PRODUCTION OF THE LYTIC POLYSACCHARIDE MONOOXYGENASE *Tr*Cel61A FROM *Trichoderma reesei BY Komagataella phaffii* IN BIOREACTOR

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ABSTRACT

Cellulose is the most abundant natural biopolymer on the earth. However, its recalcitrance is one of the main limiting factors for the complete deconstruction of this polysaccharide in monomers. Hydrolytic enzymes derived from fungi and bacteria as cellulases and hemicellulases have been used for glucose production and subsequent alcoholic fermentation. In addition, oxidative enzymes have been used to increase the release of glucose together with the hydrolases. Lytic polysaccharide monooxygenases *(LPMOs) present an oxidative activity and are central component of commercial enzyme cocktails used for the industrial production of lignocellulosic ethanol. The LPMO TrCel61A from Trichoderma reesei is a model LPMO and was already produced by different hosts including Komagataella phaffii in bioreactor. In this work, we investigated the recombinant production of this enzyme by K. phaffii cells using a mineral salts medium, methanol feed and pO_2 fixed at 15% and 30%. The results obtained in the cultures A (pO_2 15%) and B (pO_2 30%) were 37.9 and 33.0 g/L for cell biomass and 0.23 and 0.33 g/L to extracellular protein, respectively. Molecular mass calculated from the SDS PAGE analysis showed a protein with approximately 60 kDa. LPMO produced in fixed pO_2 at 30% and methanol feed of 3 g/L per hour is promising approach to obtain this protein at high levels.*

1. INTRODUCTION

Several scientific studies have focused on the deconstruction of agro-industrial residues for production of biofuels and chemicals. Cellulose is the most abundant natural biopolymer on the earth. However, the recalcitrance of this biopolymer is a limiting factor for the completely deconstruction of the cellulose in monomers. Enzymatic hydrolysis is a great solution for cellulose breakdown (Carere et al., 2008).

Several enzymes derived from fungi and bacteria, such as cellulases and hemicellulases, have been used for glucose production and subsequent alcoholic fermentation. These enzymes have been applied in enzyme cocktails for the industrial production of lignocellulosic ethanol, but new enzymes are required to obtain higher level of sugar, for example oxidative enzymes, such as the lytic polysaccharide monooxygenases (LPMOs). LPMOs present an oxidative activity which increases the release of glucose together with the cellulases and hemicellulases. These oxidative enzymes acting in that breakdown of the recalcitrance of the lignocellulose biomass. LPMOs are a central component of the commercial enzyme cocktails used for the industrial production of lignocellulosic ethanol (Rodrigues et al., 2017).

LPMOs are produced by microorganisms, specially fungi and bacteria. The heterologous production of enzymes with industrial application is a promising strategy to obtain proteins in greater quantity. *Komagataella phaffii* (former *Pichia pastoris*) is a methylotrophic yeast commonly used as a host for the production of recombinant proteins. *K. phaffii* present an ability to produce high amounts of biomass and functional enzymes (Juturu et al., 2018). The LPMO *Tr*Cel61A from the cellulolytic fungus *T. reesei* was previously produced by *K. phaffii* in bioreactor (using a construct with the AOX1 promoter, a codon-optimized gene and the protein's native secretion signal) (Tanghe et al., 2015). In the present work, we used a *K. phaffii* recombinant strain to produce the native form of the LPMO *Tr*Cel61A in bioreactor using a mineral salts medium and methanol.

2. METHODOLOGY

High cell-density production of *Tr*Cel61A: The *K. phaffii* recombinant strain producing the LPMO *Tr*Cel61A from *T. reesei* was pre-cultivated in 200 mL YPD medium for 24 h at 30°C. Cells were centrifuged at 1500 x *g* and inoculated in a 2 L Minifors bioreactor (Infors) vessel 1 and 2 containing 1.0 liter of mineral medium supplemented with 4% (w/v) glycerol, PTM_1 trace salts (Sigma-Aldrich), and Antiphoam 204 (Sigma Aldrich) (Maurer et al., 2006). The pH was adjusted to 5.5 using ammonium hydroxide 28%. Dissolved oxygen concentration (pO_2) was maintained fixed at 15% (culture A) and 30% of the saturation (culture B) by controlling the stirrer speed (400 and 1200 rpm), the airflow (0.1 and 3.0 vvm) and gas mix of the pure O_2 (25 and 100%). After glycerol depletion, methanol transition phase started with an increase addition of methanol 100% (v/v) (1.0 g/L - 5.0 g/L). Methanol feed started by addition of 3.0 g/L per hour of methanol 100% (v/v). Samples were taken along the cultures for quantification of glycerol and methanol, cell density (OD_{600}), and protein concentration. After the culture, the cells extract were centrifuged at 4,500 x *g* for 30 minutes to obtain the crude extract with LPMO *Tr*Cel61A.

