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Optimization of fermentation conditions for the production of ethylene-diamine-disuccinic acid by *Amycolatopsis orientalis*

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The production of EDDS (ethylene-diamine-disuccinic acid), a potential substitute for EDTA, has been optimized up to a product concentration of 20 grams per litre in fermentations of *Amycolatopsis orientalis*. Decisive steps for the increase in productivity were variation of the synthetic medium composition, investigation of the influence of metal ions on product formation, controlled feeding of carbon and nitrogen sources in fed-batch fermentations and improvement of the downstream processing steps.

Keywords: *Amycolatopsis orientalis*; EDDS; cation chelator; synthetic medium; fed-batch fermentation; metal ions

Introduction

S,S-EDDS (ethylene-diamine-disuccinic acid) is a cation chelator isomeric to EDTA (ethylene-diaminetetraacetic acid) and was found during screening to be an inhibitor of phospholipase C [15]. The actinomycete strain *Amycolatopsis orientalis* produces EDDS exclusively in the biodegradable S,S-configuration [9]. It is a possible substitute for the almost undegradable substance EDTA (Figure 1), which is a chelator with extensive industrial application [10]. In addition to cleaning processes for tank and tube systems, EDTA is used in the pharmaceutical, paper and cosmetic industries. Due to the persistence of EDTA [2,6], biodegradable alternatives like nitrilo-triacetic acid (NTA), gluconic acid, citric acid and EDDS have been tested. With the exception of EDDS, all these substances have to be rejected because of their lower capacity for complexing cations, eg in washing agents [14,16]. A further advantage of S,S-EDDS is its low toxicity [14].

Preliminary investigations have shown a strong influence of trace elements such as Fe(III), Fe(II), Cu(II) and Zn(II) ions on the production of EDDS [3]. Zinc concentrations in the region of 3 μM totally inhibit EDDS formation without significantly affecting growth rate and biomass formation [4]. Since commonly used media components like yeast extract, meat extract and peptone contain various trace elements at similar concentration ranges it was necessary to use a defined synthetic medium for all investigations. The effect of carbon, nitrogen and inorganic phosphate sources as well as cultivation method were studied as these

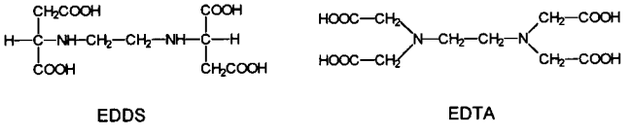

Figure 1 Structures of EDDS and EDTA.

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are known to have a strong impact on cell growth and product formation [1,8,12,19].

In view of the influence of downstream processing to overall process efficiency, it is necessary to optimize the purification steps also. Besides investigations on the composition of a synthetic production medium and feeding strategies, this paper deals with the improvement of the isolation procedure of S,S-EDDS.

Materials and methods

Organism, media and growth conditions

The actinomycete strain *Amycolatopsis orientalis* MG417-CF17 [15] was used in all investigations. The strain was stored as a lyophilisate at room temperature. Stock cultures were grown on agar plates (glycerol 20 g L^{-1}, soybean meal 20 g L^{-1}, agar agar 15 g L^{-1}; pH 7.5) and incubated for 4–5 days at 27°C. This stock culture could be used for about 2 months to inoculate 500-ml Erlenmeyer flasks with one baffle, containing 100 ml of a complex pre-culture medium, consisting of glycerol (20 g L^{-1}) and soybean meal (20 g L^{-1}) at pH 7.5. The cultures were grown for 48 h on a rotary shaker (RC 106, Infors, Basle, Switzerland) at 120 rpm and 27°C. Five per cent of the pre-culture was used to inoculate 500-ml Erlenmeyer flasks under the same conditions, containing a synthetic medium. For fermentation studies, this step was used as a second pre-culture. The fermenters were inoculated with 5% inoculum from the shaken cultures.

