An integrative approach for high level production of glucuronoyl esterase in Pichia pastoris.

CURRENT STATUS: ACCEPTED

BMC Research Notes

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DOI:
10.21203/rs.2.12831/v1

SUBJECT AREAS
Biotechnology and Bioengineering

KEYWORDS
Glucuronoyl esterase, cellobiose dehydrogenase, methanol-free constitutive expression system, Pichia pastoris, lignocellulose biorefinery enzymes, enzyme bioprocess technology
Abstract

**Objective:** The commercial availability of lignin-modifying and accessory enzymes is limited, which delays the investigation of their functionality and development of industrial applications. Glucuronoyl esterase (GE) has been shown to improve fractionation of lignin-carbohydrate complexes, yet no pure commercial enzyme preparations are available. To improve accessibility to this enzyme for emerging research, this study reports a simple and effective heterologous expression strategy, coupled with a methanol-free production protocol in a bioreactor for high-level production of GE. This strategy was further validated by production of cellobiose dehydrogenase, a lignocellulose degrading enzyme which is scarcely available at high cost. **Results:** The secreted recombinant enzymes were functionally produced in excess of levels previously reported for constitutive production (1489-2780 mg.L\(^{-1}\)), and were secreted at moderate to high percentages of the total extracellular protein (51-94 %). The constant glycerol feed, implemented during fed-batch fermentation, lead to a decline in growth rate, and plateaued productivity. Tangential flow ultrafiltration was used to concentrate cell-free enzyme extracts 5-6-fold, in a two-step filtration process. This study describes a workable, cost-effective, and commercially scalable production strategy to improve the ease of GE-based research.

Introduction

Enzyme production and enzymatic hydrolysis steps are still bottlenecks to the feasibility of second generation biorefineries [1]. The inclusion of accessory enzymes to cellulase preparations, at appropriate ratios, could improve yields of fermentable sugars while providing a lignin-enriched residue more suited for further applications [2]. Glucuronoyl esterase (GE) is a recently defined carbohydrate esterase that has been isolated from several microorganisms [3-8]. GE has potential for application in lignocellulose biorefineries (excluding alkali-based lignin extraction), where lignin-carbohydrate complexes hamper separation of lignin and polysaccharides [9-13]. Recent reports have proven that the removal of glucuronic acid branches from the hemicellulose, xylan, significantly improved release of fermentable sugars for biofuels production [10, 14].

GE is an exciting production target since, despite its industrial potential, very limited recombinant production has been reported. Pure commercial preparations of GE do not exist. This is an impedance
in the development of GE-based bioprocesses, since each study must first produce and purify the enzyme before it is possible to determine its lignocellulosic degrading capabilities. Some commercial enzyme cocktails exist with low GE side activity [15, this study], but these cocktails do not allow for the determination of specific catalytic roles of each enzyme. To harness the catalytic ability of GE, understand its specific role, and determine optimum dosages, pure enzyme preparations are required. Notably, the recent increase in publications which evaluate application of GE, show an urgent need for GE production [9–12].

In light of the above restriction in GE availability, this study sought to create a ‘plug-and-play’ system for production of large amounts of GE. The use of a constitutive, patent-free, PGAP-\textit{P. pastoris} expression system, to produce \textit{Hypocrea jecorina} GE at bioreactor scale was investigated. For robust proof of concept, a correspondingly applicable enzyme, \textit{Neurospora crassa} cellobiose dehydrogenase (CDH, CAZy Auxiliary Activity family AA3), was investigated in parallel with GE. CDH has a similarly limited availability in terms of commercial options as well as cost, and it improves the saccharification of pre-treated lignocellulosic materials through reduction of end-product inhibition and catalytic activation of lytic polysaccharide monooxygenase (LPMO) [16]. Both the enzyme production itself as well as downstream processing of the enzyme into a stable product were considered. This study is the first report of GE secretion yields under constitutive expression, and the second report of bioreactor heterologous production [11].

