Ulrike Jost¹Björn Andreeßen²Dirk Michalik³Alexander Steinbüchel^{2,4}Udo Kragl¹

Research Article

Downstream processing of serinol from a glycerol-based fermentation broth and transfer to other amine containing molecules

¹Department of Analytical and Technical Chemistry, Institute of Chemistry, University of Rostock, Rostock, Germany

²Institute of Molecular Microbiology and Biotechnology, Westphalian Wilhelms-University, Münster, Germany

³Leibniz Institute for Catalysis (LIKAT Rostock), Rostock, Germany

⁴Environmental Sciences Department, Faculty of Metrology, Environment and Arid Land Agriculture, King Abdulaziz University, Jeddah, Saudi Arabia

A possible application of glycerol, which is produced in large amounts as a by-product from the biodiesel industry, is its fermentation to serinol (2-amino-1,3-propanediol), a glycerol derivative. The downstream processing of this glycerol-based fermentation broth was investigated. The challenge of the isolation of serinol was the complex media and the solubility of the desired substance in aqueous media. In this study, the isolation of serinol was investigated by an appropriate reversible derivatization method. Serinol was isolated by protecting the amino group with diethyl ethoxymethylenemalonate directly in the aqueous phase, followed by extraction of the 2,2-bis(ethoxycarbonyl)vinyl-serinol (BECV-serinol) with ethyl acetate resulting in an isolated yield of 63%. We demonstrate the possibility of isolation of a hydroscopic amino alcohol from the fermentation broth and the comparison of the products in water as well as the cleavage of the 2,2-bis(ethoxycarbonyl)vinyl group (BECV group). The procedure can also be used for other amino group containing molecules, such as serine, glucosamine, hexylamine and amino methyl laureate.

Keywords: Amino alcohol isolation / Downstream processing / Fermentation broth / Glycerol / Protecting group / Serinol

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1 Introduction

The downstream processing of fermentation broths is a most discussed issue in the area of biotechnology and in the industry in recent years. The awareness of the use of green and renewable resources is increasing due to the fact that the population is growing and fossil fuels are depleting [1, 2]. Certainly the value of the final product plays a major role. A higher price will allow more or more complicated steps for downstream processing [3]. Especially with low-value-added products the downstream processing has to be simple and cheap. Therefore, a number of fermentation processes do not meet the criteria for a cost competitive process offering an alternative to for example for oil-based fuels. In addition, the concentration of the target product as well as its purity influences the complexity of the downstream process with respect to the number of separation steps. In most cases, several sequential steps are necessary for the separation of the product and other existing components in the media, such

as centrifugation, extraction, microfiltration, chromatography or the addition of adsorption material and further filtration and elution, crystallization and drying. All these points add to the final product costs. As a rule of the thumb, 60–80% of the production costs arise from the downstream processing [4, 5]. For example, the isolation of butanol with a boiling point of 118 °C from a fermentation broth is a very energy intensive process because of the heating of the supernatant to its boiling point and the evaporation of water. To decrease the energy consumption a simultaneous fermentation and lipase catalyzed esterification, followed by product extraction was performed [6]. The isolation of glycerol from biodiesel was described by Liu et al. (2009). Different adsorption materials like active carbon, clay minerals or zeolite were used for separation. The benefit of an adsorption material is that modifications of the surface influence the adsorption behavior and therefore the separation [7]. Anand et al. also reported the application of active carbon for the downstream processing of propane-1,3-diol from a glycerol-based fermentation broth, but in combination with microfiltration, vacuum distillation as well as silica gel chromatography [8]. The downstream processing of propane-1,3-diol and butane-1-ol by use of chemically modified silica gels is also described. However, the separation is only effective for butane-1-ol and not for propane-1,3-diol. Dia et al. (2008) investigated the

Correspondence: Prof. Udo Kragl (udo.kragl@uni-rostock.de), Department of Analytical and Technical Chemistry, Institute of Chemistry, University of Rostock, Albert-Einstein-Str. 3A, 18059 Rostock
Abbreviations: BECV, 2,2-bis(ethoxycarbonyl)vinyl

downstream processing of carboxylic acids from fermentation broths with different polymer adsorption materials, pH values and salt concentrations [9].

Reactive extraction is another possibility for the downstream processing. The separation of propane-1,3-diol by reactive extraction from diluted aqueous solution was discussed [10]. This process combines the reversible reaction of propane-1,3-diol to 2-methyl-1,3-dioxane and the extraction of the product into an organic solvent. In most of the developed techniques thermal unit operations were used such as adsorption or extraction. In the literature several adsorption materials were investigated such as active carbon or modified silica gel. The reactive extraction seems to be a highly effective method for the isolation of valuable products with the opportunity to avoid the removal of water and therefore again a possible decrease of energy consumption.

Serinol 1 (2-amino-propane-1,3-diol) is a polyfunctional glycerol derivative, first synthesized by Piloty and Ruff in 1897. An optimized procedure for the synthesis of serinol was offered in 1980, starting from 2-nitro-propane-1,3-diol with a yield of 75 % [11]. The serinol structure can be found in many bioactive compounds such as sphingosines, ceramides or medical compounds such as fingolimod (for the treatment of multiple sclerosis) [16]. Ceramide analogues, synthesized from serinol are discussed to have a positive effect against cancer. A large number of anticancer drugs affect an increase of endogenous ceramides, which induce the apoptosis of tumor cells [12, 13]. Moreover, Michaelis et al. (2004) reported the application of serinol derivatives for the preparation of pharmaceuticals for the prevention and treatment of different kinds of pain. Furthermore, it is not only possible to prepare serinol by chemical synthesis but also by biotechnological conversions [14]. Itoh et al. (2000) obtained serinol using aminoalcohol dehydrogenase [15]. Andreeßen and Steinbüchel (2012) described its synthesis by a recombinant *Escherichia coli* strain for the first time. Serinol is accumulated between 3.3 and 3.6 g/L in the supernatant [16].