The optimized synthetic medium consisted of glycerol 25 (g L^{-1}), urea (1.8 g L^{-1}), KH_2PO_4 (8 g L^{-1}), $Na_2HPO_4 \cdot 2H_2O$ (12 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (1.2 g L^{-1}), Fe(III)citric acid (60 mg L^{-1}) and $FeSO_4 \cdot 7H_2O$ (556 mg L^{-1}). As an alternative to urea, glutamic acid (8.83 g L^{-1}) or $(NH_4)_2HPO_4$ (3.96 g L^{-1}), was used to provide a total nitrogen concentration of 60 mM. Glycerol and $MgSO_4$ were sterilized separately and added to the medium before inoculation. To prevent foam formation, small quantities of Ucolub N 115 (Fragol, Mulheim, Germany) were added at regular intervals. For fed-batch fermentations various feeding solutions were applied:

Feeding solution I: glycerol (165 g L^{-1}), glutamic acid

(47 g L^{-1}), KH_2PO_4) (8 g L^{-1}), $Na_2HPO_4 \cdot 2H_2O$ (12 g L^{-1}) and Fe(III)citric acid (60 mg L^{-1}).

Feeding solution II: glycerol (165 g L^{-1}), urea (6 g L^{-1}), glutamic acid (59 g L^{-1}), KH_2PO_4 (8 g L^{-1}), $Na_2HPO_4 \cdot 2H_2O$ (12 g L^{-1}) and Fe(III)citric acid (60 mg L^{-1}).

The glycerol, urea and glutamic acid were sterilized separately for all feeding solutions and added aseptically to the medium before inoculation. To remove trace elements, the flasks and fermenters were treated first with 0.5 M HCl, and then with 5 mM EDTA for 12 h each, before medium components and distilled water were added.

Fermentations

Batch and fed-batch cultivations were carried out at 27°C in stirred tank reactors (glass tanks) with a working volume of 8–10 L (Biostat E, B Braun Biotech Int, Melsungen, Germany). The aeration was kept constant at 0.5 vvm, and agitation was 300 rpm. The pH was adjusted to 6.8 and kept constant during the fermentation processes by addition of 3 N NaOH and 3 N H_2SO_4. Feeding start and harvesting time are reported in the results.

Analytical methods

For determination of the biomass concentration, 1 ml fermentation broth was centrifuged for 5 min at $14\,900 \times g$ (Biofuge, Heraeus Sepatech, Osterode, Germany). The sediment was dried to constant weight at 100°C. The EDDS concentration was determined in the supernatant by HPLC according to the method of Cebulla [3].

For determination of glycerol, an HPLC method was applied as reported by Plaga *et al* [17], using a polymer-coated amino-phase column (Capcell NH_2, 5 μm, 125×4.6 mm; Grom, Herrenberg, Germany) and an isocratic pump system (HP 1050, Hewlett-Packard, Waldbronn, Germany). The supernatant (10 μl) was injected onto the column and separated with acetonitrile/H_2O (75 : 25) at a flow rate of 1 ml min^{-1}. The eluate was monitored using a refractive index detector (HP 1037 A, Hewlett Packard) at 40°C. Water was purified by means of a Milli-Q-system (Millipore, Eschborn, Germany).

The concentration of glutamic acid was determined quantitatively by reversed-phase HPLC after labelling the supernatant with dimethylaminoazobenzenesulphonyl chloride (DABS-C1) according to the method of Chang *et al* [5].

The ammonium concentration was determined off-line using gas-sensitive electrode (type 701, Orion, Cambridge, MA, USA). Urea was determined using a test kit (No. 542 946, Boehringer Mannheim, Mannheim, Germany).

Isolation and purification

Celit (4%) was added to the fermentation broth which was separated by multiple sheet filtration (filter type C 250 AF, Schenk Filterbau, Schwabisch Gmund, Germany) into culture filtrate and mycelium cake. EDDS concentrations higher than 2 g L^{-1} were precipitated by slow addition of HCl (conc.) to a pH of 1.5 and cooling to 4°C. After 12 h acidic treatment EDDS was removed by filtration. Further purification was achieved by resuspending the dried EDDS in ethanol (70%) at 78°C; 30 ml ethanol were added per gram EDDS. Pure EDDS was obtained by a repeated recrystallisation from water at pH 1.5.

Results

Optimization of the medium composition

Preliminary results have shown that EDDS production was best using glycerol as carbon and energy source [3]. For the optimization of the initial concentration of glycerol, a computer program based on genetic algorithms for multi-parameter optimization was used [7,11]. The initial substrate concentration was varied in linear steps between 0 and 50 g L^{-1}. The optimal glycerol concentration with respect to product formation was found to be 25 g L^{-1}, which was chosen for further studies.

