SEVEN
publicações acadêmicas

Molecular Characterization and Cloning of the enzymatic portion of a chitin synthase from *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora

Crossref doi
https://doi.org/10.56238/uniknowindevolp-142

Edjane Bastos Ferreira
Master's Degree in Biotechnology, State University of Feira de Santana
LATTES: http://lattes.cnpq.br/3465031695215053,
E-mail: edjanebferreira@gmail.com

Catiane Sacramento Souza
PhD in Biotechnology, State University of Feira de Santana
LATTES: http://lattes.cnpq.br/7368708603630128
E-mail: casouza2010@yahoo.com.br

Luiz Henrique Machado Oliveira
Master's Degree in Biotechnology, State University of Feira de Santana
LATTES: http://lattes.cnpq.br/8445169113030900
E-mail: henriquebiomol@gmail.com

Raquel Guimarães Benevides
PhD in Biochemistry, State University of Feira de Santana
LATTES: http://lattes.cnpq.br/7633115237379209
E-mail: raquelgb@gmail.com

ABSTRACT
Moniliophthora perniciosa is a phytopathogenic basidiomycosis that affects cacao; alternatives for its control and resistance for its development have not given significant results. A new control alternative is the use of enzyme inhibitors from the chitin synthesis route. Therefore, this work aims to characterize and clone the enzymatic portion of Moniliophthora perniciosa chitin synthase as a tool to enable in vitro tests of the potential inhibitors of this phytopathogen. The complete chitin synthase sequence was analyzed; the transmembrane region, introns and UTRs were removed to design specific primers. PCR, sequencing, plasmid linearization (pET28a and pET42a) and their digestion of inserts for ligation were performed. The transformation was carried out by electroporation and thermal shock into E.coli DH5α and BL21 strains. Colony PCR, plasmid extraction and PCR were performed to confirm the transformed ones. There was positive amplification of the interest region with the designed primers, confirmed by sequencing and alignment with the chitin synthase sequence from GenBank. Digestion and binding to the pET42a vector were efficient. Despite the success in amplification, digestion and ligation, the colonies grown and transformed only contained empty vectors. Testing new vectors and new proportions with the already validated protocols for amplification, digestion and binding of the nucleotide sequence of M. perniciosa chitin synthase will allow an efficient transformation.

Keywords: Witch's Broom, Chitin synthesis, Cloning vector, Expression vector, Escherichia coli.

1 INTRODUCTION

Chitin enzyme is the main component of the cell wall of the *Moniliophtora perniciosa* fungus (11). This phytopathogen causes witches' broom in cocoa (*Theobroma cacao*) (11) and generated numerous losses after its discovery in 1989 in Bahia (2) (1).

With the discovery of the fungus, numerous alternatives were tested to control the phytopathogen, how biological control (15), antifungal application (14), poda phytosanitary pruning (5), development of varieties more resistant to the fungus that over time new strains appear and reinfected the plant (19) (5).

An alternative for fungal control is based on analysis of the characterization of key enzymes in the metabolism of the phytopathogen and subsequent *in silico* selection of potential inhibitors for the metabolic pathway for inhibition of fungal development (11) (17) (7).

The enzyme chitin synthase (EC: 2.4.1.16) is responsible for converting UDP-N-acetylglycosamine to chitin. The study of this enzyme enabled the characterization and selection *in silico* of possible inhibitors to its metabolic pathway. (17). Thus, this work aims to characterize and describe cloning strategies for the enzymatic portion of a chitin synthase from Moniliophtora perniciosa. These results will allow the recombinant production of this interest protein, providing in vitro analysis of inhibitors tool to the biocontrol of this important phytopathogen.

2 MATERIALS AND METHODS

2.1 MOLECULAR CHARACTERIZATION OF THE SYNTHETIC GENE OF THE CHITIN SYNTHASE ENZYME

Synthetic chitin synthase gene was provided by Dr. Catiane Sacramento de Souza (LAPEM/UEFS). This gene has a transmembrane portion and an enzymatic portion inserted in the pF3a cloning vector carrying 6071 bp. The analysis of the deposited sequence was performed in GenBank (EU154354). After the removal of the transmembrane region, intros and UTRs determined by Souza (19) the enzymatic portion (1800 bp) (CHS-EP) was translated using the ExPASy (https://www.expasy.org/) and molecular mass estimation was performed by ProtParam (https://web.expasy.org/protparam/).

2.2 DESIGN OF SPECIFIS PRIMERS FOR CHITIN SYNTASE (CHS-EP)

Expression vectors pET28a and pET42a were chosen, then the restriction sites for the vectors and the insert were defined, assessing the absence in the coding sequence: For pET28a *Nhe*I (sense), B*am*HI (antisense); for pET42a *BamH*I (sense), *EcoR*I (antisense). The sequences of the designed primers were analyzed using the online tool OligoAnalyzer3.1 (https://www.idtdna.com/calc/analyzer), where the following parameters were evaluated: annealing temperatures (Tm), formation of secondary structures and amount of C/G.

2.3 PREPARATION OF COMPETENT CELLS (CHEMICALLY COMPETENT AND ELECTROCOMPETENT)

The competent *E. coli* strains used for cloning were Top10 (Thermo Fisher Scientific), DH5α (Invitrogen) and BL21(DE3) (Invitrogen). For the storage of the synthetic gene, the strain Top10 (Thermo Fisher Scientific) was used.

Chemically competent and electrocompetent strains were prepared according to the protocols described by Sambrook (16).

