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Development Of Scalable Bioreactor Process For Production, Purification And Characterization Of Human Recombinant A-Glucocerebrosidase

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ABSTRACT

The development of a scalable bioreactor process for the production in P. pastoris of recombinant human α -Glucocerebrosidase (rhGC), aiming to implement a reproducible process to obtain significant amounts of the enzyme. One of the main advantages of the use of P. pastoris in protein production is the extraordinary cell densities that can be reached in bioreactor cultures by the yeast. Indeed, >10 g/L of product titers have been documented in P. pastoris for certain proteins. Methanol-limited fed-batch (MLFB) has been one the most extended strategies for the protein production in P. pastoris. Unlike MLFB, non-limited cultures require a complex set-up for the control of cultivation parameters, representing technical and economic constraints. The methanol-limited strategy can be divided in three major phases. The first two (glycerol batch and fed-batch) have as a clear objective the fast increase of cell biomass. The process was not only useful in terms of productivity, but also allowed to decrease dramatically the consumption of pure O2 and improved notably the stability and reproducibility of the cultures. This novel strategy was then used to compare the bioreactor productivity of 2 different strains that showed a marked differences in specific productivity at the 10 mL-scale of the clone screening. Performing in vitro and in vivo studies often require high amounts of the protein to be tested, ranging usually from milligrams to a few grams. Finally, methods were defined for the characterization of the purified enzyme, with the aim to describe its properties when produced in a wild type P. pastoris strain (i.e. NRRLY-11430).

Key words: P.pastoris, Recombinant human α -Glucocerebrosidase (rhGC), Methanol-limited fed batch (MLFB), Bioreactor, NRRLY-11430

INTRODUCTION

The creation of a bioreactor procedure that is scalable for producing recombinant human α-glucocerebrosidase (rhGC) in P. pastoris with the goal of establishing a dependable method to yield substantial quantities of the enzyme ahmad et.al. Appl. Microbiol. Biotechnol. 2014; 98: 5301-

5317. The remarkable cell densities that P. pastoris can achieve in bioreactor cultures is one of the key benefits of using it to produce protein. In literature, more than ten g/Lof protein product titres were reported by Mellitzer et al., 2015; .and MLFB has been the quite successful approach in P. pastoris for protein synthesis. Non-limited cultures, in contrast to MLFB, have technical and financial limitations due to the need for a complicated setup for the management of cultivation conditions asalkar et.al J. Pharm. Innov. 12(1): 124-130.

There are three main stages to the methanol-limited approach. The rapid growth in cell biomass is the obvious goal of the first two (fed-batch and glycerol batch). Glycerol is utilised in place of methanol since it is a less favoured alternative. This substrate is known to have a greater biomass /substrate yield as a result culture resources concentrate on cell growth instead of protein expression. Methanol replaces glycerol when a significant 40-45 g/L DCW cell concentration is attained, which starts the expression of the protein. By using this novel approach, considerable volumes of enzyme were produced, which made it possible for the development devappa et.al Int. J. Adv. Res 4(4): 1468-1474 fuller et.al Mycologia. 77(3): 424-432.

MATERIALS AND PROCEDURES

The following strains were selected for cultivations:

Clone Name	Expression	Promoter sequence
GLAwt_5.1	rhGC alone	AOX
GLAwt_5.1[HAC1AOX]	rhGC and HAC1p	AOX

Getting ready for pre-inoculums:

cell growth and media culture Formulation of bioreactor media and composition of additives: All fed-batch bioreactor cultivations employed a fermentation basal salts medium (85% phosphoric acid - 26.7 mL, calcium sulphate-0.93 g, potassium sulphate-18.2 g/L, and magnesium sulphate-14.9 g/L, potassium hydroxide, glycerol-40 g/L, 4.35 milliliters/L PTM1 trace salts solution which is a composition of 6 g/L cupric sulphate, 5 litres of water, 0.08 g/L sodium iodide, 36 g/L manganese sulphate, 0.26 g/L sodium molybdate, 0.026 g/L boric acid in 2 litres of water,

Equipment for bioreactor and parameters for AlphaGC Production :

A 5 L bioreactor made of glass and can be autoclavable fitted with ez-Control was utilised for the cultivations, and an IMTECH methanol sensor system was employed to track the methanol content during the induction stage. Two automatic burettes were used for feeding methods.
A fed-batch culture technique limited by methanol. The process was run at 25°C with 30% ammonia to maintain a pH of 6. O2 was kept at 20% of saturation ambient pressure by cascade agitation (800-1100 rpm) and setting the 1 vvm flow rate. An increase in the Do value, which showed depletion of glycerol (40g/L), signaled at batch phase end.
Fed Batch cultivation technique with O2-limitation determination of biomass. In pre-weighed tubes, 3 one mL broth samples were centrifuged at 16000rpm.

