in frame with *K. lactis* α-mating factor secretion domain (α-MF) to direct the fusion protein to the general secretory pathway, resulting in secretion of the recombinant protein into the medium. The construction was confirmed by cleavage with the EcoR I and Nde I restriction endonucleases, resulting in DNA fragments with the expected size of approximately 1056 base pairs (Figure 4).

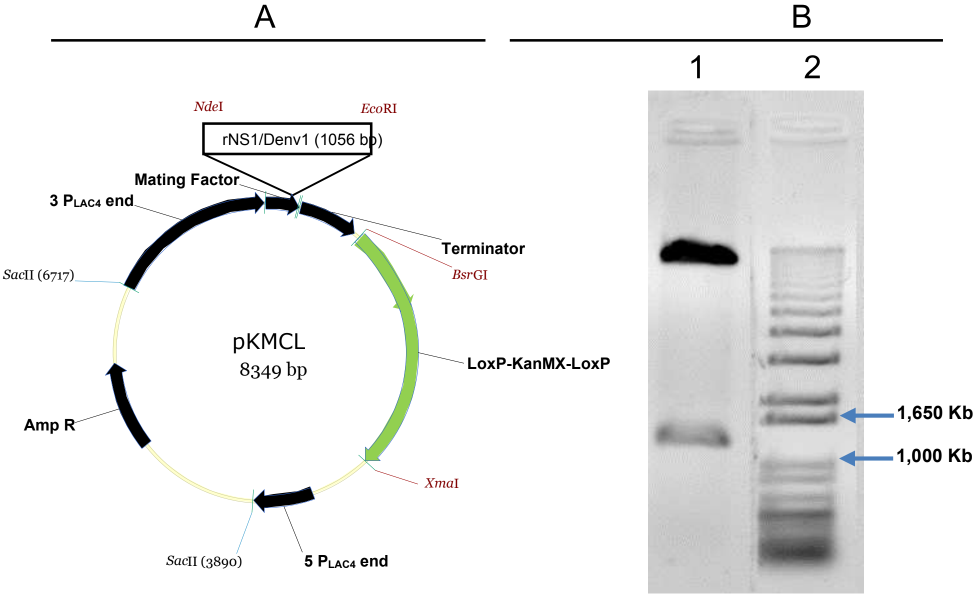


Figure 4. Confirmation of construction vector. (A) pKMCL-rNS1/Denv1 expression vector map. (B) Agarose gel electrophoresis 1,2% (w/v); (1) Insert released after cleavage the construction with the EcoR I and Nde I restriction endonucleases; (2) 1 Kb DNA ladder plus (Invitrogen).

3.2 Transformation of *K. marxianus* UFV-3 with pKMCL-rNS1/Denv1 and mitotic stability of recombinants

Before transformation of *K. marxianus* UFV-3, with the constructed and checked vector pKMCL-rNS1/Denv1, this vector was linearized with the Sac II enzyme. Cleavage resulted in a cassette containing the sequence of interest flanked by regions 3'PLAC4 and 5'PLAC4, directing cassette integration by homologous recombination in the promoter region of the *LAC4* locus. The NS1 gene was fused in frame with *K. lactis* α-mating factor secretion domain (α-MF), which directs the fusion protein to the general secretory pathway. We selected transformants which were grown in the presence of 200 μg mL^{-1} geneticin and further verified the presence of the integrated NS1 gene by direct colony PCR. Transformation efficiency was 504 transformants per μg of plasmidial DNA. To analyze if the integration of the expression fragment at the *LAC4* locus in the *K. marxianus* UFV-3 genome was in the correct location, DNA extraction from clones was performed to PCR amplification with the primers 1 and 2 (Table 1), which resulted in amplification of a diagnostic DNA fragment of 2.4 Kb product (Figure 5) from a putative clone. No nonspecific amplification band was found. As the next step, we chose several of these PCR-positive *NS1* gene-harboring clones, and tested them for mitotic stability. All recombinants analyzed were stable (data not shown).