2.4 STORAGE AND EXTRACTION OF THE SYNTHETIC GENE

For synthetic gene stocking, top 10 strain was used. It was placed in a 1.5 ml microtube 1µl (240 ng) of the synthetic gene and 40 µl of electrocompetent cell. The cells were homogenized and then transferred to a 1 mm electroporation cuvette of the Multiporator electroporator (Eppendorf®) previously frozen and kept on ice during the process. In sequence, electroporation was performed in the prokaryotic mode with a 1700 mV pulse. After the shock, 959 µl of L.B (1%Lubria Bertani Luria-Bertani-Agar-Triptona; 0.5%Yeast extract; 1% NaCl) medium was added, homogenized, transferred to a 2 mL microtube and incubated at 37 ° C for 40 minutes under 900 rpm agitation. Then the culture was centrifuged at 9000 rpm for 1 minute and 900 µl of the supernatant was removed; the precipitate (pellet) formed was resuspended with the remaining supernatant and used for inoculation in plates containing L.B e 12% Agar- plus Ampicillin at 100 µg/mL for selection of transformed colonies. After 24 hours at 37 ° C, the grown colonies were reinoculated in 5 mL of L.B with Ampicillin at 100 µg / mL for another 24 hours at 37 ° C under agitation. Then 750 µl aliquots were removed and 250 µl of 100% glycerol were added and stored at -20 ° C and -80 ° C. The remainder was used for the extraction of the plasmid.

For the plasmid extraction, the transformed colonies were centrifuged at 13,000x*g* for 2 minutes. The supernatant was discarded and the precipitate (pellet) formed was used according to the protocol of the PureLink® Quick Plasmid Miniprep extraction kit (ThermoFisherScientific®). After extraction, the presence of nucleic acids in electrophoresis in 1% (m / v) agarose gel in TBE 1X Buffer (89 mM Tris pH 8.2 / 89 mM boric acid / 2 mM EDTA) was evaluated. Electrophoresis was performed usind the following parameters: 100 V, 90 mA for 30 minutes. The visualization of the extracted synthetic gene was through the ultraviolet light running transluminator where they were photographed with a KODAK EDAS 290® digital photography system. Then, the purified plasmids were analyzed by PCR to guarantee validation.

2.5 AMPLIFICATION OF THE ENZYMATIC PORTION OF THE SYNTHETIC GENE

The amplification of the synthetic gene was performed with specific restriction enzymes for vector pET28a or pT42a, using the PCR technique. The reaction was prepared in a 200 µl microtube, containing 25 µL Toptaq Master Mix (Quiagen®), primer (sense) 0,5 µL, primer (antisense) 0,5 µL, purified synthetic plasmid (135ng) 1 µL and water in a final volume of 50 µL. Amplification was performed using a thermocycler *Mastercycler* 5333 (Eppendrof®). The reaction was carried out according to the following specifications: 1 initial denaturation cycle 95 ° C for 5 minutes, followed

by 35 cycles of denaturation 95 ° C for 45 seconds, annealing 60 ° C for 45 seconds and extension 72 ° C for 90 seconds. Electrophoresis was performed on 1% (m / v) agarose gel in TBE 1X Buffer (89 mM Tris pH 8.2 / 89 mM boric acid / 2 mM EDTA).

2.6 SEQUENCING

The sample was sequenced to confirm the amplification of the enzymatic portion of chitin synthase following the recommendations of Myleus (http://myleus.com/) to prepare the sample for sequencing. The electro spherogram was analyzed using the program Geneious Prime (versão 2020.0.4), and the nucleotide sequence was alined using the online tool Clustal (https://www.ebi.ac.uk/Tools/msa/clustalo/) then it was translated and aligned using the online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) translated nucleotide for protein.

2.7 DIGESTION OF LIGATION VECTORS AND INSERTION

The vectors pET28a and pET42a were provided by Dr. Humberto Fonseca de Freitas (UFBA), were extracted from *E. coli* DH5α strains using the Kit PureLink® Quick Plasmid Miniprep (ThermoFisherScientific®).

Digestion of the vector and insertion of pET28a was used 1,5 ug was digested with the restriction enzymes *Nhe*I and *BamH*I (20 U), the buffer 10x Anza (Invitrogen) and with (1 U) of the FastAP Thermosensitive Alkaline Phosphatase (Invitrogen). The reaction was maintained at 37 ° C for 15 minutes then there was heating at 80 ° C for 20 minutes to Alkaline Phosphatase inactivation. Then, the sample was visualized on a 1% agarose gel, purified from the gel and quantified. Digestion of the insert occurred in the same way, however there was no addition of Alkaline Phosphatase.

For digestion of the vector and insertion of pET42a was used 1,5 ug was digested with the restriction enzymes *BamHI* and *EcoRI* (20 U), the buffer K (Invitrogen) and with (1 U) of the FastAP Thermosensitive Alkaline Phosphatase (Invitrogen). The reaction was maintained at 37 ° C for 4 hour then there was heating at 80 ° C for 20 minutes to Alkaline Phosphatase inactivation. Then, the sample was visualized on a 1% agarose gel, purified from the gel and quantified. Digestion of the insert occurred in the same way, however there was no addition of Alkaline Phosphatase.

The ligation reaction was made using the vector with its respective insert. The following proportions were used 1:3; 1:5; 1:8; 1:20; 1:40 e 1:50 vector / insert according to the formule: (Mass of Vector / Size of Vector) / (Mass of Insert / Size of Insert) = 1. The reaction was performed according to the manufacturer's recommendation T4 DNA Ligase (Invitrogen®), adding 1U of the T4Ligase (1U/ µL) and 4 µL of 5x buffer (Invitrogen®). The reactions were incubated at 14 ° C for 23 hours. After incubation, the reactions were visualized on a 1% agarose gel.

