UNLOCKING BACTERIAL BIOCATALYTIC POTENTIAL: ENZYME ENGINEERING FOR SUSTAINABLE BIOPROCESSING

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ABSTRACT

Over the last ten years, our laboratory has focused on studying the enzymes involved in the degradation of hemicellulose and cellulose in *Caulobacter crescentus* a bacterium capable of expressing several enzymes related to the use of lignocellulosic biomass. Its genome contains genes for cellulase and xylanase, as well as five genes for β-xylosidases. The genes *xynB1, xynB2, xynB5* β-xylosidases, *xynA1-2* for xylanase and *celA* for cellulase were overexpressed in *Escherichia coli*, and their enzymes were characterized. β-xylosidase II (xynB2) was studied in detail, showing resistance to inhibitors of the fermentative process and a probable regulatory role in the expression of hydrolases. Xylanase I (xynA1) was integrated into a vector and reintroduced into bacteria, improving baking characteristics. Xylanase II (XynA2) was applied in the biotreatment of jeans, resulting in effective cleaning and degumming. CelA cellulase was also efficient in the hydrolysis of corn straw and cobs and is an alternative to produce biofuels. In summary, the research carried out has expanded our understanding of the biocatalytic capabilities of this bacterium and provides exciting perspectives for its bioeconomy and environmental sustainability.

Keywords: *Caulobacter crescentus*. bioengineering. biofuels. textile industry. food industry

1 INTRODUCTION

Over the last ten years, our laboratory has been dedicated to studying the enzymes involved in the degradation of hemicellulose and cellulose in *C. crescentus*. During this period, it was observed that the bacteria can express several enzymes related to the use of lignocellulosic biomass and are therefore promising microorganisms for biotechnological applications. The *C. crescentus* genome contains one gene encoding cellulase [1], two genes encoding xylanase [2-4] and five xynB1-5 genes encoding β-xylosidases [5-11]. The *xynB1*, xynB2, xynB3, xynA1-2 and celA1 genes from *C. crescentus* were cloned and overexpressed in *Escherichia coli*, and their enzymes were characterized [2, 8, 9 ,11].

2 MATERIAL & METHODS

The data presented in this review involve published and/or ongoing work with *C. crescentus* hydrolases in recent years at the Unioeste Molecular Biochemistry Laboratory, Cascavel, PR.

3 *DEVELOPMENT*

All other xynB genes for β-xylosidases in *C. crescentus* are encoded as independent mRNAs and are likely monocistronic [5,9]. Among these genes, the genes xynB1, xynB2 and xynB3 for β-xylosidase, in addition to the two genes that encode xylanases and the only gene that encodes cellulase, were cloned and expressed in different expression vectors in our laboratory. The kinetic parameters of the purified recombinant proteins were characterized, and the data from these analyses are summarized in Table 1.

Among the genes encoding β-xylosidases in *C. crescentus* (strain NA1000), *xynB2* (CCNA_02442), which encodes the enzyme β-xylosidase II, has been the most thoroughly studied to date [6, 8-12] due to the particular importance of this enzyme. As xylose does not regulate the *xynB2* gene, it was cloned under the control of an artificial promoter to create the PxylX O-xynB2 strain, which overexpresses the enzyme in the presence of xylose [9]. Furthermore, a Δ-*xynB2* null mutant was created through two homologous recombination events in which the chromosomal *xynB2* gene was replaced with a copy interrupted by a spectinomycin resistance cassette. Interestingly, the absence of the β-xylosidase II enzyme in *C. crescentus* cells can upregulate the expression of the *xynB gene*, increasing the global activity of β-xylosidases. Transcriptional analysis of the *xynB1* (RT–PCR analysis) and *xynB2* (lacZ transcriptional fusion) genes, for example, revealed a significant induction of the expression of these genes in the *xynB2* gene null mutant strain when C. crescentus cells were cultured in the presence of various agro-industrial residues. Furthermore, greater β-xylosidase II activity was detected in the null mutant strain than in the wild-type strain. In contrast, overexpression of the *xynB2* gene caused a decrease in β-xylosidase activity in bacteria. These data suggest a regulatory role for β-xylosidase II in *C. crescentus*. However, the regulatory participation of β-xylosidase II in *C. crescentus* has not yet been experimentally proven; that is, there is no experimental evidence that β-xylosidase II binds to the bacterial chromosome. Therefore, experimental validation of this hypothesis still needs to be performed through DNA-ChIP-seq assays.