α -Glucocerebrosidase activity test

GC production using a methanol-limited induction technique :

For the bioreactor production of rhGC Clone GC wt_5.1 was chosen as a model strain. Under the control of the AOX promoter it produced the enzyme. Glycerol batch phase came to a conclusion following a 24-hour culture at 24.4 g L-1 DCW. Cell concentration increased to 47.25 grams L-1 DCW over the 5 hr glycerol fed batch haveri et.al Phytochem. 8(4): 510-513. In conclusion, the fed

batch phase of methanol was extended for 137 hours, partial bioreactor draining was done after 73 hours. At conclusion of the fermentation, the highest possible quantities of cells and rhGC (155.8 g) were attained.

Oxygen-limited induction technique for GC production:

An innovative O2-limited strategy (Berdichevsky et al., 2011) was investigated as a substitute for the methanol-limited strategy. Despite the fact that other comparable methods based on O2 limitation were documented, the simplicity, usability, and apparent scalability of this technology made it appealing. The clone GCwt_5.1 was utilised once more to ensure a proper comparison of the results obtained from the two techniques (OLFB vs. MLFB). The concentration of cells stayed relatively constant Machenahalli et.al . J. Mycopathol. Res. 50(2): 273-277. Maheshwari,et.al Mc Kinney et.al J. Agric.Res.26: 195-218

Steady (varying between 77.25 and 69.25 g L-1 DCW) over the duration of the induction phase. Conversely, during the first 115.5 hours of induction, the product concentration rose linearly and reached a plateau that lasted for the final 54 hours. Significant operational benefits were obtained from the application of the O2-limited strategy, including significantly lower cooling and stirring speeds, decreased methanol consumption Mishra et.al. Mater. Sci. Eng. A: 812: 825.

Evaluating and contrasting O2- and MetOH-limited approaches:

Both techniques were thoroughly analysed using data from the O2- and methanol-limited fermentations. A trustworthy performance comparison was made possible by using the same clone, medium formulation, and bioreactor configuration Naik, G et.al J. Chem Stud. 5(5): 1210-2121. In all the metrics that directly affect process productivity, the O2-limited induction technique performed noticeably better than the methanol-limited one. In particular, it was predicted that overall production was 1.7 times higher than total methanol consumption.

In addition to the previously mentioned performance parameter improvements, the O2-limited induction approach was found to have a number of other benefits over the MetOH limited strategy: With all the benefits experienced and discussed above, Bioingenium adopted the O2-limited induction strategy as standard procedure for producing a large number of high productive recombinant proteins in P. pastoris.

GLA production in conjunction with HAC1 under O2-limited circumstances:

After determining that the O2-limited approach was the best way to produce rhGC in a bioreactor. There was a discernible relationship between the strain's performance in the fermenter and the particular productivity values found in the expression tests. As methanol is injected in pulses, the O2-limited induction profile closely resembles the traditional approach used to perform expression tests at the 10-mL or shake flask-scale Pandya, U. et.al lett. 115(3): 195-204.

Table 2 explainsO2-limited cultivation performance parameters of clones GCwt_5.1 (producing rhGC alone) and GCwt_5.1[HAC1AOX]. The entire induction period was used to calculate productivities. Green dots indicate the parameters that distinguish the clone that co-expresses rhGC+HAC1 from the clone that expresses rhGC only.

Findings:

A recently published fermentation technique for the effective synthesis of human α-GC has been successfully applied. It is based on O2-limitation during the induction phase. -O2-limited induction demonstrated significant operational benefits and higher productivity yields than the traditional methanol-limited induction approach.
The O2-limited method outlined in this chapter was used to preserve the varied productivity profiles for the two strains that were found at the 10-mL clone screening stage. Consequently, the variations in clonal production shown at the shake flask scale could be replicated at the bioreactor scale.

