Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 256 (2003) 199–206
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Process for producing the potential food ingredient DFA III from inulin: screening, genetic engineering, fermentation and immobilisation of inulase II

Ulrich Jahnz, Milada Schubert, Heike Baars-Hibbe, Klaus-Dieter Vorlop*

Federal Agricultural Research Centre, Institute of Technology and Biosystems Engineering, Bundesallee 50, 38116 Braunschweig, Germany

Received 10 July 2002; received in revised form 27 November 2002; accepted 12 December 2002

Abstract

Difructose anhydride (DFA III) is a new potential sweet food additive. A screening was undertaken to isolate bacterial strains for conversion of inulin to DFA. Of special interest were thermotolerant enzymes. Some 400 strains were investigated, among four of them produce DFA and strain Buo141 expresses an extracellular enzyme which is stable at elevated temperatures. Based on metabolic data and 16S-rRNA-sequencing, the strain was identified as a new *Arthrobacter* species. For increased enzyme production, the inulase gene was cloned into *E. coli* XL1-blue, inulase II was expressed and its activity detected. After identifying the cleavage site, the sequence coding for a signal-peptide was eliminated from the plasmid and a beneficial amino acid exchange introduced by error-prone PCR. The recombinant *E. coli* was fermented to 10.5 g/l and after disruption an activity of 1.76 Mio U/l was observed. The enzyme was flocculated from supernatant and entrapped in calcium alginate hydrogels. To enable production of uniform and small beads JetCutter technology was used with a production rate of 5600 beads/(s nozzle). The influence of bead diameter on activity was investigated. An activity of 196 U/g was measured for 600-μm beads.

Keywords: Inulin; DFA; Food additive; Screening; Thermotolerant enzyme; Genetic engineering; Immobilisation; JetCutter

1. Introduction

1.1. Properties of DFA III

The disaccharide difructose anhydride III (DFA III; Fig. 1) is derived from the vegetable storage compound inulin. DFA is a non-reducing sugar which is very well soluble in water and has a melting point of 162 °C. Due to an intramolecular dioxane ring, the molecule is very stable and is not hydrolysed in the stomach. It has approximately half the sweetness of sucrose, is not cariogenic, and is not metabolised by the human body. As is known from oligofructoses, it influences the composition of the intestinal flora positively as was shown in feeding experiments with rats (Saito and Tomita, 2000). Likewise, in rats an increased uptake of calcium could be observed in the intestine after nourishing on DFA-enriched food (Suzuki et al., 1998). The authors conclude that the risk of osteoporosis could be lowered by such a diet. Investigations regarding the effects in humans were not made yet. However, as DFA is also a small by-product obtained in crystallisation it is not expected to have negative effects. So far, DFA is not produced on a commercial scale as a food additive.

* Corresponding author. Tel.: +49-531-596-4101; fax: +49-531-596-4199.
E-mail address: klaus.vorlop@fal.de (K.-D. Vorlop).

0378-5173/03/$ – see front matter
doi:10.1016/S0378-5173(03)00078-4

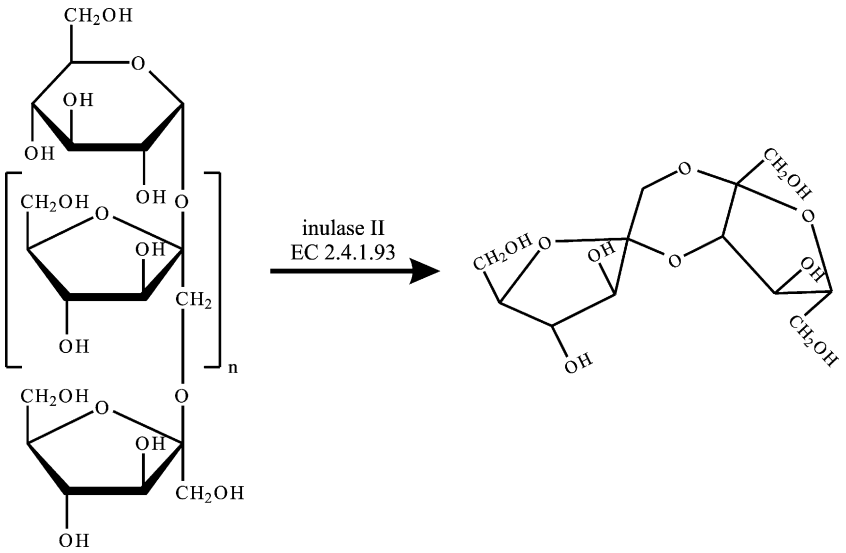

Fig. 1. Chemical structure of inulin (left) and the enzymatic product difructose dianhydride III (DFA III).

