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Production of nodulation factors by *Rhizobium meliloti*: fermentation, purification and characterization of glycolipids

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Lipooligosaccharides, synthesized by soil bacteria of the genera *Rhizobium,* are known to have multifunctional effects on a wide variety of plants as signal substances in symbiosis initiation, cell response elicitation and growth regulation. These so called nodulation (Nod-) factors represent interesting biotechnological products with respect to fundamental studies of symbiotic interactions as well as for potential applications. Therefore, a batch fermentation process on a scale of 30 l has been developed by means of the *Rhizobium meliloti* strain R.m. 1021 (pEK327) strongly overexpressing the genes for the synthesis of Nod factors. Induction by the flavone luteolin led to growth associated production of the lipooligosaccharides. Ultrafiltration was used for separating the biomass from the filtrate containing the extracellular Nod factors. Simultaneously, ultrafiltration reduced the amount of lipophilic substances, which would otherwise interfere with processes downstream. The second separation step consisted in adsorption on XAD-2, a nonspecific hydrophobic adsorptive resin. Adsorption of Nod factors was carried out by batch operation of a stirred tank. Desorption was performed by elution with methanol in a fixed bed column. A semi-preparative reversed phase HPLC (Polygoprep 100-30 C18) was chosen as the final purification step. The Nod factors were obtained after evaporation and lyophilization. Thus, about 600 mg of Nod factors were produced from 20 l of fermentation broth. The Nod factors produced by *Rhizobium meliloti* R.m. 1021 (pEK327) were identified by liquid secondary ion mass spectrometry and by reversed-phase HPLC as fluorescent derivatives of 2-aminobenzamide. The biological activity of the products was demonstrated by means of the root hair deformation (HAD-) assay.

***Keywords*: *Rhizobium meliloti*, nodulation factors, fermentation, ultrafiltration, adsorption, reversed phase (RP) chromatography, fluorescence labelling, LSIMS, HAD-assay**

***Abbreviations*: ads, adsorption; 2-AB, 2-aminobenzamide; BDA, borane dimethylamine complex; Da, Dalton; DMSO, dimethyl sulfoxid; HAC, root hair curling; HAD, root hair deformation; HPLC, high performance liquid chromatography; LSIMS, liquid secondary ion mass spectrometry; MeOH, methanol; Nod, nodulation; OD, optical density; R.m., *Rhizobium meliloti*; RP, reversed phase; Tc, tetracycline; TY, trypton-yeast; UF, ultrafiltration; UV-Vis, ultraviolet-visible**

Introduction

The forage plant alfalfa (*Medicago sativa*) in symbiosis with the soil bacterium *Rhizobium meliloti* is one of the most commonly cultivated symbiotic systems in the world [1]. Lipooligosaccharides synthesized by *Rhizobium meliloti*, called nodulation factors (Nod factors), play an important role during initiation of the symbiosis [2]. In addition, it has been demonstrated that Nod factors and similar substances [3–5] elicit various responses on non-host plants, indicating that these oligosaccharides may have general functions in plant development, morphogenesis and defence. The chemical structures of the first purified Nod factors were determined by Lerouge *et al.* [6]. *Rhizobium meliloti* produces a family of related lipooligosaccharides [7].

The structural features of these Nod factors are summarized in Figure 1. Their sugar moiety consists of β-1,4 linked *N*-acetylated glucosamine building blocks. The number of the glucosamine units varies from three to five. The fatty acid moiety consists of a carbon chain of 16 carbon atoms with up to three double bonds. The molecule is eventually decorated with a sulfate group at the C6 position of the reducing end-glucosamine unit. The hydroxyl group at the C6 position of the non-reducing sugar may be

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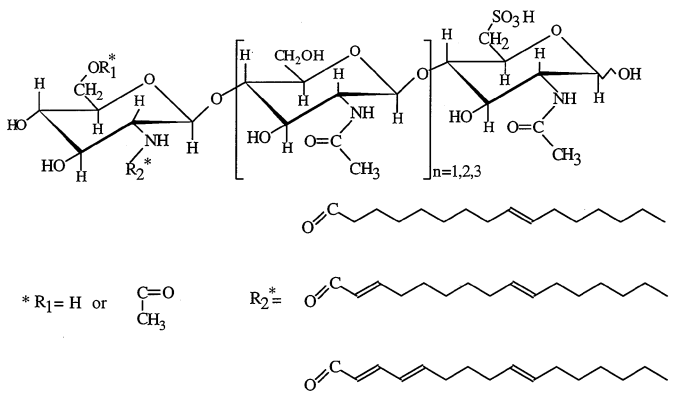

Figure 1. Structures of the Nod factors produced by the soil bacterium *Rhizobium meliloti*.

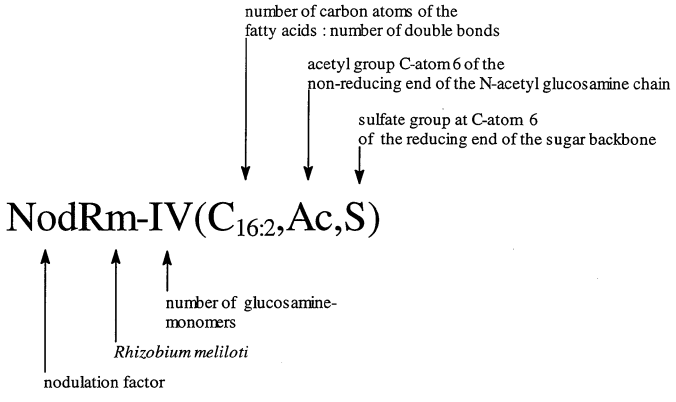

Figure 2. Nomenclature of Nod factors according to the Third Congress of the International Society for Plant Molecular Biology, held on 6–11 October 1991.