Here, we describe the development of a separation method of serinol from the complex composition of supernatant of different fermentation broths. The challenge of the downstream processing of serinol from the fermentation broth was to develop a method which works in aqueous media. The investigation was orientated on the work of Ilangovan et al. (2010), who describe the protection of amines with diethyl ethoxymethylenemalonate. The benefit of this kind of protection is its high selectivity. The amino group could be protected selectively while other functional groups present in the molecule do not react. Furthermore, literature reports the deprotection of the 2,2-bis(ethoxycarbonyl)vinyl group using ethylene diamine [17]. The biotechnological conversion of glycerol to serinol and the subsequent isolation of the derivative offer an alternative way to transfer glycerol to a higher value-added compound.

2 Materials and methods

2.1 Material

The fermentation of the recombinant microorganisms was performed at the Institute of Molecular Microbiology and Biotechnology, University of Münster, Germany.

Fed-batch fermentations of recombinant *E. coli* and *Shimwellia blattae* strains harboring the bifunctional enzyme dihydroxyacetone phosphate transaminase/rhizobitoxin synthase RtxA (Sugawara et al. 2007 [18]; Yasuta et al. 2001 [19]) from *Bradyrhizobium elkanii* USD94 were carried out in a 2 L Biostat B plus (Sartorius AG, Göttingen, Germany) fermenter. Temperature, pH, foam and dissolved oxygen were monitored online during fermentation. Optical density at 600 nm, soluble metabolites and NH_4^+ concentration were measured in samples withdrawn from the cultures. The dissolved oxygen (DO%) was set to 25% (vol/vol) and maintained with increasing agitation and constant air flow. The formation of foam was controlled by the addition of a 50% (vol/vol) Struktol (Schill & Seilacher GmbH, Böblingen, Germany) solution. A pH of 6.9 was maintained by addition of a 7.5% (vol/vol) ammonium hydroxide solution and 1 M HCl. For fermentation of *S. blattae* strains a modified basal medium was used (MMB, Sato et al. 2015 [21]). *E. coli* strains were cultivated in Riesenberger-medium (Korz et al. 1995 [20]) at the 2 L scale. For both organisms, the feeding solution consists of 1021 g/L glycerol, 20 g/L $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 13 mg/L EDTA, 4 mg/L $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$, 23.5 mg/L $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$, 2.5 mg/L $\text{CuCl}_2 \cdot 2 \text{H}_2\text{O}$, 5 mg/L H_3BO_3 , 4 mg/L $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$, 16 mg/L $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2 \text{H}_2\text{O}$, 40 mg/L Fe(III)citrate (Korz et al. 1995 [18]). After the consumption of the initial substrate, the feeding solution was added to sustain a final glycerol concentration of 30 g/L and to maintain the concentration during the further cultivation time between 10 and 30 g/L. The inoculation procedure was the following: based on a cryo-stock a fresh agar plate containing adequate antibiotics was prepared. With one colony a tube containing 5 mL Lysogeny Broth (Sambrook et al. 1989 [22]) was inoculated and incubated for 12 to 15 h at 30 °C. 1 mL of this first preculture was used to inoculate 100 mL of the fermentation medium in a 1 L Erlenmeyer flask and incubated for further 14 h. The 2 L fermenter was filled with 1.5 L fermentation medium and inoculated with 100 mL of the second preculture. Antibiotics were added at the following concentrations: kanamycin at 50 µg/mL and chloramphenicol at 34 µg/mL.

As a reference material and starting material serinol from Alfa Aesar (98%) was used. For further syntheses, diethyl ethoxymethylenemalonate and hexylamine from Aldrich Chemistry (99%) and glucosamine hydrochloride (99%) and bromine from Sigma were used. The 12-amino lauric acid was ordered from TCI (>98%).

2.2 Amino group protection

2.2.1 Synthesis of 2,2-bis-(ethoxycarbonyl)vinyl-serinol 2

To 50 mL fermentation broth, which contained about 350 mg of serinol (0.004 mol, 1 eq), diethyl ethoxymethylenemalonate (0.019 mol, 3.877 mL, 5 eq) was added. The reaction mixture was stirred overnight at room temperature (23 °C). After completion of the reaction, the aqueous solution was transferred to a separation funnel and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were dried over cotton wool and the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography with an eluent system of ethyl acetate-heptane, 2:1, and increasing the polarity to ethyl acetate-methanol, 9:1. The excess of solvent was removed under

reduced pressure whereupon colorless crystals were formed. The pure product of 2,2-bis(ethoxycarbonyl)vinyl-aminopropane-1,3-diol **2** was obtained with an isolated yield of 63 % (R_F = 0.14, ethyl acetate-heptane, 2:1). M_p = 92–94 °C

$^1\text{H-NMR}$ (300 MHz, DMSO- d_6) δ_H : 1.19 (t, 3H, 3J = 7.1 Hz, CH_3); 2.85 (t, 3H, 3J = 7.1 Hz, CH_3); 3.37–3.58 (m, 5H, CH_2CHCH_2); 4.04 (dd, 2H, 3J = 7.1 Hz, CH_2); 4.09 (dd, 2H, 3J = 7.1 Hz, CH_2); 4.96 (t, 2H, 3J = 5.0 Hz, OH); 8.04 (d, 1H, 3J = 14.5 Hz, $\text{CH}_{\text{olefine}}$); 9.20 (dd, 1H, 3J = 7.8 Hz, NH).

$^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) δ_C : 14.0, 14.1, 58.4, 58.5, 60.4, 62.3, 159.4.

Elemental analysis: calculated: C: 50.57 H: 7.33 N: 5.36, found C: 50.525 H: 7.392 N: 5.459.

