Exploration of *Vibrio natriegens* as a host strain for high-yield heterologous production of chitinolytic enzymes

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Introduction

Heterologous production of secreted proteins in bacterial expression often gives low yields (<10 mg/L culture) and present limitations for experiments that require large protein quantities. *Vibrio natriegens* is a Gram-negative bacterium of marine origin and is a lesser-known expression host compared to *E. coli.*
V. natriegens has a faster growth rate, a higher cell density and an efficient protein secretion system. In this work, an inducer-based expression system with signal peptides for protein secretion was used with *V. natriegens* for high-yield production (>0.3 g/L) of two enzymes; a chitinase from *Serratia marcescens* (*Sm*Chi18C, aka: SmChiC) and a lytic polysaccharide monooxygenase from *Vibrio anguillarum* (*Va*LPMO10A, aka: Va-LPMO).

Growth conditions for optimal expression of recombinant protein

The first step of the project involved finding cultivation conditions for optimal protein secretion. It was observed that minor changes in media composition had major impacts on expression and secretion of the target proteins. This was especially visible for SmChiC, where analysis of the secreted protein showed that growth medium 1 gave substantially larger yield compared to the more nutrient rich growth media 2, 3 and 4 (Fig. 1; see legend for info on the media). Multiple repetitions of this experiment (with minor variation) all showed the same trend.

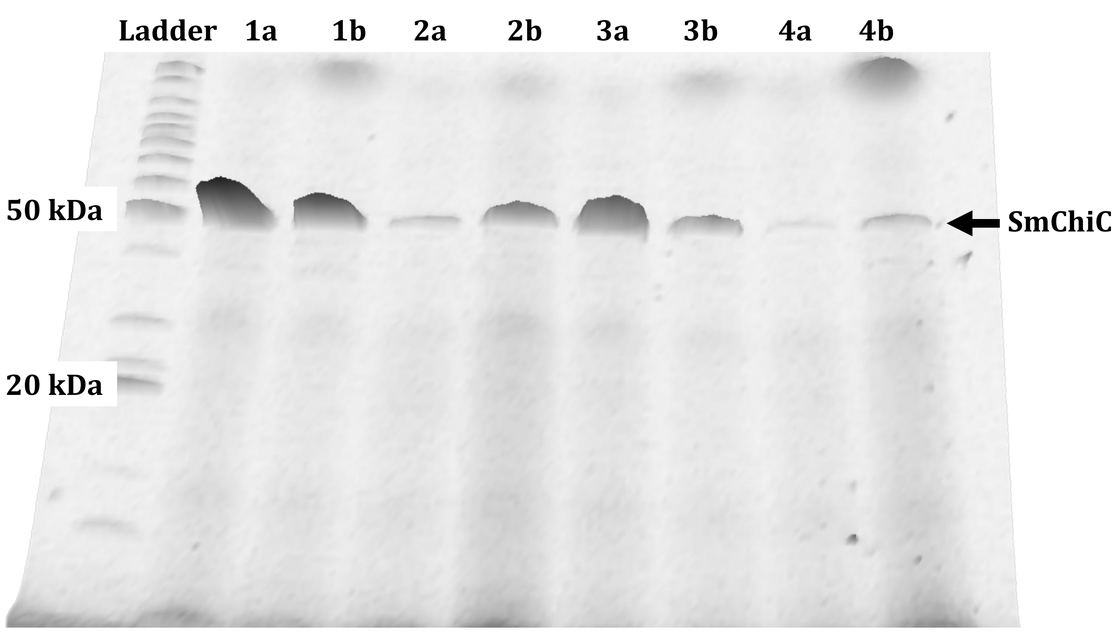

Figure 1. SDS-PAGE of secreted SmChiC from eight separate cultures, the image is shown in a 3-dimensional aspect. *The numbers 1-4 correspond to four increasingly nutrient rich growth media, and the letter 'a' represents a culture induced at OD = ~0.5, while 'b' represents a culture induced at OD >2.0. All cultures were grown at 30 °C in the LEX-48 Bioreactor (Harbinger Biotech) and induced with 1 mM IPTG.* ***Growth medium 1:*** *16 g/L tryptone, 10 g/L yeast extract and 10 g/L NaCl.* ***Growth medium 2*** *= 16 g/L tryptone, 10 g/L yeast extract and 30 g/L NaCl.* ***Growth medium 3*** *= 16 g/L tryptone, 10 g/L yeast extract and V2 salts (V2 salts per liter: 204.0 mM NaCl, 4.2 mM KCl and 23.14 mM $MgCl_2$).* ***Growth medium 4*** *= 32 g/L tryptone, 20 g/L yeast extract, 17 g/L NaCl, 2.0 g/L glucose, 17.6 mM $NaH_2PO_4 \cdot 7H_2O$, medium pH adjusted to 7.4.*