Experiments in shake flasks gave best results using KH_2PO_4 as phosphate source. Surprisingly, the highest EDDS production was found at high concentrations of KH_2PO_4, although the product does not include phosphate or phosphorus in the molecule. As can be seen from Figure 2, KH_2PO_4 concentrations of 13 g L^{-1} or more, resulted in a stable EDDS formation, whereas lower initial concentrations led to a significant drop in production.

In contrast to carbon and phosphate, various nitrogen sources were suitable for the production of EDDS. Best results were obtained with urea, glutamic acid and ammonium phosphate. For each nitrogen source, measurements showed an optimal EDDS production with an initial concentration of 60 mM nitrogen. Supplementation with additional amino acids had no influence on biomass or production yield.

Due to the repression of EDDS formation by zinc, it was investigated whether this effect could be reduced or replaced by addition of other cations. Figure 3 demonstrates the impact of low cation concentrations. The growth requirement of the organism for iron was served by Fe(III)citric acid. Using Fe(III) ions in citric acid, the iron is masked and can pass the cytoplasmic membrane without interacting with EDDS. In addition to the effect of zinc, which was expected from results of Cebulla and coworkers [4], an influence of divalent iron (Fe(II)) was determined (Figure 3). A simultaneous variation of both parameters, Zn(II) and Fe(II), showed a slight increase in product for-

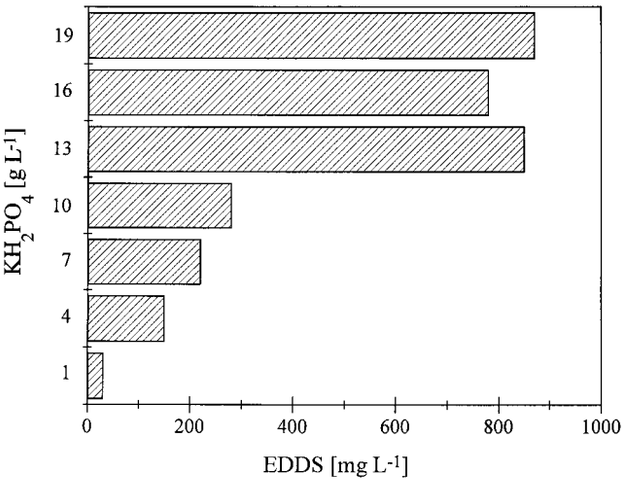

Figure 2 Effect of phosphate concentration in the medium on EDDS production.

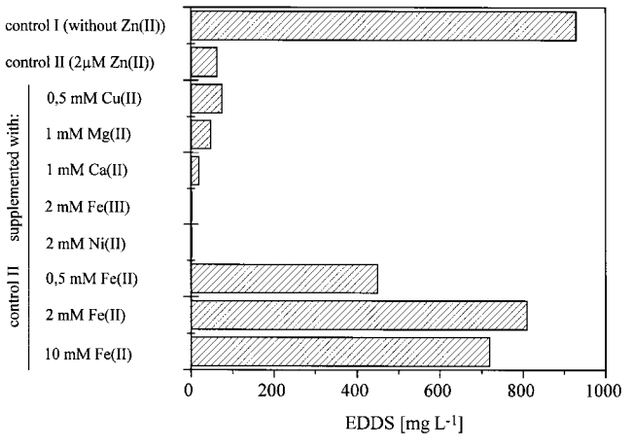

Figure 3 Influence of different divalent and trivalent ions on EDDS production.

mation after addition of 2 mM Fe(II)sulfate, which is shown in Figure 4.