2.2.2 Synthesis of 2-deoxy-[bis(ethoxycarbonylvinyl)amino]-D-glucopyranose [30]

Glucosamine hydrochloride (0.01 mol, 2.16 g, 1 eq) and sodium carbonate (0.005 mol, 0.53 g, 0.5 eq) were dissolved in 8 mL water. After complete dissolution diethyl ethoxymethylenemalonate (0.021 mol, 4.1 mL, 2.1 eq) was added and the solution was gently stirred at room temperature. After 10 min a white precipitate was formed. The reaction was stirred for further 10 min and then the precipitate was filtered off and washed with 50 mL diethyl ether. After drying in air 4.9 g of the crude product were obtained. The white solid was purified by recrystallization from ethanol (3x). 1.96 g (56%) of the pure compound was isolated.

$^1\text{H-NMR}$ (300 MHz, D_2O) δ_H : 1.26 – 1.41 (m, 6H, 2 x CH_3), 3.14 – 3.26 (dt, 1H, $^3J_{1,2}$ = 8.3 Hz, H'-2), 3.44 – 3.60 (m, 1H, H-2), 3.73 – 4.01 (m, 4H, H-3, H-4, H-6a,b), 4.14 – 4.35 (m, 5H, 2 x CH_2 , H-5), 4.93 (d, 1H, $^3J_{1,2}$ = 8.3 Hz, H'-1), 5.38 (dd, 1H, $^3J_{1,2}$ = 3.6 Hz, H-1), 8.25 (s, 1H, $\text{CH}_{\text{olefine}}$).

$^{13}\text{C-NMR}$ (75 MHz, D_2O) δ_C : 13.5, 60.5, 60.6, 60.9, 61.0, 63.6, 67.0, 69.4, 69.7, 71.5, 71.7, 73.5, 75.9, 88.9, 89.3, 91.1, 94.3, 160.5, 161.3, 168.6, 169.2.

HRESI-TOF/MS: found $[\text{M}+\text{Na}]^+$ 372.12687 m/z .

2.2.3 Synthesis of 2,2-bis(ethoxycarbonyl)vinyl-hexylamine

Hexylamine (5 mmol, 500 mg, 0.655 mL, 1 eq) was dissolved in 5 mL distilled water at r.t. (24 °C) and sodium carbonate (2.5 mmol, 265 mg, 0.5 eq) was added. After dissolution diethyl ethoxymethylenemalonate (10 mmol, 2162.31, 2 mL, 2 eq) was added and the reaction mixture was stirred 30 min at r.t. (24 °C). The reaction was controlled by TLC (heptane-ethyl acetate, 2:1). After complete conversion the reaction was stopped, the mixture diluted with 50 mL distilled water and extracted with ethyl acetate (4 x 30 mL). The combined organic layers were dried over cotton wool and the excess of solvent was removed under reduced pressure. 2.49 g of a mixture of two compounds were obtained. The separation was performed by using column chromatography (heptane-ethyl acetate, 2:1) and the product was isolated with 95% (1.295 g).

$^1\text{H-NMR}$ (300.13 MHz, CDCl_3) δ_H : 0.88 (t, 3J = 6.8 Hz, 3H, CH_3 aliph.), 1.23–1.40 (m, 12H, CH_2 aliph., CH_3 bis(ethoxy).), 1.51–1.67 (m, 2H, $\beta\text{-CH}_2$), 3.26–3.38 (dt, 2H, $\alpha\text{-CH}_2$), 4.17 (q,

3J = 7.0 Hz, OCH_2), 4.23 (q, 3J = 7.0 Hz, OCH_2), 7.99 (d, 1H, 3J = 14.2 Hz, CH olef.), 9.20 (m, 1H, NH).

$^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ_C : 13.9, 14.3, 14.4, 22.4, 26.1, 30.6, 31.3, 49.8, 59.5, 59.7, 89.2, 160.00, 166.2, 169.4.

HRESI-TOF/MS: found $[\text{M}+\text{Na}]^+$ 294.16793 m/z .

2.2.4 Synthesis of 2,2-bis(ethoxycarbonyl)vinyl-amino lauric acid methyl ester

12-Amino lauric acid (4.64 mmol, 1 g, 1 eq) was dissolved in abs. methanol (5 mL). The mixture was stirred and concentrated sulfuric acid (100 μL) was added. The solution was heated under reflux for 4 h using an oil bath at 90 °C. The solution was stirred further at r.t. overnight whereby a white precipitate was formed.

The methanol was removed under reduced pressure and the white precipitate was again used as starting material. The white solid was dissolved in 5 mL absolute methanol and concentrated sulfuric acid (100 μL) was added. The solution was heated under reflux for 1 h and further at r.t. The methanol was removed under reduced pressure and the obtained white solid was dissolved with 5 mL methanol and diluted with 50 mL ethyl acetate. The product was precipitated with saturated sodium bicarbonate solution. The precipitate of 12-amino lauric acid methyl ester was filtered off and washed with ethyl acetate and dried under air. The desired product was obtained with a yield of 84%.

$^1\text{H-NMR}$ (300.13 MHz, MeOD) δ_H : 3.77 – 3.56 (m, 3H), 2.95 (dt, 2H), 2.32 (d, 3J = 7.4 Hz, 2H), 1.82 – 1.50 (m, 4H), 1.50 – 1.22 (m, 14H).

$^{13}\text{C-NMR}$ (75 MHz, MeOD) δ_C : 26.2, 28.0, 30.4, 30.6, 30.6, 30.73, 30.8, 30.8, 35.0, 52.2, 176.2.

HRESI-TOF/MS: found $[\text{M}+\text{H}]^+$ 252.19384 m/z .

2.2.4.1 Conversion of 12-amino lauric acid methyl ester in water. 12-Amino lauric acid methyl ester (0.87 mmol, 0.200 g, 1 eq) was dissolved in 50 mL water and 100 mg of sodium carbonate was added and the mixture was stirred at r.t. After complete dissolution diethyl ethoxymethylenemalonate (0.200 mL, 0.216 g, 1.1 eq) was added and the reaction mixture was stirred overnight. The reaction was controlled by TLC (heptane-ethyl acetate, 1:1). After complete conversion the reaction was stopped. The aqueous phase was extracted with ethyl acetate (3 x 30 mL). The combined organic layers were dried over cotton wool and the excess of solvent was removed under reduced pressure. 436 mg of the crude product were obtained. For purification column chromatography with heptane-ethyl acetate in a ratio of 2:1 was used. 242 mg (69.5%) of the pure product were obtained.