O-acetylated. The nomenclature of Nod factors is shown in Figure 2. An aqueous solution of Nod factors applied to the root tips of young alfalfa germs induces the deformation, branching, and curling of root hairs observed, when rhizobial cultures are applied to root hairs [8, 9]. The extent of this reaction depends on the concentration (0.01 μM to 0.01 nM) and the structure of the Nod factor applied [7, 9].

Nod factors have to be produced in adequate amounts in order to enable the action of Nod factors in symbiosis and plant response to be investigated. Fermentation of *Rhizobium meliloti* and separation by liquid extraction has been the method of choice so far for producing Nod factors [4, 7, 10]. The present paper describes the development of novel techniques for Nod factor production and characterization focusing on downstream processing. It shows that the production of Nod factors by *Rhizobium meliloti* is strictly growth associated. The purification of Nod factors is achieved by combining ultrafiltration for biomass removal and adsorption on hydrophobic resins for Nod-factor recovery followed by semi-preparative reversed-phase chromatography. The Nod factors are analysed by liquid secondary ion mass spectrometry (LSIMS) and HPLC of fluorescent 2-aminobenzamide derivatives. The biological activity is screened by means of the root hair deformation (HAD-) assay.

Materials and methods

Organisms

The strongly overexpressing strain *Rhizobium meliloti* R.m. 1021 (pEK327) was kindly supplied by M. Schultze. The plasmid (pEK327) carries a 23.6 kb *Eco*RI-DNA-fragment of the *nod-nif* region, taken from a genomic library of the strain AK631. It comprises the host specific nodulation genes EFGHPQ and the positive regulatory genes for the nodulation-factor synthesis. This fragment was ligated into the 21.6 kb cosmid vector p(LAFR1). The resulting plasmid was introduced into the strain *Rhizobium meliloti* R.m. 1021, a streptomycin-resistent derivative of the wild-type SU 47 [7]. The strain was maintained at 4 °C on TY-agar petri dishes with 5 mg l^{-1} tetracycline added and was transferred every 4 weeks.

Small-scale culture experiments

Small-scale cultures of *R. meliloti* were studied in Erlenmeyer flasks of 500 ml total volume. The flasks were shaken on a rotary shaker at 200 rpm. The media used for these experiments consisted of: mannitol 10 g l^{-1}; disodium succinate 2 g l^{-1}; L-sodium-glutamate 1 g l^{-1}; KH_2PO_4 1 g l^{-1}; $K_2HPO_4 \times 3H_2O$ 1.3 g l^{-1}; $MgSO_4 \times 7H_2O$ 0.125 g l^{-1}; $CaCl_2 \times 2H_2O$ 0.1 g l^{-1}; $KNO_3 \times 3H_2O$ 0.6 g l^{-1}; Fe-(III) citrate $\times H_2O$ 0.01 g l^{-1}; trace elements: plant nodulation medium D [11] 1 ml; (+)-biotin 0.5 mg l^{-1}; and luteolin 286 μg l^{-1}. Tetracycline was added at a concentration of 5 mg l^{-1}. All chemicals were from Fluka, D, except Luteolin (Roth, D), succinate, mannitol, glutamate, trypton and yeast extract (Sigma, D). The cell density was analysed by measuring the optical density of the culture broth at the wavelength of 580 nm (OD_{580}), eventually after appropriate dilution. Samples of 1 ml were taken and clarified by centrifugation at 14 000 rpm for 6 min. The supernatant was directly applied for analytical reversed phase HPLC in order to quantify the concentration of Nod factors.

Batch fermentation

Fermentation experiments were carried out in a bioreactor with a total volume of 30 l (pilot-scale bioreactor, LAB 732, MBR, CH). The fermenter is shown in Figure 3 together with the micro- or ultrafiltration unit (Centrasette GH830, Filtron, D), which served for biomass removal. The medium used was the same as already described above. The fermenter was inoculated with 200 ml of a culture of *Rhizobium meliloti* R.m. 1021 (pEK327) grown for 36 h on a TY-Tc medium. The stirrer speed was adjusted to 500 rpm and the temperature was controlled at 28 °C as was the pH at 6.8. The oxygen supply was controlled by means of the