1.2. Production of DFA

DFA can be produced either chemically or enzymatically from inulin. The prior method uses diluted sulfuric acid (McDonald, 1947) or pyrolysis conditions (Defaye et al., 1985) and yields of up to 40% can be found in inulin-derived caramels (Richards, 1996). The enzymatic route in contrast is more specific. The enzyme inulase II (EC 2.4.1.93) accomplished the DFA formation by an intramolecular transfructosylation. This enzyme was first described in *Arthrobacter ureafaciens* (Uchiyama et al., 1973) and has meanwhile been found in various other bacteria (Kawamura et al., 1988; Yokota et al., 1991; Kim and Lee, 2000). However, the cited enzymes are not long-term stable at elevated temperature which is eligible for a process on industrial scale.

1.3. Enzymes 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, i.e. the enzyme in the present case, in the bioreaction vessel. Various methods have been developed for this purpose as is shown in Fig. 2 (Klein and Vorlop, 1985). In addition to the advantage of easy retention immobilised catalysts often also show an increased stability regarding for instance pH-value and temperature. Moreover, in the case of entrapment, the catalysts are protected against other bacteria and thus processes can run under non-sterile conditions since potential contaminations are washed out while the favoured catalyst specifically is protected.

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

The resulting alginate-matrix is a hydrogel, i.e. it principally consists of water with only a minor content of polymer. The cut-off for these gels is normally not low enough to retard common enzymes so that these enzymes have to be treated first. They can be linked to each other or co-crosslinked with a spacing agent. As a consequence, the molecular weight of the enzyme is increased. The reaction conditions and used chemicals have to be tested and optimised anew for each enzyme.

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

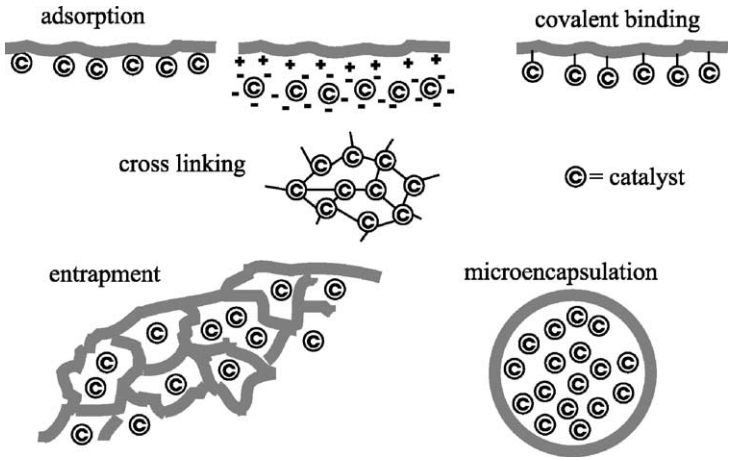

Fig. 2. Schematic representation of different immobilisation methods.

effect, particles have to be kept as small as possible and reasonable for the later application.

Continuous processes are preferably run in packed bed reactors. Since these build up significant pressure drop it is important that beads used for this purpose have a high mechanical stability. The stability of beads can be increased by using higher concentrated polymer solutions. However, the technical problems to make the desired small particles from the resulting highly viscous polymer solutions are immense.

1.4. Aims of the work

A broad screening programme was started to isolate strains producing inulase II enzymes. Of special interest were thermotolerant enzymes, i. e. enzymes which are stable at leastwise 60 °C for a prolonged period of time. By means of genetic engineering, the production of this enzyme should be enhanced. For this purpose, the *ift*-gene encoding for inulase II was cloned and expressed in *E. coli* as a new host. To facilitate the use of the enzyme in a future industrial process, the basics for its immobilisation were investigated.