2.2.4.2 Conversion of 12-amino lauric acid methyl ester in Riesenberg medium. 12-Amino lauric acid methyl ester (0.87 mmol, 0.200 g, 1 eq) was dissolved in 50 mL Riesenberg medium and 100 mg of sodium carbonate were added and the mixture was stirred at r.t. After complete dissolution diethyl ethoxymethylenemalonate (0.200 mL, 0.216 g, 1.1 eq) was added and the reaction mixture was stirred overnight. The reaction was controlled by TLC (heptane-ethyl acetate, 1:1). After complete conversion the reaction was stopped. The aqueous phase was extracted with ethyl acetate (3 x 30 mL). The combined organic layers were dried over cotton wool and the excess of solvent was removed under reduced pressure. 374 mg of a mixture of two

compounds were obtained. For purification column chromatography with heptane-ethyl acetate in a ratio of 2:1 was used. 120 mg (34.5 %) of the 2,2-bis(ethoxycarbonyl)vinyl-amino lauric acid methyl ester and 100 mg (28%) of 2,2-bis(ethoxycarbonyl)vinylamine were obtained.

2.2.4.3 2,2-bis(ethoxycarbonyl)vinyl-amino lauric acid methyl ester. $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ_{H} : 1.43 – 1.15 (m, 20H), 1.70 – 1.50 (m, 4H), 2.29 (t, $^3J = 7.5$ Hz, 2H), 3.32 (d, $^3J = 6.6$ Hz, 2H), 3.66 (s, 3H), 4.17 (q, $^3J = 7.1$ Hz, CH_2), 4.23 (q, $^3J = 7.1$ Hz, CH_2), 7.98 (d, $^3J = 14.3$ Hz, 1H), 9.43 – 8.94 (m, 1H).

$^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ_{C} : 14.3, 14.4, 24.9, 26.4, 29.1, 29.1, 29.2, 29.3, 29.3, 29.4, 30.7, 34.0, 49.8, 51.4, 59.5, 59.7, 89.2, 156.0, 166.2, 169.4, 174.3.

HRESI-TOF/MS: found $[\text{M}+\text{Na}]^+$ 422.25186 m/z .

2.2.4.4 2,2-bis(ethoxycarbonyl)vinyl-amine. $^1\text{H-NMR}$ (300.13 MHz, CDCl_3) δ_{H} : 1.27 (t, $^3J = 7.1$ Hz, 1H), 1.33 (t, $^3J = 7.1$ Hz, 1H), 4.17 (q, $^3J = 7.1$ Hz, 1H), 4.24 (q, $^3J = 7.1$ Hz, 1H), 6.18 (brs, 1H, NH_a), 8.26 – 7.97 (dd, 1H, CH_{ole}), 8.46 (brs, 1H, NH_b).

$^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ_{C} : 14.3, 14.3, 59.7, 59.8, 91.9, 157.9, 166.1, 168.6.

HRESI-TOF/MS: found $[\text{M}+\text{Na}]^+$ 210.07389 m/z .

2.2.5 Synthesis of 2,2-bis(ethoxycarbonyl)vinyl-serine

L-serine (1.9 mmol, 0.2 g, 1 eq) and sodium carbonate (0.95 mmol, 101 mg, 0.5 eq) were dissolved in 2 mL water. After dissolution diethyl ethoxymethylenemalonate (2.1 mmol, 0.45 g, 0.42 mL, 1.1 eq) was added and the reaction mixture was stirred overnight at r.t. The solution was diluted with 50 mL distilled water and extracted with ethyl acetate (3×30 mL). The product 2,2-bis(ethoxycarbonyl)vinyl-serine remained in the aqueous phase. After removal of the water 536 mg of the product and sodium carbonate were obtained (yield 86%).

$^1\text{H-NMR}$ (300 MHz, DMSO-d_6) δ_{H} : 1.18 (t, 3H, $J = 7.1$ Hz, CH_3), 1.21 (t, 3H, $J = 7.1$ Hz, CH_3), 3.35 (s, 19H), 3.81 – 3.53 (m, 2H), 4.19 – 3.93 (m, 4H), 5.14 (s, 1H), 8.01 (d, $J = 15.3$ Hz, 1H), 9.55 (dd, $J = 15.3$, 6.5 Hz, 1H).

$^{13}\text{C-NMR}$ (75 MHz, DMSO-d_6) δ_{C} : 14.4, 14.4, 58.4, 64.2, 78.5, 79.0, 79.2, 79.4, 87.3, 158.0, 165.7, 167.6.

2.3 Cleavage of the amino protecting group [17], [30]

2.3.1 With bromine

2.3.1.1 BECV-serinol. BECV-serinol (0.81 mmol, 211 mg, 1 eq) was dissolved in 5 mL methanol. After complete dissolution bromine (1.95 mmol, 100 μL , 2.4 eq) was added dropwise. The reaction mixture was stirred for 3 h at r.t. The reaction was controlled by TLC. After complete conversion the solution was diluted with 50 mL of water and extracted with chloroform (4×30 mL). 125 mg (90%) of serinol hydrobromide were isolated.

$^1\text{H-NMR}$ (300.13 MHz, DMSO-d_6) δ_{H} : 2.97 – 3.16 (m, 1H, CH), 3.45 – 3.66 (m, 4H, 2 × CH_2), 5.13 (t, 2H, $^3J = 4.9$ Hz, 2 × OH), 7.82 (brs, 3H, NH_3).

$^{13}\text{C-NMR}$ (75 MHz, DMSO-d_6) δ_{C} : 54.1, 58.5.

HRESI-TOF/MS: found $[\text{M}+\text{Na}]^+$ 92.07101 m/z .