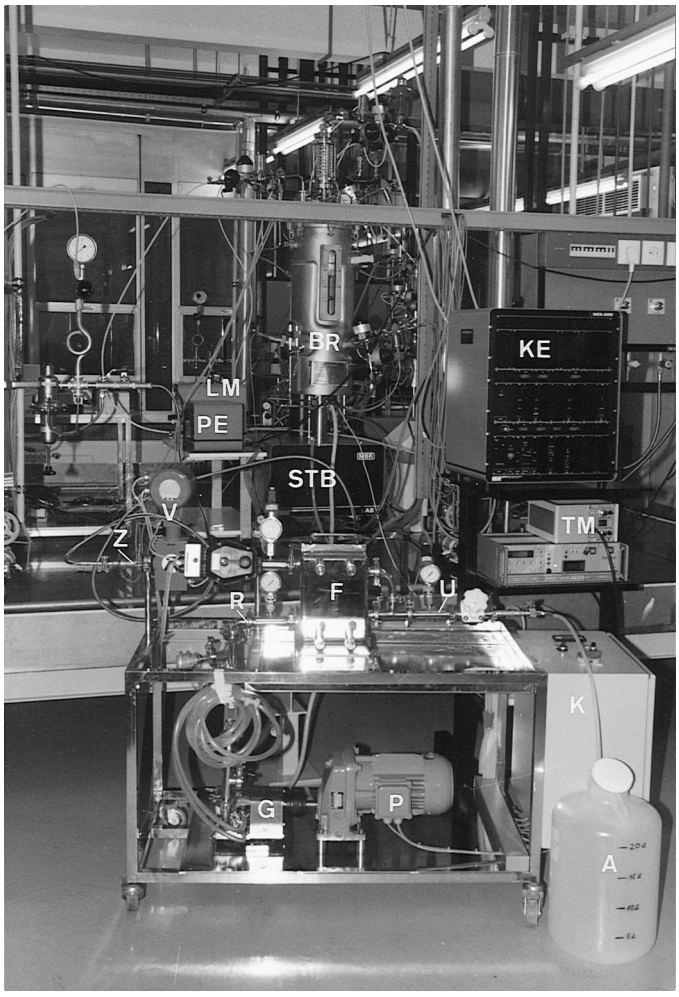

Figure 3. Equipment for batch fermentation experiments. A bioreactor (LAB 732, MBR, CH) of 30 l total volume is coupled with an ultrafiltration unit (Centrasette GH830, Filtron, D) for biomass removal. Abbreviations: **BR**, bioreactor; **STB**, stirrer, thermostat, balance; **PE**, peristaltic pumps for adding acid, base, and antifoam; **TM**, turbidity probe; **LM**, mass-flow meter; **KE**, control unit of the reactor system; **F**, ultrafiltration module; **Z**, inlet line with pressure gauge; **R**, retentate line with pressure gauge; **U**, filtrate line with pressure gauge; **V**, flow meter; **P**, pump motor; **G**, pump head; **K**, control unit; **A**, filtrate tank.

flow rate of air (in the range of 4–6 $N\,\mathrm{min}^{-1}$) by keeping the partial pressure of oxygen in the medium at 90% saturation.

The biomass concentration was followed by measuring the turbidity on-line (type AS82, Aquasant, CH) as well as the optical density (OD_{580}) and the dry-mass concentration off-line. For the determination of dry mass empty Eppendorf tubes (1.5 ml) were equilibrated for 8 h in a desiccator prior to weighing. Three times, 1.5 ml of the fermentation broth was pelleted by centrifugation for 8 min at 14 000 rpm. Each time the supernatant was drained off. The cell mass was dried for 4 h in a block thermostat (5320, Eppendorf, D) at 95 °C, equilibrated again for 8 h in the desiccator before weighing.

Biomass removal by ultrafiltration

The ultrafiltration unit is shown in Figure 3. It consisted of a circulating pump connected to an ultrafiltration module (Centrasette, Filtron, D). A magneto-inductive flow meter indicated the flow rate in the retentate line. The pressure was manually controlled by means of a valve in the retentate line. Experiments were performed at room temperature with two different membranes, Omega Open Channel with a cut-off of 10 kDa or 1 MDa. Two modules of 0.46 m^2 each were used for treating 10 l of fermentation broth each time. The recirculating flow rate was fixed at 500 $l\,h^{-1}$ (pressure in the retentate line = $3.2{\cdot}10^5$ Pa).

Recovery of Nod factors by means of adsorptive resins

Adsorption onto nonspecific hydrophobic resins was used for the primary recovery of Nod factors from the filtrate of fermentation broths. A resin from Toso Haas, XAD-2 pract. (Serva, D), was applied for this purpose. For pre-conditioning, 1 l (800 g wet weight) of the resin was washed twice with 400 ml acetone (p.a., Grüssing, D) followed by equilibration with 400 ml methanol (MeOH) (p.a., Baker, D) and twice with 400 ml water. Prior to adsorption, the filtrate was supplemented with methanol until reaching a concentration of 2%. The resin beads were suspended in 20 l of filtrate by stirring with a propeller at 875 rpm in a PE tank of 25 l capacity and a diameter of 340 mm. The suspension was stirred at room temperature. Samples taken from the tank were treated by centrifugation prior to analysis by analytical reversed-phase HPLC. The resin was separated from the liquid phase by sedimentation and subsequent transfer into a chromatographic glass column. It was washed with 1 l of demineralized water prior to recovering the nodulation factors by elution with 400 ml methanol and 300 ml acetone. Both, the methanol and the acetone fractions were evaporated at 45 °C under reduced pressure in order to reduce the volume to 10 ml.

Semi-preparative reversed-phase LC

Semi-preparative reversed-phase LC was performed by using a fixed bed of 125 ml of the stationary phase Polygoprep 100-30 C18 (Macherey Nagel, D) in a glass column, the length of which was 500 mm and the diameter 20 mm (Latek, D). Samples of the reduced methanol and acetone fractions obtained by elution of the adsorptive resins were applied *via* a Luer-adapter in fractions of 3 ml. Separation was achieved by applying a linear gradient from 20 to 100% of methanol in 120 min and flushing the column with 100% methanol for a further period of 30 min at a flow rate of 2.6 $ml\,min^{-1}$. A UV-Vis detector (type 757, Applied Biosystems, USA) was used for analysing the elution of substances at 220 nm. Eluates appearing in the time window of 90 and 120 min were pooled by means of a fraction collector (FC204, Gilson, D). The pooled eluates were concentrated by evaporation at 45 °C under reduced pressure prior to lyophilization.