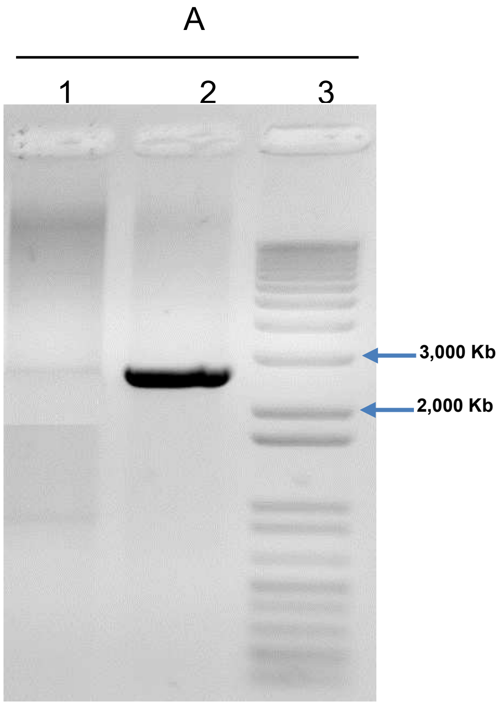


Figure 5. Agarose gel electrophoresis 1,2% (w/v) of genomic DNA PCR product from wild type (1) and recombinant strain. The fragment inserts into the promoter of the *LAC4* locus. Single-copy integration at the *LAC4* locus was detected using primers 1 and 2 to amplify a 2.4 kb diagnostic fragment (2), 1 Kb DNA ladder plus (Invitrogen) (3).

3.3 Selection of *K. marxianus* UFV-3 recombinant strain and expression and function of the NS1 protein

In order to analyze the expression of the rNS1/Denv1 protein, an induction test of the transcript and recognition of proteins secreted by dot-blot immunoassay with positive human serum for dengue virus was performed. It was chosen several of recombinant clones, and tested them in a small-scale expression assay in induction medium. DNA fragments of approximately 1056 bp obtained by RT-PCR (Figure 6 A) confirmed the transcription of the inserted sequences into the genome of *K. marxianus* UFV-3. Cell-free culture supernatants from recombinant cells was used to perform dot-blot immunoassay, which revealed that the recombinant strain was able to secrete rNS1/Denv1 (Figure 6 B), demonstrating that the signal sequences cloned upstream the *ns1* gene indeed targeted the recombinant protein to secretion.

To identify the best expressing clone, logarithmically growing test tube cultures of 10 clones were galactose-induced for 72 hours, and supernatants were assayed for rNS1 SDS-page (data not shown). One clone, #1 (a putative multi-copy clone), which expressed maximal levels of rNS1 among all clones tested, was used for further study. Since it was our objective to develop costeffective production of the viral protein, we sought to explore culture conditions that would help maximize rNS1 production.