2.8 TRANSFORMATION

For each ligation reaction, 4 different transformations were performed using chemically or electrocompetent competent strains of the *E. coli* DH5α (cloning strain) or *E. coli* BL21 (expression strain).

2.8.1 Transformation (chemically competent)

Strains of chemically competent *E. coli* DH5 or BL21 were transformed with the ligation products (pET28a + CHS-EP) or (pET42a + CHS-EP) as described below. 5µl of the ligation product was homogenized and incubated in 50 µl strains of competent *E. coli* (DH5α or BL21) on ice for 30 minutes. Then the cells were subjected at 42 ° C for 90 seconds, followed by 2 minutes on ice. After this process 960 µl LB medium was added and subjected to stirring at 200 rpm, 37 ° C, 45 minutes. The cells were centrifuged for 1 minute at 1200 rpm, the pellet formed was resuspended in 50 µl of L.B medium, the recovered cells were plated in L.B agar medium plus 30 µg/mL kanamycin for selection of transformed colonies.

After this procedure by the method using chemically competent cells, colonies grown with proportions 1/20 and 1/40 using pET28a and pET42a vectors and DH5 and BL21 strains were submitted to colony PCR to confirm positive clones. Each colony was removed from the plate and placed in a 1.5 microtube with 100 µL of water, later homogenized and subjected to heating at 95 ° C for 5 minutes, then PCR was performed with the lysate serving as a template. The reaction was prepared in a 200 µL microtube, containing 25 µL Toptaq Master Mix (Quiagen®), 0.5 µL of each primer (sense and antisense), 1 µL of purified synthetic plasmid (135 ng) and water in a final volume of 50 µL. Amplification was performed in a thermocycler *Mastercycler* 5333 (Eppendrof®). The reaction followed the same parameters as described (2.5). After amplification, the inserts were submitted to 1% agarose gel electrophoresis.

2.8.2 Transformation (electrocompetent)

Electrocompetent cells of *E. coli* DH5 and BL21 were used in different proportions: 1/20 and 1/40 as described in the topic (2.4). After colonies growth, plasmid extraction was performed as topic (2.4). To confirm the insert / plasmid transformation, a PCR was performed according to the topic (2.5).

3 RESULTS

3.1 MOLECULAR CHARACTERIZATION AND PRIMERS DESIGN

The chitin synthase sequence was analyzed at GenBank (EU154354), subsequently the intros, transmembrane region and UTRs were removed, maintaining the enzymatic portion (1800 bp) were

the three classic signatures of fungal chitin synthases were identified at translated sequence (QNFEY, EDRIL, QRRRM) (19). The enzyme region translated (CHS-PE) by the ExPASy covered the entire reading window. Then the Protparam tool was used for sequence analysis where it had a molecular mass of approximately 67.56 kDa, pI of 8 and a total of 600 amino acid residues. Primers were designed according to Table 1 for CHS-EP.

Table 1: Primers design

Primer	Restriction Site*	Sequence (5' → 3')	S.I.E.**	Tm
CHS-01	*Nde*I	GGAATC **GCT AGC** ATG GCG AAT CGC CCG CC	7169	69.4°C
CHS-02	*BamH*I	GGA **GGATCC** TTA ATC ACC GGG CAG GTT GGG AAG		66.9 °C
CHS-03	*BamH*I	GGAATC **GGA TCC** ATG GCG AAT CGC CCG CC	7730	65.5 °C
CHS-04	*Eco*RI	GGA **GAA TTC** TTA ATC ACC GGG CAG GTT GGG AAG		64.4 °C

* Primers with the restriction sites NdeI, BamHI are compatible with pET28a vector; restriction sites BamHI and EcoRI with pET42a .
** S.I.E. (Size of the insert expected next to the plasmid).
Underline / bold (restriction sites); red (stop codons of the reverse primers) and gray (nucleotide sequence of CHS-EP).

3.2 PLASMID EXTRACTION WITH THE SYNTHETIC GENE AND PCR

There was a positive transformation of *E. coli* top10 cells by electroporation with the synthetic gene (6071bp) confirmed by plasmid extraction (Figure 1) with a fragment of the expected size appeared of approximately 6000 bp.

After plamidial extraction, a PCR was performed to amplify CHS-EP, using the primer sets CHS-01/CHS-02 or CHS-03/CHS-04 presenting an insert with expected size of 1800 bp (Figure 2).

Figure 1: Electrophoretic analysis of plasmid extraction of the synthetic gene in 1% agarose gel. The race followed the following standards: 100 V, 90 mA for 30 minutes. M: 1 kb molecular marker, plus DNA Ladder (invitrogen). A: sample with a fragment between 5,000 to 7,000 base pairs.