2. Materials and methods

2.1. Screening for microorganisms

For selective enrichment, a mineral salt medium with inulin from dahlia tubers (Sigma Chemicals Co., St. Louis, MO) 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, respectively. Grown colonies were then subcultured to liquid media and screened for formation of DFA III by HPLC analysis (Jahnz, 2001). One unit of enzyme activity was defined as the formation of 1 μmol of DFA per minute. Supernatant of positive strains was tested after incubation at different elevated temperatures to determine temperature stability.

2.2. Genetic engineering

For cloning of the *ift* gene, a genomic library was constructed from partially digested *Arthrobacter* genome in phage λ. 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 hybridising 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 min 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 subsequently the entire region coding for the transfer-peptide was deleted by means of specific endonucleases. Additionally, a point-mutation in the coding region of the *ift* gene was generated by error-prone PCR.

2.3. Fermentation

To obtain large quantities of the enzyme, the genetically modified organism was fermented in 10-l scale (fermenter NLF22, Bioengineering, Wald) using as starting medium technical yeast extract (15 g/l, Ohly, Hamburg) and glycerol (2.5 g/l). Based on HPLC-measurements of residual glycerol (Jahnz, 2001), new medium was fed to the fermenter during the course of the fermentation. Afterwards cells were harvested and disrupted with a high-pressure homogenizer (LAB60, APV Gaulin, Lübeck; three passages of 65 MPa; 0.5 l/min, $T_{in} = 5\,^{\circ}C$, $T_{out} = 21\,^{\circ}C$).

2.4. Enzyme immobilisation

For immobilisation, the enzyme was co-crosslinked. The exact amounts of chemicals needed for this procedure are listed below in the Section 3, the composition of the chemicals was as follows. Chitosan (type EC, 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, crosslinking was accomplished over 24 h at 4 °C while stirring vigorously and afterwards 3% sodium alginate (LF20/60, FMC Biopolymer, Drammen) was added. Small droplets of 500–850 μm in diameter were formed with a JetCutter (geniaLab, Braunschweig; nozzle 300 μm; flow of liquid 0.9 g/s; cutting tool 48 wires of 100 μm; rotation speed 7000 rpm) and hardened for 15 min in a 2% calcium chloride solution. To evaluate the effect of diffusional limitation, the activity of beads of different diameters was measured.

Alginate beads (0.5 g) were dissolved for 30 min in 5 ml of a mixed solution of 100 mmol/l sodium citrate and 100 mmol/l sodium chloride.

3. Results and discussion

3.1. Screening for microorganisms

Some 400 strains were investigated in pure culture. Four strains were found to produce DFA III, the strain Buo141 expresses an enzyme which is stable for weeks at elevated temperatures of 60 °C. Using metabolic data and 16S-rRNA-sequencing, the strain was identified to be a new *Arthrobacter* species. It grows aerobically at ambient temperatures. Inulase II is secreted as an extracellular enzyme.

3.2. Cloning of ift gene

To increase the production of the enzyme, the gene encoding for the inulase II (*ift* gene) should be transferred to an *E. coli* host and expressed. To gain access to the bacterial gene, suited primers for a PCR-reaction were needed. For the primer design, only two highly divergent sequences of inulase proteins were known and published in the databases:

- 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).

Phylogenetic analysis of both these sequences resulted in two conserved regions for design of appropriate primers. One of them at the N-terminal end and the other one approximately in the middle of the inulase protein. Using this universal *ift* gene-specific primer pair, a region of approximately 500 bp could be amplified from the *Arthrobacter* chromosomal DNA which mirrors about one-third of the total *ift* gene. The described primer-design extremely accelerated the isolation of *ift* gene. The complete *ift* gene was obtained by screening the genomic library with this probe. As a result, a plasmid was constructed which expressed an enzyme of 477 amino acids when transferred to *E. coli*. A cell-free extract of such a culture showed an activity of 3000 U/l, whereas the majority of this activity was detected intracellularly.