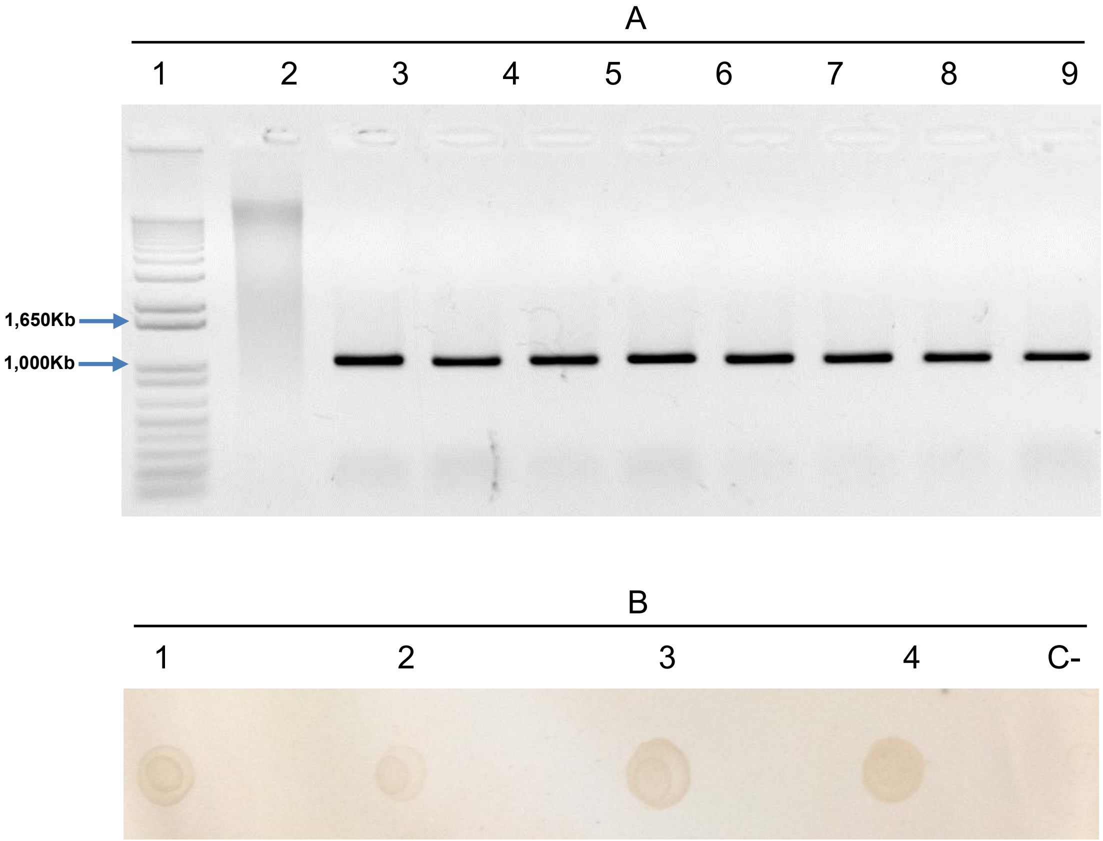


Figure 6. Induction test of the transcript and recognition of proteins secreted by dot-blot immunoassay. (A) Agarose gel electrophoresis 1,2% (w/v) of RT-PCR product of mRNA from recombinant strains induced with galactose. Detecting the mRNA encoding the peptides of interest confirmed the rNS1transcription introduced into the genome of *K. marxianus* UFV-3. (1) 1 Kb DNA ladder plus (Invitrogen), (2) wild type, negative control, (3-10) *K. marxianus* UFV-3 transformed with the rNS1 gene. (B) Dot-blot analysis of rNS1 secreted by *K. marxianus* UFV-3. (1-4) culture supernatant (100 μL) of recombinant strains and wild type (C-) were spotted onto a nitrocellulose membrane and immunodetected with positive human serum for dengue virus. All the recombinants analyzed were able to secrete the rNS1 peptide.

3.4 Growth of recombinant *K. marxianus* UFV-3 and production of rNS1 protein in shake-flask and bioreactor

Recombinant protein expressing in laboratory-scale is generally performed using complex medium at shake-flasks culture. However, for producing protein in bioreactor, the composition of media seems to be an important strategy to ensure both cell growth and rNS1 protein production. To synthesize the rNS1 protein by *K. marxianus* UFV-3 recombinant strain, the culture was carried out in two stages. In the first, a cell mass of approximately 10 g·L^{-1}, corresponding to OD_{600} of 20 was obtained. This stage was conducted in YPD medium for 16 hours of a culture starting with OD_{600} of 2. In the second stage, the recombinant cell mass was harvested by centrifugation and resuspended in induction medium [YNB buffered at pH 5.0, containing 4 g·L^{-1} of galactose, yeast extract 0,5 % (w/v) supplemented with biotin 4 x 10^{-5}% (w/v)]. The experiments were conducted in Bioflo® & Celligen® 310 – Fermentor/Bioreactor (*New Brunswick*). Shake-flask experiments were performed. In 250 mL-Erlenmeyer flasks containing 50 mL of the same cultivation medium. Samples of the supernatants of induction medium were evaluated by the total proteins and proteolytic activity. Total protein was analyzed during the induction phase from culture aliquots, withdrawn at various time points. It was observed that the recombinant strains secreted a greater amount of total proteins when galactose pulse was applied every 24 hours (data not shown). The quantitation measured according to Bradford revealed that their concentrations reached a maximum of ~1,2 mg mL^{-1} after 72 hours of induction. The accumulation of total proteins in the culture medium revealed the stability of secreted proteins and the absence of proteases, which was confirmed by analysis of protease activity assay analyzed in the supernatant of both recombinant and wild type strains cultured in bioreactor and shake flasks (Tables 2-5).

