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Heterologous Expression and Biochemistry Characterization of a putative *a*-amylase from *Clostridium thermocellum* B8 isolated from goat rumen

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ABSTRACT

Enzymes, mainly hydrolases, are considered important tools in biotechnological processes, nowadays. Among them, amylases occupy a remarkable quota in enzymes' world market and have been frequently used to degrade starch into fermentable sugars, which could be later used in a different set of industrial processes, as bioethanol production. In the present study, the main goal is obtain an α-amylase from C. thermocellum and carry its kinetic and molecular characterization aiming to evaluate its potential use in industrial processes. A putative α-amylase encoding gene (Cthe_2191) presenting carbohydrate binding module and two others conserved domains only described for α-amylases superfamilies which encodes a enzyme with theoretical molecular mass of 93858.85 Da and isoelectric point of 4.9 was identified in C. thermocellum B8 genome. In the present work, this gene was amplified and cloned in E. coli using pGEM T-Easy. Next steps of subcloning and heterologous expression are under development to obtain the heterologous protein and carry purification and kinetics characterization.

Keywords: a-amylase; heterologous expression; Clostridium thermocellum

INTRODUCTION

Amylases are among hydrolases most frequently used in biotechnological processes. They can be used in textile, food, detergent, papermaking and biofuel industries. Bacteria and fungus are easy to manipulate, and their enzymes show stability in a variety of temperatures and pH values, which make them attractive for use in different industrial fields (De Souza & Magalhães, 2010). Amylases could be also applied in biofuel production, indeed they can be used in starch liquefaction and hydrolysis to produce fermentable sugars which further would be fermented to bio ethanol. α-Amylases presented a huge interest in world enzyme market and termophiles produced robust enzymes adapted to industrial processes, thus there is a crescent interest in thermo amylases. Regarding this, our research group has been focusing efforts on the study of amylases produced by the termophile bacterium *C. thermocellum* B8.

MATERIAL AND METHODS

Bioinformatics analysis

Clostridium thermocellum B8 was completely sequenced, assembled and annotated. Amylases encoding genes were identified, one of them identified as an α-amylase enconding gene (Cthe_2191). This gene was searched against data sequence banks using "blastx" and

"blastn" tools in NCBI platform. The theoretical protein's molecular mass and isoelectric point was determined by the "Compute pI/Mw" tool in ExPASy platform, and of signal peptide presence was searched using "SignalP" tool, also found in ExPASy.

Cultivation conditions

C. thermocellum B8 from goat rumen was cultivated under anaerobic atmosphere (Freier *et al.*, 1988) in liquid reducing medium supplemented with crystalline cellulose 1% (m/v), as carbon source. The culture flasks after inoculation were incubated at 65°C. After 24 hours of growth period, the cells were collected by centrifugation and used for DNA extraction.

DNA extraction and gene amplification

C. thermocellum's chromosomal DNA was extracted using Wizard® SV Genomic DNA Purification System (Promega) kit according to the manufacturer instructions, and further used as a template in PCR reactions. Primers were designed using α-amylase encoding gene Cthe_2191, as template. PCR reactions were carried using: 10ng of chromosomal DNA, dNTPs 2.5 mM, 1U of LongAmp Taq DNA Polimerase GoTaq (Promega), LongAmp Taq Reaction Buffer 5M and 10mM of forward and reverse primers in a total volume of 25 µl. Amplification cycle was carried as follow: 95°C/1min; 94°C/0.5min, 51°C/1min, 65°C/2min - 5 cycles; 94°C/0.5min, 56°C/0.5min, 65°C/2min-30 cycles; final extension at 65°C/7min. PCR result was analyzed by electrophoresis in 0.8% (m/v) agarose gel. PCR product was purified using illustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare Life Sciences) and quantified by NanoDrop (Thermo Scientific).

α-amylase cloning and expression

Purified PCR product was linked to pGEM®-T Easy Vector (Promega). *E. coli* strain DH10B transformation was made by electroporation transformed clones were plated in LB selective medium containing Ampicillin/X-GAL/IPTG. Expression experiments will be carried using pET21a as vector and *E. coli*'s BL-21 as host cell. Heterologous α-amylase production will be induced using 0.5Mm IPTG for 5 hours at 37°C under shaking at 200rpm.

Purification and biochemical characterization of the heterologous protein

Heterologous protein's purification will be made using a His-Trap HP affinity column, coupled to AKTA Purifier (GE) apparatus, in flow rate of 1 mL/min. Mobile phases will be: Ligation buffer A (TrisHCl pH 8.0 20mM, 20mM Imidazol) and the Elution buffer B (TrisHCl pH 8.0 20mM, 500mM Imidazol), both containing NaCl 100mM. Resulting fraction will be analyzed to detected amylolytic activity and fractions presenting this activity will be polled and its purity evaluated by SDS-PAGE electrophoresis gel at 12% (m/v). Amylolytic activity will be determined using Fuwa (1954) and Miller (1954) methods. Protein concentration will be quantified using Bradford (1959) method.