Fermentation studies

Batch fermentations were carried out with the different nitrogen sources. The data obtained using ammonium phosphate are shown in Figure 5. Ammonium was exhausted after 60 h of cultivation, whereas the carbon source, glycerol, was still present in the medium until the end of the fermentation course. As expected the exhaustion of nitrogen coincided with the end of the growth phase. Nevertheless, product formation continued and the EDDS concentration had doubled by the end of the fermentation. Plots from batch fermentations using urea and glutamic acid, respectively, showed similar profiles. Table 1 summarizes the data of the yield coefficients Y_{XS} (g dry weight g substrate^{-1}), Y_{PS} (g product g substrate^{-1}) and Y_{PX} (g product g dry weight^{-1}) from fermentations using these different nitrogen sources. The data emphasize that fermentations with urea resulted in lower yields compared to ammonium or glutamic acid. In addition, Figure 5 shows that product formation occurred nearly in parallel to biomass formation. To distinguish exactly between product formation depending on the bacterial growth (dc_X/dt) or on the

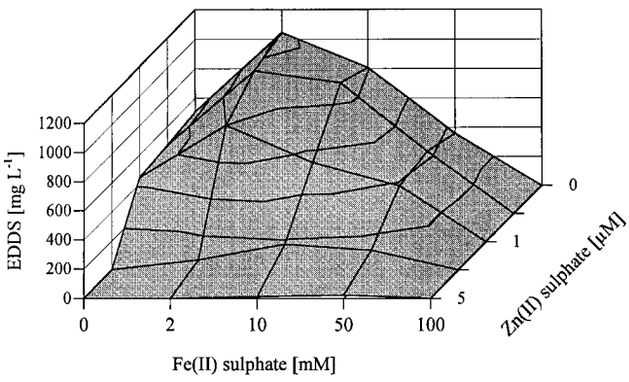

Figure 4 Interdependency of Fe(II) and Zn(II) on EDDS production.

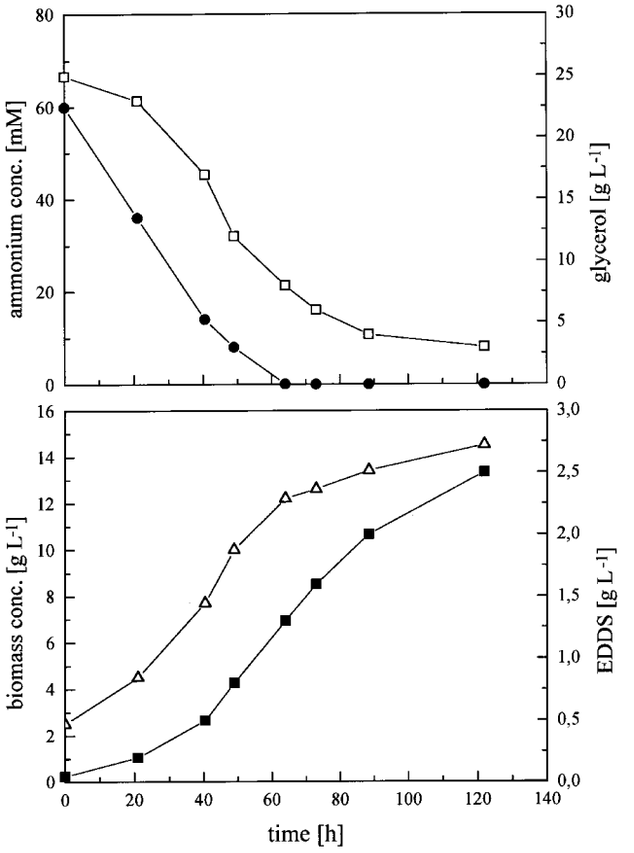

Figure 5 Batch fermentation of *Amycolatopsis orientalis* with ammonium phosphate as sole nitrogen source. ●, Ammonium; □, glycerol; ■, EDDS; △, biomass.

Table 1 Yield coefficients Y_{XS} (gram dry weight per gram substrate), Y_{PS} (gram product per gram substrate) and Y_{PX} (gram product per gram dry weight) during batch fermentations with different nitrogen sources in the synthetic medium

Nitrogen source	Y_{XS} [g g^{-1}]	Y_{PS} [g g^{-1}]	Y_{PX} [g g^{-1}]
$(NH_4)_2HPO_4$	0.55	0.11	0.20
Urea	0.37	0.04	0.10
Glutamic acid	0.62	0.08	0.15

biomass concentration (c_X), the expression of Luedeking and Piret [13]

$$dc_P/dt = \alpha\, dc_X/dt + \beta\, c_X$$

was used. The parameters α and β are constants of proportionality, and c_P is the product concentration (mg EDDS L^{-1}). To evaluate the constants, the equation was divided by the biomass concentration c_X. Since by definition, the specific growth rate $\mu = (1/c_X)\,(dc_X/dt)$ and the specific