Table 2. Protease activity assay of the recombinant strain supernatant in bioreactor with additional/no additional 4% (w/v) galactose and 4 x 10^{-5}% (w/v) biotin pulse at 24 hours intervals. The cell mass (OD_{600} nm) corresponds only the cells cultured on induction medium with additional galactose and biotin pulse.

Time (h)	**Cell mass (OD_{600} nm)**	**Specific protease activity (units/mL/OD)**	
		Additional galactose and biotin pulse	**No additional galactose and biotin pulse**
24	11,5	0,00431	0,00821
48	14,9	0,00273	0,00567
72	17,4	0,00260	0,00334
96	24,3	0,00418	0,00357

U=A630 divided by the total protein concentration in mg·L^{-1} per unit time.

Table 3. Protease activity assay of wild type strain supernatant in bioreactor with additional/no additional 4% (w/v) galactose and 4 x 10^{-5}% (w/v) biotin pulse at 24 hours intervals. The Cell mass (OD_{600} nm) corresponds only the cells cultured on induction medium with additional galactose and biotin pulse.

Time (h)	Cell mass (OD$_{600}$ nm)	Specific protease activity (units/mL/OD)	
		Additional galactose and biotin pulse	No additional galactose and biotin pulse
24	12,27	0,00256	0,00302
48	15,30	0,00297	0,00377
72	18,15	0,00543	0,00439
96	24,92	0,00428	0,00427

U=A630 divided by the total protein concentration in mg·L^{-1} per unit time.

Table 4. Protease activity assay of the recombinant strain supernatant in shake-flasks with additional/no additional 4% (w/v) galactose and 4 x 10^{-5}% (w/v) biotin pulse at 24 hours intervals. The Cell mass (OD$_{600}$ nm) corresponds only the cells cultured on induction medium with additional galactose and biotin pulse.

Time (h)	Cell mass (OD$_{600}$ nm)	Specific protease activity (units/mL/OD)	
		Additional galactose and biotin pulse	No additional galactose and biotin pulse
24	9,13	0,00812	0,00562
48	12,66	0,00331	0,00596
72	13,5	0,00259	0,00403
96	13,64	0,00593	0,00391

U=A630 divided by the total protein concentration in mg·L^{-1} per unit time.

Table 5. Protease activity assay of wild type supernatant in shake-flasks with additional/no additional 4% (w/v) galactose and 4 x 10^{-5}% (w/v) biotin pulse at 24 hours intervals. The Cell mass (OD$_{600}$ nm) corresponds only the cells cultured on induction medium with additional galactose and biotin pulse.

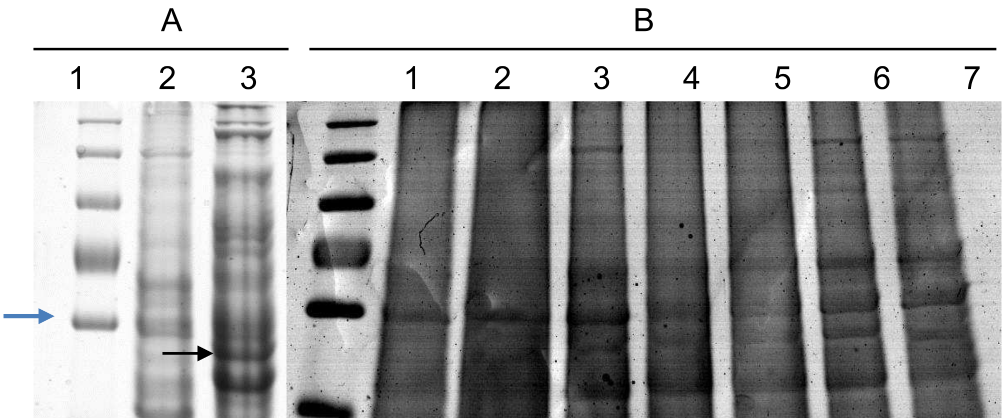
Time (h)	Specific protease activity (units/mL/OD)

	Cell mass (OD_{600} nm)	Additional galactose and biotin pulse	No additional galactose and biotin pulse
24	9,27	0,00812	0,00562
48	12,82	0,00331	0,00596
72	13,12	0,00259	0,00403
96	13,56	0,00593	0,00391

U=A630 divided by the total protein concentration in mg·L^{-1} per unit time.

