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Production of nikkomycin by immobilized *Streptomyces* cells – Physiological properties*

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Summary. The antibiotic nikkomycin can be produced by calcium alginate immobilized mycelium of *Streptomyces tendae* Tü 901 in batch and continuous culture.

Scanning electron micrographs show the porous structure of the matrix and the distribution of the cells in the gel.

Some physiological properties of free and immobilized mycelia were compared. Immobilization does not change the relative amounts of nikkomycin compounds in the culture broth. DNA and protein content were the same in free and immobilized cells. The specific activity of fructosediphosphate aldolase dropped during fermentation and was lower for entrapped than for free cells. The specific activity of mannitol dehydrogenase increased up to the end of the fermentation and was the same for free and immobilized mycelium.

In continuous culture the relative amount of mannitol consumed decreased with increasing flow rate. When the medium was supplemented with amino acids mannitol consumption increased significantly.

Introduction

Although the immobilization of cells has been intensively investigated few reports have been published on the production of antibiotics, secondary metabolites that are produced via multistep pathways.

Candicidin (Venkatasubramanian and Vieth 1979), penicillin (Morikawa et al. 1979a) and cephalosporins (Freeman and Aharonowitz 1981) were produced with immobilized cells in batch culture. Morikawa et al. (1979b, 1980) reported about the production of bacitracin by immobilized *Bacillus* sp. in batch and continuous fermentation. Deo and Gaucher (1983) published the semi-continuous fermentation of patulin by immobilized cells of *Penicillium urticae.*

In previous papers (Veelken and Pape 1982, 1983) we described the production of the nucleoside peptide antibiotic nikkomycin (Dähn et al. 1976) with mycelium of *Streptomyces tendae* Tü 901 immobilized in calcium alginate gel. Besides batch fermentation continuous production was carried out in an air-bubbled reactor. Continuous fermentation could be performed for more than 640 h (Veelken and Pape 1983). The production rate increased linearly with increasing flow rate of the medium. Addition of L-isoleucine and L-leucine increased the production rate significantly.

In this paper some physiological properties of nikkomycin production with free and immobilized cells are described.

Materials and methods

Organism, growth conditions and immobilization. Streptomyces tendae Tü 901 was cultivated as described recently (Veelken and Pape 1982).

The cells were immobilized by entrapment into calcium alginate gel (Veelken and Pape 1982).

Determination of nikkomycin compounds. The compounds of nikkomycin fermentation were determined by hplc with a Spectra-Physics liquid chromatograph Model SP 8000-10, a Spectra-Physics autosampler Model SP 8010 and a Schoeffel variable wavelength detector Model SF 770. A reversed-phase column packed with Octodecylsilan (particle size 5 µm) of dimensions 125 × 4.6 mm I.D., supplied with a pre-column of dimensions 10 × 4.6 mm I.D. was obtained from Shandon. Solvent A was 10 mmol/l hexansulfonic acid containing 2 ml/l acetic acid. Solvent B consisted of four volumes of demineralized water and six volumes of acetonitrile with 10 mmol/l hexansulfonic acid and

* Dedicated to Prof. Dr. L. Acker on the occasion of his 70th birthday

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2 ml/l acetic acid. For the elution of nikkomycins solvent A and B were mixed according to a time program:
0–9 min: 85% A + 15% B; 9–10 min: 55% A + 45% B; 10–14 min: 85% A + 15% B.

The flow rate was set to 2 ml/min and the wavelength to 290 nm. The fermentation broth was centrifuged and a 10-µl sample of the supernatant injected onto the column.

Determination of mannitol. D-mannitol was determined by hplc with a Hewlett-Packard liquid chromatograph Model 1084 B with terminal 79850 B and a refractive index detector. The column (dimensions 200 × 4 mm I.D.) and pre-column (dimensions 30 × 4 mm I.D.) were packed with Lichrosorb NH_2 (particle size 10 µm). The mobile phase was acetonitrile and destilled water (80/20; v/v). The flow rate was set to 1 ml/min.

A 10-µl sample of the centrifuged fermentation broth was injected onto the column.

Determination of DNA. DNA was determined according to Herbert et al. (1971).

Determination of protein. Protein was determined according to Bradford (1976).

Preparation of cell-free extracts. Mycelium was harvested by centrifugation and washed three times with tap water.

