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A Continuous Lipase-Catalyzed Acylation Process for the Large-Scale Production of Vitamin A Precursors

Bernard Orsat[a]*, Beat Wirz[b]), and Stefan Bischof[a])

Abstract. A continuous enzyme-catalyzed acylation process for the selective preparation of monoacylated Vitamin A precursors starting from a 1,6-diol on a large scale is reported. Screenings led to the selection of the commercially available immobilized lipase *Chirazyme L2-C2* (lipase B from *Candida antarctica*) as the biocatalyst, different vinyl acylates as the acylating agents, and acetone as the co-solvent. Using a mixture of 70% (*v/v*) acetone and acylating agent allowed to increase the substrate concentration from 10 to 30% (*w/w*). Using a small fixed-bed reactor, this continuous process produced monoacylated product with >99% yield and >97% selectivity for the primary hydroxy group. The robustness of this system under different conditions was investigated. Consequently, the stability of the biocatalyst could be greatly improved by adding a protective pre-column and by adding small amounts of organic base and antioxidant to the substrate solution.
This optimized laboratory process was used to selectively prepare monoacylated compounds in kilogram scales over one hundred days with only a minor decrease in conversion efficiency. The process was also implemented in an up-scaled miniplant for the continuous production on a kilogram-per-day scale, reproducing the results previously obtained on smaller laboratory scales.

1. Introduction

The science of vitamins is generally considered to be one hundred years old. At the end of the last century, it was shown that the growth and vital functions of experimental animals required small supplements of essential substances: vitamins. Today, the substances that should be regarded as vitamins are all considered known. They belong to thirteen groups of compounds classified according to their solubility: the fat-soluble are differentiated from the water-soluble vitamins.

Their metabolic functions are mainly catalytic and/or regulatory. Therefore, an inadequate supply of them leads to typical pathological deficiency symptoms and can severely damage the organism.

Vitamins must be regularly consumed with the diet, either as such or as precursors (provitamins), but there are periods and populations for which an adequate supply of these important diet components is still not possible. In industrial countries, vitamin requirements could theoretically be covered by the food consumed, but the increased preservation time of food results in a considerable loss of vitamins due mainly to oxidation processes. To compensate for this loss, fortification of basic foods (margarine and milk products), cereals, diet foods, and fruit juices has therefore gained importance.

Vitamins currently command a market of 3.0–3.5 billion USD/year (4.5–5.0 billion CHF/year). In terms of value, the feed industry accounts for *ca.* 50%, the pharmaceutical industry for *ca.* 30%, and the food industry for *ca.* 20%. Vitamins and their precursors are produced on large scale by four major industrial technologies (isolation, chemical synthesis, fermentation, and mixed processes) [1].

The 'History of Vitamins' continues with a changed orientation of R&D in the field. Currently, new applications, production alternatives, and process optimization are the main focuses.

2. Vitamin A

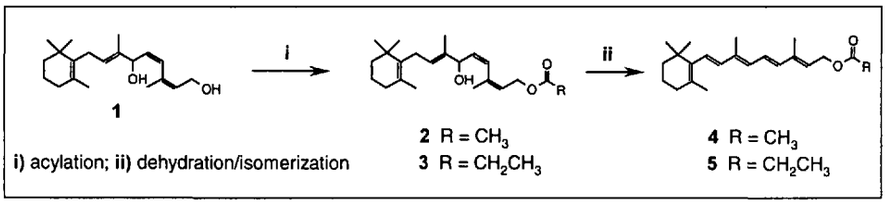
Although it was known from antiquity that night blindness could be diet-related and that fresh liver had an effect against it, the identification of Vitamin A and its role in the sight process was recognized only at the beginning of this century. Its structure was elucidated by *P. Karrer* in 1931. The first synthesis of retinol was carried out by *R. Kuhn* in 1937, and *O. Isler* completed the first industrial chemical synthesis of vitamin A in 1946. Since then, numerous strategies have been developed, and there are at least nine different approaches, the key building block of all industrial syntheses being β-ionone.

Vitamin A is involved in the regulation of cell differentiation, in the stimulation of growth, and in the visual process, which is the most sensitive to a lack of it. The deficiency symptoms are nightblindness, xerophthalmia, impaired reproductivity, and growth retardation. Still today, in the third-world countries, Vitamin A deficiency is the most frequent cause for blindness and concerns mainly children, who also often show a deficient growth and an increased vulnerability to infectious diseases. Therefore, both treatment and prevention are fundamentally important.