production rate $q_P = (1/c_X)\ (dc_P/dt)$, the equation simplifies to

$$q_P = \alpha\ \mu + \beta.$$

The growth associated parameter α (mg EDDS g dry weight^{-1}) and the non-growth associated constant β (mg EDDS g dry weight^{-1} h^{-1}) were determined when the experimental values of q_P were plotted against μ for the growth phase. Despite the variation of the experimental data which are shown in Figure 6, a calculation using data from different batch fermentations showed that the product formation is a function of biomass concentration only and not dependent on the growth rate. Therefore, the growth-rate dependent parameter α could be neglected and the constant β, dependent on biomass concentration, was estimated to be 2.51 (mg EDDS g dry weight^{-1} h^{-1}). Moreover, Figure 6 emphasizes the influence of the nitrogen source upon the maximal specific growth rate of *Amycolatopsis orientalis* in the synthetic medium.

As a result of the calculation, our further objective was to increase biomass concentration in order to enhance EDDS production. Fed-batch and/or repeated fed-batch fermentations are the usual tools to increase both, biomass concentration by extension of the growth phase, and product yields through extension of the production phase. Glutamic acid was found to give better results (higher EDDS yields) than urea or ammonium phosphate when used as sole nitrogen source in fed-batch fermentations. The feeding (feeding solution I) was started after 95 h of batch fermentation, when glutamic acid was exhausted in the fermentation broth. During two feeding phases, the biomass concentration increased to 48 g L^{-1} and EDDS reached a concentration of 12.5 g L^{-1} (Figure 7). The feeding was controlled by the concentration of glutamic acid. It was intended to keep the value above a level of 3 g L^{-1} to prevent nitrogen limitation. From the beginning of the second feeding phase the growth rate decreased due to an accumulation of glycerol (data not shown). Consequently, with the dilution from feeding and concurrent reduced growth, biomass concentration dropped to 43 g L^{-1} at the end of the fermentation.

Results from shake flask experiments indicated that a mixture of different nitrogen sources in the feeding solution would be possible. However, when urea was used for additional feeding, as in case of feeding solution II, it was necessary to start with urea as nitrogen source in the batch phase, although biomass and product formation were reduced. It can be seen from Figure 8, that during 550 h of feeding the biomass increased continuously to about 50 g L^{-1} and EDDS concentration reached nearly 16 g L^{-1}. The medium flow was controlled by off-line determination of the glycerol concentration. In contrast to urea, glutamic acid could not be detected and indicates that glutamic acid was the limiting parameter. To increase the glutamic acid content in the fermentation broth, the feed was raised, regardless of the glycerol concentration. Therefore, the concentration of glycerol accumulated as expected and biomass formation dropped, but EDDS concentration reached a concentration of 20 g L^{-1}. During this period the glutamic acid provided in the feed was completely metabolized and used for product formation. Glycerol and urea accumulated in the medium and seemed to be responsible for the deceleration of growth.

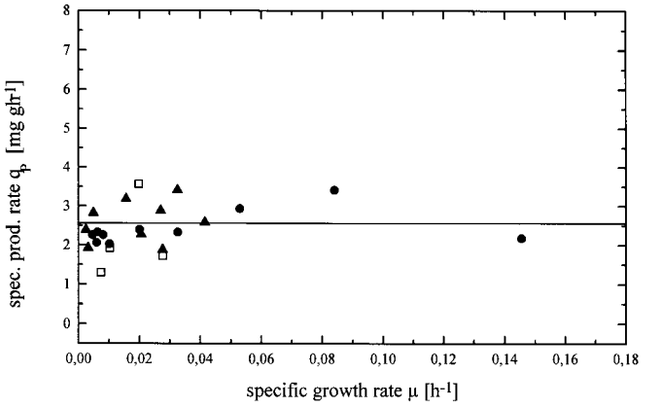

Figure 6 Specific production rate q_P as a function of the specific growth rate μ for different nitrogen sources. The experimental data led to an average value of approximately 2.5 mg EDDS g^{-1} h^{-1} for all growth rates. □, Urea; ●, glutamic acid; ▲, ammonium phosphate.

