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Treatment of waste gas from piggeries with nitrogen recovery

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Effective development of a biotechnical process:
Screening, genetic engineering, and immobilization for the enzymatic conversion of inulin to DFA III on industrial scale

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Abstract

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

A broad screening programme was carried out to isolate bacterial strains with the necessary enzymatic activity (inulase II). Of special interest were thermotolerant enzymes, i. e. enzymes which are stable at leastwise 60° C for prolonged time. Using a mineral salt medium with inulin as the sole source for carbon and energy some 400 strains were investigated. Four strains were found to produce DFA III and the strain Buo141 expresses an enzyme which is stable for weeks at elevated temperatures. Using metabolic data and 16S-rRNA-sequencing the strain was identified to be a new Arthrobacter species. Inulase II is secreted as an extracellular enzyme.

To increase the formation of enzyme the inulase gene was cloned into *E. coli* XL1-blue, inulase II was expressed and its activity was detected. After identifying the cleavage site the DNA sequence coding for the original signal peptide was eliminated in the expression vector. An amino acid exchange was induced by error-prone PCR. The recombinant *E. coli* was fermented to 10,5 g/L (dry matter) and cells were disrupted. An activity of 1.76 Mio U/L was observed.

The enzyme was flocculated from cell-free extract and entrapped in calcium alginate hydrogels for better retention under continuous operation. 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 m beads.

The presented work covers the complete range of developing a biotechnical process and provides the basics for establishing an industrial process for producing DFA from inulin at low cost.

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

Zusammenfassung

Entwicklung eines biotechnischen Prozesses: Screening, genetic engineering und Immobilisierung zur enzymatischen Umsetzung von Inulin in DFA III im industriellen Maßstab

Die Biokonversion nachwachsender Rohstoffe für industrielle Anwendungen erfordert die Bereitstellung geeigneter Enzyme und Verfahren. Die vorliegende Arbeit präsentiert die Ergebnisse der Entwicklung eines Prozesses zur Umsetzung von Inulin zu einem Difructoseanhydrid (DFA III).

Zur Isolierung bakterieller Stämme mit der gesuchten Enzymaktivität (Inuase II) wurde ein Screeningprogramm durchgeführt. Von besonderem Interesse waren thermotolerante Enzyme, die bei 60° C langzeitstabil sind. Von ca. 400 isolierten Stämmen zeigten vier DFA-III-Bildung. Stamm Buo141 produziert ein Enzym, das über Wochen hinweg bei erhöhter Temperatur stabil ist. Aufgrund von Stoffwechseldaten und der 16S-rRNA-Sequenz wurde er als neue Art der Gattung Arthrobacter eingestuft. Die Inulase II wird als extrazelluläres Enzym sekretiert.

Zur Steigerung der Enzymbildung wurde das Inulasegen in *E. coli* XL1-blue kloniert, exprimiert und seine Aktivität nachgewiesen. Eine Aktivitätssteigerung wurde durch Entfernung der DNA-Sequenz eines in *E. coli* funktionslosen Signalpeptides und einen Aminosäureaustausch erreicht. Der rekombinante *E. coli* Stamm wurde fermentiert (10,5 g/L BTM) und die Zellen aufgeschlossen. Die Aktivität betrug 1,76 Mio U/L.

Das Enzym wurde aus dem zellfreien Extrakt flokkuliert und in Calciumalginathydrogel eingeschlossen, um eine kontinuierliche Prozessführung zu ermöglichen. Zur Herstellung gleichgroßer, kleiner perlenförmiger Partikel wurde hierzu die neuartige JetCutter technologie mit einer Perlenbildungsfrequenz von 5600 Perlen/(s·Düse) eingesetzt. Der Einfluss des Perlendurchmessers auf die Aktivität wurde untersucht und für 600- m-Perlen ein Wert von 196 U/g gemessen.

Die vorgestellte Arbeit beschreibt die Entwicklung eines biotechnischen Prozesses und stellt die Grundlagen für eine industriellen Herstellung von DFA aus Inulin bereit.