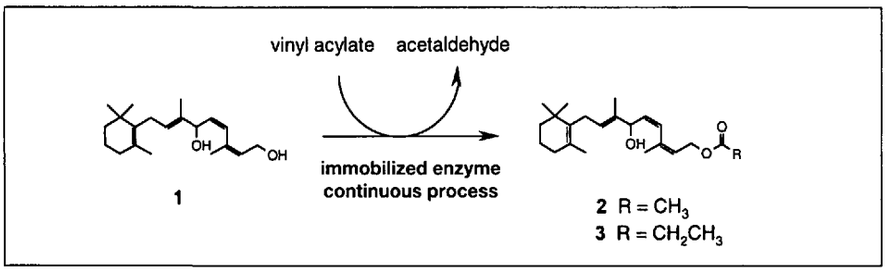
In nature, Vitamin A is found in animal tissues only, whereas the precursor carote-

*Correspondence: Dr. B. Orsat[a])
F. Hoffmann-La Roche Ltd., VFCN, Bau 86/327
CH-4070 Basel
Tel. +41 61 688 93 46, Fax +41 61 688 13 05
E-Mail: bernard.orsat@roche.com
[a]) Vitamins and Fine Chemicals Division, Chemical Process Technology
[b]) Pharmaceutical Division, Preclinical Research, Dept. of Biotechnology

Scheme 1. *The Last Steps in the* Roche *Synthesis of Vitamin A Esters* [1]



Scheme 2. *Principle of the Proposed Enzyme-Catalyzed Acylation of* **1**



noids, known as provitamin A, are found in the plant world. Oranges, green fruits, and vegetables are sources of carotenoids, while milk, eggs, cheese, and blood plasma are sources of vitamin A. But the richest sources of vitamin A are livers, most notably those of some fishes (halibut, cod, shark). Due to its high storage capacity for retinyl esters, almost 90% of the vitamin A body content is located in the liver. When required, mobilization from this organ occurs through hydrolysis and distribution to different tissues, including the eyes. Tissues where vitamin A is known to have its main functions, such as the eye and epithelial tissues, have very low concentration in comparison with the liver .

Today, pharmaceutical preparations, which are identical with natural sources both chemically and biologically, are almost exclusively containing esters (acetate, propionate, and palmitate) which are more stable than retinol. About 80% of their production is used in animal feed, the remainder in food, cosmetics, and pharmaceutical sectors [1][2].

3. Project

In the *Roche* plant process, the synthesis of **2** and **3** is performed *via* acylation of **1**. The acylated products are then subjected to acid-catalysed dehydration and isomerization of the double bonds to the (*E*)-configuration to yield the vitamin A esters **4** or **5** (*Scheme 1*) [1].

O. Isler and co-workers reported in 1949 the use of an acylation procedure employing acetyl chloride in the presence of pyridine in benzene for partial acetylation of **1** [3]. Later, *U. Schwieter* and co-workers described the same reaction in dichloromethane [4]. Such procedures generally produced mixtures of monoacylated and diacylated compounds.

It was shown that the yield could be improved when the dehydration/isomerization reaction was carried out with pure **2** or with enriched mixtures. Consequently, the selective preparation of **2** and **3** from mixtures of monoacylated and diacylated compounds or directly from the diol **1** has gained interest. As a possible procedure to achieve this, an enzyme-catalyzed acylation of **1** was investigated (*Scheme 2*).

Generally speaking, enzyme-catalyzed acylation reactions have been widely reported in the literature [5]. Porcine pancreatic lipase (PPL) was used to catalyze the regioselective transesterification reactions between various diols and ethyl carboxylates in organic solvents [6]. The use of enol esters and oxime esters for the acylation of various diols in tetrahydrofuran in presence of PPL was compared and described [7]. Lipases were used in various organic solvents with methyl carboxylates, trifluoroethyl carboxylates or cyclic anhydrides for the regioselective acylation of chloramphenicol and thiamphenicol [8]. The PPL-mediated acylation of diols and triols in various organic solvents was reported [9]. The lipase-catalyzed synthesis of esters in organic solvents using lipase from *Chromobacterium viscosum* and from *Pseudomonas* sp., solubilized in microemulsions or immobilized in gelatin-containing microemulsion-based gels, was described [10]. In the total synthesis of oudemansin X, a lipase-catalyzed acetylation of a diol in diisopropyl ether with isopropenyl acetate and lipase *Amano P* (*Pseudomonas* sp.) was performed [11]. The synthesis of monoesters from diols with vinyl acetate and lipase from *Aspergillus niger* was reported [12]. Chemical alternatives were also investigated. The conversion of a mixture containing **1** in the presence of various acylating agents and catalysts to obtain **2** and/or **3** was also described [13]. Furthermore, other chemical catalytic reactions were reported, *e.g.*, the use of distannoxane with vinyl acylates for the acylation of primary hydroxy groups [14][15].