RESULTS AND DISCUSSION

Bioinformatics analysis

Cthe_2191 gene presented three conserved domains: carbohydrate binding module and two others specific for α-amylases superfamily C (Figure 1). Putative α-amylase also showed that theoretical molecular mass of 93858.85 Da and isoelectric point of 4.9. It wasn't detected a signal peptide sequence.

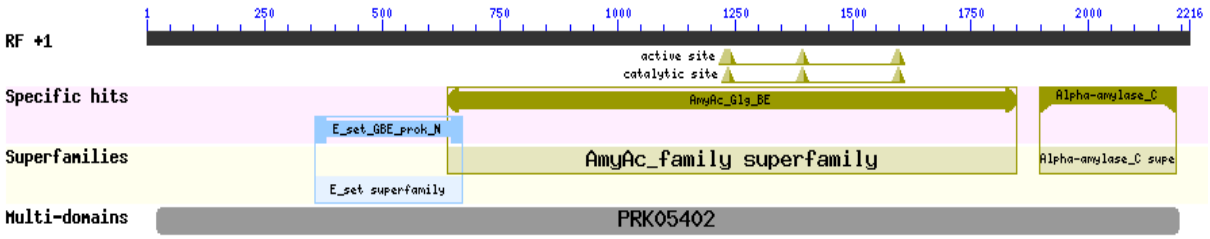

Figure 1. Blast x search's result showing amino acid conserved domains of Cthe_2191 (α-amylase encoding gene).

Gene amplification by PCR and cloning in pGEM T easy vector

PCR reaction resulted in the amplification of a unique band presenting the length (2.2Kb) which is in agreement to Cthe_2191 gene length (Figure 2).

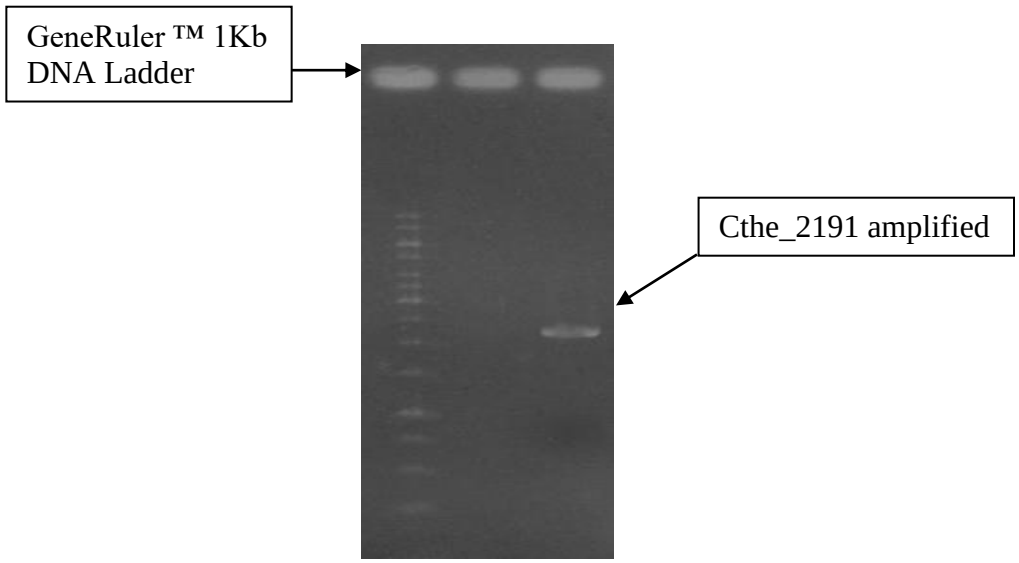

Figure 2. PCR products analysis in agarose gels 0.8% (m/v). Reaction was carried using extracted chromosomal DNA from *Clostridium thermocellum* B8 as a template and the primers were: 5'- CCT TCA TAT GAA CAC AAC AGC AAA TAT TGA -3' (Forward) and 5'- CCT TCT CGA GAT CCT TCC TGT CAA ACC TTG -3' (Reverse).

E.coli cells harboring pGEMT+amy were selected after growth in LB solid medium Ampicillin/X-GAL/IPTG. All transformed white colonies harbored pGEMT+amy presenting

restriction fragments of 3Kb and 2.2Kb, as expected as a result of a double restriction with NdeI and XhoI. They also presented restriction fragments of 3Kb, 0.867Kb and 1.347Kb, as expected as a result of a triple restriction with EcoRI.

PERSPECTIVES

Next steps of subcloning and heterologous expression are under development to obtain the heterologous protein and carry purification and kinetics characterization.

CONCLUSIONS

Cthe_2191, α-amylase encoding gene, was amplified and cloned into *E. coli* using pGEM-T Easy as a vector. Besides that, our research group main goal is contributed to the improvement of biotechnological processes applied to bio ethanol production from starch as raw material and the increase in the basic knowledge concerning amylases produced by the extremophile bacterium C. *thermocellum* B8.

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