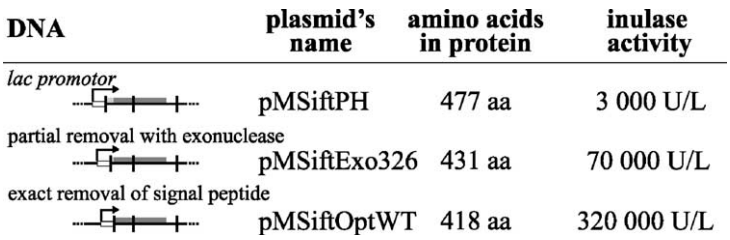

Fig. 3. Partial and exact removal of sequences coding for the transfer-peptide and the resulting effects in terms of number of amino acids and measured activity.

3.3. Enzyme design for increase in inulase activity

In *Arthrobacter*, the inulase II enzyme is expressed as an extracellular enzyme. The transport via the cell membrane is accomplished by means of a specific signal transfer-peptide which is part of the *ift* gene. The cleavage site for this signal-peptide was identified by database comparison and verified by sequencing the N-terminus of the matured wild-type protein. Due to phylogenetic differences between the species of *Arthrobacter* and *Escherichia*, the transfer-peptide does not work in *E. coli*. In *E. coli*, the enzyme remains intracellular as was shown by activity analysis of disrupted cells and supernatant of cultivations. By stepwise removal of the region coding for the signal-peptide it was evidenced, that not only the inulase II remains in good order but rather an increase in activity could be observed, as is displayed in Fig. 3. An exact removal of the complete transfer-peptide resulted in a hundred fold increased activity. Probably the leading signal-peptide and remaining parts of it have a negative effect on the folding of the following protein which leads to the low activities observed afore.

A further increase in activity of approximately 35% was possible due to a point-mutation which was induced by error-prone PCR. On position 221 of the enzyme, a glycine residue was exchanged with arginine. Fig. 4 shows the probable effect of this exchange on the assumed secondary structure of the protein chain. A new α-helix region can be generated in the region of amino acids 216 to 224 according to the model of Garnier et al. (1978). The clone bearing this plasmid was named *E. coli* pMSiftOptR.

3.4. Fermentation of E. coli pMSiftOptR

The recombinant *E. coli* pMSiftOptR was fermented using an inexpensive technical medium. During the fermentation, the inulase activity was monitored. A final biomass concentration of 10.5 g/l (dry weight) and an overall activity of 1,760,000 U/l was measured. The majority of the activity (82%) was measured as being intracellular, only 18% of the activity were present in the supernatant.

Since high-density fermentations of *E. coli* are known to reach biomass concentration of about 110 g/l (Cutayar and Poillon, 1989), it is reasonable that by optimising the fermentation step an activity of at least 15 million units per litre could be achieved.

3.5. Co-crosslinking of inulase II

Initially, it was tested if the covalent binding of the enzyme inulase II has an influence on its activity. If, for

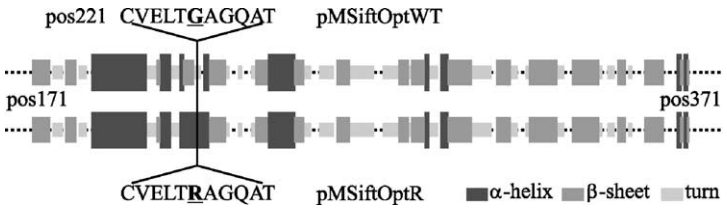

Fig. 4. Assumed secondary structure of native and mutated enzyme for the region around the amino acid exchange (position 221).

example, lysine residues are located in the enzyme's active site a reaction of their amino groups may lead to a readily inactivation. As was shown in experiments, our enzyme is not susceptible to glutardialdehyde over a broad range of concentration and thus the envisaged method of co-crosslinking was applicable. The optimal concentrations for the individual chemicals in the reaction mixture were found to be as follows:

- protein concentration 5.6 g/l,
- chitosan concentration 4.5 g/l,
- glutardialdehyde concentration 140 mmol/l.

3.6. JetCutter technology

To accomplish the task of producing the desired small droplets from the highly viscous alginate–enzyme solution, JetCutter technology was employed. 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, which is shown in Fig. 5 (Prüße et al., 1998).