Immobilized mycelium was detached from the matrix by solubilizing the calcium alginate gel with 2% sodium hexametaphosphate.

The cells were resuspended in one volume of 0.1 mol/l tris-(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5) containing 1 mmol/l mercaptoethanol. The suspension was sonicated (Branson Sonifier B-12, microtip) in an ice-bath eight times in 15-s intervals. The resulting suspension was centrifuged for 20 min at 0° C. The supernatant was used as enzyme source.

Enzyme assays. Fructosediphosphate aldolase (E.C. 4.1.2.13) was determined according to Bergmeyer (1970).

NAD-dependent mannitol dehydrogenase (E.C. 1.1.1.67) was measured by a modified procedure according to Mehta et al. (1977). The assay mixture consisted of 350 µl Tris-HCl buffer (pH 7.5; 0.1 mol/l), 50 µl NAD (10 mmol/l), and 50 µl of cell-free extract. As substrate 50 µl of D-mannitol (500 mmol/l) were added. The change of adsorbance at 340 nm was followed with a Zeiss PM 6 spectrometer at 25° C.

The assays of other enzymes of mannitol metabolism (mannitol kinase, mannitol-1-phosphate dehydrogenase and NADP-dependent mannitol dehydrogenase) were performed according to Mehta et al. (1977).

Electron microscopy. Samples for electron microscopy were quickly frozen in liquid nitrogen. The frozen gel particles were split with a scalpel. Under permanent cooling with nitrogen the surface of the particles was coated with gold. The samples were analyzed with a scanning electron microscope PSEM 505 (Philips).

Results and discussion

Scanning electron microscopy

Figures 1–2 show electron micrographs of calcium alginate immobilized mycelium.

Calcium alginate gel itself has a porous structure that resembles a sponge (Figs. 1, 3). The filaments of the mycelium are inserted in this porous matrix.

The surface of the gel particles is coated by a membrane-like layer of calcium alginate (Fig. 2). This membrane results from the contact of the sodium alginate drop with the calcium chloride solution at the beginning of the formation of the gel.

Mycelium covers the surface of the gel particles as well as the layer just beyond the surface (Fig. 2). As there were no free cells detected in the supernatant fluid the filaments of the mycelium seem to be well attached to the surface by the membrane-like layer of calcium alginate.

Quantitative distribution of nikkomycin compounds

The relative amounts of nikkomycin compounds in the fermentation broth were analysed in batch cultures with immobilized and free cells (Fig. 4).

During batch fermentation with free cells the amount of biologically inactive nikkomycins C and D increased up to 54%, up to 40% in cultures with immobilized mycelium. The main increase was observed for nikkomycin C, whereas nikkomycin D was produced in a constant amount of 7%.

The amounts of biologically active nikkomycins Z, X, and I reached a constant proportion after 48 h that was the same for free and immobilized cells: Z : X : I = 16 : 57 : 27.

In continuous nikkomycin production the amount of biologically inactive nikkomycins C and D reached about 48–50% (Fig. 5A). This is somewhat more than in batch cultures with immobilized cells. Nikkomycin D was again produced in a constant amount of 7%.

The biologically active nikkomycins Z, X, and I were synthesized nearly in the same ratio as in batch culture (Fig. 5B). The constant proportion was Z : X : I = 16 : 54 : 30.

The continuous flow of the medium, therefore, does not seem to have any influence on the quantitative relation of the nikkomycin compounds in the fermentation broth.

DNA and protein content of the cells

If one takes into consideration that there is a relatively great tolerance in the determination of protein there could not be found a significant dependence of the DNA and protein content of the cells upon the period of fermentation. There was also no significant difference between free and immobilized mycelium.

Fructosediphosphate aldolase (E.C. 4.1.2.13)

As an enzyme of primary metabolism the activity of fructosediphosphate aldolase was determined in