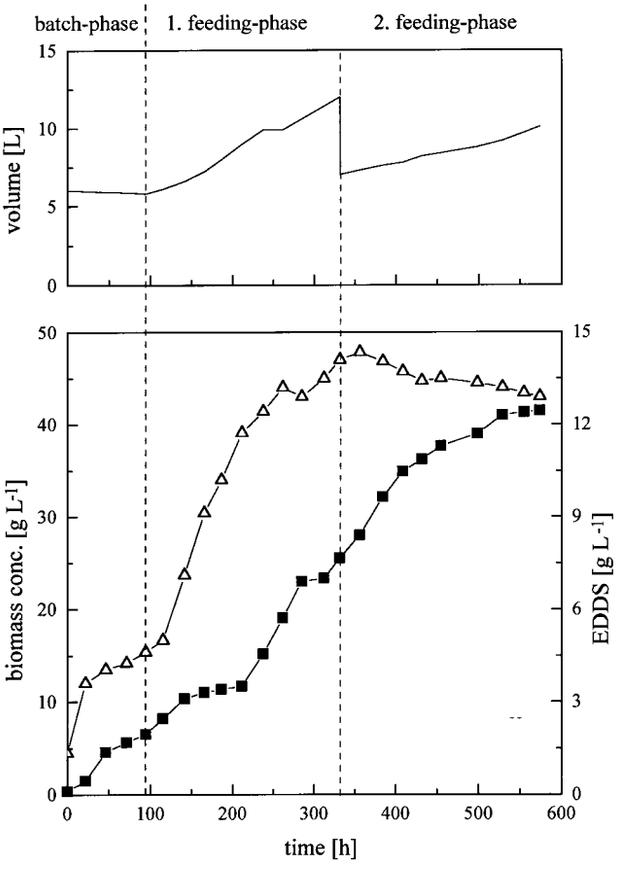

Figure 7 Repeated fed-batch fermentation of *Amycolatopsis orientalis* with glutamic acid as nitrogen source (feeding solution I). ■, EDDS; △, biomass.

Product purification

A fast isolation and purification procedure for EDDS was reported earlier [3]. The method consisted of a filtration step to obtain cell-free medium and an acid treatment to

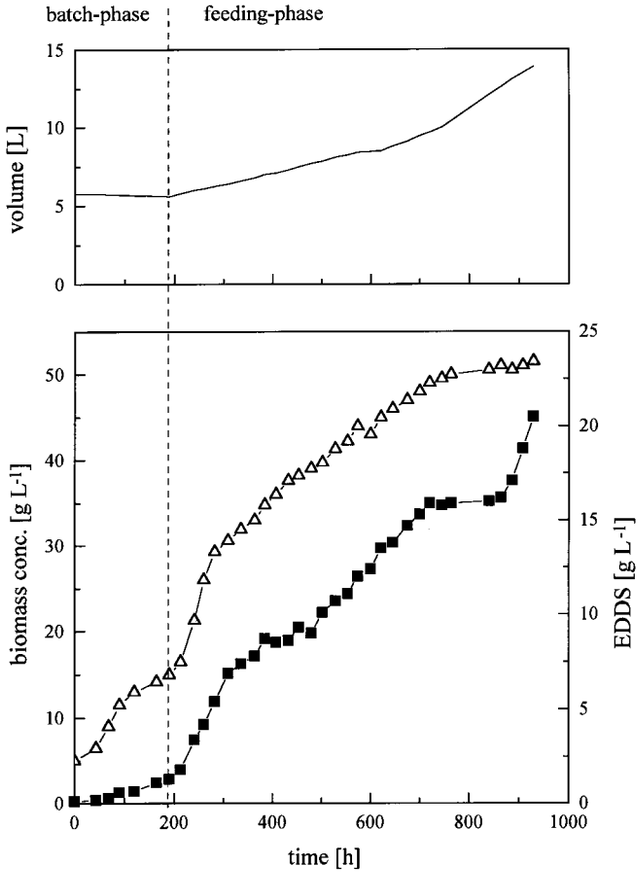

Figure 8 Fed-batch fermentation with urea as sole nitrogen source during the batch phase, and feeding of a mixture of glutamic acid and urea (feeding solution II). ■, EDDS; △, biomass.