Analytical reversed-phase HPLC

The stationary phase Licrospher 100 RP-18 endcapped (E. Merck, D), contained in a steel column of 250 mm length and 4 mm diameter was used for analytical HPLC. The HPLC system consisted of two pumps (type Σ871, Irika, ERC, J), an autosampler (Marathon, Spark, NL), and a UV-Vis detector (type 757, Applied Biosystems, USA). Samples of 20 or 100 µl were applied. The elution was performed by means of a linear gradient of 20–100% of aqueous methanol in 20 min followed by a further period of time of 5 min at 100% methanol at a flow rate of 1.2 ml min^{-1}. Alternatively, the samples were analysed under isocratic conditions with 36% aqueous acetronitrile eluent containing 40 mM ammonium acetate.

HPLC analysis of fluorescence-labelled Nod factors

2-Aminobenzamide (2-AB) (98% +) and borane-dimethylamine-complex (BDA) (p.a.) were purchased from Aldrich, DMSO was from Grüssing and acetic acid (100% p.a.) from E. Merck. 2-AB was crystallized twice from ethanol: water (1 : 1). For derivatisation 5 mg samples of dry preparations of nodulation factors were weighed into screw-topped glass ampoules of 1 ml. 2-AB was taken up in a DMSO:acetic acid mixture (2 : 1) at a concentration of 0.3 M. Four hundred µl of the dye solution was added to the lyophilizates, the ampoules were closed and placed in a thermoblock at 80 °C for 2 h under constant shaking. After 1 h 300 µl BDA in DMSO (2.1 mol l^{-1}) was added. Samples (50 µl) were taken before start of the reaction, after 1 h and 2 h. The samples were stored at − 20 °C and diluted by a factor of 5 : 1000 prior to analysis by reversed-phase HPLC as described above by applying aliquots of 20 µl. Two detectors, a UV-Vis- and a fluorescence detector (RF 551, Shimadzu, J) were used at a wavelength of 220 nm and an excitation wavelength of 330 nm combined with an emission wavelength of 420 nm, respectively.

Liquid secondary ion mass spectrometry

LSIMS was carried out on a Fison's Autospec VG. Samples were prepared from dry preparations of Nod factors in a glycerol (99%, E. Merck, D) matrix. The energy of the primary cesium-ion source was set at 35 keV and the voltage for the acceleration of charged fragments at 8 kV.

Root hair deformation (HAD-) assay

For the HAD assay *Medicago sativa* seeds of the variety 'Du Puits' (Deutsche Saatveredelung, D) were used after having been treated by surface sterilization [11]. Seeds were spread on plant nodulation agar [11] petri dishes and allowed to germinate at room temperature in the dark for 36 h. A number of 4–5 alfalfa sprouts were transferred onto new slant agar petri dishes. These dishes were placed vertically in an incubator for 3 d under temperature cycling of 22 °C (day) and 16 °C (night) at constant humidity of 70% and an illumination period of 16 h per d. A sample of 25 µl containing a concentration of $1.62 \cdot 10^{-5}$ M of Nod factors was applied onto each tip of the young alfalfa germs. The dishes were kept in the incubator for a further period of 24 h under the same conditions described above. For visual inspection the roots of the induced alfalfa seedlings were separated by cutting, transferred into a drop of physiological solution of sodium chloride (0.9%) on a microscope slide protected by a cover slide. Photographic images were taken on an Olympus BH-2 microscope.

Results and discussion

A typical experiment of growing *R. meliloti* in shake flasks is given in Figure 4. Biomass concentration is given in terms of optical density. The concentration of Nod factors was determined by analytical reversed-phase HPLC. It is clearly visible that Nod factor formation is linked to growth.

Batch fermentation in the fermenter leads to similar results as shown in Figure 5. Three different means were used for measuring the development of biomass. Since *Rhizobium meliloti* is a microaerophilic bacterium, fermentations may be performed at constant stirrer speed and relative constant oxygen flow rate. This was required for reliable on-line turbidity measurements. As demonstrated in Figure 5, the turbidity may be a convenient means for estimating the biomass concentration on-line.

The fermentation broth from a batch fermentation experiment characterized by a final optical density of 3.28 was submitted to ultrafiltration applying two different micro- or ultrafiltration membranes – one with a cut-off of 1 MDa and one with 10 kDa. As shown in Figure 6, which contains chromatograms of both filtrates obtained from analytical reversed-phase HPLC, the membrane with the cut-off of 10 kDa was able to reduce the amount of hydrophobic

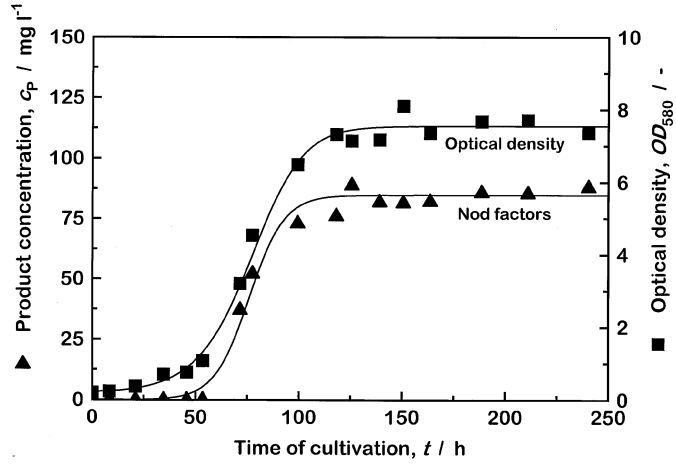

Figure 4. Cultivation of *R. meliloti* in a shake flask.

