artes white paper

Efficient Production of Secreted and Active Invertase

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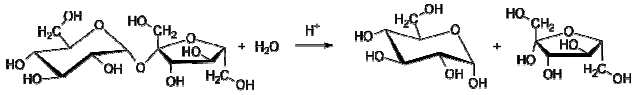
In our today's society, sugar plays an eminent roll. In industrialized countries, it serves as energy provider and it is covering 20% of the total needed energy of the population. 2015/2016 the total annual need of sugar was 37.8 kg per person in Europe. Most of the industrially produced sugar is gained for that matter from sucrose, a disaccharide that is found in many plants and their parts. Industrial relevant as sucrose producers are beet and cane. In 2013 worldwide 175 million metric tons had been produced.

Sucrose is the raw material for an enzymatic production of the monosaccharides glucose and fructose, a process for which the enzyme **Invertase** is needed.

If sucrose is converted to its monosaccharides (Figure 1) the direction of plain polarized light rotation changes from right to left - it becomes „inverted". This so-called invert sugar has many applications in the life science and food industry. It is used for example in the manufacturing of candies and preserves, in the production of lactic acid or in the production of ethanol. The inversion provides sweet syrups, which less likely crystallize than the pure sucrose syrups and thus are more stable what is needed for the further processing.

Industrially, many types of inverted sugar syrups are produced by the use of the *SUC2* encoded yeast enzyme Invertase (EC 3.2.1.26; ß-fructofuranosidase).



Sucrose **Glucose** **Fructose**

Figure 1. The Invertase catalyzed reaction. The disaccharide sucrose (left) is hydrolyzed into its monosaccharides glucose and fructose (right). The rotation direction of plain polarized light is thereby shifted from right to left (graphic adopted from https://en.wikipedia.org/wiki/Inverted_sugar_syrup with modifications).

Recombinant production as a source of Invertase for industrial use

How to produce Invertase as stable enzyme in a robust, reliable and commercially affordable process? One way is for sure to isolate the enzyme from its natural host *Saccharomyces cerevisiae*.

In *S. cerevisiae*, the Invertase is a large, glycosylated secretory protein of 532 amino acids, including a 19 amino acids N-terminal leader

peptide cleaved off during ER uptake and secretion. The unmodified mature protein has a theoretical molecular weight of 58.63 kDa and contains 14 potential N-glycosylation sites. In the active form the enzyme is highly glycosylated and dimeric.

For commercial and industrial use of Invertase, it is much easier to produce the enzyme in a recombinant process than to isolate it from the originator organism. The production of recombinant protein in a quality certified process ensures that the enzyme can be produced in feasible amounts and in reproducible quality. This is the reason why there have been numerous approaches to produce Invertase recombinant in a variety of expression systems like yeasts (others than *S. cerevisiae*), filamentous fungi and bacteria. In many cases, the recombinant expression host cells have been immobilized.

Highly active Invertase from *Hansenula polymorpha*

ARTES Biotechnology´s expression system - the methylotrophic yeast *Hansenula polymorpha* - was used for recombinant Invertase production. Genomic analytics confirmed that the proteom of wild type *H. polymorpha* does not contain an equivalent to the secretory Invertase of *S. cerevisiae*. For our studies, we used host strain RB11, which is well acknowledged for its robustness, the high mitotic stability of the introduced expression cassette, but also for its ability of high-level secretory expression of target proteins.

High-copy integrated expression cassettes

To obtain high transcript level, we have placed a codon optimized *SUC2* ORF under the control of the strong carbon-source regulated *FMD* promoter derived from the formate dehydrogenase gene of *H. polymorpha*. Termination of transcription is provided by the *MOX* terminator derived from the methanol oxidase gene. Expression cassettes are integrated as multiple (approx. 40 per cell) head-to-tail copies into the *Hansenula* genome – resulting in genomically stable production cell lines.

Selection of production cell lines secreting active Invertase

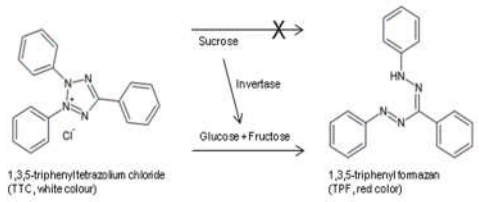
In the given example, we selected genomic integrants with highest expression level by plate overlay assay and in batch cultivation within approx. 4 weeks.