RESULTS AND DISCUSSION

α-Glucocerebrosidase, a human recombinant, is purified and characterised:

Following the investigation of strain enhancement for rhGC synthesis in P pastoris and the formulation of tactics for its excess production in a bioreactor, techniques for recombinant enzyme's purification and characterization were created. Every protein purification procedure can be split into two categories: chromatographic purification and early downstream (product recovery) Patel, A.et.al Microbiol. App. Sci. 9(8): 1297-1302. Scholten, O. E et.al Euphytica. 156 :345-353 .While the target protein is typically separated from the cellular fraction in the early downstream phase, its concentration is determined, and a subsequent buffer exchange is required, chromatographic techniques are typically used in the late downstream phase.
Both in vitro and in vivo experiments frequently need large concentrations of the protein under investigation, typically a few grams or milligrams.

Sample extraction from the bioreactor and preparation :

The bioreactor cultivation culture broth was centrifuged at 5000rpm for 15 minutes at 4°C. After being combined with 1L volume of storage buffer (100 mM Sodium phosphate dibasic, 0.50 mM phenylmethylsulfonyl fluoride), 1L of supernatant was kept at -20°C. Depth filtration and tangential flow filtration are two types of microfiltration procedures investigated for secondary clarification of the bioreactor harvest. Then, using an ultrafilter the filtered sample was again filtered Berlec. J. Ind. Microbiol. Biotechnol. Et.al 2013; 40: 257-274. Through this diafiltration process, the buffer was changed to 10 mM Bis-tris at 8°C and 6 pH, and the supernatant was concentrated to 250 mL. Ultimately, the chromatographic purification of untagged rhGC.

Initial Step:

Anion exchange chromatography (AIEX):

The whole bioreactor batch's worth of purification required loading the sample into an XK26/20 column. Thus, 570 mL of concentrated buffer was adjusted and cleared supernatant was transferred onto the Capto Q column at 3 ml/min flow rate after the column had been previously equilibrated with binding buffer After three CV of the same buffer were used to wash the column, by using linear gradient of elution the enzyme was eluted using 0% to 20% (200 mM NaCl) buffer in one CV, with a step at 200 mM NaCl for three CV. Ultimately, five CV of elution buffer were used to wash the column. The fractions containing enzyme activity were pooled after 10 mL of each were collected García-Ortega et.al García-Ortega. Hammerschmidt et.al Biochem.2016; 51: 325-332.

Final step: Size exclusion chromatography (SEC) or gel filtration (GF) *:*

GE Life Sciences instrument was used for third chromatographic phase. Using centrifugal ultrafiltration, the dialyzed enzyme solution from the HIC stage was reduced to 8.5mL. To put the sample into the SEC column in three separate runs, it was separated into three parts each of 2.5ml Using a single mobile phase (25mM monobasic sodium phosphate, 150mM sodium chloride at 6.0 pH) and 0.5 mL/min flow rate using isocratic mode. After gathering 2 mL of fractions, the ones exhibiting enzymatic activity were combined and concentrated to 10.5 mL using centrifugal ultrafiltration Modi S et.al Biochem. 1996; 35:4540-4550.

Quantification of proteins:

The α-Glucocerebrosidase activity test was utilised to analyse the enzymatic activity of the materials. For both partially and fully purified samples, the Bradford (ThermoFisher, #23200) test was used to calculate the total protein concentration. In 96-well plates, absorbance was measured using a Powerwave XS spectrophotometer (Biotek) at 595nm. The concentration of pure rhGC samples was determined using a quartz cuvette and UV 280nm absorbance Soliman NA et,al Acta Oceanol Sin. 2012; 31(6):117-126.

Determination of protein size using DLS:

Using a Zetasizer Nano ZS for DLS, aggregates of rhGC were detected on the purified sample. To determine the molecular size, 30 µL of the sample were added to a Low-volume quartz batch cuvette Zhu Yanbing et.al Sin. 2012; 31(6):117-126.Various amounts of Tween® 20 were added to the sample as a disaggregant agent. The chromatogram with linear gradient elution represents the entire process of AIEX capture and purification. Blue line: signal at UV 280 nm. Green line: elution buffer percentage (100 percent equals 1 M sodium chloride). GC activity is shown by red bars.