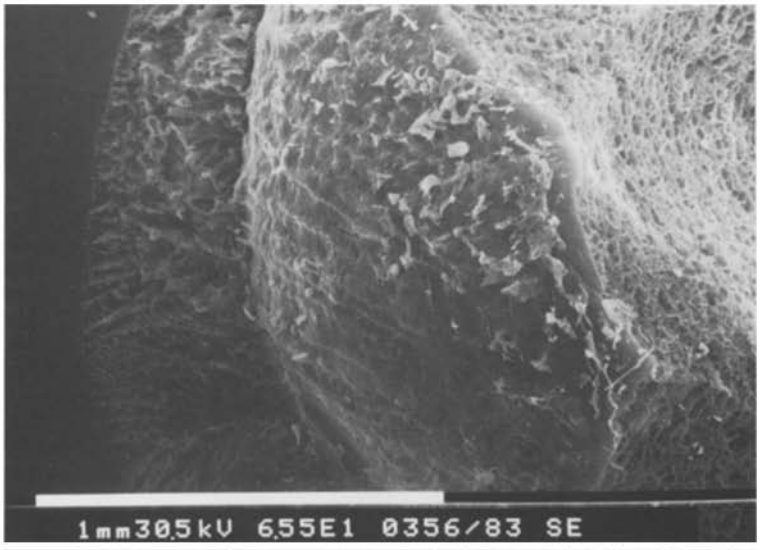


Fig. 1. Surface of a gel particle and surface of the fracture

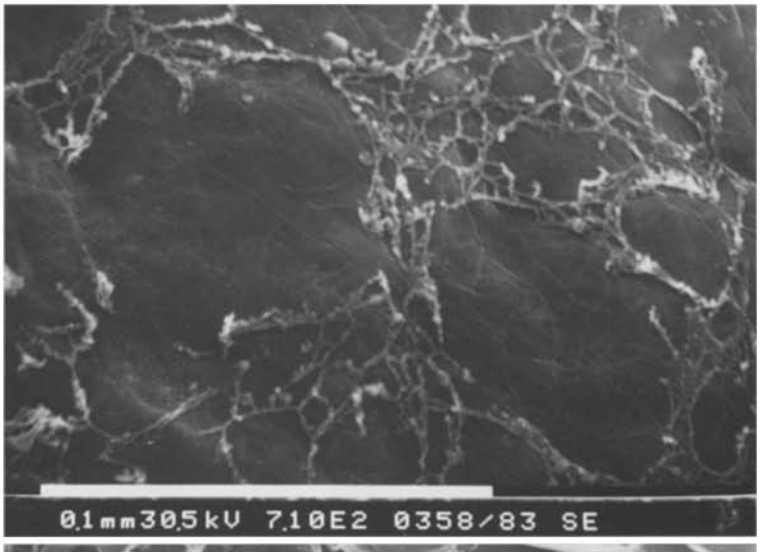


Fig. 2. Surface of gel particle with mycelium

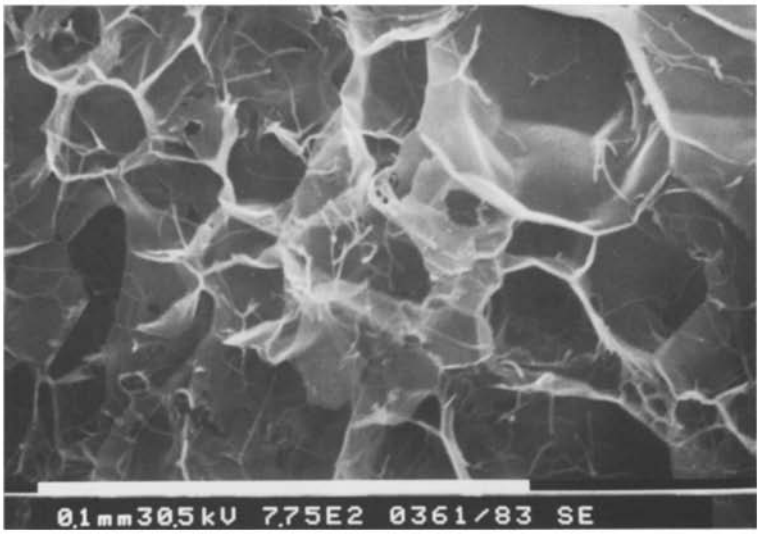


Fig. 3. Center of the gel particle

Figs. 1–3. Scanning electron microscopy of calcium alginate immobilized mycelium of *Streptomyces tendae* Tü 901