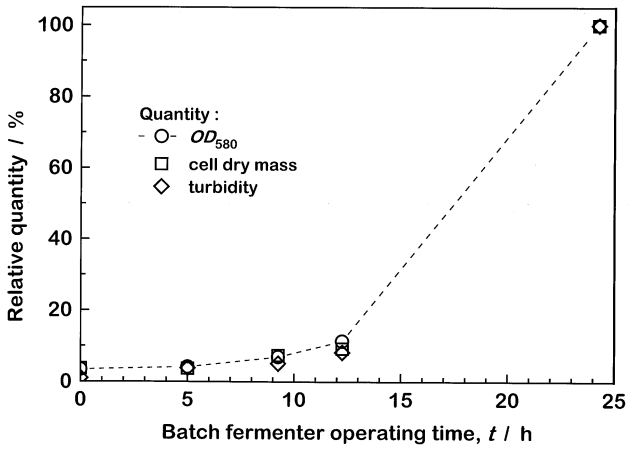

Figure 5. Comparison of the methods for monitoring the growth rate of *Rhizobium meliloti* R.m.1021 (pEK327). The experimental values were normalized with respect to the maximum values of each quantity – turbidity: 10.7 U; cell dry mass: 1.18 g l^{-1}; OD_{580}: 3.28.

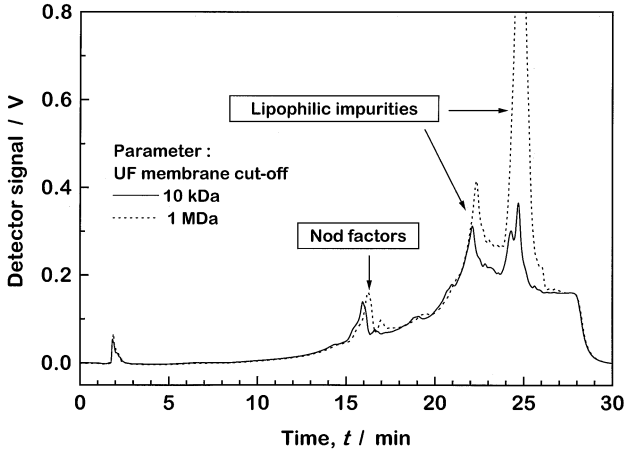

Figure 6. Analytical reversed-phase HPLC of samples from filtrates obtained by treating fermentation broth with membranes of different cut-off. The detector signal corresponds to absorption at 220 nm.

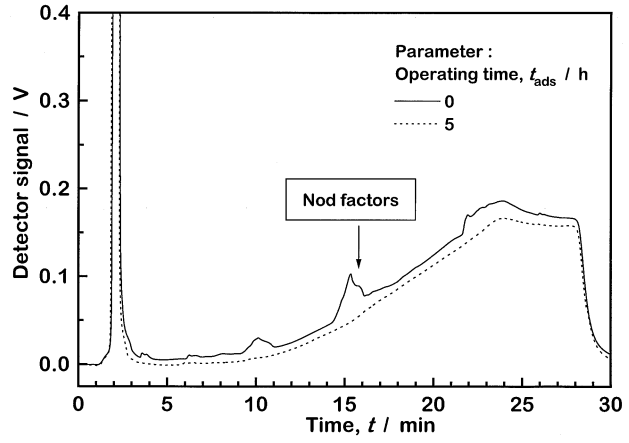

Figure 7. Reversed-phase HPLC analyses at the beginning and the end of the batch adsorption process. The detector signal corresponds to absorption at 220 nm.

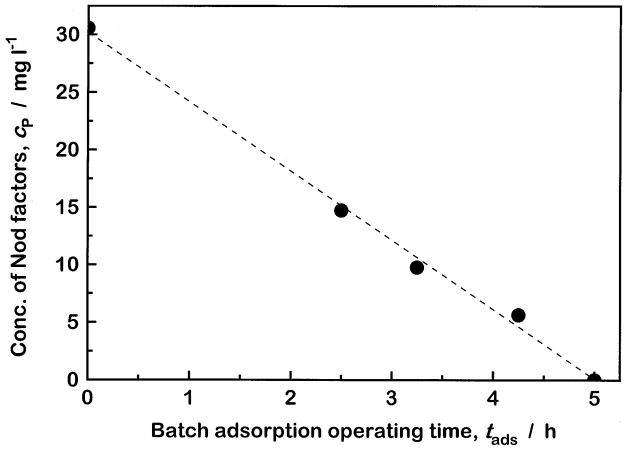

Figure 8. Adsorption kinetics of Nod factors on XAD-2 pract in suspension.

biproducts considerably. Therefore, ultrafiltration may represent an interesting technique for combining biomass removal with an additional purification effect not obtained by simple centrifugation [7, 10]. The absence of competing lipophilic substances had beneficial effects on the following downstream processes. A rough estimation of the detector signals suggested that the content of impurities might be reduced by 70%.

The filtrate obtained from ultrafiltration with the membrane of nominal cut-off of 10 kDa was treated with the hydrophobic resin XAD-2 by suspending the resin in the filtrate. The success of this adsorption process may be seen from Figure 7 showing reversed-phase HPLC analyses of the liquid phase at the beginning as well as the end of the process.