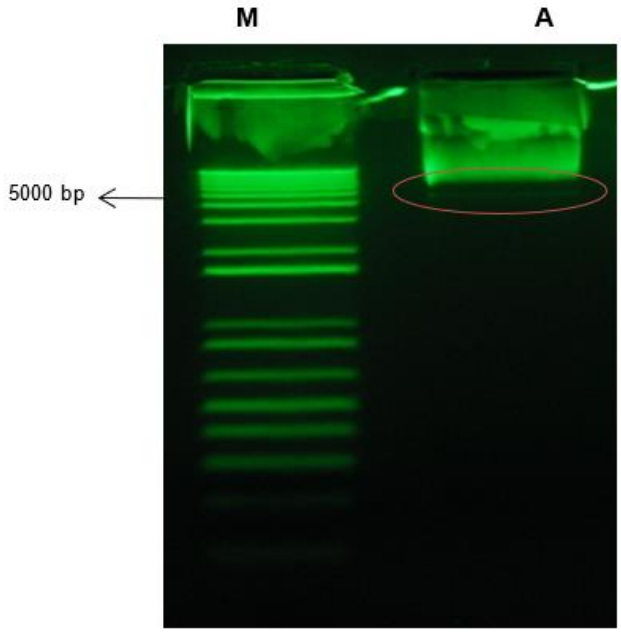
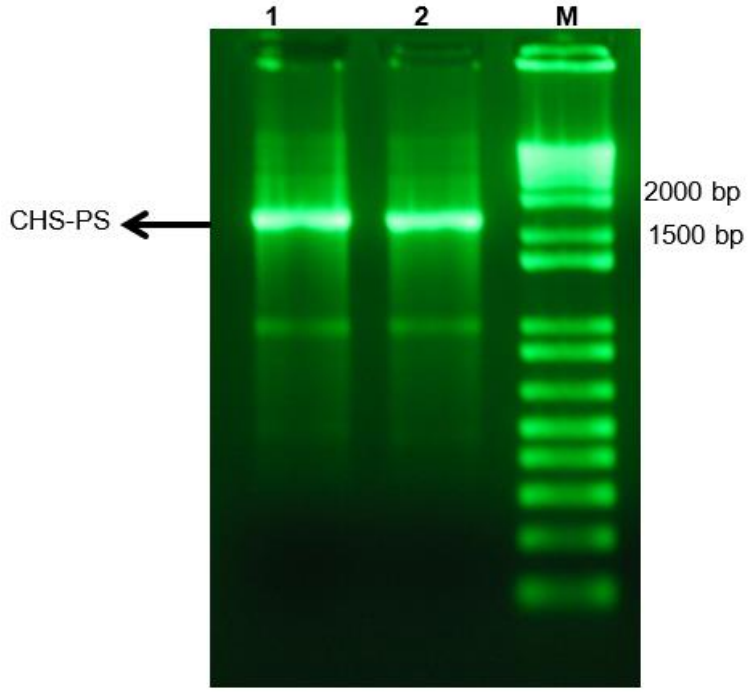


Figure 2: 1% agarose gel with PCR result of the synthetic gene (CHS-EP) with the designed primers. The race followed the following standards: 100 V, 90 mA for 30 minutes. 1: Amplification of the synthetic gene (CHS-EP) using primers CHS-01 and CHS-02. 2: Amplification of the synthetic gene (CHS-EP) using primers CHS-03 and CHS-04. M: 1Kb DNA Ladder molecular mass marker (Invitrogen).



3.3 SEQUENCING

The spherogram generated after sequencing evaluated using the Geneious Prime program (version 2020.0.4) showed a significant recovery of 64.7% of the CHS-EP sequence with 567 bp and 54% with 530 bp using sense and antisense primer respectively.

To confirm the sequencing with the chitin synthase gene, a consensus sequence was performed with the sense and the antisense sequencing (Supplementary Material 1); after submission at Blast, there was confirmed 100% of identity with the deposited sequence of the *M. perniciosa* chitin synthase (EU154354). The consensus sequence was aligned with the chitin synthase (EU154354), where there was 100% identity including 195 amino acids residues (Supplementary Material 2).

3.4 DIGESTION AND LIGATION

The digestion process was efficient for pET28a and pET42a vectors and inserts (Figure 3). After confirmation of digestion, the vectors were purified from the gel and then ligation was performed and evaluated on agarose gel (Figure 4). Three bands are presente, the first lowest represents the unbound insert, the second the empty vector and the third the insert ligation to the vector. It is noticed that there was a bond in the proportions 1/20 and 1/40 in the vector pET42a, however the ligation was not efficient for pET28a in the proportions performed. Even so, the transformation to pET28a was carried out.

Figure 3: Electrophoresis on 1% agarose gel. The race followed the following standards: 100 V, 90 mA for 30 minutes. Gel A - 1 and 2: CHS-EP after digestion with NdeI and BamHI. 3 and 4: CHS-EP after digestion with BamHI and EcoRI. M: Molecular marker of 1Kb DNA ladder (Invitrogen). 6: Digestion of pET42a with BamHI and EcoRI.Gel B- M: Solis biodyne molecular marker 100pb DNA ladder. 1: pET28a digestion with NdeI and BamHI.

