**Aus dem Institut für Technologie und Biosystemtechnik**

**Ulrich Jahnz**
**Milada Schubert**
**Klaus-Dieter Vorlop**

# Process development for production of DFA from inulin on an industrial scale : screening, genetic engineering and immobilisation

Manuskript, zu finden in www.fal.de

Published in: Landbauforschung Völkenrode Sonderheft 241, pp. 113-119

**Braunschweig**
**Bundesforschungsanstalt für Landwirtschaft (FAL)**
**2002**

# Process development for production of DFA from inulin on an industrial scale: Screening, genetic engineering and immobilisation

Ulrich Jahnz, Milada Schubert and Klaus-Dieter Vorlop[1]

**Abstract**

The bioconversion of renewable resources for industrial applications increasingly often demands the availability of suitable technologies for the usage of enzymes. This paper presents results obtained from the development of a process designed to convert inulin into difructose anhydrid (DFA III).

In a broad screening programme, strain Buo141 was isolated which expresses a thermo stable enzyme carrying out the desired conversion. To increase the formation of enzymes the inulase gene was cloned into *E. coli* XL1-blue, inulase II was expressed and its activity was detected. After optimising the enzyme with genetic engineering techniques, the GMO was fermented and an activity of 1.76 Mio U/L was observed.

To allow permanent immobilisation the enzyme was flocculated from cell-free extract by co-cross-linking with chitosan and glutardialdehyde. Subsequently the enzyme was entrapped in calcium alginate hydrogels. To enable the production of uniform and small bead shaped particles, novel JetCutter technology was used with a production rate of 5600 beads/(s·nozzle). The influence of bead diameter on the activity was investigated and an activity of 196 U/g was measured for 600 µm beads. The figures obtained from the experiments were in wide consistency with theoretical data.

*Keywords: inulin, DFA, screening, thermotolerant enzyme, genetic engineering, immobilization, JetCutter*

## 1 Introduction

### *1.1 Bioconversions for industrial raw materials*

The growing awareness of limited fossil resources and environmental problems connected to carbon dioxide have caused an increased interest in renewable resources. For many applications, crop-based products have to be refined first. Refining steps can either be chemical or use whole cells or isolated enzymes. Before establishing a process to convert agricultural products for non food applications, proper cells or enzymes have to be found and optimised for optimal operation. This paper shows the schedule of such a development for the conversion of inulin into a disaccharide called DFA III.

### *1.2 Origin and usage of inulin*

Inulin is a linear β-2,1-linked polyfructan terminated with a glucose residue. Large amounts of inulin are contained as reserve carbohydrates in the roots and tubers of such crops as chicory and Jerusalem artichoke. The degree of polymerisation depends on the originating plant and is usually in the range from nine to some thirty fructose units. Inulin has various applications in food and non-food areas.

Carboxylated inulins act as tensides and can be used as replacements for polyacrylates in washing agents or can be further functionalised with sulfonic groups and employed as chelating agents. Ether-products of long-chained inulins with epoxides can be used as plasticisers for thermoplasts or in textile industries. Further chemical products from inulin like furandialdehyde or furandicarbonic acid are accessible via the intermediate 5-hydroxymethylfurfural.

Numerous works deal with the bioconversion of inulin to obtain products for the non-food-market. Possible fermentation products are butanol (*Clostridium pasteurianum*), acetone (*C. acetobutyricum*), and 2,3-butanediol (*Bacillus polymyxa*) (Oiwa et al., 1987). Ethanol for fuel purposes can be produced by *Klyuveromyces marxianus* without the need for prior hydrolysing of the inulin chains (Bajpai and Bajpai, 1989).

The uses of inulins in human nutrition have long been known, and they have, since the 19th century, for instance, been used in coffee-surrogates. Fructooligosaccharides are added to various food products, mainly in the dairy area as dietary fibres in probiotics. As an alternative to high-fructose corn syrups (HFCS) high-fructose syrups can be gained from either chemical or enzymatic hydrolysis of inulins or inulin-containing crops like the Mexican agave and their juices are fermented directly to yield alcoholic drinks like tequila.