4. Experimental Work and Results

When the project was initiated, we were aware that the main concerns, besides environmental, regulatory, and technical issues, would be economical ones. Therefore, the stability of the enzyme and the substrate concentration became important arguments and the product/biocatalyst ratio the major factor to be considered.

In screening experiments for commercially available hydrolases, *Chirazyme L-2* on *C2* carrier (*L2-C2*) exhibited not only the usual advantages of an immobilized enzyme, but also the highest conversion efficiency for **1**. The conversion yields were determined by reversed-phase HPLC and expressed in percent of the HPLC area (% HPLC area) (*Table 1*).

Screening also revealed the superior acylating behavior of vinyl acylates (*Fig. 1*). However, to avoid crystallization problems, the concentration of **1** in the acylating agent had to be kept below 10% (*w/v*). Vinyl acetate and *L2-C2* were selected for the first enzyme stability experiment. More than twenty solvents were tested in the presence of vinyl acylates. Among them, acetone, methylfuran, tetrahydrofuran, formaldehyde dimethylacetal, diisopropyl ether, *tert*-butyl methyl ether, toluene, dichloromethane, *n*-hexane, and cyclohexane showed the most promising results.

Although a batch procedure was suitable for screening, it would make the monitoring of the enzyme stability tedious and difficult. Therefore, a continuous process including a thermostatic fixed-bed reactor with continuously monitored flow-rate was used.

During preliminary runs to approximately 99% conversion under diverse conditions, this simple set-up made the observation of negative as well as positive changes on the enzyme stability fast and easy. However, unexpected factors, probably arising from the substrate solution, usually caused a severe deactivation within a few days. To solve this problem, a protec-

tive pre-column, filled with commercially available alkaline, complexing and/or filtering solid materials (EDTA tetrasodium salt), was introduced. Adding 100 ppm of organic base (triethylamine or ethyl(diisopropyl)amine) and 100 ppm antioxidant (hydroquinone or 2,6-di(*tert*-butyl)-*p*-cresol) to the substrate solution and keeping it under an inert atmosphere and protected from light also had positive effects.

Two first long-term continuous syntheses using vinyl acetate were simultaneously run at different temperatures. The fixed-bed reactors contained 2.5 g *L2-C2* (at 50°) and 6.0 g *L2-C2* (at 22–24°), were protected by identical pre-columns (EDTA tetrasodium salt), used the same flow-rate (1 ml/min), and similarly concentrated substrate solutions (10% (*w/v*) **1**) containing 100 ppm organic base and 100 ppm hydroquinone. At 50°, the conversion efficiency dropped severely within a few days, whereas only a –0.54% decrease was observed over 100 days (linear regression) at 22–24°. This minor decrease illustrated the intrinsic stability of *L2-C2* at room temperature. The pre-column was then removed and the run continued with the same load of enzyme for another hundred days. The conversion efficiency decreased by –9.6% over this period, highlighting the significantly positive influence of the EDTA-tetrasodium-salt pre-column on the stability of *L2-C2* (*Fig. 2*). Further improvements included the use of a co-solvent to increase the substrate concentration and a reduction of both the organic base and the antioxidant concentrations; 100% conversion was required to be maintained over the complete duration.

Among the co-solvents commercially available in large quantities, which are inexpensive and compatible with the reaction and the biocatalyst, only acetone, cyclohexane, tetrahydrofuran, and *tert*-butyl methyl ether allowed concentrations of over 20% (*w/w*) of **1** to be achieved at operating temperatures and were thus selected for further long-term experiments with vinyl acetate. Two control experiments with vinyl acetate and with vinyl propionate used as solvents were also included. The six continuous experiments were simultaneously started at 22–24° with a reduced flow-rate (0.1 ml/min), identical pre-columns (5.5 g EDTA tetrasodium salt), but different biocatalyst quantities and differently concentrated substrate solutions containing 15 ppm hydroquinone and 100 ppm triethylamine. The extrapolated decreases in conversion efficiency were then compared (*Table 2*).