Purification of rhGC variants by galactose-affinity chromatography:

Galactose affinity chromatography was used to separate supernatants from cultures expressing rhGC, rhGCΔKDLL, rhGCC-HIS, and rhGCC-RGD using the same conditions previously established for purification rhGC (GC with the natural peptide sequence). Western blot verified the presence of rhGC in the eluted fractions, which displayed a high degree of purity. After determination of GC activity and protein concentration in each sample, the specific activity of the different variant could then be calculated.These findings verified that when rhGCC-HIS was changed to pH 7.5 (52% of initial activity concentration), there was a noticeable drop in activity, as reported in the literature for rhGC (Ioannou et al., 2001). At pH 7 and 6.5, the activity loss was lessened (68% and 89% of the starting concentration, respectively). In relation to the filtration stage, an extra 11% was lost for samples with pH values of 6.5 and 7, which might be the result of some protein aggregation. Still, only pH 6.5 was chosen for additional process enhancement.

Table 1: Comparison between MetOH-limited and O2-limited strategies

	Induction strategy		**Fold-change (O_2-lim. vs. MetOH-lim.)**
	MetOH-limited*1	**O_2-limited*2**	
Time of induction (h)	137 (partial draining at 73 hours)	115.5	0.84 X
Broth volume (L)	4.9	4.6	0.9 X

Supernatant volume (L)	2.5	3.6	1.4 X
Cell concentration (DCW, g/L)	155.8	72.8	0.47 X
Activity concentration (AU/mL)	3135	3720	1.2 x
rhGC concentration (mg protein/L)	20.8	24.7	
Total production (mg protein)	52	88.9	1.7 X
End-point productivity (mg protein L^{-1} h^{-1})	7.8 · 10-2	0.17	2.2 X
End-point specific productivity (mg protein g X^{-1} h^{-1})	5.0 · 10-4	2.3 · 10-3	4.6 X
MetOH consumption (L)	2.2	0.85	0.4 X
Product/Substrate yield, YP/S (g protein / g MetOH)	2.4 · 10-2	0.10	4.4 X

*1 Both during partial bioreactor draining and at conclusion of cultivation, parameters are calculated with the addition of the acquired enzyme.
2 parameters were computed using the culture's maximal activity concentration as a starting point.

Table 2: Performance parameters calculated from O2-limited cultivations of clones GCwt_5.1

Parameter	**Clone GCwt_5.1***	**Clone GCwt_5.1[$HAC1_{AOX}$]***	**Fold-change (rhGC+HAC1 vs. rhGC alone)**
Time of induction (h)	115.5	102	0.9 x
Broth volume (L)	4.6	4.3	0.9 x
Supernatant volume (L)	3.6	3.4	0.9 x
Cell concentration (DCW, g/L)	72.8	70.8	~ 1 x
Activity concentration (AU/mL)	3720	12228	3.3 x
rhGC concentration (mg protein/L)	24.7	81.2	

* The moment at which the maximum activity concentration in culture was obtained was used to determine the parameters

Table 3: Total activity and percentage recovered (with respect to the loaded sample) in the AIEX capture purification step eluted by linear gradient.