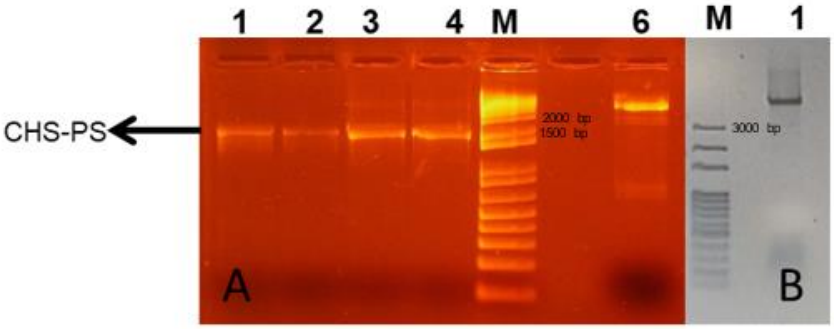
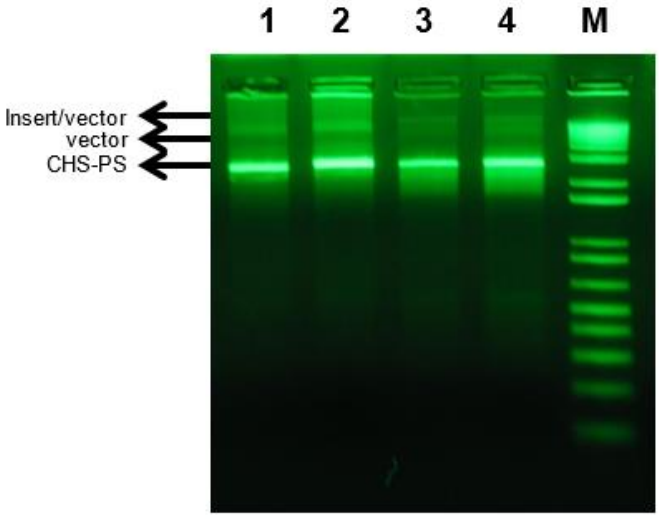


Figure 4: Electrophoresis on 1% agarose gel. The race followed the following standards: 100 V, 90 mA for 30 minutes. 1- pET28a ligation 1:20 ratio; 2- pET28a ligation at 1:40 ratio; 3- pET42a ligation at 1:20 ratio; 4- pET42a ligation at 1:40 ratio; M: 1 kb molecular marker, plus DNA Ladder (Invitrogen).



3.5 TRANSFORMATION

There was transformation in the BL21 and DH5α strains used with the 1/20 and 1/40 proportions to the pET28a and pET42a vectors using *E. coli* strains chemically and electrocompetent competent (Figure 5).

Colonies grown with plasmids pET28a and pET42 transformed by heat shock were analyzed by colony PCR (Figure 6) were identified only bands below the expected (between 100 and 300 bp).

E. coli strains transformed by electroporation were selected and subjected to plasmid extraction as topic (2.4). Figure 7 shows the result of plasmid extraction where the presence of the plasmid is

observed (bands compatible with its size between 5000 and 7000 bp appear). The transformation was efficient also for the positive control plasmid pET42a in BL21.

After confirming the plasmid extraction, a PCR of the plasmid extraction was performed as described in the topic (2.5) to confirm the transformation with the insert of interest. Figure 8 shows an agarose gel electrophoresis from the PCR of plasmid extraction transformed by electroporation.

After amplification of the region of interest for none of the strains transformed by electroporation, there was no amplification in any of the proportions performed (Figure 8).

Figure 5: Plates LB+Antibiotic grown after transformation. A: BL21 (DE3) + pET42a + CHS-EP 1/40 electroporated plates. B: DH5α + pET24a + CHS-EP 1/20 thermal shock plates

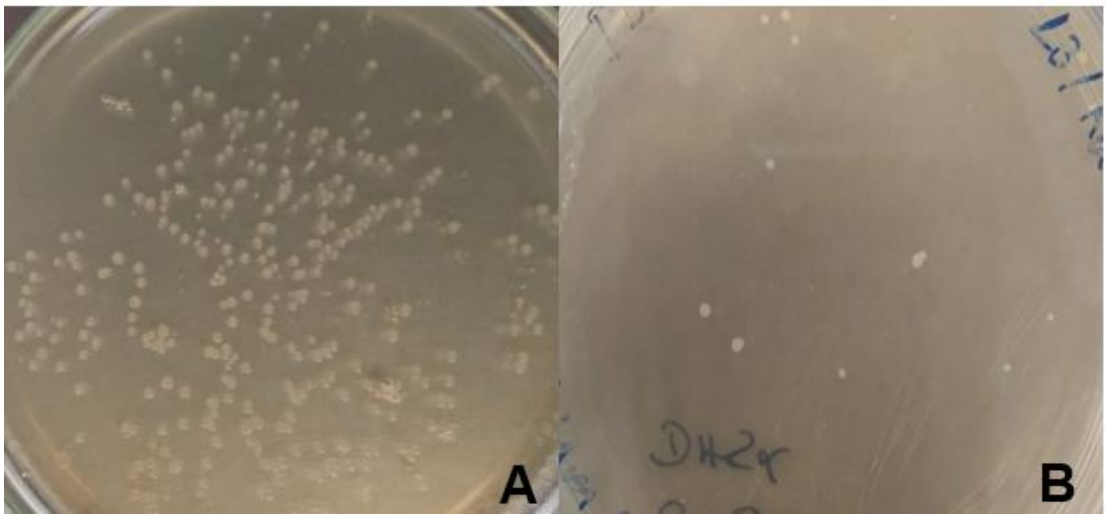


Figure 6: Thermal shock colonies PCR. Electrophoresis in 1% agarose gel as a result of the connection. The race followed the following standards: 100 V, 90 mA for 30 minutes. From right to left. M: 1 kb molecular marker, plus DNA Ladder, invitrogen, 1- (1/20 BL21 (DE3) + pET28a), 2- (1/40 BL21 (DE3) + pET28a), 3- (1/20 DH5α + pET28a), 4- (1/20 BL21 (DE3) + pET42a), 5- (1/40 BL21 (DE3) + pET42a) and 6 (1/20 DH5α + pET42a).