To confirm activity of the Invertase expressed by different selected strains we performed a

qualitative assay based on the reduction of tetrazolium salt 1,3,5-triphenyl tetrazolium chloride (TTC), a soluble colorless substance. In presence of a reducing agent it converts into 1,3,5-triphenyl formazan (TPF) which is an insoluble red-coloured substance. In a mixture of sucrose together with TTC and the active Invertase enzyme a conversion starts. The non-reducing sucrose will be cleaved into the reducing monosaccharides glucose and fructose and these start the reduction of TTC (colourless) to TPF (red) (Figure 2A).

The cell spots of the Invertase negative transformants and the negative control strain remained pale in this assay while the spots of the Invertase positive transformants became intensively red (Figure 2B). This indicates qualitatively active Invertase produced by the production cell line candidates tested.

A



Ogur M. et al, 1957

B

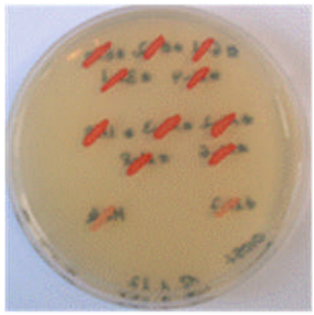


Figure 2. TTC reaction.

A) Principle. The soluble colourless TTC reacts in presence of a reducing agent towards TPF, a red substance, which is insoluble. Reducing sugars are good electron donors and thus support the reaction.

B) TTC assay. As test strains five strong strains (upper five dots), five weak strains (middle five dots) one negative strain of the Invertase series (lower right dot), and a negative control strain (lower left dot, respectively) have been grown for 16 h on a YP agar plate containing 2 % sucrose as a carbon source. A TTC overlay mixture (1,5% agar; 0,067M phosphate buffer pH7) has been generated by autoclaving. This solution was cooled down to 50 °C and 2,4,5-triphenyltetrazolium chloride (TTC) (0,05% (w/v)) was added. The plate has been overlayed with 10 ml of this solution and incubated for 20 min at 37 °C.

Productivity of secreted Invertase

A selected production cell line was cultivated at 3 ml test tubes scale to determine the concentration of Invertase secreted into the medium. Invertase concentration was determined by two

independent methods, SDS-PAGE/Coomassie Blue stain and Bradford assay. Figure 3 shows a protein gel loaded with serial dilutions of crude culture supernatant of Invertase production cell line # 11-2 (lanes 12 - 20) alongside with serial dilutions of two standard proteins BSA and Interferon α2a (lanes 2 - 6 and 7 - 11). Prior to analysis, the Invertase has been deglycosylated with Endoglycosidase H (Endo H) to focus the material in one band and to allow for a more precise quantification by consistent Coomassie staining.
Based on the Coomassie gel analysis the estimated Invertase concentration is about 200 mg/l at a cell density of an approx. O.D.600nm of 30 obtained in the 3 ml batch culture.

This result was confirmed by total protein analysis using the Bradford method. It is noteworthy, that according to the SDS-PAA gel, Invertase is the major protein in the culture supernatant with an estimated purity of approx. 95%, facilitating purification substantially.

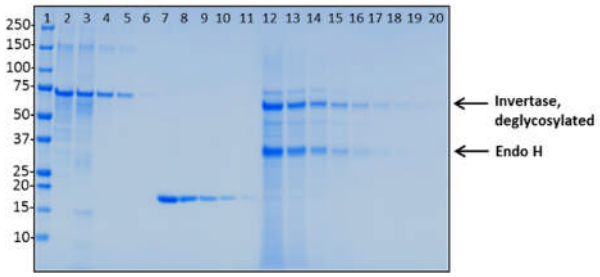


Figure 3. Quantification of Invertase by serial dilutions of culture supernatant. Strain # 11-2 has been cultivated as 3 ml batch culture under standard expression conditions. Resulting culture supernatant has been treated with Endo H to remove the glycan structures. The indicated samples have been separated on a 4 - 12 % Bis-Tris protein gel, staining has been performed with Coomassie Blue. The concentrations of the BSA and IFNα-2a standard proteins have been determined according to Bradford. Scheme of loading:

1	Precision Plus Standard
2 - 6	BSA standard 2/1/0,5/0,25/0,125 µg
7 - 11	IFNα-2a standard 2/1/0,5/0,25/0,125 µg
12 - 19	RB11/pFPMT-H1-SUC2 # 11-2 11,2/5,6/2,8/1,4/0,7/0,35/ 0,175/0,088 µl sample volume

For the determination of volumetric activity of the enzyme, we used an Invertase assay kit of Sigma-Aldrich (catalog Number MAK118). The assay is based on the detection of glucose released from sucrose by the action of Invertase. One unit of enzyme activity is defined as the amount of enzyme that will catalyze the formation of 1 µmol of glucose from sucrose per minute at pH 4,5 under assay conditions. Culture supernatant of production cell line # 11-2 was diluted (1:20.000, 1:80.000, 1:320.000, 1:1.280.000, and 1: 2.560.000) and subjected to the assay.
The mean value of the 1:80.000 diluted sample was used for calculating the volumetric activity at 270.000 U/l in batch culture (normalized to undiluted crude culture supernatant of production cell line # 11-2).

Rodriguez et al. (1996) reported a specific activity for crude Invertase present in the culture supernatant of *H. polymorpha* of 1.500 U/g and 2.700 U/g for the respective purified enzyme. Based on the specific activity for crude Invertase reported, we calculate approx. 180 mg/l of active Invertase in batch culture supernatant – a value well in line with the data derived from SDS-PAGE and Bradford analyses.

Invertase production in *Hansenula*: benchmarking against data reported in the literature

Invertase production from *H. polymorpha* under control of the *Pichia AOX1* promoter is reported at approx. 1 g of enzyme per liter culture present in the periplasm of which approx. only 1 %, i.e. approx. 10 mg/l was found in the cell-free culture supernatant (Rodriguez et al., 1996). These data were obtained in high cell density (approx. 80 g/l) fed-batch fermentation (1.5 – 50 liter) for approx. 90 – 150 hours using continuous feeding of methanol.
With our current *H. polymorpha* production cell line expressing the Invertase under control of the strong homologous *FMD* promoter, we have obtained preliminary yields of approx. 200 mg/l active enzyme secreted into the cell-free culture supernatant within 72 hours batch cultivation at 3 ml scale.

Thus with our expression cell line Invertase secretion performs much more efficiently than with the cell line described by Rodriguez et al. (1996).

A comparison of volumetric productivities resp. product formation rates of Invertase secreted into the cell-free culture supernatant (SN) for the production cell line generated and for a reference reported is provided in Table 1.

	Rodriguez et al. 1996	current study
host strain	*H. polymorpha* LR9 (Ura-)	*H. polymorpha* RB11 (Ura-)
promoter	*P. pastoris AOX1*	*H.polymorpha* FMD
cultivation	50 l scale fed-batch fermentation	3 ml scale batch cultivation
volumetric productivity (SN) [mg/l]	10	200
product formation rate (SN) [mg/(l x hr)]	0,1	2,8

Table 1. Productivities of active Invertase secreted into the cell-free culture supernatant.

Outlook

A high cell density fermentation process will be developed based on the production cell line generated. The preliminary productivity reported here for a simple small scale batch cultivation allows for forecasting a productivity for Invertase at large scale routine manufacturing in the high g/l range as obtained a. o. for phytase (Mayer et al., 1999).

References:

- Rodriguez et al (1996) Invertase secretion in *Hansenula polymorpha* under the AOX1 promoter from *Pichia pastoris*. Yeast 12(9), 815-822.
- Mayer et al (1999) An Expression System Matures: A Highly Efficient and Cost-Effective Process for Phytase Production by Recombinant Strains of *Hansenula polymorpha*. Biotechn. and Bioeng. Vol. 63, 373-81.
- Ogur et al (1957) Tetrazolium overlay technique for population studies of respiration deficiency in yeast. Science 125(3254):928-9

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