Fraction	**Total AU (nmols MU/h)**	**% recovered**

Load	105302	
FT+WO	3978	4
Elution	94723	90

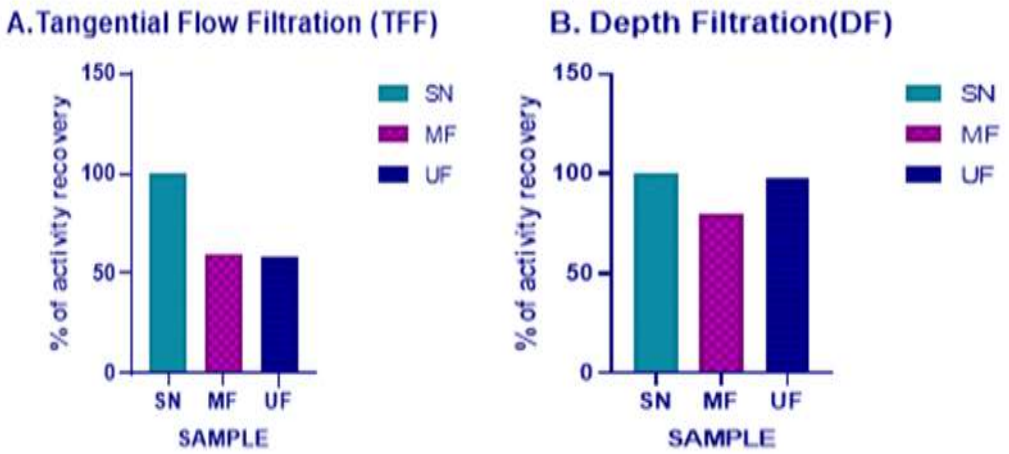

Fig 1: Recovery yield comparison between using (A) tangential flow filtration (TFF) and (B) depth filtration (DF) for the microfiltration of rhGC bioreactor supernatants.

SN: supernatant; MF: microfiltered sample; UF: ultrafiltered sample and wash-out (WO) fractions were acceptably low (4% of the loaded activity).

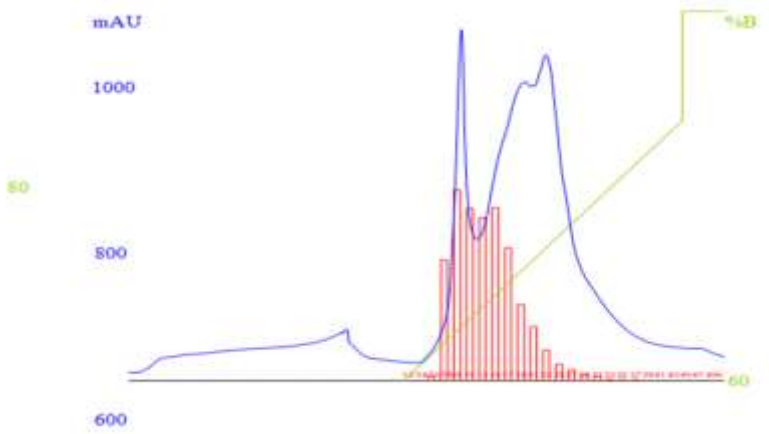

Fig 2: Chromatogram corresponding to the whole AIEX capture purification step. A linear gradient elution was performed.

Blue line: UV280nm signal; Green line: % of elution buffer (being 100% equal to 1 M NaCl); Red bars: GC activity (arbitrary units).

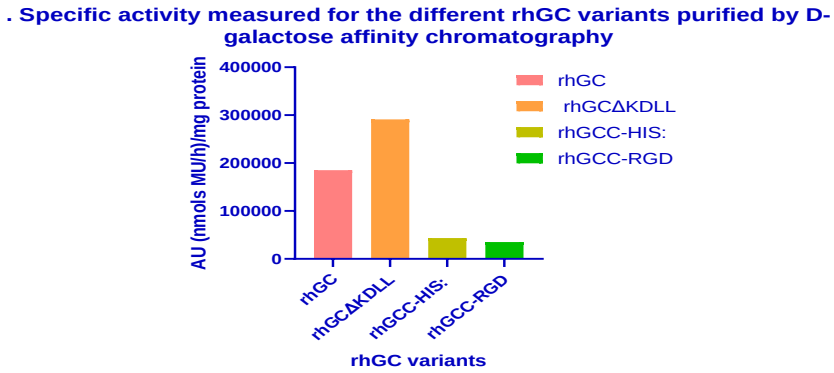

Fig 3: Chromatography column

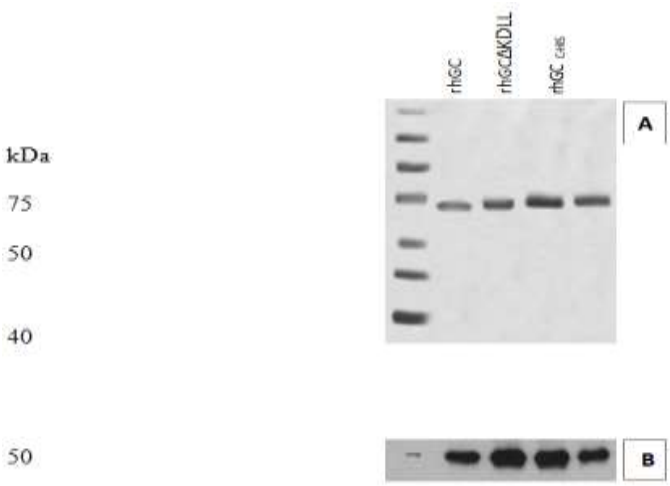

Fig 4: SDS-PAGE (silver-stained) (A) and Western-blot (B)