\textbf{Methods}

\textit{Construction of the production strains.} The \textit{P. pastoris} type strain DSMZ 70382 (CBS704) (DSMZ German Collection of Microorganisms and Cell Cultures) was selected as the expression host. The commercial pjexpress 905 (pj905) vector (ATUM, USA) containing the GAP promoter and AOX1 terminator, was used to generate constructs for GE (AAP57749.1) and CDH (XM_951498.2) expression and secretion. Details of cloning, transformation, confirmation and screening procedures are described in [17]. The transformant showing the best production during screening in shake flasks for each enzyme was selected for production at bioreactor scale.

\textit{Bioreactor cultivations.} The inoculum preparation and fermentation procedure were conducted as
described in Invitrogen guidelines [18, 19]. Fermentations were conducted in 14 L New Brunswick BioFlo 110 bioreactors, using BioCommand® software (Version 3.30 Plus, New Brunswick Scientific Co. Inc.). The fermentation conditions were identical for all fermentations: 30 °C, pH 5 (combination glass pH probe Mettler Toledo), DO maintained at 30 % (polarographic DOT probe, Mettler Toledo), and an aeration rate of 1 vvm. The fed-batch stage was initiated after depletion of glycerol, where 4 L of 50 % (w.v⁻¹) glycerol feed, supplemented with PTM₁ trace salts solution (1.2 % v.v⁻¹), was fed at a constant rate of 72.6 ml.hr⁻¹. The fed-batch stage was concluded after approximately 48 hours, when the feed was depleted. Samples taken (10ml) during fermentation were analysed for biomass, enzyme activity as described in [20, 21], and glycerol concentration [22] (during fed-batch stage). The final samples were used to determine total protein concentrations [bicinchoninic acid (BCA™) microassay (Sigma-Aldrich, USA)] and target protein concentrations (densitometry of tris-tricine SDS-PAGE [23] image using ImageJ® software). Maximum growth rate ($\mu_{\text{max}}$) was graphically calculated during the exponential growth phase of the batch phase. All trends were fitted to a linear regression with $R^2$ values of above 0.98.

**Concentration of crude enzyme extracts.** The culture was harvested immediately after the fed-batch phase to minimize protease degradation of the enzyme product. The total culture was centrifuged (8000 rpm; 4 °C; 10 minutes) and subjected to a two-step filtration process. The Pellicon 2.0 tangential flow filtration apparatus (Merck, South Africa) was used as per the manual instructions. The feed and retentate pressure were maintained at a maximum of 10 and 2 bar, respectively. The transmembrane pressure (TMP) was 2.5 bar. The supernatant was first filtered through a 0.22 μm filter cassette (Durapore® PVDF, Merck Millipore), then concentrated by ultrafiltration using a 5 kDa filter cassette (Biomax™ 5, Merck Millipore). Once the volume of the retentate/feeding reservoir was decreased 10-fold, the process was complete. Samples of each permeate and retentate were analysed for volumetric activity. Volumes of the feed, permeate and retentate of each filtration process were measured.