3.5 Detection and purification of rNS1 on supernatant culture

To analyze the distribution of the rNS1 protein, the culture were fractionated in supernatant, cell-bound and cell wall protein and separated by SDS-polyacrylamide gel electrophoresis (Figure 7). The results confirmed the rNS1 secretion (Figure 7A) and that the rNS1 appear in the three fractions of the recombinant strain culture and it was a consequence of the expression of the cloned synthetic gene. The concentration of rNS1 released by cell wall and cell-bound protein (Figure 7B) was lower than the total protein secreted in the supernatant. In particular, the secretion efficiency of rNS1 using the pKMCL expression vector adapted from pKLAC2 was confirmed by western blot (Figure 8). No similar band appeared in negative control.



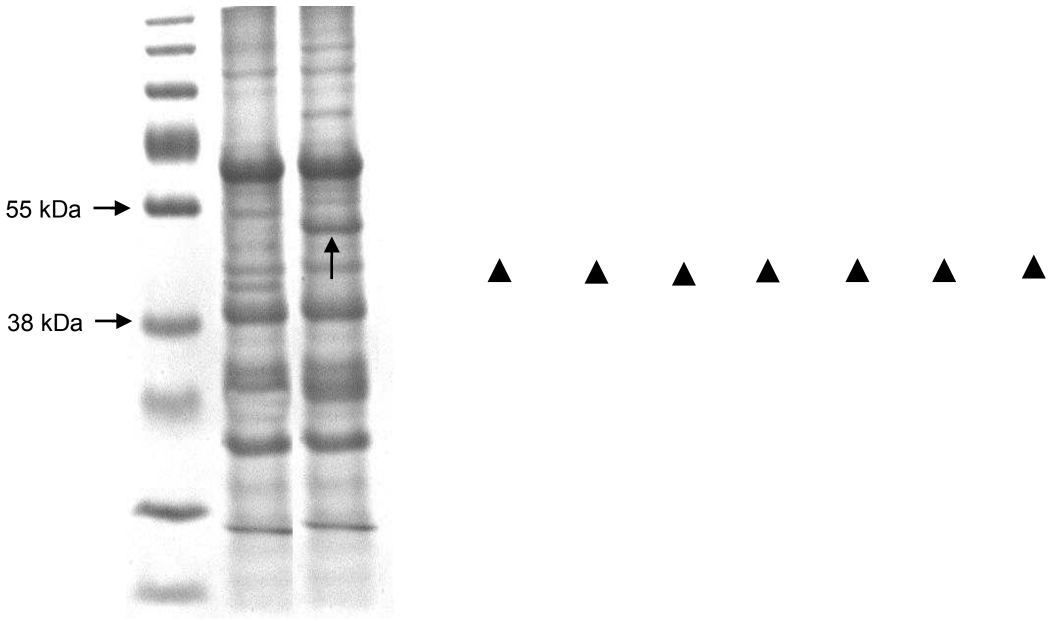


Figure 7. Coomassie blue stained SDS-PAGE 12% (w/v) for the analysis of the rNS1 expressed in *K. marxianus* UFV-3. (A) *K. marxianus* supernatant. (1) Prestained protein ladder (Thermo Scientific); (2) Wild type; (3) recombinant strain producing rNS1. (B) Fractionation of cultures for rNS1 detection in *K. marxianus*. (1) Prestained protein ladder (Thermo Scientific); (2) negative control; (3-4) *K. marxianus* cell-bound rNS1; (5-8) *K. marxianus* cell wall associated rNS1. The arrow and the arrowheads identify rNS1.

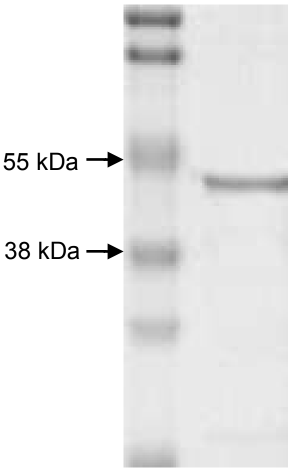


Figure 8. Expression of dengue virus type 1 rNS1 in *K. marxianus* UFV-3. (1) prestained molecular weight standard (Thermo Scientific). (2) Western blot analysis of rNS1 protein using positive human serum for dengue virus.