It is possible to work with liquids which have a viscosity of up to several pascal seconds. 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 12,000 rpm. Due to these parameters, the JetCutter has a tremendous throughput and, in parallel, a very narrow distribution of particle diameters. Based on the individual parameters, particles can be generated in the range from approximately 120 μm up to 4 mm in diameter. A typical industrial JetCutter setup with 10 nozzles has a daily output of 1.2 tons of beads (diameter 1 mm) and needs only little supervision (Fig. 5). The monodisperse distribution of diameters is advantageous for most applications of spherical particles. For instance, all particles show the same concentration profile and in packed bed reactors no demixing due to different sizes occurs.

JetCutter technology was originally developed for preparation of beads from aqueous systems like alginate, pectinate and chitosan solutions for bioencapsulation purposes. Meanwhile, this technology stood the test and was successfully applied to other areas where highly viscous solutions have to be transformed into small droplets. The following examples are taken from the area of neutraceuticals and pharmaceuticals: preparation of food additives like encapsulated vitamins or masked amino acids, the preparation of

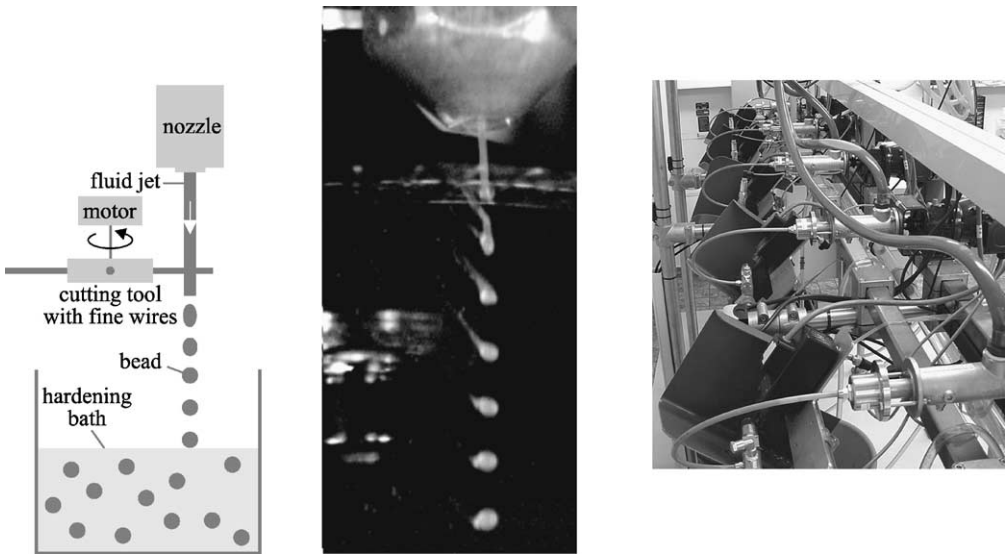

Fig. 5. Principle of JetCutting (left), high-speed motion picture of the cutting process (middle) and an industrial installation with a daily capacity of 1.2 tons of 1-mm beads (right).

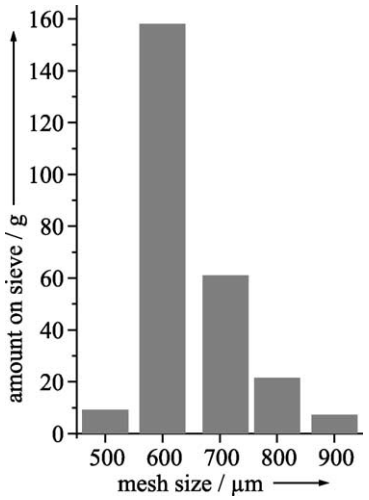

Fig. 6. Particle diameter distribution of alginate beads with entrapped inulase II produced with JetCutter technology.

starter cultures with entrapped bifidobacteria and the processing of hotmelts. Especially, the later can find application in the pharmaceutical industry, since JetCutter technology allows the generation of excellent particles for entrapping of or subsequent coating with active substances, respectively, or production of beads for controlled and/or retarded release applications with therapeutics and health food compounds.

