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white paper

Heterologous production of holoproteins in *Hansenula polymorpha*

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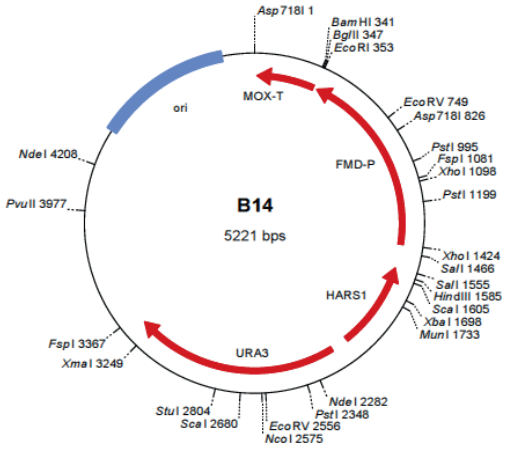

Fig. 1: ARTES´antibiotic resistance gene free vector backbone B14

Features of ARTES´ *Hansenula polymorpha* protein production platform

Hansenula polymorpha is a ubiquitous yeast species present in soil, water and on fruits. It is non-pathogenic to animals and humans, does not produce toxins and is an established platform for production of recombinant proteins. Examples of products marketed and produced in ARTES´ *Hansenula* expression platform are diversified in application. Clients products include e.g. HEPLISAV B by Dynavax, Germany and USA, HEPAVAX-Gene by Berna Biotech Korea Corp., Genevac-B by Serum Institute of India and the HBsAg component of multivalent vaccines (e.g. HEXYON resp HEXACIMA, Sanofi Pasteur, France), Insulin (Wockhardt Ltd., India), Interferon alpha2a and Hirudin (Minapharm Biogenetics, Egypt).

Hansenula host cell line RB11 and its derivatives are designed with one to three auxotrophic marker (ura-, ade-, leu-;) allow for multi-step targeted genetic engineering to improve productivity (e.g. by increase of integrated expression vector copies) and/or product secretion (e.g. by chaperone engineering). As a result, recombinant products localized intra- or extra-cellular as soluble, active proteins are produced in the *Hansenula* platform at high titres of up to 25 g/l.

For the manufacturing of feed/food products, ARTES has developed a host/vector toolbox termed “B14”. B14 is free of any antibiotic resistance genes and the fermentation processes are free as well of antibiotics and of animal derived components. These features and several safety studies provide our customers the basis for large scale manufacturing of enzymes applied in the feed and food industry (e.g. Hexose oxidase and Lipase, Danisco/ DuPont; Fig. 2). As of today, *H. polymorpha* derived products have been registered in more than 60 countries worldwide, including FDA approval, and have been recommended by the WHO for mass vaccination programs.

Fig. 2: Grindamyl bakery enzymes; source: additive-concepts.ch

Holoproteins

A class of commercially important products are expressed as apoproteins, which require a co-factor or a prosthetic group to form a stable and active holoprotein complex.

Examples of heterologous holoproteins expressed in yeast by recombinant DNA technology have been reported earlier for *Saccharomyces cerevisiae* (1), *Pichia pastoris* (2) and *Hansenula polymorpha* (3).

Here we report the high-level intracellular expression of different holoproteins carrying either metal ions as co-factor or organic compounds as prosthetic group.

Production of holoproteins containing a co-factor in *Hansenula polymorpha*

The iron storage protein ferritin is a member of the non-heme iron protein family. It can store and release iron, therefore it prevents the cell from damage caused by iron-dioxygen reactions as well as it provides iron for biological processing. To study whether the human ferritin heavy chain (FTH1) can be expressed in *Hansenula polymorpha*, we integrated an expression cassette for FTH1 and analyzed the protein expression. We found very efficient expression of FTH1 and obtained yields up to 1.9 g/L under non-optimized conditions (4).

Similar results were obtained for a FTH1-para-thyroid hormone fusion protein to successfully express the parathyroid hormone fragment 1–34 (PTH) for the first time intracellular in *H. polymorpha*. ARTES ferritin ´platform is protected by international patents (EP 2 484 766).

Production of holoproteins containing a prosthetic group in *Hansenula polymorpha*

Holoproteins carrying a prosthetic group include a.o. carboxylase enzymes (biotin) and catalases and globins (both with heme). A previous example of the expression of heme containing catalase in *Hansenula* for whole-cell catalysed bio-transformations was reported by Gellissen et al. 1996. Examples for the recombinant expression of heme containing globins in Saccharomyces were reported for hemoglobin (1) and for myoglobin as holoprotein at 1% of total cellprotein (5). Soy leghemoglobin is manufactured in Pichia pastoris to enrich ethical food with meaty flavour (6).

We have applied the *Hansenula polymorpha* protein production platform for expression of different types of globin proteins. After optimization of the codon usage and of other structural elements of the recombinant gene relevant for improved transcription and translation, the newly synthesized genes were cloned into ARTES´ standard expression vectors with (pFPMT121) or without (B14) ampicillin resistance marker. Expression was placed under control of a strong inducible promoter (FMD or MOX), respectively a constitutive promoter (TPS, TEF). Following transformation of episomal globin expression vectors, the transformants were subjected to a series of cultivation steps under selective and non-selective conditions to enforce stable multi-copy integration into the host genome (7).

In each case, high levels of soluble intra-cellular product could be determined upon cultivation with or without methanol induction. With more than 10% of total cell protein the target protein was one of the most abundant proteins present in extracts. Host cell derived proteins could be largely removed in a single Ni-NTA chromatography step from His-tagged variants of the individual globins.

Binding of the prosthetic group heme to the globin apoprotein was first indicated by a red-brownish color of the Ni-NTA eluate fraction protein. For both tagged and untagged globins, it was also confirmed by measurement with a Heme Assay Kit that the majority of the expressed product did incorporate heme.

Summary and outlook

For all examples described above, we have successfully transferred the processes to pilot scale fermentation in animal-free media and without methanol induction, showing production of biologically active and authentic holoproteins with either a metal ion co-factor or the required prosthetic group heme incoprorated. This indicates the native potential of *Hansenula polymorpha* to provide co-factors and the prosthetic group heme in amounts sufficient for large scale and high level production of respective holoproteins.

ARTES will expand its internal research to evaluate the potential of the *Hansenula* platform to produce other holoproteins, addressing especially the growing demand in the emerging field of ethical food production.

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