Table 1 - Enzymatic properties of the recombinant enzymes from *Caulobacter crescentus*.

Properties	XynB1 (GH43) β-xylosidase I	XynB2 (GH39) β-xylosidase II	XynB5 (GH3-BglX Family) β-xylosidase V	XynA1 (GH10) Xylanase I	XyA2 (GH10) Xylanase II	CelA1 (GH 9) Cellulase
Substrates	*o*NPX *p*NPA	*p*NPX	pNPG pNPX pNPA	*xylan from beechwood*	*xylan from beechwood*	
Optimal pH	6	6	6	6	8	5.5
pH Stability 24h	3-10	4.5-7.5		6	4-10	6
Optimal T	45 °C	55 °C	50 °C - Glucosidase and Arabinosidase; 60 °C -Xylosidase	50 °C	60 °C	40 °C
Thermal stability	45 °C 4 h	60 °C 4 h	-	50 °C 4h	65 °C 2h	35 °C 4h
MW (KDa)	65	60	95	50	43	73
Vmax	1.4 U mg^{-1}	402 U mg^{-1}	0.002 µM min-1 mg1 0.055 µM $min^{-1}mg^{-1}$ 0.091µM $min^{-1}mg^{-1}$	10.20 µM $min^{-1}mg^{-1}$	54.64 µmol min^{-1} mg^{-1}	2.41 U $mg^{-1}min^{-1}$
Km	2.89 mM	9.3 mM	0.24 mM - glucosidase 0.64 mM- xylosidase 1.45 mM - arabinosidase	3.77 mg mL^{-1}	5.78 µmol min^{-1} mg^{-1}	0.66 mg mL^{-1}
Kcat/Km mM^{-1} s^{-1}	0.55	43.3	0.27—pNPG 0.14—pNPX 0.10—pNPA	2.26	7.02	4.44
Source	Graciano et al. 2012	Corrêa et al. 2012	Justo et al. 2015	Graciano et al. 2015	Jacomini et al. 2020; 2023	Bussler et al. 2021

(-): not determined; T: temperature; MW: molecular weight; *p*NPX: *p*-nitrophenyl-β-D-xylopyranoside; *o*NPX: *o*-nitrophenyl-β-D-xylopyranoside; *p*NPA: *p*-nitrophenyl-α-L-arabinopyranoside; pNPG *p*-nitrophenyl-β-D-glucopiranoside

In addition to its conserved regulatory role, the enzyme β-xylosidase II has been shown to be highly stable and resistant to compounds that inhibit fermentative processes[12]. *C. crescentus* XynB2 (CcXynB2) activity was evaluated in the presence of several compounds known to inhibit the hydrolysis and fermentation of lignocellulosic biomass. CcXynB2 was shown to be 61% more tolerant than 200 mM methanol during a 48-hour incubation at 37 °C. The specific activity of CcXynB2 was determined in the presence of phenolic, hydroxymethylfurfural, ferulic, acetic and coumaric acids; arabinose; glucose; xylose; and pectin. After 48 hours, the activity of CcXynB2 in the presence of these compounds was equal to or greater than its activity in their absence. When the inhibitors were used in combination, CcXynB2 retained 67% of its initial activity after 48 hours at 37 °C. Enzymatic hydrolysis of hemicellulose from corn cobs was carried out using CcXynB2 alone or in combination with recombinant xylanase and β-glucosidase-β-xylosidase-α-arabinosidase from C. crescentus to produce reducing sugars. The immobilized CcXynB2 was more active than the soluble enzyme at the two tested temperatures, 37 and 50 °C. Furthermore, immobilized CcXynB2 retained more of its enzymatic activity after incubation at 50 °C than after incubation at 37 °C, maintaining more than 70% of its initial activity after 1 h of incubation. In general, CcXynB2 has potential for application in bioprocesses, as it has demonstrated robust resistance in the presence of many chemical compounds generated during the fermentation and physicochemical pretreatment stages of biomass, considering the simultaneous process of saccharification and fermentation in a biorefinery[12].