The 96 hours cell-free culture supernatant from bioreactor cultures was diluted with an equal volume of binding buffer pH 8.0 (20 mM sodium phosphate, 500 mM NaCl) and clarified by filtration through a 0.45 µm membrane, and then loaded onto an ion chromatography which yielded four distinct peaks (Figure 9 A). The recombinant protein emerged on peak 2 with other proteins found in supernatant (Figure 9 B).

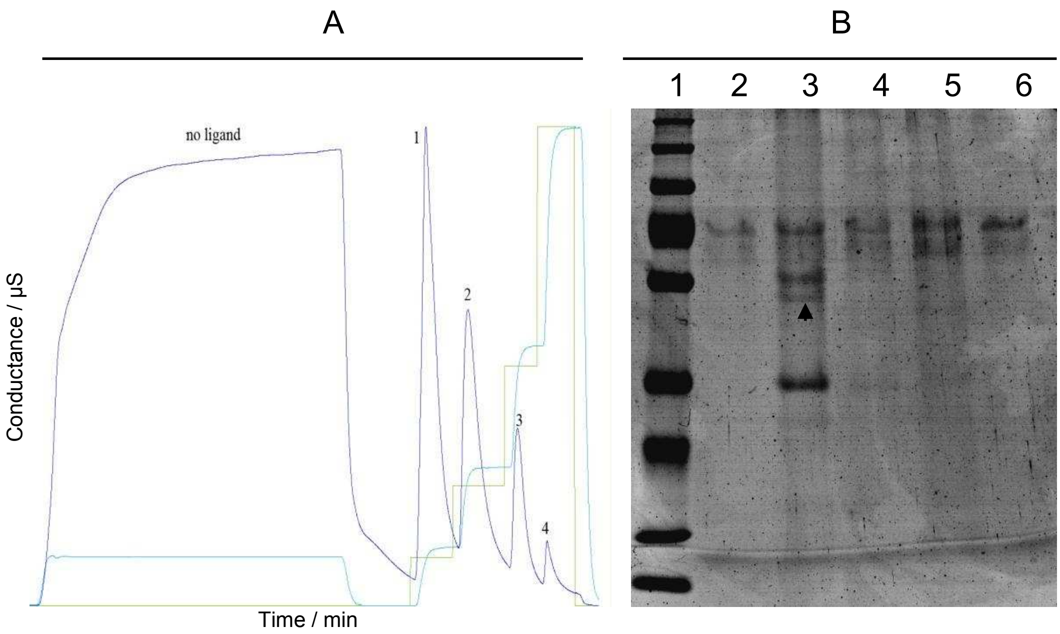


Figure 9. Ion exchange chromatography profile of 10 mL *K. marxianus* UFV-3 bioreactor supernatant containing rNS1. (A) Chromatogram of the IC. Four distinct peaks are shown. Peaks 1 to 4 were eluted with 10, 25, 50 100% of elution buffer respectively. (B) SDS-page gel electrophoresis analysis of peaks fractions collected. (1) Prestained protein ladder (Thermo Scientific); (2-5) peaks 1 to 4 respectively; (6) non-specifically bound proteins. The arrowhead indicates the position of the rNS1 protein.

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4. Discussion

In the last years, successful production of dengue virus protein has been reported in several vector systems (Batra et al. 2010; Blaney et al. 2005; Block et al. 2010; Jaiswal et al. 2004; Saxena et al. 2008; Zhou et al. 2006). Although prokaryotic expression system such as *E. coli* is simple to perform, lack in this system the modification mechanism of eukaryotic expression, like post-translational modifications that many eukaryotic proteins require for optimal biological activity and stability (Coller et al. 2011). Furthermore, it is difficult to purify recombinant protein from inclusion bodies (Wei et al. 2003). Therefore, the heterologous expression system of eukaryotic proteins in prokaryotes led a search for more suitable hosts. In the present study, the *K. marxianus* UFV-3 yeast was used to produce dengue-1 NS1 protein because of the simplicity of techniques needed for molecular genetic manipulation, its ability to express foreign proteins either intra or extracellularly, its high growth rate and capacity to produce cell mass and its status of generally regarded as safe (GRAS), which convey for biological synthesis of protein for public health purposes.