However, inulins have a very limited market thus far in both the food and the non-food-area. This is

[1] Ulrich Jahnz, Milada Schubert and Klaus-Dieter Vorlop, Institute of Technology and Biosystems Engineering, Federal Agricultural Research Centre (FAL), Bundesallee 50, 38116 Braunschweig, Germany

mainly due to the high cost connected with expensive separation and purification steps. For 1.5 to 2 €/kg it is about four times as costly as competing glucose, starch or sucrose. This also explains why short oligofructoses for probiotic products are synthesized enzymatically from sucrose rather than obtained by partial hydrolysis of inulins.

Future use of inulin-derived products is thus either in high-value markets like the functional food segment or by converting inulin into intermediates which can be separated and purified at lower costs.

### *1.3 DFA III*

One promising compound derived from inulin for this purpose is di-D-fructofuranose-1,2':2,3'-dianhydride (DFA III). The formation of DFA III is catalysed by the enzyme inulase II as a kind of an intramolecular transfructosylation (Tanaka et al., 1975). DFA III can be used as a substitute for sucrose in human nutrition. It has half the sweetness of sucrose and thus gives a comparable volume when used as a food-additive. Since it is not metabolised by the human body it is clearly reduced in calories. Like the fibre inulin itself, DFA III has a positive effect on the intestinal microbial flora and was shown to enhance the uptake of calcium (Suzuki et al., 1998).

On the other hand, DFA III can be the basis for plastics and tensides. After ion exchange it can be crystallised as easily as sucrose and hence it can be produced at a price well below that of inulin. So far DFA III has not been introduced onto the market since no efficient enzyme and biotechnical process was available for the necessary bioconversion of inulin.

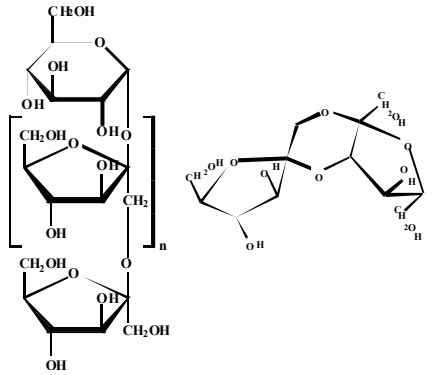

Figure 1:
Chemical structure of inulin (left) and the enzymatic conversion product DFA III (right)

### *1.4 Catalysts in continuous processes*

To establish a large scale process based on a biochemical reaction it is preferable to have means available to hold back the catalyst in the bioreaction vessel. By immobilising catalysts like growing, resting or dead cells or enzymes it is possible to retard them. Besides the advantage of easy retention, immobilised catalysts often also show an increased stability with regard, for instance, to pH-value and temperature.

As a matrix for entrapment, the biopolymer alginate can be chosen. Sodium alginate is mixed with the catalyst solution and then solidified by dripping into a solution of calcium chloride. The resulting particles are bead-shaped and the biocatalyst is equally distributed throughout the volume of the bead.

However, encapsulation of catalysts also has disadvantages. Depending on the used matrix system the catalyst may be inactivated during the process of matrix formation. Even if this is not the case, the overall activity of the bead in case of calcium-alginate entrapment is less than that of the free catalyst due to diffusional limitations. To minimise this negative effect, particles have to be kept as small as possible and as is reasonable for the later application.

Continuous processes are preferably run in packed bed reactors. Since these build up significant pressure, it is important to have as stable beads as possible. The stability of the beads can be increased by using higher concentrated polymer solutions, which simultaneously makes it more difficult to fulfil the above claim for small particles.

### *1.5 Enzyme immobilisation*

Using hydrogels, the majority of enzymes used in bioconversion processes shows a too low molecular weight and is thus not suitable for direct entrapment. In natural form, the enzyme would readily diffuse from the matrix. To increase the molecular weight, several enzymes can either be linked with each other or co-crosslinked covalently with polymers. Among suitable materials, the naturally derived polymer chitosan is favourable since its amino groups provide a good reaction site for linkage to the amino groups of lysine residues of enzymes as depicted in Figure 2. The exact reaction conditions depend on the enzyme and have to be optimised specifically.