Results And Discussion
Enzyme production yields and process productivity

The growth and enzyme production characteristics of the created recombinant strains were evaluated using bioreactor cultivations. GE side-activity of three commercial enzyme cocktails, namely Celluclast® 1.5 L (Novozymes, 27.47 U.L⁻¹), Depol™ 740 L A (Biocatalysts, 10.70 U.L⁻¹) and Depol™ 740 L B (Biocatalysts, 7.72 U.L⁻¹) were tested to serve as a comparative baseline. The bioreactor cultivation for expression of GE returned a final volumetric activity yield of 238.17 U.L⁻¹, and a final recombinant protein titre of 2778.01 mg.L⁻¹ (Fig. 1a; Table 1), well in excess of previous attempts involving methanol induction of GE [7, 24]. Notably, the volumetric activity was well above the side activities of the commercial enzyme cocktails tested here (7.72—27.47 U.L⁻¹). Bioreactor cultivation for CDH production returned a final mean volumetric activity of 329.49 U.L⁻¹ and a final mean recombinant protein concentration of 1489.30 mg.L⁻¹ (Figure 1b; Table 1). Although the protein concentration of secreted CDH reported here (1489.30 mg.L⁻¹) is an improvement on previous attempts at methanol-induced expression, the volumetric activity and specific activity is lower than previously reported [21, 25–27]. Lowered specific activity has been observed in recombinant CDH production by *P. pastoris* due to a sub-stoichiometric occupation of catalytic sites within the FAD cofactor, as well as hyper-glycosylation [28]. Further, hyper-glycosylation may have affected the specific activity of both target enzymes, since the reported protein sizes (CDH: 127 kDa; GE: 78 kDa) are larger than the expected, *in silico* protein sizes (ExPASy: CDH: 88.46 kDa; GE: 81.29 kDa) [4, 29].

The general trends observed for the fermentations are as follows (Fig. 1a-b). During the batch phase, an initial lag phase of less than 6 hours was observed, corresponding to minimal volumetric activity, followed by an exponential growth phase, where linear increases in volumetric activity were observed until the end of the batch phase (between 20–22 hours). During the glycerol fed-batch stage, there was a brief exponential growth phase of approximately 6 hours, followed by an extended growth phase. During this continuous growth phase, the agitation of the bioreactor remained at the
maximum (1000 rpm), and oxygen sparging was required throughout to maintain sufficient availability of dissolved oxygen (30%) in the culture.

The maximum specific growth rate was determined during the batch phase under identical growth conditions (Table 1). GE- and CDH-expressing *P. pastoris* strains had similar $\mu_{\text{max}}$ values of 0.15 h$^{-1}$ and 0.16 h$^{-1}$ respectively, within expected values reported for recombinant *P. pastoris* strains using glycerol (0.15—0.20 h$^{-1}$) [30, 31]. Similarly, the biomass yields obtained (120.94, 136.47 g$_{\text{DCW}.L^{-1}}$; Table 1) were comparable to previous bioreactor cultivations of recombinant *P. pastoris* [18, 32].

A decrease in specific growth rate, modelled as a power regression (GE: $20979x^{-3.63}; R^2 = 0.9871$; CDH: $885.76x^{-2.852}; R^2 = 0.9882$), was observed during the fed-batch process (Fig. S1), during which the levels of glycerol remained below detectable HPLC levels. This is attributed to the fact that the amount of biomass in the fermentation vessel increased with time (Fig. S1), while the amount of glycerol fed into the bioreactor remained constant, effectively decreasing the ratio of available carbon source per gram of biomass. This growth rate decline has been suggested to be the concluding stage of an ideal growth rate time course for a number of secreted protein fermentations in *P. pastoris* [30, 33, 34].

Specific productivity describes the efficiency of the process by quantifying the amount of recombinant enzyme secreted per gram of biomass per hour. Maximum values of specific productivity ($q_{p,\text{max}}$) of protein secretion were reached during the batch phase (1.22, 0.76 mg recombinant protein.biomass$^{-1}.h^{-1}$ for GE and CDH, respectively; Table 1). During the fed-batch stage, specific productivity remained constant at a lowered value close to the mean specific productivity value ($q_{p,\text{mean}}$) (0.52 and 0.43 mg recombinant protein.biomass$^{-1}.h^{-1}$ for GE and CDH, respectively), resulting in GE and CDH accumulation throughout the fed-batch stage (Fig 1a-b). This has been observed in previous constant fed-batch cultivations of recombinant *P*$_{\text{GAP}}$. *P. pastoris* [33, 35]. The productivity could be increased by manipulating the growth rate such that an extended growth phase at $\mu_{\text{max}}$ is maintained before the end-phase decline in growth rate [31, 33, 34, 36]. This is achieved by an increasing (linear
or exponential) glycerol feeding rate during the fed-batch stage. The optimal duration of the phase at which $\mu_{\text{max}}$ is maintained differs between recombinant strains and should be optimised on a case-by-case basis.