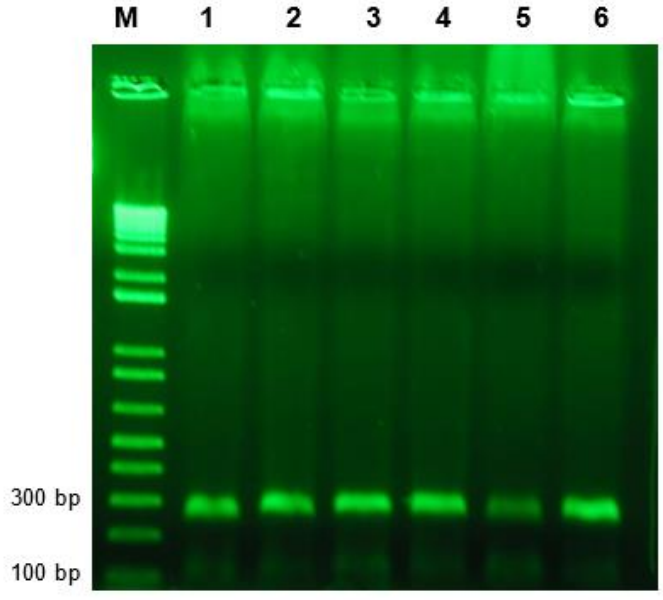


Figure 7: 1% agarose gel electrophoresis with extraction of post-transformed plasmid with CHS-EP by electroporation. The race followed the following standards: 100 V, 90 mA for 30 minutes. 1- pET28a + DH5α (1:40), 2- pET28a BL21 (DE3) (positive cloning control), 3- pET42a BL21 (DE3) (1:40), 4- pEt42a BL21 (DE3) (1:20) , 5- pEt28a BL21 (DE3) (1:20) and M: 1 kb molecular marker, plus DNA Ladder (Invitrogen).

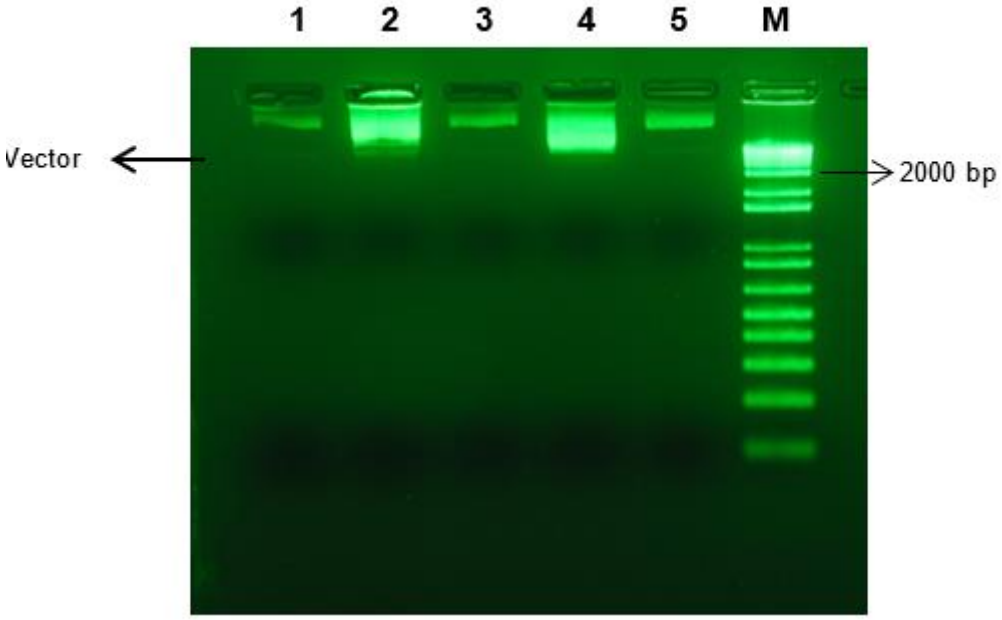
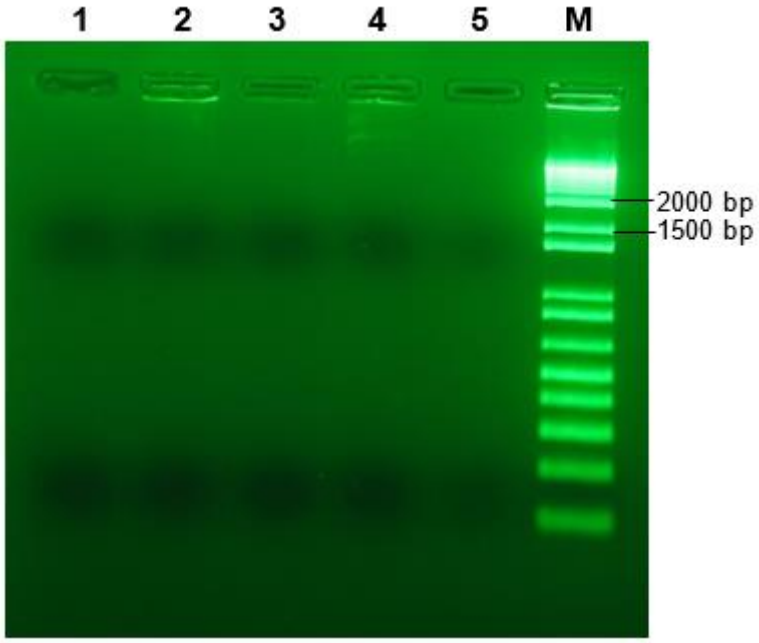


Figure 8: PCR of plasmid extractions from colonies grown after transformation by electroporation. Electrophoresis on 1% agarose gel followed the following standards: 100 V, 90 mA for 30 minutes. From right to left. 1- pET28a + DH5α (1:40), 2- pET42a BL21 (DE3), 3- pET42a BL21 (DE3) (1:40), 4- pEt42a BL21 (DE3) (1:20), 5 pEt28a BL21 (DE3)) (1:20) and M: 1 kb molecular marker, plus DNA Ladder, invitrogen,



4 DISCUSSION

In the primers design the annealing temperatures were very high because the restriction sites have polindromic sequences. These sequences can form staples (13), influencing the quality of PCR.