Schlüsselworte: Inulin, DFA, Screening, thermotolerantes Enzym, genetic engineering, Immobilisierung, JetCutter

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1 Introduction

1.1 Bioconversions for industrial raw materials

The growing awareness of limited fossil resources and environmental problems connected to the emission of carbon dioxide has caused an increased interest in the usage of renewable resources. For many applications crop based products have to be refined first before they can be put to an industrial usage. Refining steps can either be done chemically or by means of whole cells or enzymes. These biological catalysts may carry out very complex reactions which bring along an increased value of the material. However, before establishing a process to convert agricultural products for non-food applications proper cells or enzymes have to be found and optimized 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 plasticizers 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 are dealing 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 of prior hydrolysis of the inulin chains (Bajpai and Bajpai, 1989).

Uses of inulins in human nutrition have long been known and since the 19th century it has, 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 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 catalyzed by the enzyme inulase II as a kind of an intramolecular trans-fructosylation (Tanaka et al., 1975).
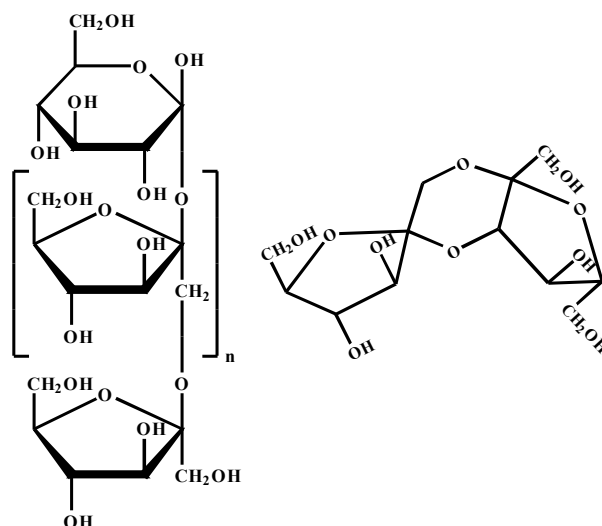

Fig. 1
Chemical structure of inulin and DFA III.

DFA III can either 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 via the chemistry of 5-hydroxymethylfurfural (HMF). It can be crystallised as easily as sucrose after an ion exchange step and hence it can be produced at a price well below that of inulin. So far DFA III was not introduced to the market since no efficient enzyme and

biotechnical process was available for the necessary bio-conversion of inulin.

1.4 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 in the bioreaction vessel. By immobilising catalysts like growing, resting or dead cells or enzymes it is possible to retard them. Various methods have been developed for this purpose as is shown in figure 2 (Klein and Vorlop, 1985). Besides 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.
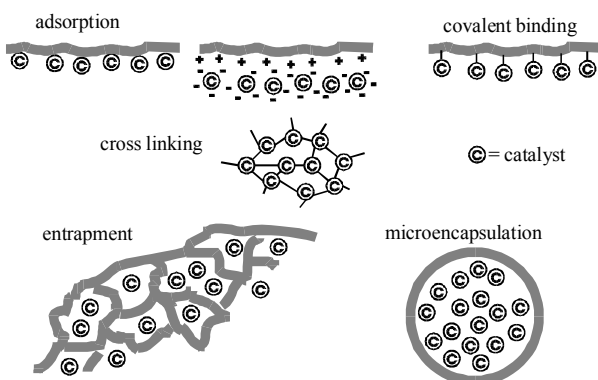

Fig. 2
Different ways for immobilising biocatalysts.

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 lesser than that of the free catalyst due to diffusional limitations. To minimize this negative 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 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 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 at least 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 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 sub-cultured to liquid media and screened for formation of DFA III by HPLC analysis. 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 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 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.

To obtain large quantities of the enzyme the genetically modified organism was fermented in 10-L-scale using a medium consisting of technical yeast extract and glycerol

as the carbon source. Cells were harvested and disrupted with a high pressure homogenizer.