The first difficult found on utilizing the *K. marxianus* as a host strains to synthetize recombinants proteins is due to the absence of commercial cloning and expression system available for this strains. When the system was used on the selection of recombinant *K. marxianus* UFV-3, the wild type strains were able to utilize acetamide, the selective marker, a sole nitrogen source. We have tested several concentrations of acetamida (5 mM to 40 mM) and in all concentrations tested was observed growth of the *K. marxianus* strains. In order to use this vector in *K. marxianus* UFV-3 cells, it was necessary to replace the acetamidase selectable marker (*amdS*) from pKLAC2 by a LoxP-KanMX-LoxP cassete that would confers resistance to the geneticin. To transform the *K. marxianus* UFV-3 cells several protocols were tested but only the protocol (with modifications) described in material and methods was successful with transformation efficiency of 504 transformants per µg of plasmidial DNA.

An interesting feature of *K. marxianus* and *K. lactis* strains as a host system is that vectors that replicate extrachromosomally (episomal vectors) or that integrate into the genome (integrative vectors) can be used to transform cells. Both vector systems have been used to direct expression of recombinant proteins as describe in OOYEN *et al.*, (2006) and ROCHA, SAUL N *et al.*, (2010). Episomal vectors provide higher copy numbers of expression constructs but can be unstable in the absence of selection. This instability leads to expression problems of some heterologous proteins, especially in large industrial applications which cells are often subjected to prolonged growth in the absence of selection. ROCHA, SAUL N *et al.*, (2010) employed different episomal and integrative constructs and demonstrated that according to plasmid stability studies, after 48 hours of cultivation, both cells of *K. marxianus* and *K. lactis* nearly lost their episomal expression plasmids. In other studies, after growth of a *K. lactis* strain secreting human lysozyme expressed from a pKD1 vector, only 17.3 % of cells retained the vector contrasted with > 91.5% of cells retaining an integrated expression vector producing the same enzyme under the same growth conditions (Iwata et al. 2004). The integration cassette used in this work has regions of homology with the *LAC*4 promoter, which reduces but does not eliminate the possibility of ectopic integration. In order to identify the correct genomic integration of a linear expression cassette into the promoter region of the *LAC4* locus in the *K. marxianus* genome was used specific primers designed to *K. lactis*. Our results demonstrates that these primers can be used to *K. marxianus*, because nonspecific bands were not amplified. The analysis of *K. marxianus* UFV-3 recombinant strains by RT-PCR and the protein detection using positive human serum for dengue virus confirmed the transcription and secretion of rNS1. A major benefit of the using integrative expression vector was the increased genetic stability of recombinant strain, confirmed by mitotic stability test, since recombinant cells mitotically stable reduces the difficulties with maintaining of selective pressure in the culture medium for the large-scale industrial application.