Protein Analysis and purification: The extracellular concentration protein was obtained by precipitation with acetone (v/v) for 60 min at -80°C and determined using a Bicinchoninic acid (BCA) protein assay Kit with bovine serum albumin (BSA) (Sigma-Aldrich) ranging from 0.0625 to 1 mg/mL as standard. Protein quantification was performed in triplicate. The protein purification was carried out using a size exclusion chromatography (SEC) on the Superdex 75 column type (GE Helthcare Life

Sciences) equilibrated with 50 mM Tris HCl, pH 7.0 buffer containing 0.15 M NaCl. The proteins samples were applied and eluted from the column using a rate of the 0.5 mL/min. All the fractions collected were analyzed by SDS-PAGE 12% according to the manufacturer's recommendations (Bio-Rad Laboratories) and the bands were stained with silver nitrate solution (Nesterenko el al., 1994).

3. RESULTS AND DISCUSSION

K. phaffii recombinant strain producing the *Tr*Cel61A LPMO was cultivated in bioreactor using 15% (Figure 1-A) and 30% (Figure 1-B) fixed pO_2. Cell density, extracellular protein and SDS-PAGE analysis were monitored during the 67 hours of cultivation. At the end of glycerol phase, biomass concentrations obtained were 12.5 and 15.7 g/L, respectively. Additionally, at the end of methanol phase the biomass concentrations were 37.9 g/L and 33.0 g/L while extracellular protein concentrations were estimated at 0.23 ± 0.01 and 0.33 ± 0.03 g/L, respectively. In both strategies (15 and 30% pO_2) *Tr*Cel61A LPMO was produced, however, only the protein amount obtained in the second strategy exhibited a similar value to that obtained by Tanghe et al. (2015).

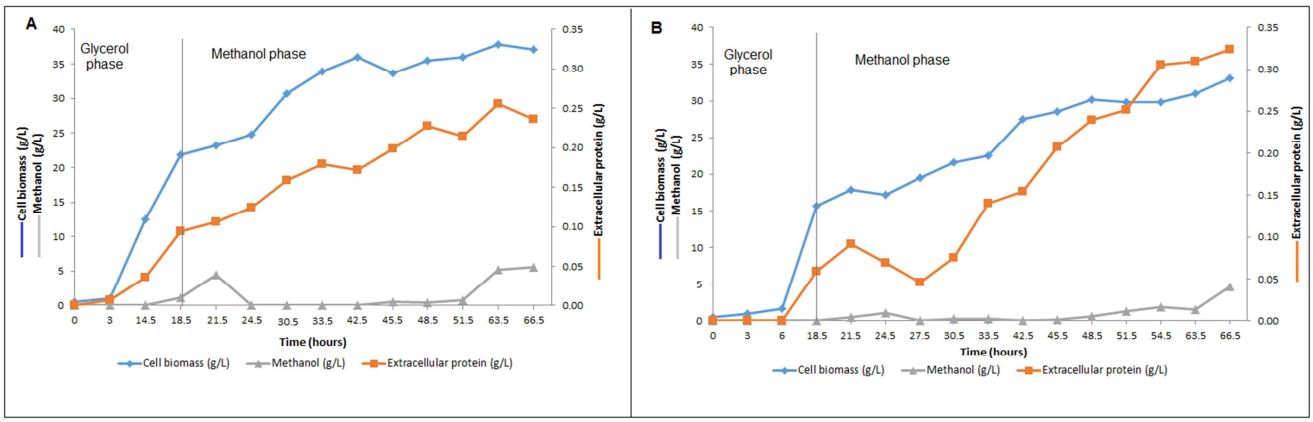

Figure 1. Fermentation parameters of the *K. phaffii* recombinant strain producing the *Tr*Cel61A LPMO from *T. reesei* in bioreactor at pO_2 fixed in 15% (A) and 30% (B). Cell biomass (g/L) (blue line), Protein concentration (g/L) (orange line) and methanol (g/L) (gray line) were monitored in different hours of cultivation (0 – 66.5 hours). Glycerol phase: 0 to 18 hours, and Methanol phase: 20 to 66.5 hours.

The LPMO was purified with the only intention of performing some functional assays and therefore the yield could not be calculated. The enzyme presented a molecular size of approximately 60 kDa (Figure 2A-C), which was similar to profile described previously by Tanghe et al. (2015). This result indicates that the protein was glycosylated, since the estimated size for *Tr*Cel61A is approximately 35 kDa. The band of 100 kDa (Figures 2A and B) corresponds to a native protein from *K. phaffii.* Our results showed that the enzyme production in fixed pO_2 30% and the continuous methanol feed using 3 g/L is promising to obtain the recombinant LPMO *Tr*Cel61A at high levels.