### *1.6 Aims of the work*

To isolate strains producing inulase II enzymes, a broad screening-programme was started. Of special interest were thermo-tolerant enzymes which are stable at a temperature of at least 60°C for a prolonged period of time. By cloning the corresponding *ift* gene into *E. coli* the production of this enzyme should be enhanced. To facilitate the use of the enzyme in a future industrial process, the basics for its immobilisa-

tion were investigated and the practical results compared to theoretical values.

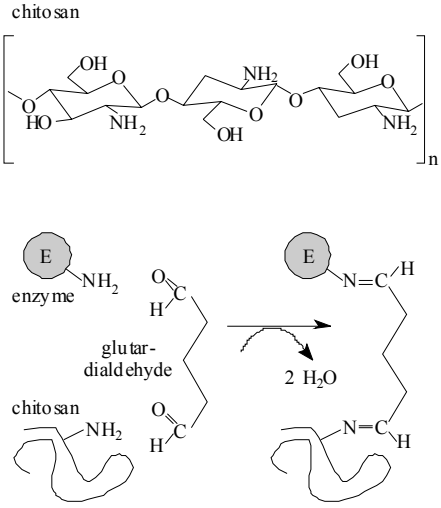


Figure 2:
Chemical structure of chitosan and reaction with glutardialdehyde and enzyme-bound amino groups, simplified scheme

## 2 Materials and methods

### *2.1 Screening for microorganisms*

For selective enrichment, a mineral salt medium with inulin from dahlia tubers as the sole source for carbon and energy was used. Samples were plated on a solidified medium and incubated at 30, 45 and 60°C under aerobic and anaerobic conditions. Grown colonies were then subcultured to liquid media and screened for formation of DFA III by HPLC analysis (column CHO611, Interaction Chromatography, San Jose; eluent 1 mM NaOH; 0.5 mL/min; RI detector). Supernatant of positive strains was tested after incubation at different elevated temperatures to determine temperature stability. DFA was verified by NMR measurement.

### *2.2 Genetic engineering*

For cloning of the *ift* gene, a genomic library was constructed from partially digested Arthrobacter genome in phage l. By phylogenetic analysis of published data, a universal *ift* gene-specific primer pair was designed and used to amplify a homologous *ift* gene-specific probe from Arthrobacter chromosomal DNA. This probe was used to screen the genomic library and a hybridizing clone, bearing an approximately 15 kBp large genomic fragment with the complete *ift* gene, was isolated. The genomic subfragments were subcloned into the plasmids pUC18 and pUC19, respectively. Based on these *ift*-subclones, expression vectors were constructed and the enzyme expressed in *E. coli* XL1-blue and its activity detected. Activity tests were done by measuring the amount of DFA III formed in 30 minutes from a 10 % (w/v) solution of inulin at 50°C.

Enzyme design was accomplished on the DNA level in two steps: The sequence for the original signal peptide was partially removed by exonuclease activity. Based on results obtained from these experiments, the entire region coding for the transfer peptide was subsequently deleted by means of specific endonucleases. Additionally a point mutation in the coding region of the *ift* gene was generated by error-prone PCR.

To obtain large quantities of the enzyme, the genetically modified organism was fermented in 10-L-scale (NLF22, Bioengineering, Wald) using a medium consisting of technical yeast (Ohly, Hamburg) extract and glycerol as carbon source. Cells were harvested and disrupted with a high pressure homogenizer (LAB60, APV Gaulin, Lübeck; 3 passages of 65 MPa; 0.5 L/min).