Cyclohexane, *tert*-butyl methyl ether, and tetrahydrofuran exhibited severe loss-

Table 1. *Conversion Efficiencies Obtained With Different Lipases within 16.5 h at 21–23° in the Presence of 10%* (w/v) **1** *in Vinyl Acetate*

Biocatalyst	Supplier	Conv. [% HPLC area]
Chirazyme L-2 on C2 (L2-C2)	formerly *Boehringer Mannheim*	97
PL on DEAE-*Toyopearl*	*Meito Sangyo*	96
Chirazyme L-2	formerly *Boehringer Mannheim*	96
PLC	*Meito Sangyo*	88
QLG	*Meito Sangyo*	88
QLC	*Meito Sangyo*	87
QLGA	*Meito Sangyo*	84
Chirazyme L-1	formerly *Boehringer Mannheim*	83
Chirazyme L-9 on C2 (L9-C2)	formerly *Boehringer Mannheim*	82
Chirazyme L-9	formerly *Boehringer Mannheim*	76
PS on *Toyonite-200-P*	*Amano*	50
PS on *diatomite*	*Amano*	50

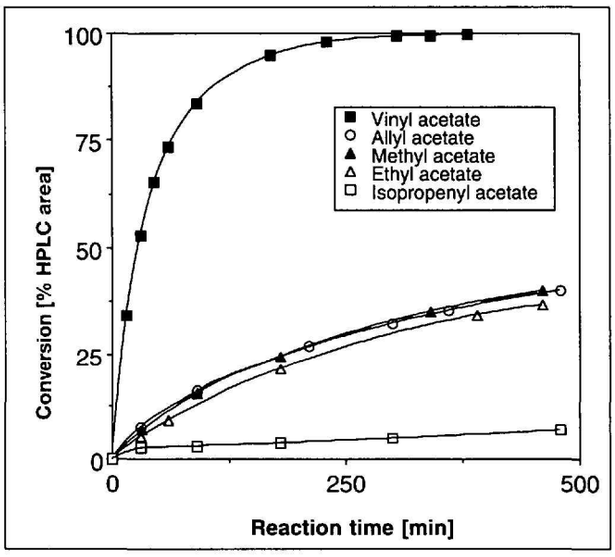


Fig. 1. *Comparison of five acetylating agents used in lipase-catalyzed batch reactions at 50° in the presence of 10%* (w/v) *of* **1**

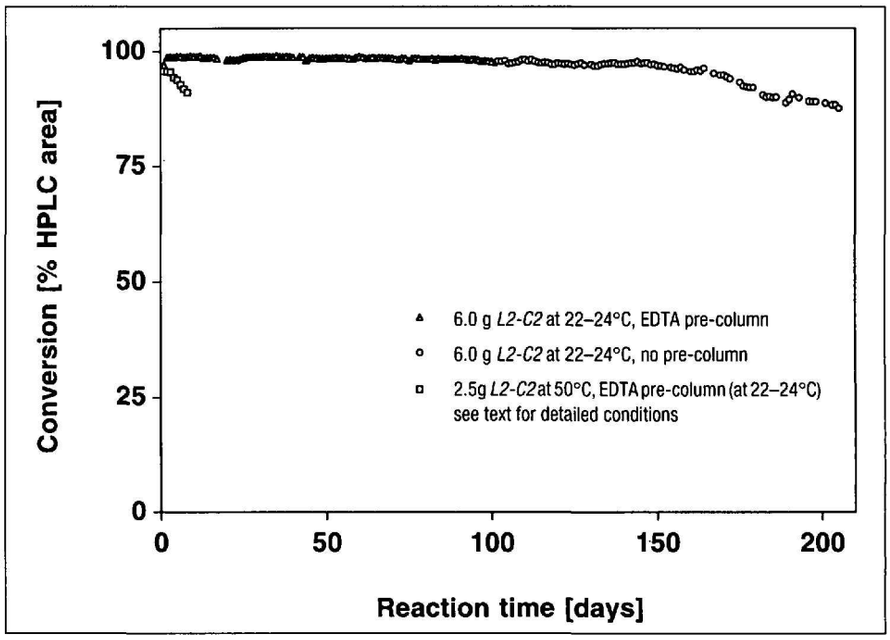


Fig. 2. *Continuous acetylation catalyzed by* L2-C2 *in the presence of 10%* (w/v) *of* **1** *in vinyl acetate:* (Δ) *at 22–24° with a pre-column;* (❑) *at 50° with a pre-column* (at 22–24°); (O) *at 22–24° without a pre-column*