Different conditions to maximize rNS1 production were evaluated. Early experiments at our laboratory showed that of the different media tested, highest yields were obtained using YNB containing 4 $g \cdot L^{-1}$ of galactose and yeast extract 0,5 % (w/v). In this work, a variety of induction conditions were evaluated, including pH and galactose and biotin pulse to enhance the cell mass and level of rNS1 induction in YNB, rather than in YPGal [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) galactose]. We observed that the induction performed in YNB [containing 5 $g \cdot L^{-1}$ ammonium, 4 $g \cdot L^{-1}$ of galactose, yeast extract 0,5 % (w/v) supplemented with biotin 4 x 10^{-5}%] buffered at pH 5.0, increased cell mass and rNS1 levels approximately 50-fold, compared to YPGal medium. Periodic monitoring of the cell density during the induction phase revealed that the cells grew better at the pH 5.0. Since in this pH value, cell viability increased and no proteolysis was detected, there was a higher accumulation of recombinant protein in *K. marxianus* UFV-3 cultured in bioreactor. When recombinant cells were cultured in shake-flasks, the maximum DO_{600} 13 was reached during 96 hours of induction in buffered medium. In contrast, when the recombinant cells were performed in bioreactor under the same conditions described above for comparing, the maximum DO_{600} 24 was reached. These results suggest that the use of bioreactors for recombinant proteins production is recommended, since the production of recombinant protein is directly related to the cell mass in the medium. We also evaluated the effect of pH on the rNS1 production from *K. marxianus* UFV-3. As the pI (isoelectric point) of NS1 is predicted to be ~5.7, we used YNB at pH 3, 4 and 5, to preclude isoelectric precipitation of the recombinant protein. The YNB buffered at pH 5.0 was selected. The effect of galactose concentration on the induction of gene expression and cell mass was also evlauated. Several studies used 2% (w/v) galactose or lactose as inducer for heterologous proteins production (Bartkeviciute and Sasnauskas 2003; Donnini et al. 2004; Ganatra et al. 2011; Kooistra et al. 2004; Li et al. 2011), but we have detected that the use of 4% (w/v) galactose increased the concentration of cell mass and hence the recombinant protein in bioreactor.

rNS1 synthesis by *K. marxianus* UFV-3 recombinant strain was performed in two stages and conducted in bioreactor and shake-flasks containing the same cultivation medium. In the first stage, glucose was used as carbon source for obtaining cell mass. A culture complex medium provides high rates for *K. marxianus* growth. However, this culture medium can compromise the recovery of recombinant peptides due to the presence of self-peptides in this culture medium. Set a culture medium for maximum cell mass production is crucial in order to improve rNS1 yields. In this sense, it was defined a culture medium to maximize production of cell mass and consequently increasing the production of rNS1. In the second step, the induction of rNS1synthesis in 4 $g \cdot L^{-1}$ of galactose showed that the induction parameters are also important and can directly affect the yield of the recombinant protein. Two induction conditions were tested: pulse and no pulse of galactose and biotin every 24 hours during 96 hours of induction, since biotin is an essential vitamin for the growth of many microorganisms (Jungo et al. 2007). The concentration of extracellular proteins in the step of galactose and biotin pulse was higher compared to the condition without pulse of galactose and biotin. Therefore, it can be inferred that the inductor is consumed over time and the addition of galactose maintains the synthesis and secretion of proteins. In this way, the galactose and biotin pulse must be considered as a strategy for increasing rNS1 yields. The accumulation of total proteins in the supernatant revealed the stability of secreted proteins and absence of proteases, which was confirmed by protease activity assay. This result is important considering the costs of the process, because the absence of proteases in the supernatant prevents higher expenses for the proteases inactivation, since they could compromise the obtaining of the interest proteins. Sugrue et al. (1997) showed that expression of the dengue protein in *P. pastoris* was accompanied by extensive proteolytic degradation, difficulting downstream operations.

Currently, most *P. pastoris* fed-batch cultivations for the production of recombinant proteins, using AOX1 expression system, consist of three or four distinct phases, which include glycerol batch, glycerol fed batch, starvation and methanol fed batch. However, we have recently demonstrated a simple two-step strategy, which only includes a glucose batch phase, to generate sufficient cell mass, directly followed by the induction phase with a galactose for the recombinant protein synthesis. In this work, we have successfully employed this two-step strategy for the secretion of rNS1. Recombinant NS1 protein was obtained at the first 24 hours of cultivation. SDS-Page analysis showed that large quantities of secreted rNS1 were present in the supernatant than retained inside the periplasmic space or cell-bound. The cell-free culture supernatant obtained was loaded onto an ion chromatography for rNS1 isolation, but other proteins were also isolated in the same fractions.

Our rNS1 protein exhibited the same molecular size as authentic dengue virus protein. A novel approach to generate recombinant NS1 protein was performed using *K. marxianus* UFV-3. To the best of our knowledge, this is the first study about the viral protein production with immunogenic potential of pharmaceutical interest using the yeast *K. marxianus*. The rNS1 produced has a potential application for detection of anti-dengue IgM and IgG antibodies as well a further studies in vaccine development for dengue virus infection. By using this strategy, other recombinant viral proteins can also be produced. Further study of the structure and functions of the rNS1 protein will be performed.

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