**Concentration of crude enzyme extracts by tangential flow ultrafiltration (TFU)**

The heterologous proteins were secreted at moderate (CDH: 50.89 %) to high (GE: 93.61 %) percentages of the total extracellular protein, as determined by densitometry analyses (Fig. 1c). CDH and GE were, therefore, the major proteins present in the fermentation broth. While native, extracellular *P. pastoris* proteins were present in the fermentation broth (Fig. 1c), these were devoid of any contaminating enzymatic activity (as determined with the use of a negative control strain), which allows for simple and economical downstream processing, contributing to the affordability and accessibility of the process.

The harvested supernatant was concentrated using TFU (Table 2). The microfiltration process returned satisfactory volumetric activity yields (GE: 86.84 %, CDH: 86.54 %; Table 2), and the loss in activity units can be explained by the inherent loss of sample volume in the filtration apparatus itself. The total protein concentration in the enzyme extracts was increased 3.69-, 2.84-fold for GE and CDH, respectively. In terms of volumetric activity, GE and CDH were concentrated 4.95-, and 5.20-fold, respectively. The ultrafiltration process returned volumetric activity yields that were acceptable (49.57 and 50.00 %), but lower than expected [26, 37–39] despite acceptable integrity of membrane (permeate with minimal volumetric activity 0.00—2.95 $U_{\text{Total}}\cdot \text{L}^{-1}$). It is therefore hypothesised that the recombinant protein is being retained or degraded in the filtration apparatus itself, as proteins can adsorb to membranes, often causing permanent fouling [40] and the formation of a protein monolayer on the surface of the membrane [41]. Despite these shortcomings, the final concentrated enzyme products could be used directly on lignocellulose according to enzyme dosages established in previous studies [4, 42].

**Limitations**
This study sought to facilitate emerging GE-based research and development of biorefinery processes. The production process was designed with commercial and industrial foresight, seeking to keep the process as straightforward, safe, and cost effective as possible, while limiting any proprietary restrictions on the eventual bioprocess. The bioprocess reported here returned high protein yields of active GE and shows promise for application to different enzymes as well, including CDH.

However, the flat-lining specific productivity values during fed-batch cultivation shows potential for improvement through growth rate manipulation. Tangential flow ultrafiltration concentrated the enzyme extracts to suitable enzyme dosages for lignocellulose application studies, but showed inefficient volumetric activity yields. This is significant due to the economic implications for the future production process, therefore optimization of the ultrafiltration process is recommended for future studies. Future studies should also confirm the application of the concentrated recombinant enzymes on industrially relevant substrates such as steam pretreated lignocelluloses.

**Declarations**

*Ethics approval and consent to participate:* not applicable.

*Consent for publication:* not applicable.

*Availability of data and materials:* Tabulated data of graphs reported in this study are available in reference 17 (Addendum D):

[https://pdfs.semanticscholar.org/4a02/774e4d4f01751b0140a315b617bb36d233ff.pdf](https://pdfs.semanticscholar.org/4a02/774e4d4f01751b0140a315b617bb36d233ff.pdf)

*Competing interests:* none.

*Funding:* This work was funded by the South African National Research Foundation (NRF) and the South African Biofuels Research Chair.

*Authors’ contributions:* JG, EVZ and MGA conceived the project. JG and MGA supervised the study. CC and MGA constructed the recombinant strains. CC performed the screening in shake flasks and fermentations at bioreactor scale. CC wrote first draft of manuscript with assistance of MGA. GC assisted with bioreactor experiments. All authors discussed the results, provided critical feedback and approved the final manuscript.
Acknowledgements: Novozymes and Biocatalysts are thanked for supplying the commercial enzyme preparations. Mr Arrie Arends, Dr Kim Trollope and Dr Rose Cripwell from Stellenbosch University are thanked for technical assistance with the bioreactors, molecular work and HPLC samples, respectively. Renewable and Sustainable Energy Scholarships (RSES) are thanked for their financial support.