Table 2. *Continuous Acylation Catalyzed by* L2-C2 *at 22–24° in the Presence of Vinyl Acetate, Vinyl Propionate, or Mixtures of Co-Solvents and Vinyl Acetate.* The decrease was extrapolated (to 100 d) from 22 to 35 d runs.

Solvent	b.p. [°C]	1 [% *w/v*]	Solvent [% *v/v*]	*L2-C2* [g]	Run [days]	Decrease [%]
vinyl acetate	71–73	10	0	1.0	35	–3.0
vinyl propionate	94–95	10	0	1.6	35	–8.1
cyclohexane	80–81	20	60	3.5	22	–59.5
acetone	55–57	25	70	2.5	35	–3.2
tert-butyl methyl ether	54–56	25	80	2.5	23	–88.8
tetrahydrofuran	66–67	35	60	2.0	22	–52.4

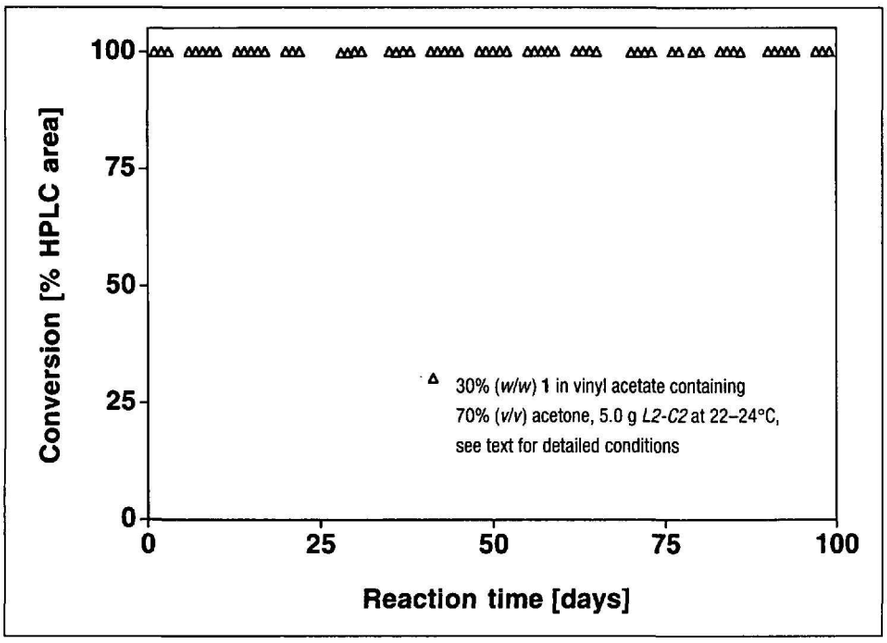


Fig. 3. *Continuous production of* **2** *catalyzed by* L2-C2 *at 22–24° in the presence of a substrate solution made of 30% (w/w) of* **1** *solubilized in 70% (v/v) acetone and vinyl acetate*