Using this technology, alginate beads with different diameters were produced with entrapped co-crosslinked enzyme inulase II. The production rate was 5600 beads per second nozzle, the distribution of particle diameters is depicted in Fig. 6.

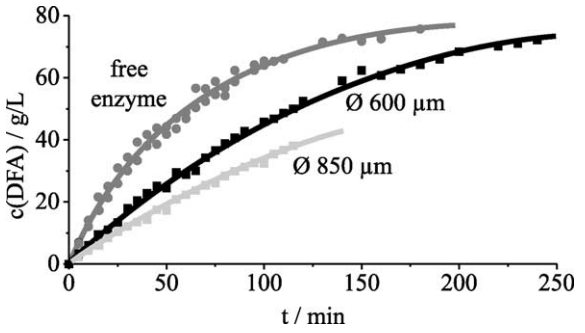

Fig. 7. Product release curve for free enzyme and enzyme entrapped in alginate beads of different diameters.

3.7. Immobilised inulase II

Beads with diameters in the range of 500–800 µm were analysed and their activity compared to the product release curve for free enzyme. Part of this data is presented in Fig. 7. While beads of 500 µm in diameter showed 54% of activity compared to the value when the same beads were dissolved, beads of 600 µm gave only 42% of the activity. For 850 µm, only one-third of the genuine activity was observed. The activity measured for 600-µm beads was 196 U/g/wet matter).

4. Conclusions

To produce DFA III in 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 genetically engineering, a standard fermentation technique is capable of producing vast amounts of enzyme which can then be encapsulated on an industrial scale by using JetCutter technology. The prospect of producing DFA III on an industrial scale has been accelerated greatly and DFA as a new food additive could be introduced to the market.

Acknowledgements

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

References

Cutayar, J.M., Poillon, D., 1989. High cell density culture of *E. coli* in a fed-batch system with dissolved oxygen as substrate feed indicator. Biotech. Lett. 11, 155–160.

Defaye, J., Gadelle, A., Pedersen, C., 1985. The behaviour of D-fructose and inulin towards anhydrous hydrogen fluids. Carbohydr. Res. 136, 53.

Garnier, J., Osguthorpe, D.J., Robson, B., 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. 120, 97–120.

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., 2001. Screening-Automation auf Basis hohlkugelverkapselter Zellen und enzymatische Bildung von Difructoseanhydrid III aus Inulin unter thermophilen Bedingungen (Screening, Charakterisierung, Immobilisierung). Ph.D. Thesis. Technical University of Braunschweig, Braunschweig.

Kawamura, M., Takahashi, S., Uchiyama, T., 1988. Purification and some properties of inulin fructotransferase (depolymerizing) from *Arthrobacter ilicis*. Agric. Biol. Chem. 52, 3209–3210.

Kim, G.E., Lee, S., 2000. Efficient production of DFA III (di-D-fructofuranose-dianhydride) from chicory root. Abstracts of the World Congress on Biotechnology 2000, vol. 272, Berlin.

Klein, J., Vorlop, K.-D., 1985. Immobilization techniques: cells. In: Moo-Young, M. (Ed.), Comprehensive Biotechnology, vol. 2. Pergamon Press, Oxford, pp. 203–224.

McDonald, E.J., 1947. The polyfructosans and difructose anhydrides. Adv. Carbohydr. Chem. 2, 253.

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.

Richards, G.N., 1996. Production of fructose dianhydride products from inulin. US-patent application US525190.

Saito, K., Tomita, F., 2000. Difructose anhydrides: their mass-production and physiological functions. Biosci. Biotech. Biochem. 64, 1321–1327.

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. Biotech. Biochem. 62, 837–841.

Uchiyama, T., Niwa, S., Tanaka, K., 1973. Purification and properties of *Arthrobacter ureafaciens* inulase II. Biochim. Biophys. Acta 315, 412–420.

Yokota, A., Hirayama, S., Enomoto, K., Miura, Y., Takao, S., Tomita, F., 1991. Production of inulin fructotransferase (depolymerizing) by *Arthrobacter* sp. H65-7 and preparation of DFA III from inulin by the enzyme. J. Ferm. Bioeng. 72, 258–261.