2.3.1.2 BECV-glucosamine. Glucosamine (0.57 mmol, 200 mg, 1 eq) was dissolved in 5 mL methanol. After complete dissolution bromine (1.37 mmol, 70.5 μL , 2.4 eq) was added dropwise. The reaction mixture was stirred for 3 h at r.t. The reaction was controlled by TLC. After complete conversion the solution was diluted with 50 mL of water and extracted with chloroform (4×30 mL). 125 mg (80%) of glucosamine hydrobromide were isolated.

$^1\text{H-NMR}$ (300.13 MHz, D_2O) δ_{H} : 3.27 – 3.40 (dd, 1H, $^3J = 3.6$ Hz, $^3J = 10.6$ Hz), 3.46 – 3.58 (m, 1H), 3.68 – 4.00 (m, 4H), 4.99 (d, $^3J = 8.5$ Hz, $\beta\text{H-1}$), 5.48 (d, 1H, $^3J = 3.6$ Hz, $\alpha\text{H-1}$).

$^{13}\text{C-NMR}$ (75 MHz, D_2O) δ_{C} : 54.3, 56.7, 60.3, 60.5, 69.6, 69.7, 71.6, 72.0, 76.2, 89.1, 92.7.

HRESI-TOF/MS: found $[\text{M}+\text{Na}]^+$ 180.08696 m/z .

2.3.1.3 BECV-amino lauric acid methyl ester. To 20 mL methanol 150 μL bromine was added. The clear brown solution was cooled down to 0 °C. Then, the BECV-amino lauric acid methyl ester (0.20 mmol, 79 mg) was added and dissolved. After 20 min the reaction was complete. The mixture was diluted with 50 mL distilled water and extracted with chloroform (4×30 mL). The combined organic phases were dried over cotton wool and the excess of solvent was removed under reduced pressure. The residue was washed with diethyl ether to obtain a white powder with a yield of 34% (22 mg).

$^1\text{H-NMR}$ (300.13 MHz, DMSO-d_6) δ_{H} : 1.15 – 1.36 (m, 14H, CH_2), 1.42 – 1.62 (m, 4H, 2 × $\beta\text{-CH}_2$), 2.28 (t, $^3J = 7.4$ Hz, 2H, $\alpha\text{-CH}_2\text{CO}$), 2.76 (t', $^3J = 7.5$ Hz, 2H, $\alpha\text{-CH}_2\text{NH}_2$), 3.57 (s, 3H, OCH_3), 7.72 (brs, 3H, NH_3).

$^{13}\text{C-NMR}$ (75 MHz, DMSO-d_6) δ_{C} : 24.4 ($\beta\text{-CH}_2$), 25.8 (CH_2), 26.9 ($\beta\text{-CH}_2$), 28.5 (CH_2), 28.6 (CH_2), 28.8 (CH_2), 28.8 (CH_2), 33.2 (33.2 $\alpha\text{-CH}_2\text{CO}$), 38.8 ($\alpha\text{-CH}_2\text{NH}_3$), 51.1 (OCH_3), 173.3 (CO).

HRESI-TOF/MS: found $[\text{M}+\text{Na}]^+$ 230.21159 m/z .

2.3.1.4 BECV-hexylamine. To 20 mL methanol 150 μL bromine was added. The clear brown solution was cooled to 0 °C. Then the BECV-hexylamine (0.30 mmol, 80 mg) was added and dissolved. After 20 min the reaction was complete and stopped. The mixture was diluted with 50 mL distilled water and extracted with chloroform (4×30 mL). The desired compound remained in the aqueous layer. The water was removed under reduced pressure to give 20 mg (37%) of hexylamine hydrobromide.

$^1\text{H-NMR}$ (300.13 MHz, DMSO-d_6) δ_{H} : 0.86 (t, $^3J = 7.0$ Hz, 3H, CH_3), 1.15–1.37 (m, 6H, CH_2), 1.43–1.63 (m, 2H, $\beta\text{-CH}_2$), 2.76 (t', $^3J = 7.2$ Hz, 2H, $\alpha\text{-CH}_2$), 7.75 (brs, 3H, NH_3).

$^{13}\text{C-NMR}$ (75 MHz, DMSO-d_6) δ_{C} : 13.8 (CH_3), 21.8 (CH_2), 25.4 (CH_2), 26.8 (CH_2), 30.7 (CH_2), 38.7 ($\alpha\text{-CH}_2$).

HRESI-TOF/MS: found $[\text{M}+\text{Na}]^+$ 102.12795 m/z .

2.3.1.5 BECV-serine. To 20 mL methanol 150 μL bromine was added. The clear brown solution was cooled to 0 °C. Then, the BECV-serine (0.30 mmol, 80 mg) was added and dissolved. After 20 min the reaction was complete and stopped. The mixture was diluted with 50 mL distilled water and extracted with chloroform (4×30 mL). The combined organic phases were dried

over cotton wool and the excess of solvent was removed under reduced pressure. The residue was washed with diethyl ether and dried in vacuo. 52 mg of a pale brown solid was isolated. The NMR-Spectra show the desired product and an additional compound which is probably ammonium bromide.

^1H -NMR (300.13 MHz, DMSO- d_6) δ_{H} : 3.78 (d, $^3J = 3.5$ Hz, 2H, CH_2), 3.89–4.01 (m, 1 H, CH), 7.33 (t, NH_4Br), 8.25 (brs, 3H, NH_3).

^{13}C -NMR (75 MHz, DMSO- d_6) δ_{C} : 54.2 (CH_2), 59.3 (CH), 169.4 (CO).

2.3.2 With chlorine

2.3.2.1 BECV-amino lauric acid methyl ester. 20 mL methanol was saturated with chlorine. The solution became yellow. The solution was cooled down to 0 °C. Then the BECV-amino lauryl methyl ester (0.20 mmol, 79 mg) was added and dissolved. After 20 min the reaction was complete and stopped. The mixture was diluted with 50 mL distilled water and extracted with chloroform (4 × 30 mL). The combined organic phases were dried over cotton wool and the excess of solvent was removed under reduced pressure. 89 mg of a crude product were obtained. For further purification the residue was washed with diethyl ether and the white precipitate filtered off. 10 mg of the pure compound could be isolated.