The pcr of the synthetic chitin synthase gene plasmid showed a drag band. Overlapping bands due to the degree of DNA compaction (13).

Amplification of the region of interest occurred, however the sequencing was not complete. The company Myleus that sequenced recommends amplifications of up to 1000 bp.

It was possible to digest the plasmid dephosphorylate the ends and prevent their recircularization using FastAP Phosphatase Alkaline from invitrogen. Vector dephosphorylation improves cleavage efficiency by preventing recircularization of the vector or incomplete cleavage (12).

It was possible to performe the ligation reaction and test different proportions of vector / insert. It is important to test different proportions to seek a balance between vector / insert concentration as well as temperature and reaction time as; these conditions are important to better activity of the DNA ligase enzyme (16) (13).

Colonies grown with chemically competent cells and was performed colony PCR. Colony PCR is a technique that allows rapid confirmation of the presence or absence of the insert linked to the vector, as well as allowing the visualization of fragments bound in the vector that are not the size of the protein of interest (4)

As there was no positive result of colony PCR with chemically competent cells, electroporation was performed. Chemically competent cells is a low cost and less efficient method compared to electroporation (20) (9).

Possibly, the transformation process was limited by the size of the fragment to be inserted in the vector. Large inserts can unstable the plasmid (3). When the DNA is large for the plasmid, a longer replication time is necessary, so plasmids with very large inserts are lost due to the very rapid increase in empty plasmids that eliminated the inserts randomly. (6). EINSFELDT (8), not obtained cells recombined in *B. subtilis*, because of the plasmid instability.

Ferreira (10) made cloning attempts with the enzymatic portion of chitin synthase in pF3a itself, where it also did not obtain satisfactory results.

Thus, selecting vectors that insert large size inserts can lead to an effective transformation and improve the stability of the plasmid.

5 CONCLUSION

There was positive amplification of the interest region with the designed primers, confirmed by sequencing and alignment with the chitin synthase sequence from genbank. Digestion and binding the the pET42a vector were efficient. Despite the success in amplification, digestion and ligation, the colonies grown and transformed only contained empty vectors, since after plasmid extraction and subsequent PCR the chitin synthase insert was not detected.

This way, testing new vectors and new proportions with the already validated protocols for amplification, digestion and binding of the nucleotide sequence of *M. perniciosa* chitin synthase will allow an efficient transformation, allowing the in vitro tests of the potential chitin synthase inhibitors and alternative control of this phytopathogen.

ACKNOWLEDGEMENTS

We would like to thank all who contributed directly or indirectly to this work and especially the Graduate Program in Biotechnology of the State University of Feira de Santana (PPGBiotec UEFS, http://www2.uefs.br/ppgbiotec), FAPESB, CAPES, CNPq.

REFERENCES

AIME M. C., PHILLIPS–MORA, W. The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. Mycologia, 97(5): 1012-1022, 2005.

ALMEIDA, L. C., ANDEBHAN, T. Recuperação de plantas de cacau com alta incidência de vassoura-de-bruxa na Amazônia Brasileira. In: CONFERÊNCIA INTERNACIONAL DE PESQUISAS EM CACAU, 10. Santo Domingo, proceeding, Lagos: CocoaProducers Alliance. p. 337-339, 1987.

BROCK, T., MADIGAN, M., MARTINHO, J., & PARKER, J. Microbiologia de Brock. 14 ed. Porto Alegre:Artmed, 2016.

CAO, MUQING et al. *Chlamydomonas* (chlorophyceae) colony PCR. Protoplasma, v. 235, n. 1-4, p. 107-110, 2009.

CEPLAC. Cacau. CARACTERÍSTICAS GERAIS DO CACAU. Disponível em: <http://www.ceplac.gov.br/radar/cacau.htm>. Acessado em: 23/01/2020.

Donald Voet, Judith G. Voet. Bioquímica. 4 ed. Porto Alegre:Artmed, 2013

DOS SANTOS, Everton Cruz et al. O patógeno Moniliophthora perniciosa promove modulação proteômica diferencial de genótipos de cacau com resistência contrastante à doença da vassoura-de-bruxa. BMC Plant Biology , v. 20, n. 1, p. 1, 2020.

EINSFELDT, Karen. SELEÇÃO, CLONAGEM E EXPRESSÃO DE UMA PROTEÍNA ASSOCIADA À VIRULÊNCIA DE Streptococcus pneumoniae. 2010. Tese de Doutorado. Universidade Federal do Rio de Janeiro.

EMBRAPA. Conceitos Básicos de Técnicas em Biologia Molecular. Disponível em: <https://ainfo.cnptia.embrapa.br/digital/bitstream/CNPA-2009-09/22214/1/DOC191.pdf>. Acessado em: 05/02/2020.