Protein concentration and purification

Since the *V. natriegens* protein secretion system is highly efficient and because the bacterium secretes few other proteins, a simple protein purification strategy was applied, using high volume desalting columns to remove medium components from the protein of interest. First, the culture medium, rich with either SmChiC or Va-LPMO, was centrifuged to remove the bacterial cells. The resulting supernatant was filtered through 0.80 and 0.45 µm pore size filters using a vacuum pump system before concentrating the filtrate to ~1/10 of its original volume using a peristaltic pump system and a Vivaflow® 30 kDa Hydrosart® membrane filter cassette (Sartorius Stedim Biotech GmbH, Cat. No.: VF20H2). Media components from the resulting concentrate were removed using HiPrep 26/10 Desalting columns (Cytiva, cat. No.: 17508701), yielding high purity of SmChiC (Fig. 2). Using the Bradford method for protein quantification we estimate a yield of ~500 mg of SmChiC and ~300 mg of Va-LPMO from each liter of supernatant. It should be noted that for SmChiC, approximately 50% of the expressed protein remained in the cell (non-secreted).

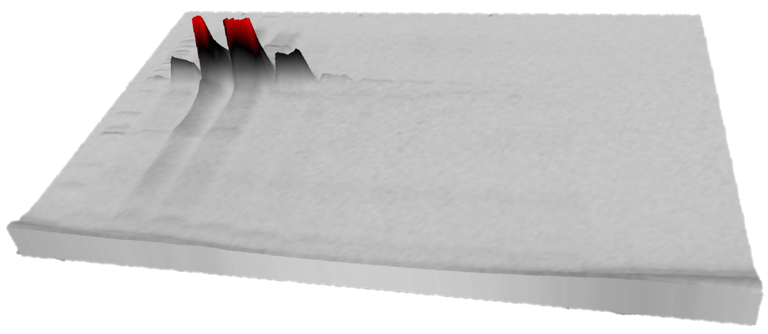

Figure 3. SDS-PAGE of purified SmChiC, the image is represented in a 3-dimensional aspect. *Each lane on the gel represents 2.5 µL sample buffer + 2.5 µL from a 7.0 mL fraction of desalted protein from concentrated supernatant. Gel load order: Ladder, fractions: 1, 3, 5, 7, 10, 14, 18, 23.*

Activity assays and optimal storage of purified protein

After purification, enzyme activity was verified using relevant enzyme assays. Activity assays were used to monitor protein activity following storage for six weeks at 12 °C, which established that SmChiC can be stored for long periods with little loss of activity (Fig. 3). To find long term storage options, SmChiC samples were frozen at -20, and -80 °C, snap-frozen in ethanol/dry ice or freeze-dried and resuspended with sterile water. The activity of these samples was, at worst, marginally lower compared with SmChiC stored at 4 °C (Fig. 4).
Va-LPMO showed lower stability than SmChiC, as storage at 4 °C resulted in visible protein degradation within weeks (results not shown). Experiments on the storage of Va-LPMO at -80 °C exhibited minimal evidence of protein degradation and demonstrated that the enzymatic activity was preserved after thawing (results not shown), but the extent of the activity loss has not yet been established.