analyses of the rhGC variants purified by D-galactose affinity chromatography

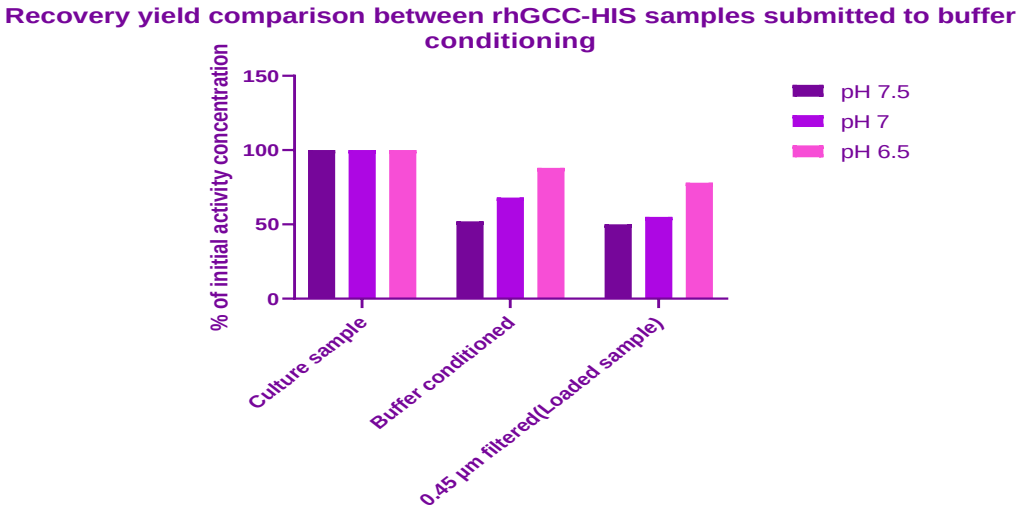

Fig 5: compares the recovery yields of rhGCC-HIS samples that were subjected to filtering (0.45 µm) and buffer conditioning (adding concentrated binding buffer at pH 6.5, 7, or 7.5).

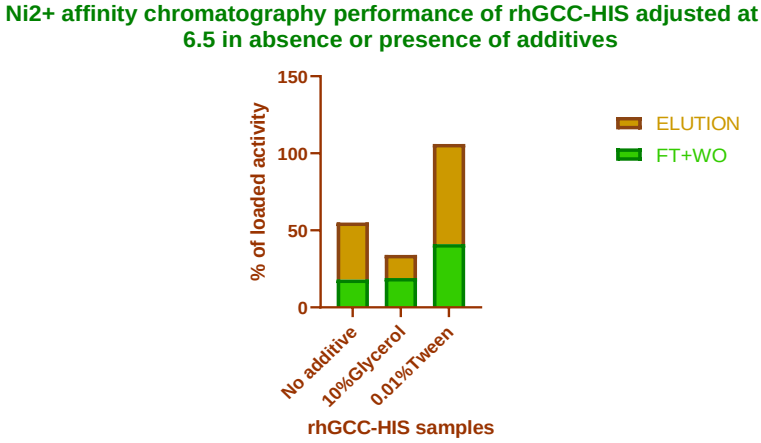

Fig 6: Ni2+ affinity chromatography performance of rhGCC-HIS adjusted at pH 6.5 in absence or presence of additives (10% glycerol or 0.01% Tween). Activity was quantified for the flow through (FT), wash-out (WO) and eluted fractions.

CONCLUSION

- A three-step chromatography system was used to establish a purification procedure for rhGC. Gaining sufficient pure enzyme using this scalable technique will eventually enable you to conduct both in vitro and in vivo testing.
- Using galactose affinity chromatography, GC variants (rhGC, rhGCΔKDLL, rhGCC-HIS, and rhGCC-RGD) were effectively separated. The quantity of enzyme recovered was sufficient for its catalytic characterisation, even with the poor yield.
- The specific activity of GC (variant rhGCΔKDLL) increases upon deletion of its four C-terminal amino acids.

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