Chromatographic analyses at different operating times revealed the profile of residual Nod factor concentrations given in Figure 8. It shows that the process seems to be characterized by an adsorption kinetics of zero order. This may have been expected, because adsorption on hydrophobic resins like XAD often is limited by the adsorptive reaction. Since a large surplus of resin had been applied, the resin was still far from saturation even at the end of the process. The rate of adsorption was 6.1 mg $^{-1}$ h $^{-1}$. A disadvantage of XAD-2 pract. was the low stability of the resin beads. However, it was stable enough to be used repetitively in stirred batch-adsorption processes. In addition, it was easy to handle and relatively inexpensive favouring its application for large-scale production of Nod factors. After semipreparative RP-chromatography of the methanol and acetone fractions, the collected eluates yielded 0.58 g of Nod

factors from 20 l of fermentation broth, which initially contained 0.75 g, after evaporation of the solvents. The Nod factors thus obtained were subjected to different additional analytical procedures. The liquid secondary ion mass spectrometry (LSIMS) was used in order to assess the different structures contained in the preparations. A typical mass spectrum is shown in Figure 9. For convenience, the expected fragmentation of Nod factors is given in Figure 10.

The signals of the mass spectrum in Figure 9 confirmed that the bacterial strain R.m.1021 (pEK327) produced a whole family of related Nod factors [2, 7, 12]. The pattern of Nod factors synthesized by R.m. 1021 (pEK327) extended from Nod factors with three glucosamine units (Nod-RmIII($C_{16:2}$,Ac,S); *m/z* 926 MH*) to glycolipids with four (Nod-RmIV($C_{16:2}$,S); *m/z* 1126, 1148 MH* + Na*; 2Na*) and five monomers (Nod-RmV($C_{16:2}$,Ac,S); *m/z* 1348 MH*. In addition to the molecule peaks, a broad range of fragments was detected corresponding to the fragmentation pattern, as shown in Figure 10. These results were substantiated by HPLC analysis of the lyophilized products shown in Figure 11. Not only several Nod factors were separated, but also partially their α- and β-anomers. Nod-RmV($C_{16:2}$,Ac,S) and Nod-RmIV($C_{16:2}$,S) could not be separated on the RP-column under the conditions described above because of their similar hydrophobicity resulting in comparable retention times. It might be a point of further investigations, why *R. meliloti* produces these different structures of Nod factors, although it had clearly been demonstrated that the Nod factor NodRmIV($C_{16:2}$,S) exhibits the highest biological activity with respect to alfalfa [7]. Experiments with the R.m. 1021 (pEK327) gave some hints that there might be a time-dependent regulation of the production of the different molecular structures during the growth phase. A possible explanation for the appearance of the different Nod factors might be that *Rhizobium meliloti* once was a symbiont with a broad host range, as experiments with different Nod factors on the non-host plant vetch had demonstrated [13].

For testing the biological activity of the preparation of Nod factors the HAD assay has been employed. The alfalfa germs, inoculated with the lyophilized preparation of Nod factors showed the typical reaction of root hairs – as shown in Figure 12 – commonly observed, when alfalfa root tips are incubated in the presence of *Rhizobium meliloti* [14]. Plants remaining after the HAD assay were placed for a further 14 days in the incubator. A fraction of 65% developed the white, bacteria-free pseudonodules on their roots, as described [15]. These observations proved the biological activity of the purified Nod factors. In addition, it has been shown that they are able to elicit the plant defence system of *Nicotiana tabacum* (see poster ECG-1; Baier, 1995).

A novel analytical technique based on reversed-phase HPLC has been worked out, which leads to a much better resolution of Nod factors. This technique relies on tagging Nod factors with 2-aminobenzamide (2-AB). The resolution of this method may be seen in Figure 13 gathering chromatograms of two tagged samples from *R. meliloti* cultures as well as of one sample of a pure Nod factor. The tagged samples were analysed by fluorescent light emission, whereas UV absorption was used for detecting the pure Nod factor. This procedure based on pre-column fluorescence tagging had a threshold of detection as low as 0.5 μM

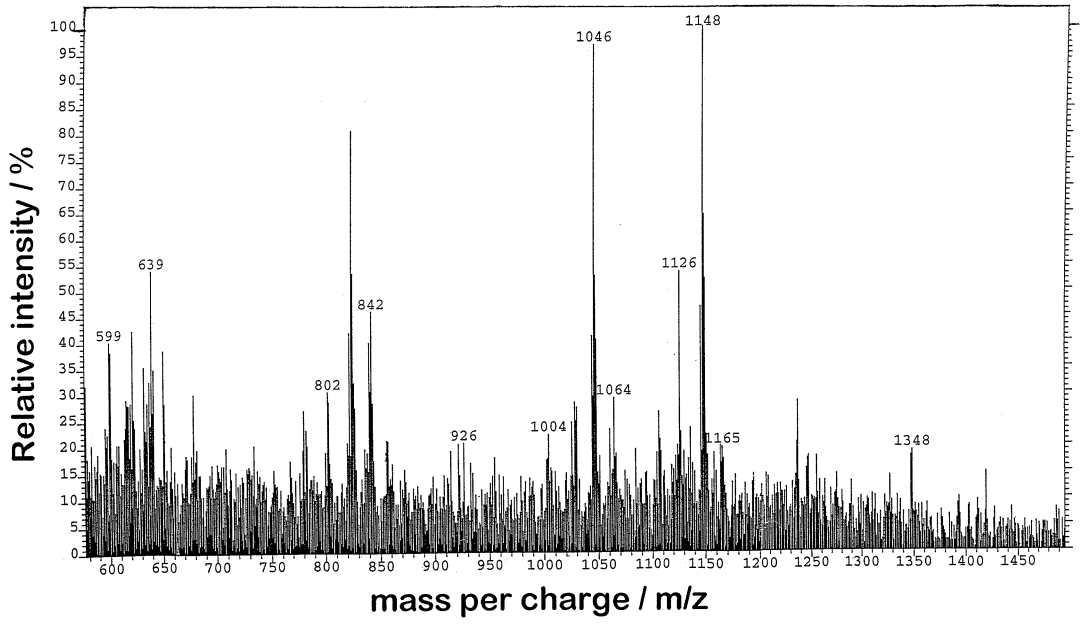

Figure 9. LSIMS in the positive mode of a preparation of Nod factors. The relative abundance of ions is given as a function of the atomic mass of ions.