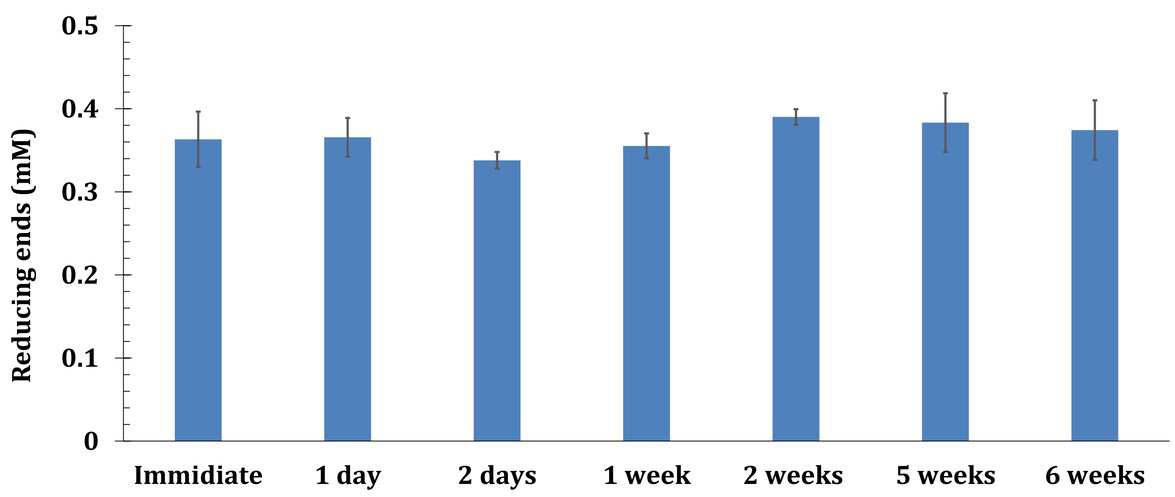

Figure 3. Activity assay with SmChiC stored at 12 °C for six weeks. *Assay reaction conditions: 500 µL seawater with 20 g/L α-chitin, 5 µM SmChiC per reaction, incubation for 1 hour at 12 °C, 800 RPM shaking in a ThermoMixer. Data for graph is based on triplicates. Product formation was quantified by the MBTH reducing end assay* (Jarle Horn & Eijsink, 2004).

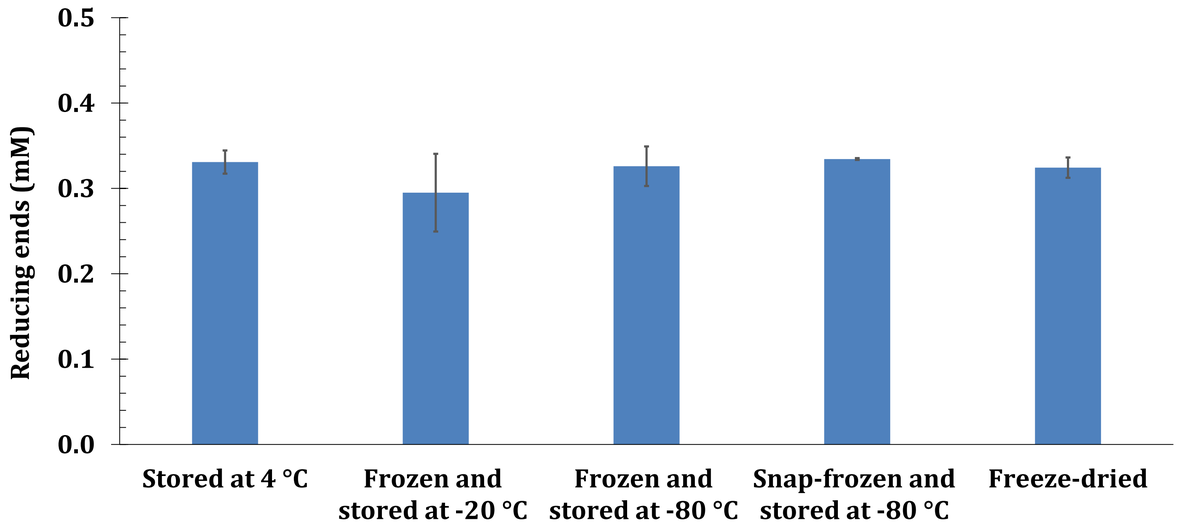

Figure 4. Activity assay results using SmChiC stored in selected conditions. *Assay reaction conditions: 500 µL seawater with 20 g/L α-chitin, 3 µM SmChiC per reaction, incubation for 1 hour at 12 °C, 800 RPM shaking in a ThermoMixer. Experiments were performed in duplicates or more. Product formation was quantified by the MBTH reducing end assay* (Jarle Horn & Eijsink, 2004).

Conclusions

- *V. natriegens* can be an excellent host strain for heterologous protein production.
- High protein yields are dependent on optimization of culture conditions and determination of the most suitable medium composition.
- The target proteins can be secreted in large amounts. In the case of SmChiC, a substantial amount of protein can be extracted from the cell fraction. The cells can be stored at -20 °C for months before purification at a suitable time.
- SmChiC is robust and maintains high enzymatic activity largely independent of storage conditions, including lyophilization.
- Assays with Va-LPMO stored at -80 °C shows high degree of residual activity. However, due to the complex nature of LPMOs, additional experimentation is needed for verification.

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