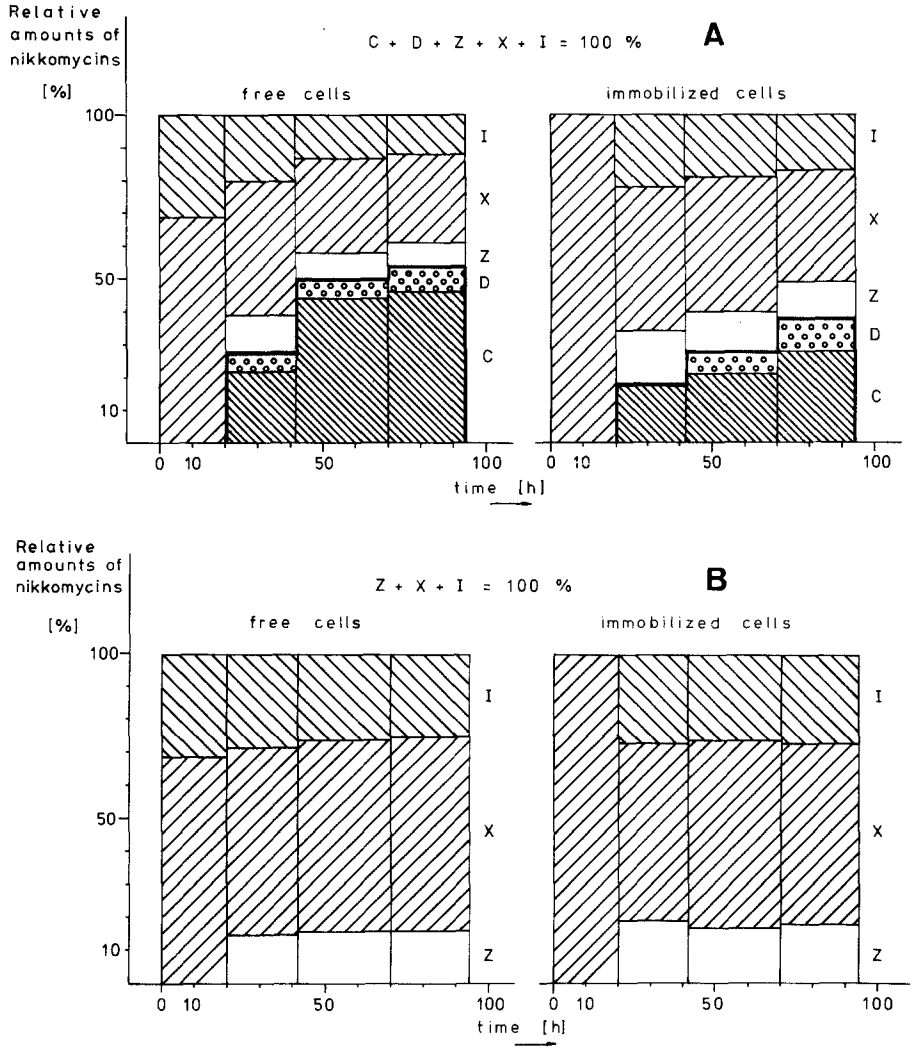


Fig. 4 A, B. Quantitative distribution of nikkomycin compounds in batch cultures with free and immobilized mycelium at different times of fermentation.
A 100% = nikkomycins C + D + Z + X + I.
B 100% = nikkomycins Z + X + I

batch cultures with free and immobilized mycelium (Fig. 6).

The specific activity of fructosediphosphate aldolase decreased rapidly during the fermentation period from 24 to 44 h and remained constant up to the end of the fermentation. Parallel to the smaller amount of nikkomycins produced by immobilized cells the activity of the enzyme was only 60% that of free mycelium after 44 h of fermentation.

Mannitol dehydrogenase (E.C. 1.1.1.67)