### *2.3 Enzyme immobilisation*

For immobilisation the enzyme was co-cross-linked. Chitosan (geniaLab, Braunschweig) was dissolved in 0.5 % (w/w) acetic acid and mixed with enzyme solution as a cell free extract. After addition of glutardialdehyde from a 50 % stock solution cross-linking was accomplished over 24 h at 4°C while stirring and afterwards 3 % sodium alginate (LF20/60, FMC Biopolymer, Drammen) was added. Small droplets of 500 to 850 µm in diameter were formed with a JetCutter (geniaLab Braunschweig; nozzle 300 µm; flow of liquid 0.9 g/sec; cutting tool 48 wires of 100 µm; rotation speed 7000 rpm) and hardened in a 2 % calcium chloride solution. To evaluate the effect of diffusional limitation, the activity of beads of different diameters was measured.

Alginate beads were dissolved for 30 min in a mixed solution of 100 mmol/L sodium citrate and 100 mmol/L sodium chloride.

## 3 Results and discussion

### *3.1 Screening and genetic optimisation*

The screening programme resulted in strain Buo141 expressing an extracellular enzyme which is stable at temperatures of 60°C. Using metabolic data and 16S-rRNA-sequencing, the strain was identified as a new Arthrobacter species, growing aerobically at ambient temperatures.

To gain access to the gene coding for inulase II (*ift* gene) primers for a PCR-reaction were needed. Suitable sequences for primers were phylogenetically reasoned from two highly divergent sequences:

- a DFA III producing inulase enzyme, in its function identical and in its phylogenetic origin closely related to our enzyme (Sakurai et al., 1997)
- a DFA I producing inulase enzyme, in its phylogenetic origin only distantly related to our enzyme (Haraguchi et al., 1995)

Using this universal *ift* gene-specific primer pair, a region of approx. 500 bp was amplified from the Arthrobacter chromosomal DNA. The complete *ift* gene was obtained by screening the genomic library with this probe. As a result a plasmid was constructed in pUC19 which expressed an enzyme of 477 amino acids when transferred to *E. coli* XL1 blue. A cell-free extract of such a culture showed an activity of 3000 U/L, whereas the majority of this activity was detected intracellularly.

For optimisation the sequence coding for a signal transfer-peptide, which is responsible for transport of the enzyme via the membrane in Arthrobacter, was identified and removed resulting in a hundred fold increased activity. A further increase in activity of approx. 35 % was achieved by a point-mutation which was induced by error-prone PCR. On position 221 of the enzyme a glycine was exchanged with arginine. A new a-helix region can be generated in the region of amino acids 216 to 224 according to the model of Garnier et al. (1978). The resulting GMO was named *E. coli pMSiftOptR*.

### *3.2 Production of enzyme*

The recombinant *E. coli* was fermented using an inexpensive technical medium. During the fermentation the inulase activity was monitored, a typical fermentation run is depicted in Figure 3. A final biomass concentration of 11 g/L (dry weight) and an overall activity of $1{,}76 \cdot 10^6$ U/L was measured.

Since high-density fermentations of *E. coli* are known to reach biomass concentration of 100 g/L, it seems reasonable that after optimising the fermentation an activity of at least 15 million units per litre seems to be possible.

### *3.3 Co-cross-linking of enzyme*

Initially it was tested if the covalent binding of the enzyme inulase II has an influence on its activity. If lysine residues are located in the enzyme's active site a reaction of their amino groups may lead to a readily inactivation. As can be seen from Figure 4, our enzyme is not susceptible to glutardialdehyde over a broad range of concentration and thus the envisaged method of co-cross-linking was viable.

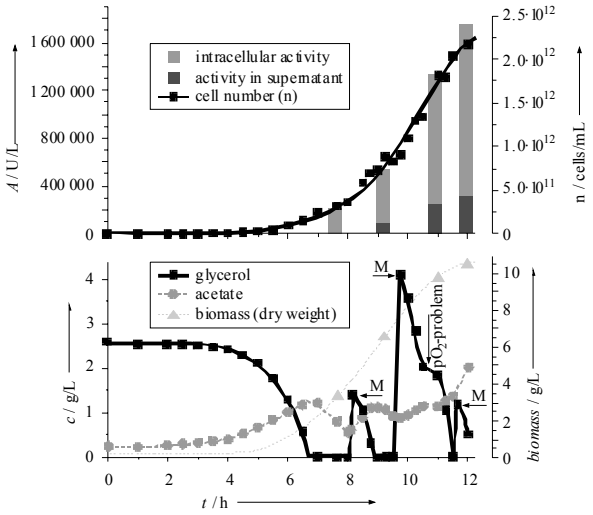


Figure 3:
Run of fermentation of *E. coli pMSiftOptR*, (M) indicates addition of new medium to the fermenter