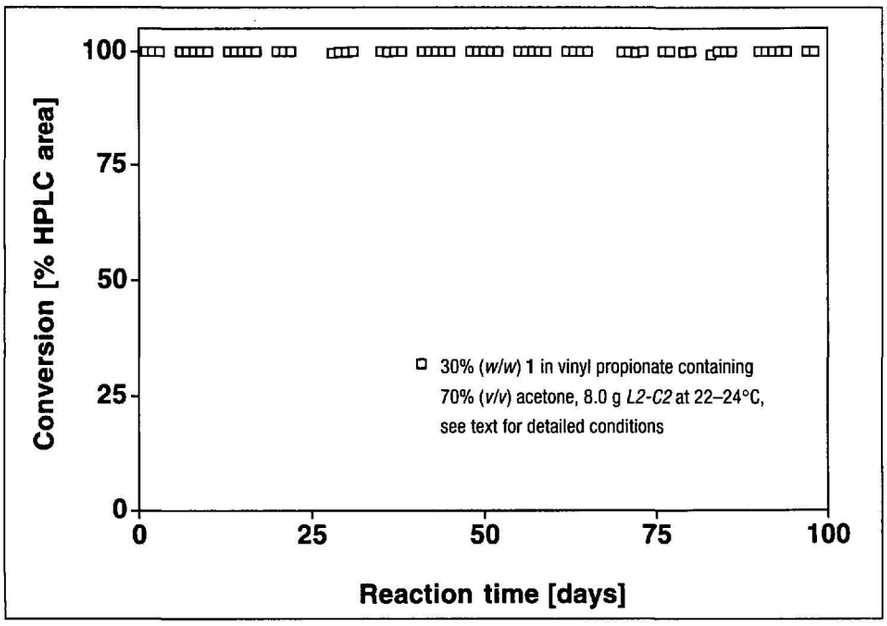


Fig. 4. *Continuous production of* **3** *catalyzed by* L2-C2 *at 22–24° in the presence of a substrate solution made of 30% (w/w) of* **1** *solubilized in 70% (v/v) acetone and vinyl propionate*

es, whereas acetone showed a decrease similar to that of the control experiments. The control experiment with vinyl acetate containing only 15 ppm hydroquinone showed an extrapolated loss almost six times higher than experienced when 100 ppm was present. We therefore considered 100 ppm hydroquinone as an adequate amount to be added.

Following these results, two continuous syntheses at 22–24° using 30% (*w/w*) **1** solubilized in mixtures of vinyl acetate or vinyl propionate and 70% (*v/v*) acetone containing 100 ppm triethylamine and 100 ppm hydroquinone and 5.5 g EDTA-tetrasodium-salt pre-columns were run over one hundred days. To reach 100% conversion with a flow-rate of 0.1 ml/min, 5.0 g *L2-C2* was used with vinyl acetate, and 8.0 g with vinyl propionate. The content of respective diacylated products reached 5–10% at the beginning and stabilized between 1–3% after 10 days. An increased starting flow-rate should solve this problem.

Under these conditions, the consumption of 43.2 g/day **1** produced 4.9 kg **2** or 5.1 kg **3** over 100 days. During this period, the conversion declined by –0.04% (linear regression) with vinyl acetate (*Fig. 3*) and –0.05% with vinyl propionate (*Fig. 4*).

Two additive factors seemed to be involved in the deactivation of the biocatalyst during continuous production. First, an irreversible and permanent one probably caused by aging or deterioration of the catalyst. Under optimized conditions, this contribution was only minor and easy to quantify over long periods by linear regression. The second one was sporadic, but reversible and most likely due to unsuitable conditions. A lack of organic base in the substrate solution, for instance, could lead to a severe impairment of conversion efficiency, which could be reverted by adding the missing amine. On-line monitoring of the conversion rate, a permanent control of the flow-rate, and of the organic base and antioxidant concentrations seem to be key factors to achieve a reliable long-term stability.

Following the laboratory work, the process was scaled up in a miniplant. For the first run, the substrate solution was made with 10% (*w/w*) **1** in vinyl acetate, containing 100 ppm triethylamine and 100 ppm hydroquinone, and was maintained under nitrogen. 120.0 g *L2-C2* (at 22–25°) and a pre-column (at 30°), containing a 1:1 mixture of EDTA tetrasodium salt and sea sand to avoid too much overpressure, were used. With a 10 g substrate solution/min throughput and 100% conversion, this mini-plant consumed 1.4 kg of **1** and pro-

duced 1.6 kg of **2** daily. The conversions were determined by a calibrated reversed-phase HPLC method and expressed in [% *w/w*].

After 74 days, the conversion was still 99.4%, the extrapolated loss (to 100 days) was –0.8%, and 100% conversion efficiency was then restored by decreasing the flow-rate by 5.0% lowering the productivity to 95.0% of the previous one. The run was then continued, and the conversion was still 99.9% after 7 days (*Fig. 5*).

This showed that 100% conversion could be maintained using on-line analytics coupled to a flow-rate controller. A continuous incremental flow-rate adjustment would also allow to optimize the loss of productivity following each flow-rate decrease.