^1H -NMR (300.13 MHz, DMSO- d_6) δ_{H} : 1.19 – 1.35 (m, 14H, CH_2), 1.44 – 1.58 (m, 4H, 2 × β - CH_2), 2.28 (t, $^3J = 7.4$ Hz, α - CH_2CO), 2.74 (t, $^3J = 7.6$ Hz, 2H, α - CH_2NH_3), 3.57 (s, 3H, OCH_3), 7.88 (brs, 3H, NH_3)

^{13}C -NMR (75 MHz, DMSO- d_6) δ_{C} : 24.4 (β - CH_2), 25.8 (CH_2), 26.9 (β - CH_2), 28.4 (CH_2), 28.6 (CH_2), 28.7 (CH_2), 28.8 (CH_2), 33.2 (α - CH_2CO), 38.7 (α - CH_2NH_3), 51.1 (OCH_3), 54.9 (CH_2 , dichloromethane), 173.3 (CO).

HRESI-TOF/MS: found $[\text{M}+\text{Na}]^+$ 230.21164 m/z .

2.4 Analysis

2.4.1 Gas chromatography mass spectrometry

For the determination of serinol content, the gas chromatography mass spectrometry (GCMS) was used. The general procedure for the preparation of trimethylsilylserinol was to dissolve 20 mg of serinol in 500 μL of trimethylsilylimidazol in pyridine. The mixture was stirred at 60 °C for 1 h. For calibration a serial dilution was prepared and measured with an ITQTM (Thermo Scientific) using the following temperature program: First 2 min heating at 70 °C, within 8 min temperature was increased to 150 °C and held for 1 min. Temperature was increased to 290 °C within the next 14 min and held for 5 min. A TG-5 SilMS column from ThermoFisher was used.

2.4.2 Liquid chromatography mass spectrometry (LCMS)

For the measurements of BECV-serinol liquid chromatography mass spectrometry (LC/ESI/MS) (Thermo Fisher Scientific, LTQ velos and LTQTM) was used. A Kinetex C18 column (150 × 3 mm, 2.1 μm (Phenomenex)) was selected for all samples. The temper-

Figure 1. GC-chromatogram of a fermentation broth (*S. blattae*, modified basal medium). 100 μL of fermentation broth was concentrated to dryness and derivatized with TMSI (Trimethylsilylimidazol).

ature of the column was 35 °C and the injection volume 5 μL . The run took 25 min and the compounds were measured in the positive scan mode.

The gradient as shown in Table 1 was used.

2.4.3 Nuclear magnetic resonance spectroscopy (NMR-spectroscopy)

The NMR-spectra were measured in DMSO- d_6 (δ_{H} 2.5 ppm, δ_{C} 39.5 ppm), CDCl_3 (δ_{H} 7.27 ppm, δ_{C} 77.0 ppm), D_2O (δ_{H} 4.8 ppm) at 20 °C by using a Bruker AVANCE 300 (300.13 und 75.48 MHz).

3 Results and discussion

3.1 Analysis of fermentation broth

The fermentation sample was controlled by liquid chromatography mass spectrometry, gas chromatography mass spectrometry and NMR measurements, whereby serinol was detected by all three methods. For the determination of serinol by using GCMS the derivatization of the sample was necessary. Trimethylsilylimidazol was used as derivatization agent. The GC-chromatogram (Fig. 1) reveals three peaks. At first the peak of the excess derivatization agent was eluted at a retention time of 6.90 min. The derivatized serinol was eluted at 8.05 min and the silylated glycerol derivative at 9.33 min. The obtained GC-chromatograms of pure serinol and serinol formed by fermentation do not show any differences in the retention time of the peaks.

The benefit of this derivatization is that only the hydroxyl groups are protected. This offers the opportunity to distinguish serinol from glycerol. This method was used for calibration of serinol and a content of 7 g/L serinol in the fermentation broth was determined (*S. blattae*, modified basal medium).

Figure 2 shows a section of the ^1H -NMR spectra from the fermentation broth. The section shows the signals for the protons of glycerol and serinol. The CH_2 -signals from serinol are overlapping with the CH-signals of the glycerol. In general, the

Figure 2. Section of the ^1H -NMR spectra after conversion of glycerol to serinol in modified basal media by recombinant *S. blattae*.

Figure 3. General procedure for the preparation of BECV-serinol in water or fermentation broth.

signals of both compounds, glycerol and serinol, show similar chemical shifts in ^1H -NMR.

The GC-chromatogram and the NMR-spectra reveal that glycerol still exists in the fermentation broth. The challenge in the isolation of serinol is the fact that both compounds have similar polarity and both are well soluble in polar solvents. To solve the isolation problem an appropriate derivatization method has to be developed to convert serinol in a more hydrophobic derivative in the presence of glycerol to extract this product in a suitable organic solvent.

3.2 Method development for isolation of pure serinol from water and fermentation broth

The literature described the selective protection of amines with diethyl ethoxymethylenemalonate in water or ethanol to obtain the corresponding product in high yields [17, 30].

This procedure was used for the protection of serinol, whereby the conditions were optimized that they could be applied to the isolation problem at hand. The general procedure for the BECV-serinol synthesis in water or fermentation broth is presented in Fig. 3.

To ensure conditions comparable to the fermentation broth, the reactions were performed in water instead of ethanol. The derivatization of pure serinol with diethyl ethoxymethylenemalonate was performed overnight at r.t. After the removal of

water 2,2-bis-(ethoxycarbonyl)vinyl-serinol remained as white solid with 72% isolated yield.