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**Tables**

**Table 1.**

1. At shake flask scale, under methanol-induced P_{AOX1} regulation

2. At 7 L bioreactor scale, under methanol-induced P_{AOX1} regulation

NR: Not reported
| Parameter                                                                 | GE (this study) | GE (previous studies) | Reference                  | CDH (this study) | CDH (previous studies) | Reference                  |
|---------------------------------------------------------------------------|-----------------|------------------------|-----------------------------|------------------|--------------------------|-----------------------------|
| Highest Volumetric Activity (U.L⁻¹)                                       | 238.17          | 5.7¹                   | Topakas et al. 2010         | 329.49           | 7955¹                    | Stapleton et al. 2004       |
| Highest Recombinant Protein Titre (mg.L⁻¹)                                | 2778.01         | -                      | -                           | 1489.30          | 633²                     | -                           |
| % recombinant protein vs total protein in supernatant                      | 93.61 %         | -                      | -                           | 50.89 %          | -                        | -                           |
| Biomass concentration (g_{DCW}.L⁻¹)                                       | 120.94          | NR                     | -                           | 136.47           | NR                       | -                           |
| μ_{max} (h⁻¹)                                                             | 0.15            | -                      | 0.16                        | -                | NR                       | -                           |
| q_{p,max} (mg recombinant protein. [g biomass•h]⁻¹)                        | 1.22            | -                      | 0.76                        | -                | -                        | -                           |
| q_{p,mean} (mg recombinant protein. [g biomass•h]⁻¹)                       | 0.52            | -                      | 0.43                        | -                | -                        | -                           |

**Table 2.**

CFE: Cell-free extract; **MFP**: Microfiltration permeate; **UFR**: Ultrafiltration retentate or concentrated enzyme; **UFP**: Ultrafiltration permeate
| Step | Volumetric Activity (U.L\(^{-1}\)) | Total Activity (U) | Yield (%), volumetric activity | Total Protein Concentration (g.L\(^{-1}\)) |
|------|---------------------------------|--------------------|-------------------------------|--------------------------------------------|
| GE   | CFE 203.92                      | 407.84             | 100.00                        | 2.93                                       |
|      | MFP 196.07                      | 352.93             | 86.54                         | -                                          |
|      | UFR 1019.61                     | 203.92             | 50.00                         | 8.31                                       |
|      | UFP 2.11                        | 2.95               | -                             | -                                          |
| CDH  | CFE 40.77                       | 81.54              | 100.00                        | 2.97                                       |
|      | MFP 39.34                       | 70.81              | 86.84                         | -                                          |
|      | UFR 202.08                      | 40.42              | 49.57                         | 10.16                                      |
|      | UFP 0.03                        | 0.04               | -                             | -                                          |

**Figures**

![Graph a](image1)

**Figure a**

Fed-Batch Stage

![Graph b](image2)

**Figure b**

Fed-Batch Stage
Volumetric activity (green diamond) and biomass yields (dry cell weight per litre) (black circle) during bioreactor cultivations of each target protein. Arrows indicate the initiation of the glycerol fed-batch phase (20 – 22h), where a 50 % (w.v-1) glycerol feed supplemented with trace salts was added at a rate of 72.6 ml/hr. A: Glucuronoyl esterase (GE) B: Cellobiose Dehydrogenase (CDH). Error bars indicate standard deviation from mean of three
fermentation replicates. C: SDS-PAGE analysis of final fermentation samples (each lane representing one replicate of a bioreactor fermentation) of 20μl supernatant containing the target proteins. Lane 1: Protein molecular weight marker. Lane 2-4: CDH-containing supernatant (rCDH). Lanes 5-7: Glucuronoyl esterase-containing supernatant (rGE).

Supplementary Files
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Graphical Abstract-BMC-05082019.pptx
Figure S1.tiff