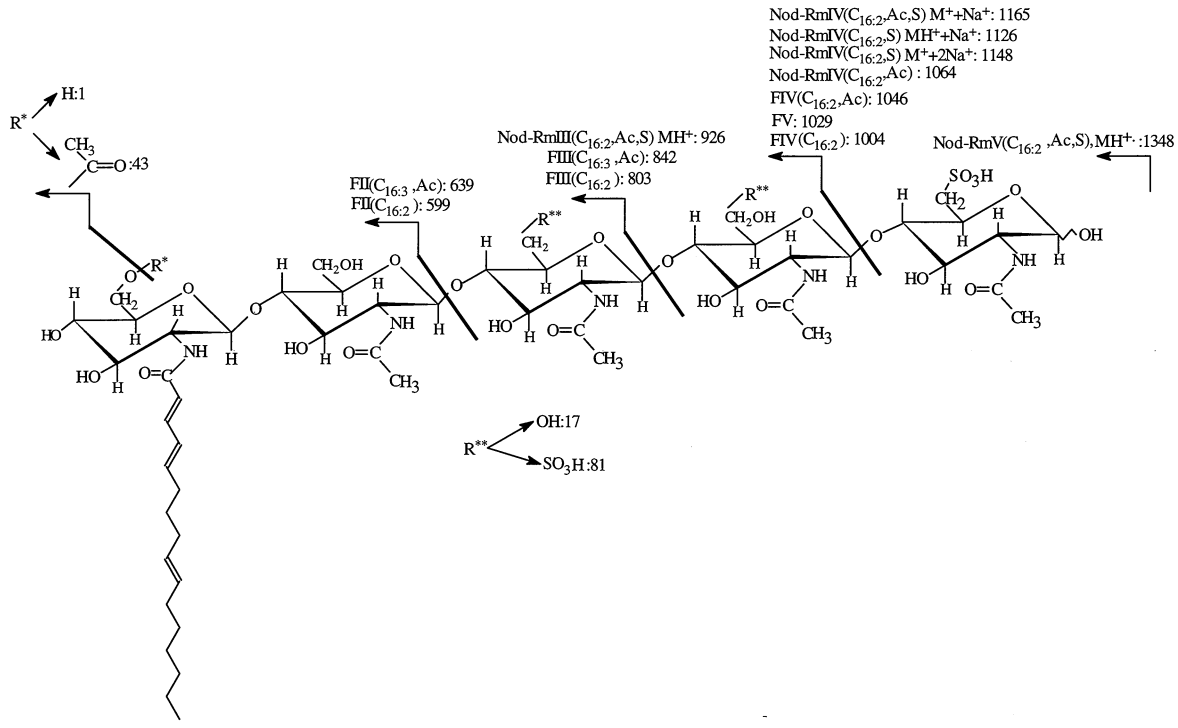

Figure 10. Expected fragmentation pattern of Nod factors under conditions of LSIMS. The arrows indicate potential fragmentation sites. The numbers represent atomic masses of the corresponding ions. For questions of nomenclature see Figure 2. *Abbreviations*: **F**, fragment; **R**, residue; **M**+, molecular ion; **MH**+, protonated molecular ion. Sodium ions (Na^+) are commonly found in mass spectrometry and might produce a single-mass signal composed of the mass of the fragment and the sodium ion.

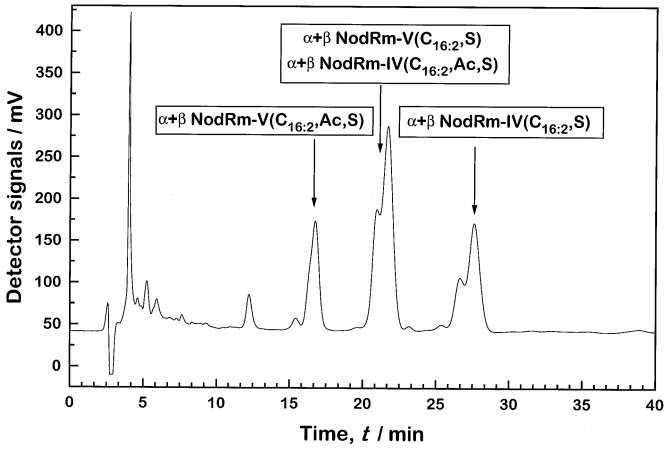

Figure 11. Elution profile of RP-HPLC of the lyophilized Nod-factor preparation under isocratic conditions with 36% aqueous acetonitrile containing 40 mM ammonium acetate. Nod factors (0.1 mg) were dissolved in the eluent. A sample volume of 100 µl was applied. The detector signal corresponds to absorption at 220 nm.

of Nod factors in the samples. The slight shift in the earlier appearance of the fluorescence labelled Nod factor signals in comparison to that of the standard UV-signal is due to the fact, that the labelled structures possess a more polar character and are eluted earlier on the C-18-column. Therefore, this analytical procedure should be a valuable tool for the quantitative analysis of further fermentation experiments as well as for following the distribution of Nod factors in downstream processes. Biological activity tests have to prove, if the fluorescence-active Nod factors are able to induce the genes in the plants that are responsible for the establishment of the symbiosis (host plant) or cell response (non-host plants) in the same way as their native derivatives do. A second step would be the coupling of fluorescence-active biotinylated aminopyridine to the lipooligosaccharides [16] in order to localize the Nod factors over streptavidin-biotin-complexes and monoclonal antibodies in plant cell tissues.