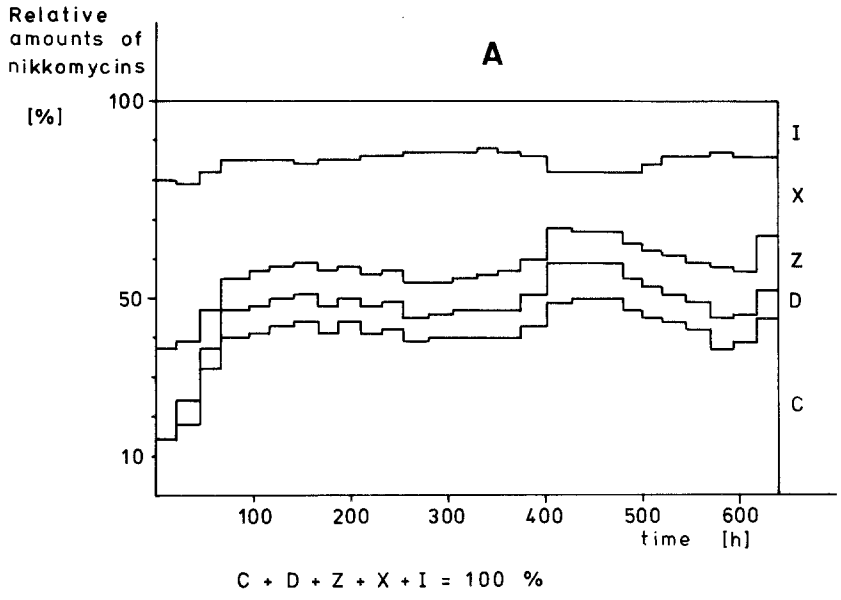
Because D-mannitol was the best substrate for nikkomycin synthesis the activity of the NAD-dependent mannitol dehydrogenase was determined. Activities of other enzymes known to be involved in alternative pathways of mannitol metabolism such as mannitol kinase, mannitol-1-phosphate dehydrogenase and NADP-dependent mannitol dehydrogenase could not be detected in *Streptomyces tendae* Tü 901.

In a complex growth medium (Veelken and Pape 1982) the specific activity of mannitol dehydrogenase of free cells was well correlated to mannitol utilization of the cells and to nikkomycin production (Fig. 7). The specific activity of mannitol dehydrogenase begins to increase slowly after 45 h of fermentation when the growth of the mycelium is nearly finished and the cells begin to utilize mannitol as source for nikkomycin synthesis.

In resting cell cultures with free and immobilized cells the specific activity of mannitol dehydrogenase increases rapidly during fermentation and there was no significant difference between free and immobilized mycelium.

Mannitol utilization by immobilized cells in continuous culture

In continuous culture mannitol utilization was dependent upon the flow rate of the medium. The



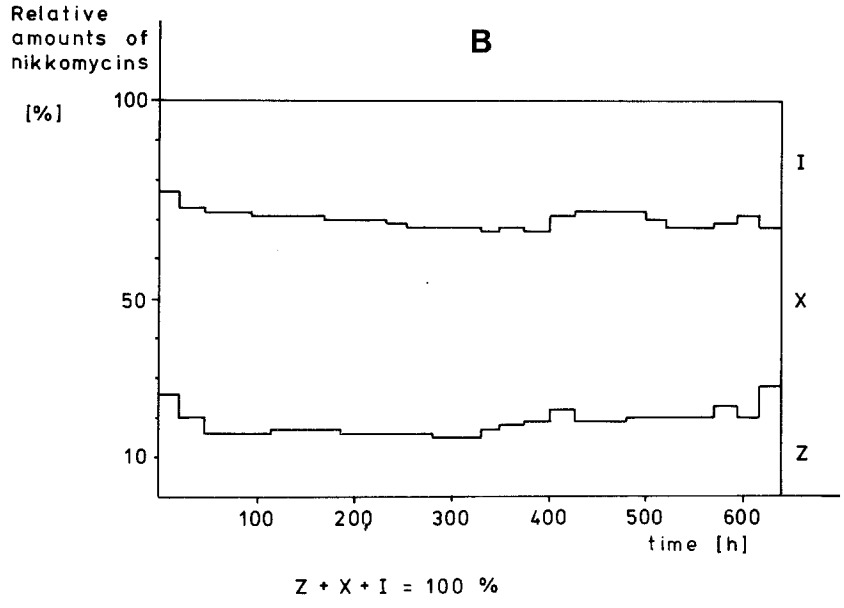


Fig. 5 A, B. Quantitative distribution of nikkomycin compounds during continuous fermentation with mycelium immobilized in calcium alginate gel.
A 100% = nikkomycins C + D + Z + X + I.
B 100% = nikkomycins Z + X + I