For product work-up, the acetaldehyde generated in the reaction had to be first separated from the mixture containing vinyl acetate and **2**. On a miniplant scale, acetaldehyde (b.p. 20.5°) had to be carefully handled because it can form explosive mixtures which are heavier than air. This removal led to a considerable loss of vinyl acetate which, however, could be regained by rectification and then recycled into the process. Subsequently, the separation of vinyl acetate and **2** was carried out and the recycled reagent was reused.

The elimination of the minor technical imperfections as well as the further exploitation of this continuous enzyme-catalyzed process in the presence of a co-solvent are currently pursued (*Scheme 3*).

5. Conclusion

An enzyme-catalyzed acylation process for the large-scale continuous production of vitamin A precursors has been developed.

The selection of adequate biocatalyst, acylating agents, and co-solvent led to a laboratory process which showed satisfactory robustness yielding **2** and **3** with >99% conversion and >97% selectivity.

This laboratory process used 15 ml fixed-bed reactors with 5.0–8.0 g of biocatalyst. Under optimized conditions, the process allowed the continuous production of 4.9 kg of **2** and 5.1 kg of **3** (containing 1–3% diacylated product) within hundred days.

This laboratory process was then implemented in a miniplant using 120 g of the biocatalyst. Under these up-scaled conditions, 1.4 kg of **1** was daily converted to 1.6 kg of **2**. After 74 days, the conversion efficiency was still 99.4%, and a 5%

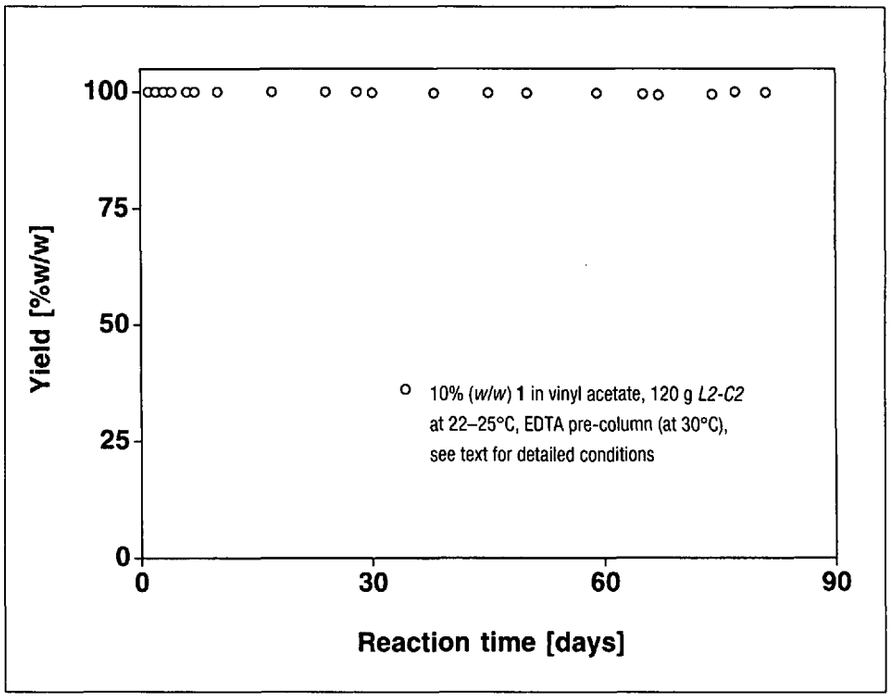
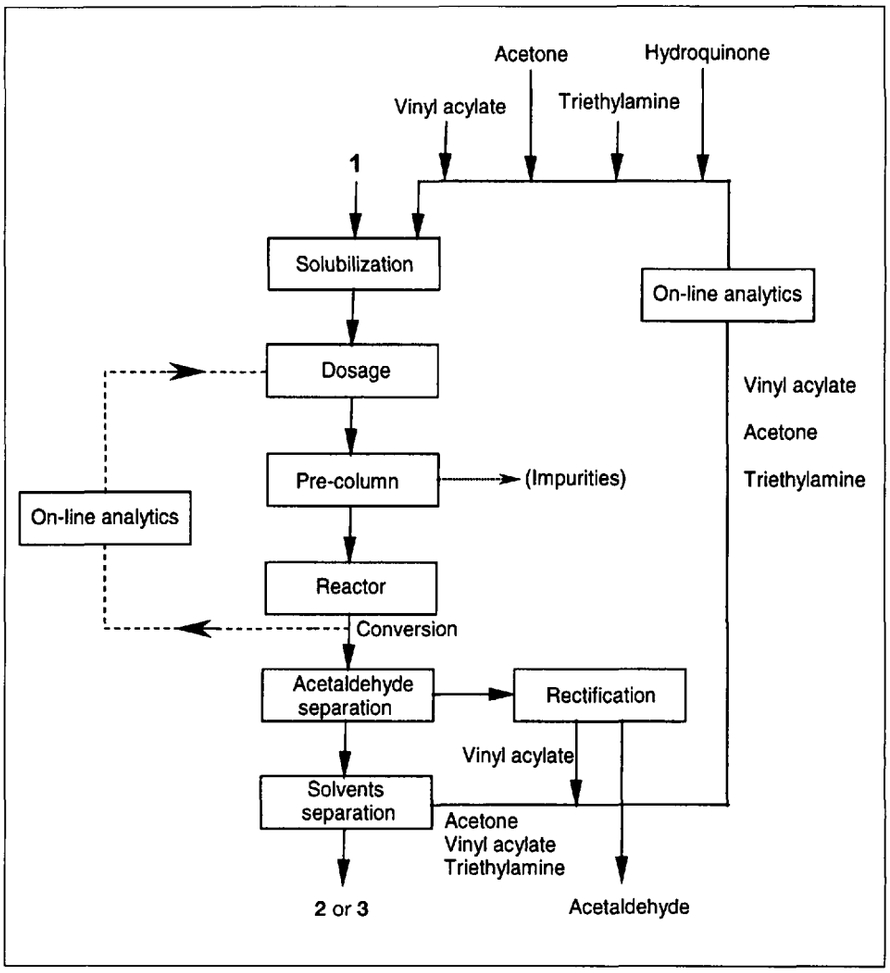