precipitate the product (as described in Materials and Methods). Purification according to the method of Cebulla [3] was completed by simple crystallization in distilled water. EDDS was purified to 89% by this method but a disadvantage was the low yield of about 19%. Most of the product (51%) was lost between the precipitation and the crystallization steps. As a consequence of this loss, a modification of the mentioned procedure was necessary. To remove hydrophilic impurities from the filtrate, several alcohols like methanol, 1-propanol and ethanol were tested at different concentrations before the crystallization step. Both parameter, product purity and product yield could be improved with an additional washing-step. Best results were obtained by using ethanol (70%). The purity was increased to 83% and a product purity of 92% was obtained after crystallization. Table 2 summarizes the data for the modified and optimized downstream processing steps.

Discussion

The results of our investigations showed that the production of S,S-EDDS by *Amycolatopsis orientalis* could be strongly controlled by the feed of an optimized synthetic medium (Figures 7 and 8). As an important parameter the optimal initial phosphate concentration was identified. Although the phosphorus content of a cell is only in the range of 2% of

Table 2 Balance of the modified isolation steps

Step	Purity (%)	Yield (%)
Culture filtrate	nd	100
Acid precipitation	77	70
EtOH washing step	83	63
Crystallization	92	49

nd: not determined.

cell dry weight, it has a tremendous impact upon limitation [8]. Most organisms use phosphate as sole phosphorus source. Taking into account the general cell composition $C_6H_{10}O_3N_1P_{0.1}$ [18], which was found as an average for several microorganisms, the optimal initial phosphate concentration for product formation (13 g L^{-1}) will correspond with a theoretical biomass concentration of about 150 gram dry weight per litre fermentation broth. Therefore, in the case of EDDS production a phosphate excess seems necessary. In addition, the choice of nitrogen source greatly influences the production of EDDS. Urea does not appear to be suitable as sole nitrogen source with respect to growth and product yield (Table 1), although it had a significant effect as a supplement in the feeding solution (Figure 8). A comparison of both feeding fermentations gave the same results for the yield Y_{PX} up to the end of the feeding phase. However, urea accumulated in the medium and had a negative effect upon cell growth, so that the yield Y_{PX} increased significantly from 0.3 g g^{-1} to 0.4 g g^{-1}, as shown in Figure 9. The calculation according to Luedeking and Piret [13] was used to show that production of EDDS is strongly dependent on the biomass concentration in the reactor. However, when urea was present in the feeding solution, the fermentation could be directed towards product accumulation once an optimal biomass concentration was reached (Figure 9).

The experiments indicated as well, that EDDS production was influenced by the concentration of divalent and trivalent cations in the medium. A tremendous interdependence could be seen between zinc ions and EDDS formation (Figures 3 and 4). A zinc concentration above 3 μM decreased the product yield by about 90%. All our investigations were carried out in glass fermenters with standard metal casing for baffles and sensors (temperature, pH, dis-

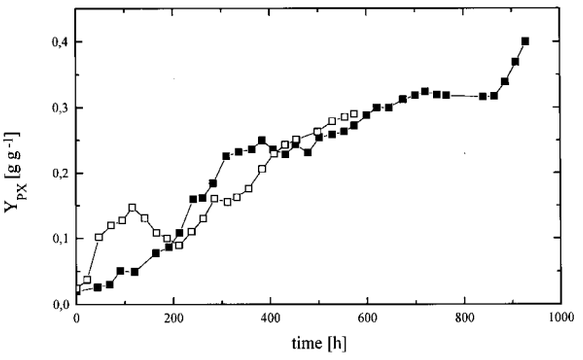

Figure 9 Calculation of the production yield Y_{PX} obtained in feeding fermentations (feeding of glutamic acid *vs* feeding of glutamic acid plus urea). ■, Glutamic acid + urea; □, glutamic acid.

solved oxygen etc). Comparative studies which were performed in steel fermenters illustrated the cation effect, because EDDS production dropped to zero (data not shown). Addition of other cations had no significant effect on the outcome of the process. This influence disables a scale-up in larger tanks at the moment.

Nevertheless, the high productivity of the process of about 20 g L^{-1} EDDS is encouraging for the possibility of replacing EDTA in industrial applications with S,S-EDDS produced by *Amycolatopsis orientalis* in controlled fermentations.

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