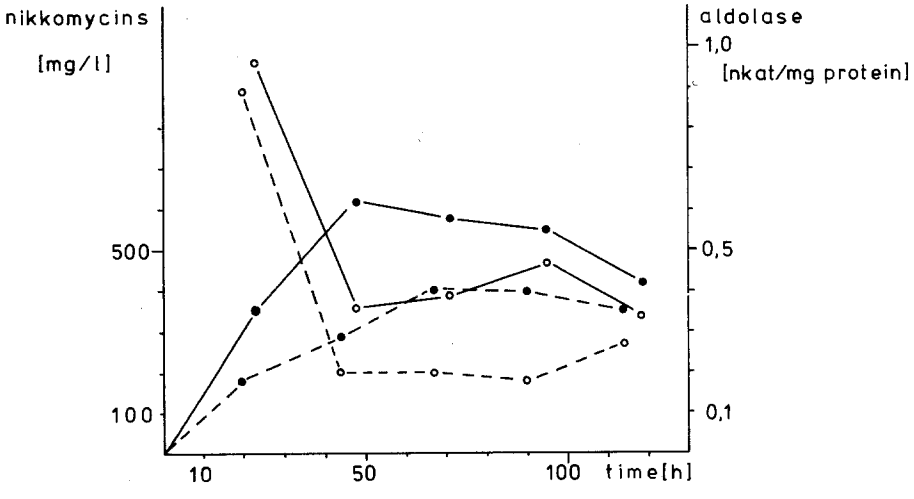


Fig. 6. Specific activity of fructosediphosphate aldolase and nikkomycin synthesis in batch cultures with free and immobilized mycelium. [1 nkat = 10^{-9} kat (Katal)]. ●———● nikkomycin, free cells; ○———○ aldolase, free cells; ●– – –● nikkomycin, immobilized cells; ○– – –○ aldolase, immobilized cells

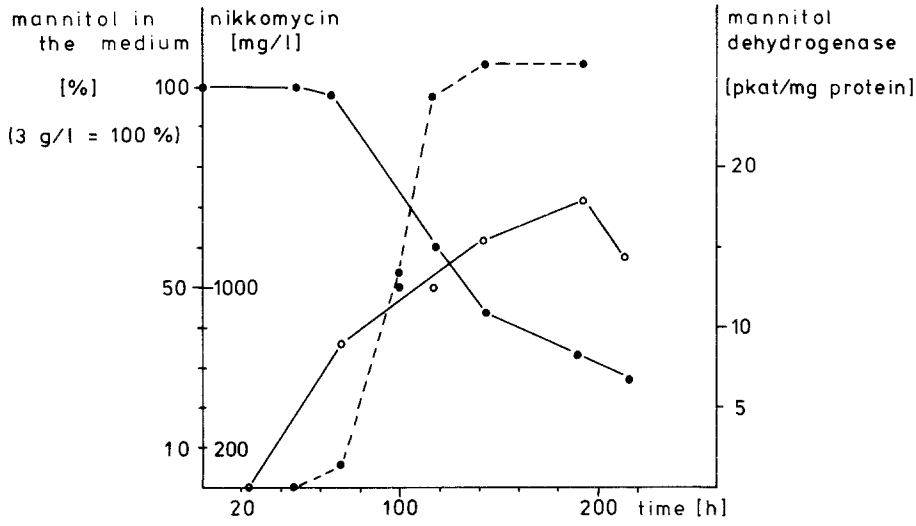


Fig. 7. Mannitol consumption, nikkomycin production and specific activity of mannitol dehydrogenase in a complex growth medium with free cells of *Streptomyces tendae* Tü 901. [1 pkat = 10^{-12} kat (Katal)]. ●———● mannitol (100% = 3 g/l); ○———○ nikkomycin; ●– – –● mannitol dehydrogenase