2.3 Enzyme immobilisation

For immobilisation the enzyme was flocculated from a cell-free extract by co-crosslinking with chitosan and mixed with 3% sodium alginate. Small droplets of 500 to 850 m in diameter were formed with the JetCutter technique and hardened in a 2 % calcium chloride solution. To evaluate the effect of diffusional limitation the activity of beads of different diameters was measured and compared.

3 Results and Discussion

3.1 Screening for microorganisms

Some 400 strains were investigated in pure culture. Of four strains found to produce DFA III the strain Buo141 expresses an enzyme which is stable for weeks at elevated temperatures of 60° C. Decline of activity for other strains is shown in comparison with enzyme from Buo141 in figure 3. 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.
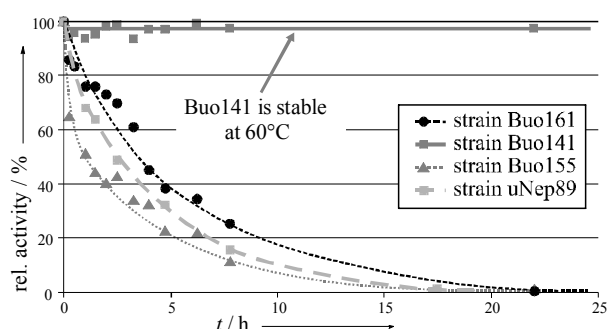

Fig. 3
Investigation of temperature stability.

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 and expressed in an *E. coli* host. 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 approx. 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.

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 figure 4. 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.
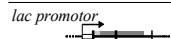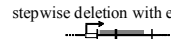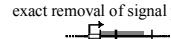
alteration DNA level	plasmid	amino acids expressed	inulase activity
lac promotor	pMSiftPH	477 aa	3 000 U/L
stepwise deletion with exonuclease	pMSiftExo326	431 aa	70 000 U/L
exact removal of signal peptide	pMSiftOptWT	418 aa	320 000 U/L

Fig. 4
Effects of the removal of DNA coding for the transfer peptide.

A further increase in activity of approx. 35% was possible due to a point-mutation which was induced by error-prone PCR. On position 221 of the enzyme a glycine was exchanged with arginine. Figure 5 shows the possible

effect this single change has on the 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).
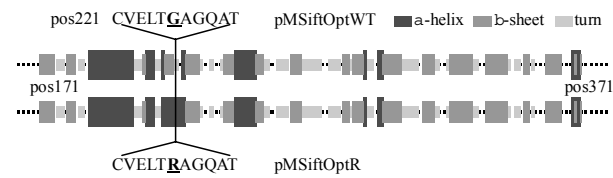

Fig. 5
Effects of the amino acid exchange on the secondy structure of the enzyme.

3.4 Fermentation of E. coli pMSiftOptR

The recombinant *E. coli* was fermented using an inexpensive technical medium. During the fermentation the inulase activity was monitored. A final biomass concentration of 11 g/L (dry weight) and an overall activity of 1.76 Mio U/L was measured.
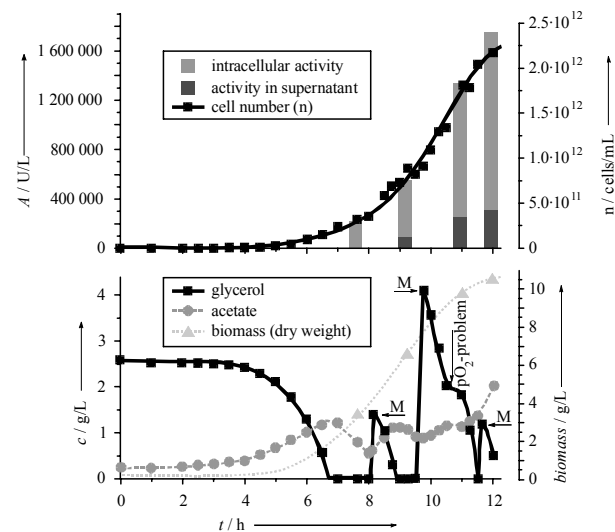

Fig. 6
Run of fermentation of *E. coli pMSiftOptR*, (M) indicates the point of time new medium was fed to the fermenter.