FERREIRA, E. B. CLONAGEM MOLECULAR DA PORÇÃO ENZIMÁTICA DA QUITINA SINTASE DE Moniliophthora perniciosa (STAHEL) AIME & PHILLIPS-MORA EM VETORES DE EXPRESSÃO. Trabalho de conclusão de curso. Universidade Estadual de Feira de Santana, 2018.

GALANTE, Rafaela S. et al. Purification, characterization and structural determination of chitinases produced by Moniliophthora perniciosa. Anais da Academia Brasileira de Ciências, v. 84, n. 2, p. 469-486, 2012.

LIU, ZHI-QIANG; YANG, PING-CHANG. Construction of pET-32 α (+) vector for protein expression and purification. North American journal of medical sciences., v. 4, n. 12, p. 651, 2012.

NELSON, D. L.; COX, M. M. Princípios de bioquímica de Lehninger. 5 ed. Porto Alegre:Artmed, 2011.

PRADO, P. F. V. D. Caracterização dos mecanismos de resistência a estrobilurinas no fungo Moniliophthora perniciosa, agente causador da vassoura de bruxa do cacaueiro. Dissertação (Mestrado). Universidade Estadual de Campinas, 2016

RUBINI, Marciano R. et al. Diversity of endophytic fungal community of cacao (Theobroma cacao L.) and biological control of Crinipellis perniciosa, causal agent of Witches' Broom Disease. International Journal of Biological Sciences, v. 1, n. 1, p. 24, 2005.

SAMBROOK, J.; RUSSELL, D. W. Molecular cloning: a laboratory manual 3rd edition. Coldspring-Harbour Laboratory Press, UK, 2001

SOUZA, C. Sintase da quitina de *Moniliophthora pernicio*sa (Stahel) Aime & Phillips-Mora: Caracterização, clonagem, expressão heteróloga e triagem virtual e teste in vitro de inibidores. Tese (doutorado). Universidade Estadual de Feira de Santana, 2012.

SOUZA, Catiane S., et al. Identification and characterization of a class III chitin synthase gene of Moniliophthora perniciosa, the fungus that causes witches' broom disease of cacao. The Journal of Microbiology. v. 47, n.4, p. 431-440, 2009.

TEIXEIRA, P. *et al*.. Time for chocolate: current understanding and New perspectives on cacao Witches' broom disease research. PLoS pathogens, v. 11, n. 10, 2015.

WEAVER, J. C. Electroporation Theory: Concepts and Mechanisms. In: Nickoloff JA, editor. Electroporation Protocols for Microorganisms. Totowa, New Jersey: Humana Press. p 1-26. 1995.

SUPLEMENTARY MATERIAL 1 - CONSENSUS SEQUENCING SEQUENCE

GATCCTTTATGCAGACCCGTTTGCAGACCGTCCTCGGCAGACCCACTTTACTGAACCCCAGCACCCTTATCCT
TCACAAGCCAGCATTCCTCGTCCTTTCGAGAGCGCGACTTCTCTGCCGCAGGAGTTTGGAGCACGGGACCA
GCAGTTCGAAGAAGATGACTACGTAGAAAAGCAACCGTTGACAGGCGGACAAGCGTTCGCTGGCGGGTTCT
ATCCACCAGGTCCTGTTGATCCTGAGGCCTATGGCGATCCTTATGCTGGGAGTCGCCCTGCTTCCGTCGTGTC
ATCGTCGACAGGCGGTGAAAAAAGTGCATGGCGACGACGACAGACCATCAAGCGTGGTGTTACCCGCAAG
GTCAAACTGACCAAGGGCAACTTCATCACCGAGTATCCAGTCCCTACACCTATTCTCAGCGCGACAGAAGCC
AAATATACTGCCACGTCCACAACCGAGTTTTCGCATATGCGATACACAGCTGCAACATGCGATCCGGATGAAT
TTTCAGAGGCCAACGGTTACTCACTGCGAACAAAGATGTACAACCGTGAGACCGAGCTTCTTATTGCCGTTA
CGTCATACAACGAAGAC

Suplementary Material- 2- Alignment of the amino acids residues sequence *Moniliophthora perniciosa* chitin synthase gene available in the GenBank database (EU154354) (1) with the amplified consensus sequence using the primers CHS-01 and CHS-02 (2).

```
EMBOSS_001    MANRPPLPSNASSSTVNDPYADPFADRPRQTHFTEPQHPYPSQASIPRPFESATSLPQEF  60
EMBOSS_002    ----------YADPFADRPRQTHFTEPQHPYPSQASIPRPFESATSLPQEF       41
                       ****************************************

EMBOSS_001        GARDQQFEEDDYVEKQPLTGGQAFAGGFYPPGPVDPEAYGDPYAGSRPASVVSSSTGGEK
          120
EMBOSS_002        GARDQQFEEDDYVEKQPLTGGQAFAGGFYPPGPVDPEAYGDPYAGSRPASVVSSSTGGEK
          101
          ************************************************************

EMBOSS_001          SAWRRRQTIKRGVTRKVKLTKGNFITEYPVPTPILSATEAKYTATSTTEFSHMRYTAATC
          180
EMBOSS_002          SAWRRRQTIKRGVTRKVKLTKGNFITEYPVPTPILSATEAKYTATSTTEFSHMRYTAATC
          161
          ************************************************************

EMBOSS_001         DPDEFSEANGYSLRTKMYNRETELLIAVTSYNEDKTLYARTLHGVMLNIRDICKTKQSKY
          240
EMBOSS_002    DPDEFSEANGYSLRTKMYNRETELLIAVTSYNED----------------------------  195
          ***********************************
```