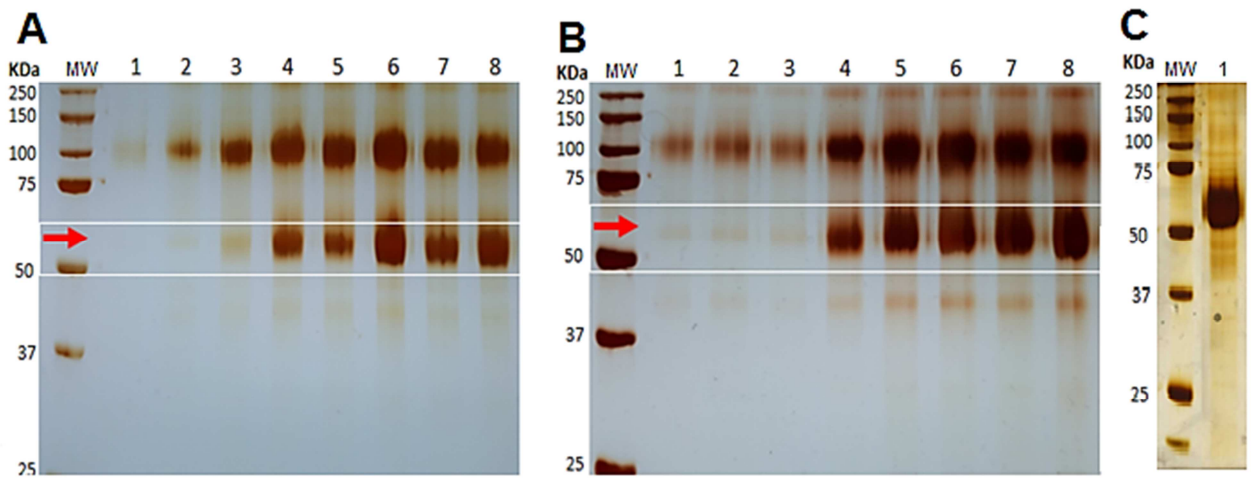

Figure 2. Electrophoretic analysis (SDS-PAGE 12%) of the supernatant containing LPMO *Tr*Cel61A from the culture A (pO_2 15%) and culture B (pO_2 30%) and LPMO *Tr*Cel61A purified. A) Supernatants of the culture A (10 uL): Lane 1-18.5 h, lane 2- 24.5 h, lane 3- 30. 5 h, lane 4- 42.5 h, lane 5- 48.5 h, lane 6- 51.5 h, lane 7- 54.5 h and lane 8- 66.5 h. B) Supernatants of the culture B (10 uL): Lane 1- 18.5 h, lane 2- 24.5 h, lane 3- 30. 5 h, lane 4- 42.5 h, lane 5- 48.5 h, lane 6- 51.5 h, lane 7- 54.5 h and lane 8- 67 h. C) LPMO *Tr*Cel61A purified by size exclusion chromatography using a Superdex 75 column presenting molecular weight of 60 kDa (Lane 1). MW- Precision Plus Protein ™ Dual Xtra-BIO-RAD Standard.

4. REFERENCES

Carere, C.R., Sparling, R., Cicek, N., Levin, D.B., 2008. Third Generation Biofuels via Direct Cellulose Fermentation. Int. J. Mol. Sci. 9, 1342-1360.

Juturu, V., and Wu, J.C., 2018. Heterologous Protein Expression in *Pichia pastoris*: Latest Research Progress and Applications. ChemBioChem 19, 7 – 21.

Maurer, M., kuehleitner, M., gasser, B., Mattanovich, D., 2006. Versatile modeling and optimization of fed batch processes for the production of secreted heterologous proteins with *K. phaffii*. Microb. Cell Fact. 5, 37–47.

Nesterenko, M.V., Tilley, M., Upton, S.J., 1994. *A* simple modification *of* Blum's silver stain method allows *for* 30 minute detection *of* proteins *in* polyacrylamide gels. J Biochem Biophys Methods. 28, 239-242.

Rodrigues, K.B., Macêdo, J.K.A., Teixeira, T., Barros, J.S., Araújo, A.C.B., Santos, F.P., Quirino, B.F., Brasil B.S.A.F., Salum, T.F.C., Abdelnur, P.V., Fávaro, L.C.L., 2017.Recombinant expression of *Thermobifida fusca* E7 LPMO in *Pichia pastoris* and *Escherichia coli* and their functional characterization. Carbohydrate research. 448,175-181.

Tanghe, M., Daneels, B., Camattari, A., Glieder, A., Vandenberghe, I., Devreese, B., Stals, I., Desmet, T., 2015. Recombinant expression of *Trichoderma reesei* Cel61A in *Pichia pastoris*: optimizing yield and N-terminal processing. Mol. Biotechnol. 57 (12), 1010–1017.

5. ACKNOWLEDGEMENTS

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