Fig. 5. *Continuous miniplant production of* **2** *catalyzed by* L2-C2 *at 22–25° in the presence of 10% (w/w) of* **1** *in vinyl acetate.* The throughput was readjusted after 74 d.

Scheme 3. *Block Diagram of the Enzyme-Catalyzed Process Using a Mixture of Acetone and Vinyl Acylate Containing Triethylamine and Hydroquinone*



decrease in flow-rate restored the conversion efficiency back to 100%. The feasibility of the work-up procedure was demonstrated, but technical modifications and improvements are still required.

The achievements described here highlight the potential of this enzyme-catalyzed process which might become an interestingly competitive alternative for the existing chemical procedure. The process is environment-friendly, robust, suitable for automation, and involves inexpensive and recyclable chemicals. Recently, a patent application covering the reported work has been filed [16].

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Enzyme and Reaction Engineering in Biocatalysis: Synthesis of (*S*)-Methoxyisopropylamine (= (*S*)-1-Methoxypropan-2-amine)

George Matcham*, Mohit Bhatia, Wei Lang, Craig Lewis, Richard Nelson, Alice Wang, and Wei Wu

Abstract. Methoxyisopropylamine is a chiral amine moiety common to the chemical structures of two important chloroacetamide herbicides, metolachlor and dimethenamid. The activity of both products lies predominantly in their (*S*)-enantiomeric forms. *Celgro* scientists have developed a high-productivity biocatalytic process to (*S*)-methoxyisopropylamine *via* transamination of methoxyacetone and isopropylamine. Biocatalyst and process optimization was achieved by integration of molecular biology, fermentation, enzymology, and engineering disciplines to identify and overcome kinetic, stability, and thermodynamic constraints on productivity. The result was a 50° vacuum reaction producing 2M (*S*)-methoxyisopropylamine (18 wt-%) at >99% ee, with 97% conversion of methoxyacetone in 7 h, meeting economic targets applicable to agrochemical manufacturing.

Introduction

The potential of biocatalysis to address the chiral chemical needs of the pharmaceutical and agrochemical industries is well recognized. For agrochemicals, much more than pharmaceuticals (which generally enjoy higher profit margins), the cost of manufacturing plays a key role in determining the commercial success of a product. This is particularly the case in the present highly competitive crop-protection market, where (established) chemical and (newer) biotechnology solutions are

*Correspondence: Dr. G. Matcham
CELGRO Corporation
661 Route 1 South
North Brunswick, NJ 08902, US
Tel.: (732) 509-2020, direct: (732) 509-2032
Fax: (732) 509-2022
E-Mail: gmatcham@celgro.com