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

3.5 JetCutter technology

To accomplish the task of producing the desired small droplets from the very viscous alginate-enzyme-solution 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, which is pictured in figure 7 (Prüße et al., 1998).
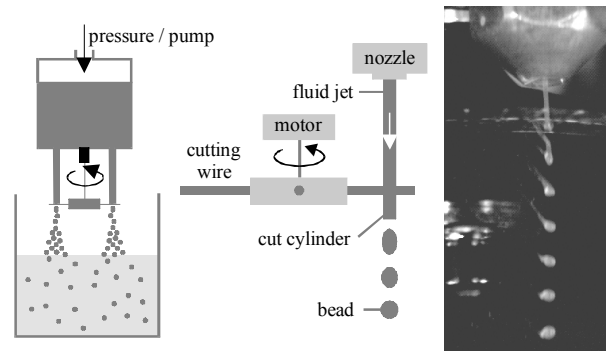

Fig. 7
Principle of JetCutting and high-speed-motion picture.

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 m 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.

Table 1
Throughput data for the production of spherical particles by Jet-Cutting

Diameter	speed of cutting tool		
mm	1250 rpm	6250 rpm	12500 rpm
0,2	0,02	0,08	0,15
0,6	0,41	2,04	4,07
0,8	0,97	4,83	9,65
1,0	1,89	9,42	18,8
1,5	6,36	31,8	63,6

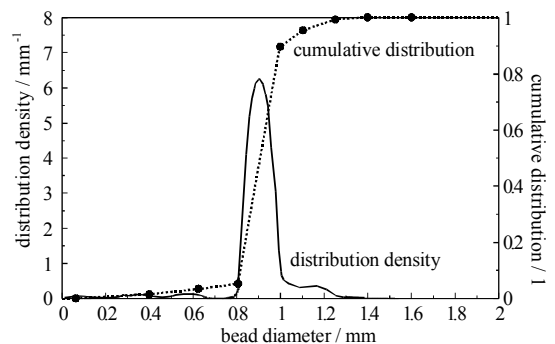throughput in kg (nozzle·h)
fluid density 1 kg/L

Fig. 8
Distribution curve for alginate beads; quantities measured by sieve analysis.

Production rates for different settings are specified in table 1. Figure 8 shows a typical distribution of particle diameters. As can be seen the distribution is very narrow which 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.

3.6 Inulase II in alginate beads

Beads with diameters in the range of 500 to 800 m were analysed and their acitivities compared, as is shown in figure 9. While beads of 500 m in diameter showed 54% of activity compared to the value when the same beads were dissolved, beads of 600 m gave only 42% of the activity. For 850 m only one third of the genuine activity was observed. This shows once more the benefits of using small enough particles when working with encapsulated systems.
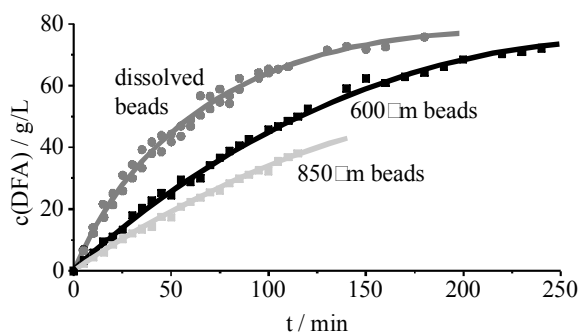

Fig. 9
Activity of beads with encapsulated inulase II.

4 Summary

We have shown the complete work starting from screening for an enzyme with the desired characteristics, i.e. a 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 organisms 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).

5 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 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.

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