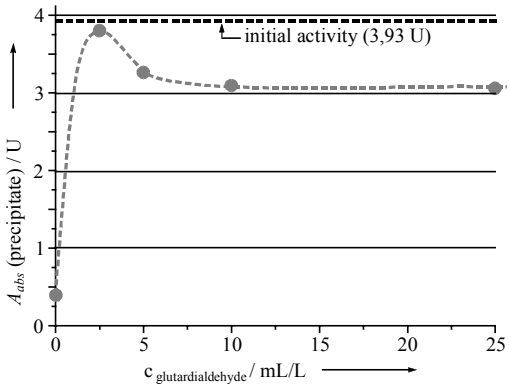


Figure 4:
Effect of glutardialdehyde on enzyme activity

Subsequently the optimal ratio of enzyme, chitosan and glutardialdehyde was determined in a matrix experiment. Using the enzyme solution derived from the above described fermentation ($1{,}76 \cdot 10^6$ U/L) precipitates were obtained in combination with varying amounts of chitosan and glutardialdehyde. To investigate the quality of the co-cross-linking alginate, beads were made in manual operation from each preparation, washed thoroughly and dissolved again for activity testing. As can be seen from Figure 5, a maximum of activity could be found at a chitosan concentration of 0.45 % (w/w) and at a glutardialdehyde usage of 25 mL/L (50 % (w/w) solution), respectively. This corresponds to approx. 30 mmol/L of reactive amino groups in the chitsoan and approx. 280 mmol/L aldehyde groups from glutardialdehyde. The protein concentration in this preparation was 2.8 g/L.

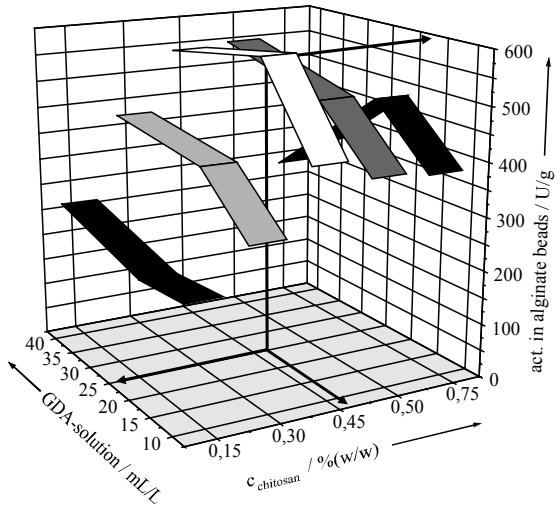


Figure 5:
Activities obtained from matrix experiments with varying concentrations of chitosan and glutardialdehyde

### *3.4 Mass production of beads*

Based on these findings, a larger batch of co-cross-linked enzyme was prepared. To accomplish the task of producing the tiny droplets from the highly viscous alginate-enzyme-mixture, novel JetCutter technology was employed (Vorlop and Breford, 1984). In comparison to other techniques like blow-off devices, vibrating nozzles or electrostatic forces, the JetCutter uses a mechanical cutting of a continuous jet of liquid to produce small droplets (Prüße et al., 1998; Jahnz et al., 2001). It is possible to work with liquids which have a viscosity of up to several Pa·s. The jet of liquid escapes from the nozzle (50 to 1200 µm in diameter) at a constant velocity of 10 to 30 m/s and the cutting tool with 48 wires rotates with a constant speed of up to 10 000 rpm. Due to these parameters the JetCutter has a tremendous throughput.