Due to the fact that compared to water the fermentation medium contains additional ingredients, the formation of the BECV-serinol was investigated in a fermentation medium. For the preliminary tests the Riesenberg medium was used. This investigation should ensure that the formation of the desired product also takes place under real conditions. Therefore, two reactions were carried out using Riesenberg medium, which did not contain serinol after fermentation: The first reaction was prepared under the same conditions like the test reaction in water. Therefore, pure serinol (1 eq) was dissolved in the fermentation medium and the derivatization agent (1.3 eq) was added. To the second reaction sodium carbonate was added to ensure basic conditions ($\text{pH} \sim 9$), and serinol is present as the amino form and not as an ammonium salt. Both reaction mixtures were stirred overnight. After complete conversion of the diethyl ethoxymethylenemalonate the excess of water was removed in vacuo and a brown residue was obtained. This residue contained beside the desired BECV-serinol still all other fermentation ingredients, the salts of the trace element solution, buffer solution and the nutrient solution. To separate the BECV-serinol from the remaining substrates both mixtures were treated with hot ethanol. The BECV-serinol and a major part of the insoluble components were filtered off. After concentration of the obtained ethanolic solution in vacuo again a brown residue was obtained. Further purification steps for the isolation of the BECV-serinol were necessary. To achieve the separation of the product from the other ingredients the column chromatography on silica gel and with heptane-ethyl acetate as eluent system was used. This method offered both the possibility to isolate glycerol and the desired product. The isolated yield of the desired product 2 was between 30 and 36%. In comparison with the reference material less than half of the product was isolated. The disadvantage of this method was on the one hand the time and energy intensive process of water removal and on the other hand the low yield.

To avoid the water removal and the treatment with hot ethanol and the following filtration a suitable extraction agent for the BECV-serinol was looked for. The product is polar due to its hydroxyl functions and therefore it is soluble in polar solvents. As extracting agent, a solvent was needed which is not miscible with water, but at the same time polar enough to isolate the product. For the extraction two solvents of medium polarity were selected: isopropyl methyl ketone and ethyl acetate.

Two reaction mixtures were prepared as described. After complete conversion of the starting materials the aqueous phase was extracted with isopropyl methyl ketone or with ethyl acetate. Both solvents could be used for the extraction of the BECV-serinol. The isopropyl methyl ketone is viscous, has an unpleasant smell and a high boiling point of 92°C . The evaporation of this solvent takes consequently almost the same time like water. In the following experiments ethyl acetate were used as extraction agent, because it belongs to the recommended “green” solvents (Astra Zeneca) [23], could be removed quickly under reduced pressure and is an excellent solvent for the BECV-serinol extraction.

A next step for the optimization of the isolation method was to increase the yield. The thin layer chromatograms of the

Figure 4. By-product (**4**) formed from ammonium salt and diethyl ethoxymethylenemalonate.

reaction showed after extractions only two products (ethyl acetate-heptane, 2:1, $R_F = 0.14$, $R_F = 0.53$). Both products were isolated and characterized using NMR and LCMS measurements. Both NMR-spectra and mass spectra showed that the BECV-serinol and 2,2-bis(ethoxycarbonyl)vinyl-amine **4** was isolated as a second product.

Consequently, the ratio of serinol to derivatization agent with 1 to 1.3 was not ideal. The small excess of diethyl ethoxymethylenemalonate **3** was not sufficient to derivatize both serinol and other amino compounds, which were also contained in the fermentation broth. The isolated second product indicates that the containing ammonium sulfate or other ammonium salts and ammonium hydroxide, which were added during the fermentation process, were derivatized as well. In the literature the formation of this amine (Fig. 4) is well known [24–26]. Due to the large amount of ammonium salt in the fermentation broth the amount of derivatization agent has been increased (excess of up to 10 eq).

As a result the isolated yield could be increased from about 30 to 60%. The test reaction, prepared in water and with pure compounds as well as ethyl acetate as extracting agent, lead to an isolated yield of 90%. For further reactions the substrate ratio of 1:5 was used, because of the higher isolated yield compared to 1:1.3 and lower costs compared to a 1:10 ratio.

The isolation from fermentation broth was performed in the same way as described before. However, for the final experiments the basal medium, which contained the biggest amount of serinol after conversion by recombinant *S. blattae*, was used. Before the protecting agent was applied, sodium carbonate was added in portions until the reaction mixture was saturated. After completion of the reaction the two products were extracted with ethyl acetate and separated using column chromatography. The desired product 2,2-bis(ethoxycarbonyl)vinyl-serinol was obtained with an isolated yield of 63% (0.62 g).

The time studies revealed that the formation of BECV-serinol is complete after 4 h. A longer reaction time only led to degradation of the diethyl ethoxymethylenemalonate. There is no difference when water or real fermentation broth is used.

To complete the whole downstream process, it is important to remove the protecting group after the isolation step to get the desired free product.

The cleavage of the BECV-group was performed using bromine as shown in Fig. 5. In the literature the cleavage of the BECV-group from glucosamine derivatives is described using chlorine [30]. The cleavage of the BECV-group from BECV-serinol was performed in methanol and with bromine within 3 h at r.t. yielding 90% of the corresponding serinol hydrobromide.

Figure 5. Cleavage of the 2,2-bis(ethoxycarbonyl)vinyl-group.

This study presents the results of the developed isolation methods for serinol from a glycerol containing fermentation broth. The isolation should be performed by derivatization of the desired compound and following extraction in an appropriate solvent. The whole process is summarized in Fig. 6.

In literature the reactive extraction of propane-1,3-diol from a diluted aqueous solution was described. The propane-1,3-diol was converted into a dioxan, a reversible reaction and simultaneous extraction into an organic solvent using a suitable extracting agent [10]. In this case the isolation of the product could be easily done without removal of water, which leads to considerable energy and time savings. The benefits of this discussed method were the base for our investigations to use a derivatization technique to solve our isolation problem. The mayor difference to the mentioned study is that we had to isolate the hydrophilic serinol from a complex medium in presence of many ingredients. Another difficulty was that the conversion of glycerol during the fermentation process was not complete and therefore a selective derivatization method for the amino alcohol in the presence of glycerol had to be developed. Nevertheless, we could demonstrate that a selective derivatization of serinol within a glycerol-based fermentation broth and following extraction and also the cleavage of the protecting group at a last step after isolation is possible. The obtained yield is comparable with data from literature as summarized in Table 2.