The *xynA1* gene, which encodes xylanase I from the nonpathogenic bacterium *C. crescentus* (strain NA1000), was integrated into the pAS22 vector with a xylose-inducible promoter and reintroduced into the bacterium, resulting in the generation of the conditional mutant BS-*xynA1*. In vitro comparison of BS-*xynA1* xylanase with three commercial xylanase mixtures commonly used in baking protocols revealed superior specific activity (163.4 U mg-1) over a wide temperature range (30-100 °C), with optimal performance at 50 °C. In practical baking tests, the addition of XynA1 from BS-*xynA1* resulted in a reduction in dough mixing time and a significant increase in loaf height compared to those of the control. Notably, the incorporation of Xylanase I improved the formation of the alveolar structure in the bread crumb. Improvements in the dough parameters, including increases in extensibility, elasticity, strain energy and strength, were observed. Furthermore, the inclusion of Xylanase I led to a reduction in the tenacity and stiffness of bread dough treated with the enzyme (not published data).

Recombinant xylanase II (XynA2) from the aquatic bacterium, previously characterized in terms of its biochemical characteristics, was applied in the biotreatment of jeans for cleaning and degumming[4]. Biotreatment performance was evaluated by tissue weight loss, the amount of reducing sugars released and scanning electron microscopy (SEM) images. Fabrics biotreated for 12 and 24 hours showed dry weight losses of 4.9% and 6.6%, respectively. The number of sugars released after the action of XynA2 on the jeans fibers was significantly different between the groups and the respective controls. SEM images revealed that the fabric treated for 12 hours had a smooth, polished surface, while the fabric treated for 24 hours had broken cotton fibers, indicating severe damage to the fabric. The best treatment for jeans was 1 U mg-1 of XynA2 at pH 8 and 60 °C for 12 hours. In conclusion, XynA2 from *C. crescentus* was satisfactorily applied in the biopolishing of jeans, offering a more sustainable alternative to chemical and abrasive processes for obtaining the same effects.

The cellulase CelA from *C. crescentus* also proved to be efficient in the hydrolysis of corn stover and corn cob (1%; m/v), releasing 2.62 µmol/mL and 1.02 µmol/mL of reducing sugars, respectively, offering an alternative to the production of chemicals and biofuels1. In addition, recombinant Xylnase II a (CelA) was applied directly to the jeans fibers, and the resulting scanning electron micrographs (SEMs) revealed that the fibers had a clean and smooth surface compared to that of the control fibers, indicating the potential of this material for the biopolishing of jeans[1].

4 CONCLUSION

In conclusion, our research has significantly contributed to revealing the biocatalytic potential of *C. crescentus* particularly in the context of sustainable bioprocessing. Through the engineering and characterization of various enzymes involved in lignocellulosic biomass degradation, such as cellulases, xylanases, and β-xylosidases, we have not only expanded our understanding of the biochemical properties of these enzymes but also demonstrated their applicability in diverse biotechnological processes. A detailed study of β-xylosidase II (xynB2), for instance, revealed its robustness and resistance to fermentation inhibitors, suggesting its viability for industrial bioprocesses. Furthermore, the successful application of xylanase I *(xynA1)* in improving baking characteristics and xylanase II (XynA2) in biotreatment processes highlights the practical relevance of our findings. Overall, these results pave the way for the development of more sustainable and efficient bioprocesses, with potential applications in biofuel production, biorefineries, and textile industries, contributing to the advancement of the bioeconomy and environmental sustainability.

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