Conclusions

The strictly growth-associated production of nodulation factors by *Rhizobium meliloti* R.m.1021 (pEK327) was demonstrated. The development of the cell density was monitored on-line by means of a turbidity probe. Turbidity did correlate well with optical density and dry biomass concentration. Ultrafiltration of the fermentation broth with membranes of relatively small cut-off did not only remove the biomass, but also retained a large amount of lipophilic substances, thereby improving the performance of

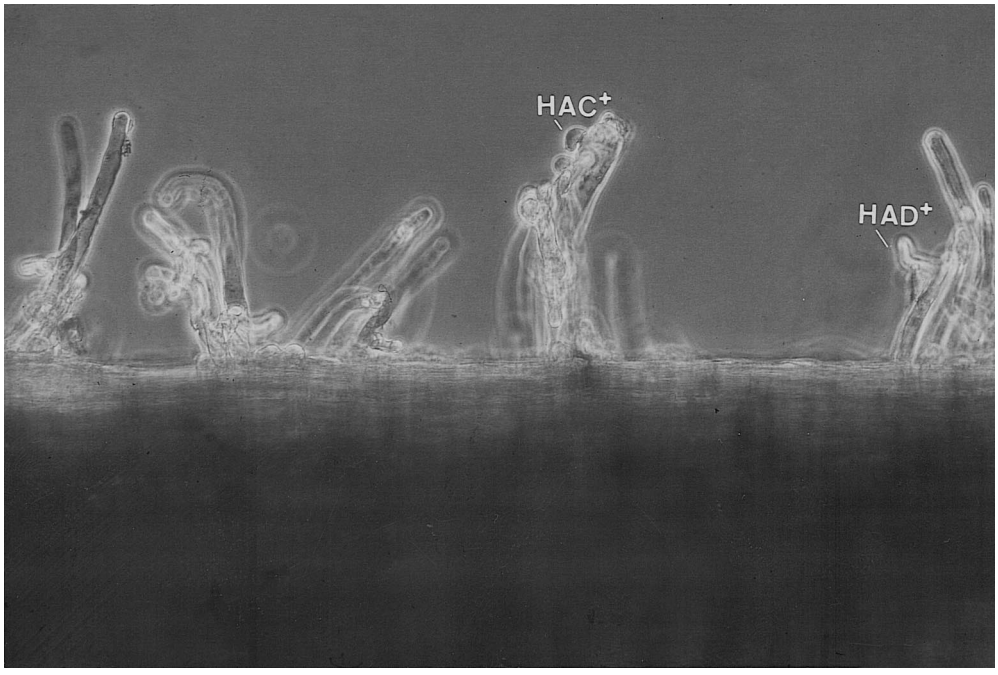

Figure 12. Photograph of the root hairs of a young alfalfa germ induced with 25 µl of an aqueous solution containing 16 µM of Nod factors. The magnification factor is 125. Dejected (deformed) root hairs (HAD^+), curled root hairs (HAC^+) are shown.

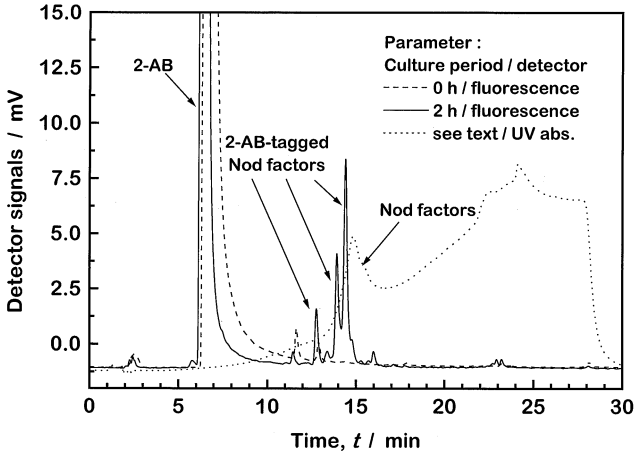

Figure 13. Reversed-phase HPLC of Nod factors and fluorescent 2-AB-tagged Nod factors. Nod factors were detected by absorption at 220 nm. The fluorescent 2-AB-tagged Nod factors were detected by light emission at 420 nm. Samples taken at different times of fermentation are compared with a pure Nod factor NodRm-IV(C16: 2,S), which had not been derivatized with 2-AB.

further purification steps. Nodulation factors have been recovered from the filtrates by adsorption to the hydrophobic adsorptive resin XAD-2. The concentrated and lyophilized preparations of Nod factors obtained exhibited biological activity verified by means of the HAD assay. The novel chromatographic analysis based on fluorescence labelled Nod factors allowed to detect Nod factors in low quantities and may help to identifying different Nod factors produced by *Rhizobium meliloti*. Process improvements are currently under investigation. These are expected from changes in fermentation conditions leading to higher growth rates as well as from novel adsorptive resins exhibiting higher rates of adsorption.

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