In literature the separation processes from complex and diluted aqueous solutions were often discussed. The isolated yields of valuable products were about 75%. The described methods consist mostly of a large number of sequential thermal unit separations such as microfiltration, adsorption, distillation, column chromatography [28,29].

The isolation process developed here combines a reversible reaction with following product extraction and a further purification step using column chromatography. The developed method is an effective process for the isolation of amino group containing compounds. The method offers the opportunities to isolate the serinol derivative by selective amino group protection whereas the hydroxyl moieties remain free and also the glycerol remains unreacted and stays in the aqueous layer. The process demonstrates a possible way for serinol isolation and therefore a new opportunity to convert glycerol to higher value added products.

3.3 Application of the developed isolation method for other amino compounds

The developed method was applied to other amino compounds shown in Table 3. The results show that the method could be

Figure 6. Flow scheme of the recovery process.

Table 1. Eluent system and time program for LCMS measurement

Time [min]	MeOH + 0.1% Fac [%]	H ₂ O + 0.1% Fac [%]	Flow [μL/min]
0	40	60	200
10	95	5	200
20	95	5	200
30	80	20	200
35	40	60	200
40	40	60	200

useful or applied to other amino compounds such as serine or 12-amino lauric acid methyl ester. This methyl ester is an important starting material in polymer chemistry, which is prepared by fermentation [31].

The BECV-derivatives could be prepared in water with yields between 50 and 95%. Furthermore, it is demonstrated that the protecting group could be split with bromine as presented in detail for serinol. The process is not only useful for the separation of serinol from a glycerol-based fermentation broth but also for different substance classes. Thus the established isolation method could be expanded to other isolation problems as well.

4 Concluding remarks

Our study presents the isolation of serinol in preparative amounts from a glycerol-based fermentation broth by derivati-

zation of the amino group with an isolated yield of 63% (0.62 g). 7 g/L serinol was obtained starting from 30 g/L glycerol with the bacterium *S. blattae* in the modified basal medium.

In case of the serinol isolation, 50 mL (about 350 mg Serinol) of this broth was used for the derivatization, whereupon a five-fold excess of the diethyl ethoxymethylenemalonate (about 3.9 mL) was applied. 0.62 g BECV-serinol and 2.4 g of the BECV-amine were isolated.

The amount of serinol the supernatant is with 7 g/L rather low. This small amount leads to additional challenges within the downstream process. Due to the low conversion of glycerol to serinol, the still high concentration of glycerol in the broth disturbs several approaches for isolation. The amount of other amino-compounds in the broth is another possibility for future optimization as it requires a surplus of the derivatization reagent. Targets are the ammonium salts in the fermentation media and especially the ammonium hydroxide, which is used during the fermentation process to adjust the pH value.

Besides for serinol and the ammonium salts as by-products the method can be applied for the isolation of a large number of other amino compounds from complex media. We could confirm that it is possible to isolate an amino acid (L-serine), a ω-amino acid methyl ester (12-amino lauric acid methyl ester), amino alkane (hexylamine) and glycosyl amine (glucoseamine HCl) from water in good to excellent yields followed by cleavage of the protecting group with bromine.

The advantage of the presented reaction is mainly the simple performance of the derivatization and of the following

Table 2. Literature examples of separation processes from complex media

Authors	Methods	Results
Malinowski et al. [10]	Isolation of propane-1,3-diol from diluted aqueous solution using reactive extraction.	98% conversion into dioxan 75% extraction of the dioxan
Liu et al. [7]	Isolation of glycerol from biodiesel wash waters using adsorption (Active carbon, clay minerals, zeolites)	60 mg glycerol/g active carbon
Anand et al. [8]	Isolation of propane-1,3-diol from glycerol-based fermentation broth using a combination of microfiltration, Active carbon adsorption, Vacuum distillation, column chromatography	Isolated yield 76%
Barski et al. [27]	Solid phase extraction for downstream separation of propane-1,3-diol and butan-1-ol using chemically modified silica gel	RP-18 material and unmodified silica gel: butanol concentration up to 83 g/L for propane-1,3-diol ineffective
Van den Berg et al. [6]	Isolation of butan-1-ol from fermentation broth using esterification and simultaneous extraction	4.9 g/L of the corresponding ester was obtained in extract phase
This study	Isolation of serinol from glycerol-based fermentation broth using derivatization method and following extraction	Isolated yield 63%

Table 3. Overview of isolation of amino compounds from aqueous solution

Compound	Synthesis	Cleavage with bromine
BECV-serinol	water 90% fermentation broth 63% yield	In methanol at r.t. 90% yield
BECV-glucosamine [25]	water 56–59% yield	In methanol at r.t. 80% yield
BECV-serine	water 86% yield	In methanol at 0 °C; only with impurities (NH ₄ Br)
BECV-hexylamine	water 95%	In methanol at 0 °C 37%
BECV-amino lauric acid methyl ester	water 70–84% yield Riesenberg medium 35%	In methanol 34% yield

extraction. Due to the fact that serinol could be derivatized in the fermentation broth directly avoiding water removal, time and energy can be saved. Furthermore, only the derivatized products were extracted with ethyl acetate. The remaining glycerol and other ingredients of the broth remain in the aqueous phase and can be discarded easily.

The target compound **2** as well as the isolated by-product **4** could be used as starting material for following chemical syntheses, for example as building blocks or as starting materials for biological active compounds such as glycolipide or ceramide derivatives.

Practical application

The downstream processing is one of the most important steps in a production, and consumes up to about 80% of the production costs. The major focus for an appropriate downstream process is an effective, easy to carry out and inexpensive method to isolate the valuable product. The challenge of the downstream process is the isolation of a product from a complex mixture with ingredients, which have similar properties like the desired product.

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