The resulting particles were sieved and the different fractions weighed. As can be seen from Figure 6 the distribution in particle size is small and in the desired range of 600 to 700 µm.

### *3.5 Kinetic investigation of entrapped inulase II*

Beads with diameters in the range of 500 to 800 µm were analysed and the activities compared as is shown in Figure 7. For beads of 600 µm diameter an activity of 196 U/g was measured for wet matter. Comparing the initial slopes of the curves an effectiveness h factor can be calculated. While beads of 600 µm in diameter showed an effectiveness factor of 0.44, for beads of 850 µm this value dropped to 0.34.

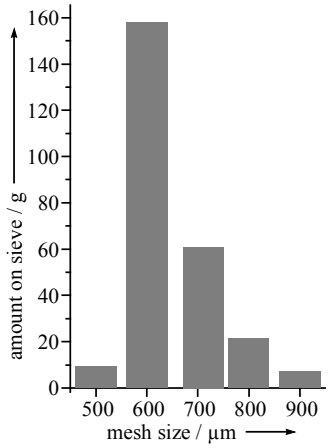


Figure 6:
Distribution of alginate beads (sieve analysis)

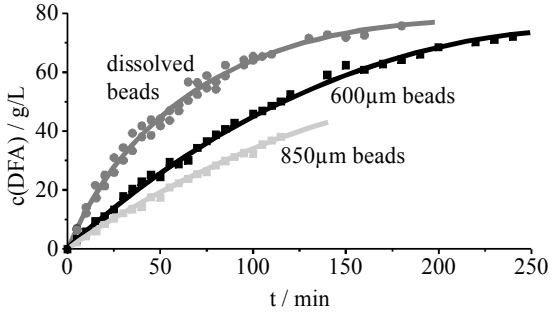


Figure 7:
Activity of beads with encapsulated inulase II at different diameters (shown are results for dissolved beads and for diameters of 600 and 850 µm, respectively)

These results were reviewed from a theoretical point of view. The diffusion coefficient for inulin in pure water at the elevated temperature of 50°C was calculated according to the following equation using the known dynamic viscosity values $\varpi_{T1}$ and $\varpi_{T2}$ for water.

$$D_{0,T2} = \frac{D_{0,T1} \cdot T2 \cdot \eta_{T1}}{T1 \cdot \eta_{T2}} = 4.64 \cdot 10^{-6}\ \mathrm{cm^2/s}$$

$D_{0,T1}$ = $2.30 \cdot 10^{-6}$ $\mathrm{cm^2/s}$ (Chaplin, 2001)
$T1$ = 293 K
$T2$ = 323 K
$\varpi_{T1}$ = 1.002 mPa·s
$\varpi_{T2}$ = 0.5468 mPa·s (Treybal, 1980)

To take into account the hindering influence of the matrix consisting of alginate and co-cross-linked chitosan, the effective diffusion coefficient was calculated according to the equation of White and Dorion (1961), where $a$ is a parameter for the molecular weight of the substrate and $Vp$ is the concentration of

solid matter in the particle. It is estimated by summing up the concentrations for alginate, protein, glutardialdehyde and chitosan:

$$D_{eff} = D_0 \cdot e^{-a \cdot Vp} = 2.11 \cdot 10^{-7}\ cm^2/s$$

$a$ = 69 (extrapolated from Vorlop, 1984)
$Vp$ = 0.0448 kg/L

Based on Michaelis-Menten kinetic the Thiele modulus Φ is calculated according to the following equation for spherical particles (data shown for beads of 600 µm in diameter):

$$\Phi = \frac{r_P}{3} \cdot \sqrt{\frac{v_{max}}{K_M \cdot D_{eff}}} = 9.63$$

$r_P$ = 0.03 cm
$v_{max}$ = $4.5 \cdot 10^{-4}$ mol/(min·g)
$K_M$ = $2.3 \cdot 10^{-3}$ mol/L
$D_{eff}$ = $2.11 \cdot 10^{-7}$ cm²/s