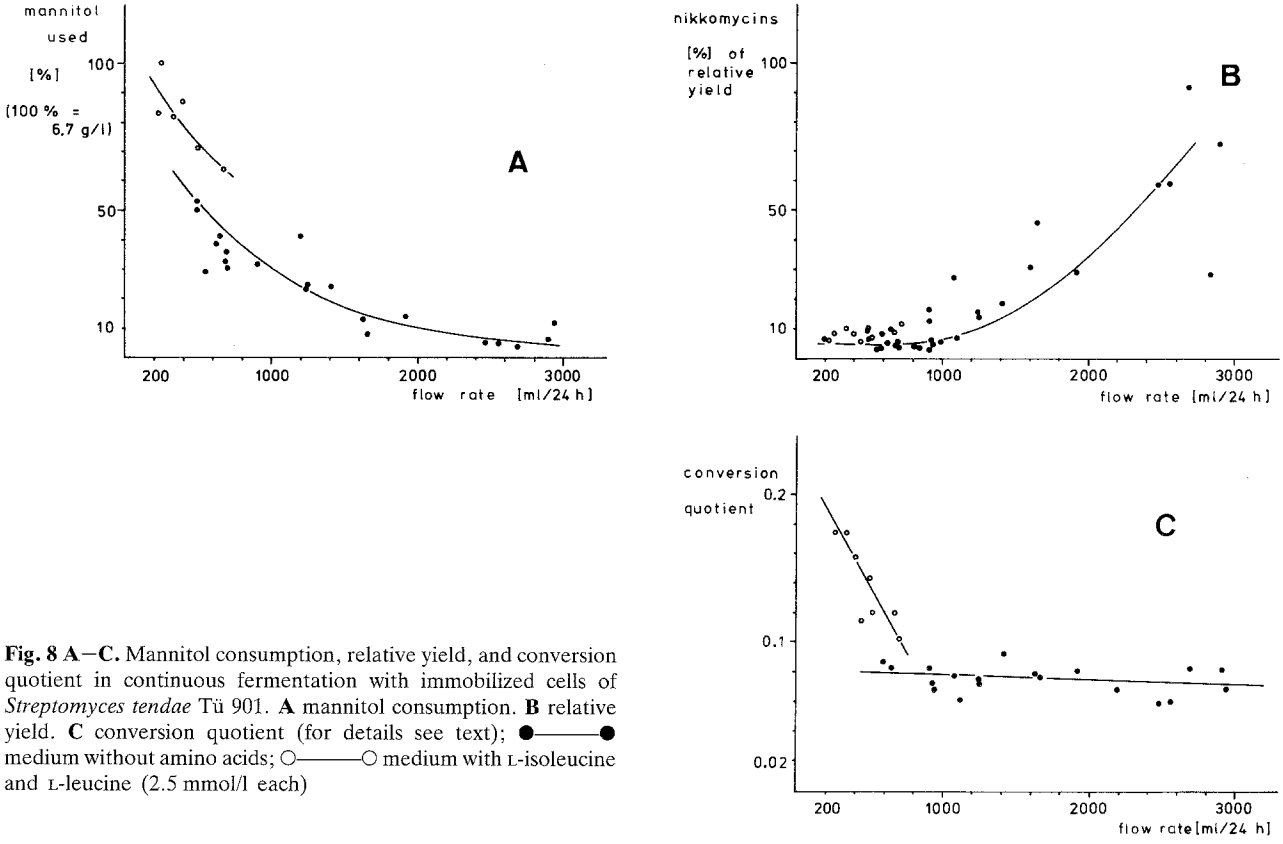


Fig. 8 A–C. Mannitol consumption, relative yield, and conversion quotient in continuous fermentation with immobilized cells of *Streptomyces tendae* Tü 901. **A** mannitol consumption. **B** relative yield. **C** conversion quotient (for details see text); ●———● medium without amino acids; ○———○ medium with L-isoleucine and L-leucine (2.5 mmol/l each)

relative amount of mannitol consumed decreased with increasing flow rate (Fig. 8A). At a flow rate of 2 l medium in 24 h (dilution rate = 0.21 h^{-1}) only 10% of D-mannitol offered were consumed by the cells. The specific mannitol consumption, i.e., the amount of substrate consumed per hour and cellmass, remained approximately constant (70 mg mannitol per hour with the cellmass content of the reactor used in these experiments). Nikkomycin production rate, however, increased linearly with increasing flow rate (Veelken and Pape 1983).

Supplementation of the medium with L-isoleucine and L-leucine increases mannitol consumption significantly (Fig. 8A). Parallel, nikkomycin production rate is also increased by addition of amino acids (Veelken and Pape 1983).

The amount of nikkomycin produced in relation to mannitol consumed (= relative yield) is therefore

the same for a medium without and with supplementation with amino acids (Fig. 8B). Surprisingly the relative yield of nikkomycins increased with the flow rate of the culture medium.

Calculation of the amount of mannitol carbon transferred to nikkomycin carbon relative to the total amount of mannitol offered (= conversion quotient) shows that this relation is independent from medium flow rate (Fig. 8C). Supplementation of the medium with L-isoleucine and L-leucine increases the quotient; under these conditions, the quotient becomes dependent on the flow rate.

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