Figure 8 shows the relation of the Thiele modulus Φ and the key figure β, which is the ratio of the concentration in the solution surrounding the particle and the $K_M$-value for the considered enzyme:

$$\beta = \frac{c_0}{K_M} = 7.7$$

$c_0$ = $1.78 \cdot 10^{-3}$ mol/L
$K_M$ = $2.3 \cdot 10^{-3}$ mol/L

Recapitulating, the following figures are calculated for different particles:

| $d_{bead}$ | $\eta_{exp.}$ | β | Φ | $\eta_{calc.}$ |
|---|---|---|---|---|
| 600 µm | 0.44 | 0.77 | 9.63 | 0.34 |
| 850 µm | 0.34 | 0.77 | 13.65 | 0.23 |

As can be seen the values obtained from the experiments and those calculated differ significantly but are in the same order of magnitude. However, it has to be considered that the following assumptions and estimations were made:

- The beads used in the experiments were selected by sieve analysis and thus they do not have an exact diameter.
- For obtaining the parameter a for the molecular weight of the substrate inulin, a far-ranging extrapolation was necessary.
- The content of solid matter within the particles was only roughly calculated.

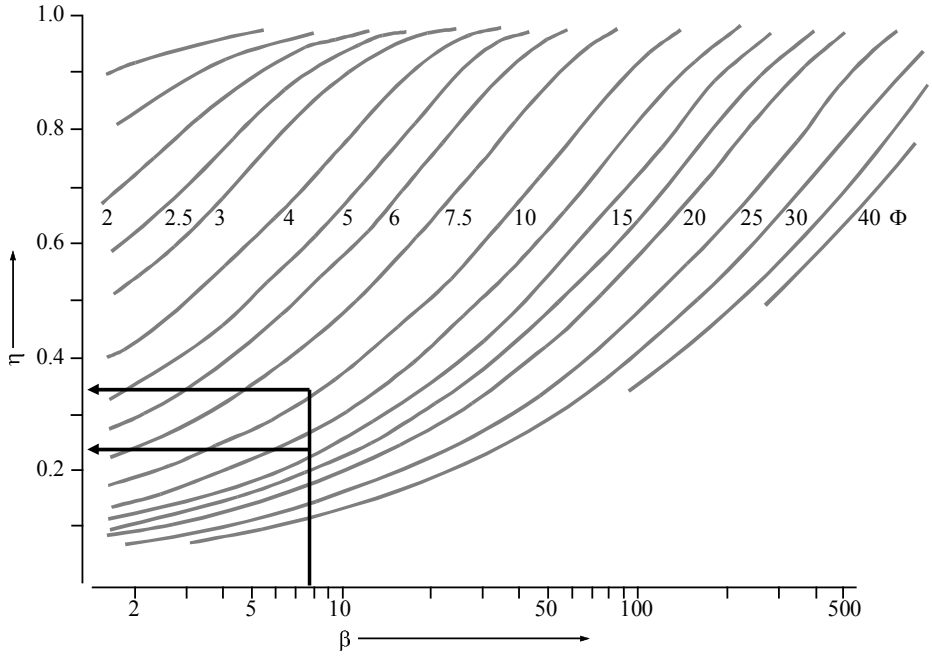


Figure 8:
Calculated effectiveness factors η with respect to different values for Thiele modulus and substrate key figures β (taken from Vorlop, 1984). The results for particles of 600 and 850 µm diameter, respectively, are pointed out.

## 4 Summary

We have shown the complete work starting from screening for an enzyme with the desired characteristics, i.e., an inulase II converting inulin to DFA III at elevated temperatures of 60°C. Next we successfully optimised the enzyme by genetic engineering and constructed a genetically modified organism which expresses the enzyme in very high numbers. We fermented this strain and showed the benefits of entrapping the enzyme in hydrogel particles which had an activity of 196 U/g (wet matter).

For different particles the effectiveness factor was measured and also calculated on a theoretical basis. The values matched each other satisfactorily.

## 5 Conclusions

To produce DFA III at a technical and industrial scale, large amounts of encapsulated enzyme are needed. The work described in this paper gives a basis for satisfying this need. Due to results from genetic engineering, a standard fermentation technique is capable of producing unlimited enzyme which can then be encapsulated on an industrial scale by using the novel JetCutter technology. The prospect of producing DFA III on an industrial scale has been accelerated greatly.

**Acknowledgements**

We are thankful to Fachagentur Nachwachsende Rohstoffe e.V. (FNR), Gülzow, and Nordzucker AG, Braunschweig, for partially financing the work.

**List of Symbols and Units**

| | |
|---|---|
| $a$ | parameter for molecular weight (-) |
| β | ratio of concentration and enzymatic activity |
| $c_0$ | concentration in solution (mol/L) |
| $D_{0,Tx}$ | diffusion coefficient at given temperature $Tx$ ($cm^2/s$) |
| $D_{eff}$ | effective diffusion coefficient within matrix ($cm^2/s$) |
| Φ | Thiele modulus (-) |
| $K_M$ | Michaelis Menten constant (mol/L) |
| η | effectiveness factor (-) |
| rpm | rotations per minute |
| $r_p$ | radius of particle (cm) |
| $Tx$ | temperature (K) |
| U | enzyme units (1 U = 1 µmol/min) |
| µ... | micro... |
| $V_p$ | concentration of solid matter (kg/L) |
| $v_{max}$ | maximum enzymatic activity (mol/(min·g)) |
| $\varpi_{Tx}$ | dynamic viscosity at given temperature $Tx$ |
| % (w/w) | weight percentage |

**References**

Bajpai P, Bajpai PK (1989), Utilization of Jerusalem artichoke for fuel ethanol production using free and immobilized cells. Biotechnol Appl Biochem 11:155-168

Haraguchi K, Seki K, Kishimoto M, Nagata T, Kasumi T, Kainuma K, Kobayashi S (1995), Cloning and nucleotide sequence of the inulin fructransferase (DFA I-producing) gene of *Arthrobacter globiformis* S14-3. Biosci Biotech Biochem 59:1809-1812

Jahnz U, Wittlich P, Pruesse, U, Vorlop K-D (2001) New matrices and bioencapsulation processes. In: Focus on Biotechnology Vol. 4, Hofmann M and Anne J (eds), Kluver Academic Publishers, Dordrecht, pp. 293-307

Oiwa H, Naganuma M, Ohnuma S (1987), acetone-butanol production from dahlia inulin by *Clostridium pasteurianum* var. I-53. Agr Biol Chem Tokyo 51(10):2819-2820

Prüße U, Fox B, Kirchhoff M, Bruske F, Breford J, Vorlop K-D (1998), New Process (Jet Cutting Method) for the production of spherical beads from highly viscous polymer solutions. Chem Eng Technol 21:29-33

Sakurai H, Yokota A, Tomita F (1997), Molecular cloning of an inulin fructotransferase (de-polymerizing) gene from *Arthrobacter sp.* H65-7 and its expression in Escherichia coli. Biosci Biotech Biochem 61:87-92

Suzuki T, Hara H, Kasai T, Tomita F (1998) Effects of difructose anhydride III on calcium absorption in small and large intestines of rats. Biosci Biotechn Biochem 62:837-841

Tanaka T, Uchiyama T, Kobori H, Tanaka K (1975) Enzymic hydrolysis of di-D-fructo-furanose 1,2':2,3' dianhydride with *Arthrobacter ureafaciens*. J Biochem 8:1201-1206

Vorlop K-D (1984) Entwicklung von Verfahren zur Polymerfixierung von Mikroorganismen und Anwendung der Biokatalysatoren zur Spaltung von Penicillin G und Synthese von L-Tryptophan. PhD-Thesis, Technical University Braunschweig.

Vorlop K-D, Breford J (1984), Verfahren zur Herstellung von Teilchen aus einem flüssigen Medium. German Patent DE4424998

White ML, Dorion GH (1961) Diffusion in a crosslinked acrylamide polymer gel. J Polym Sciences 55:731-740