BIOTECHNOLOGICAL PRODUCTION OF PODOPHYLLOTOXIN BY *Linum album* SUSPENSION CULTURES

Inaugural-Dissertation

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by

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from Kamp-Lintfort

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CONTENTS

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BR	bioreactor
ΔC	oxygen-concentration gradient
CAD	cinnamyl alcohol dehydrogenase
4CL	4-coumarate:CoA ligase
CoA	coenzyme A
DO	dissolved oxygen saturation
dw	dry weight
ε	extinction coefficient
EC	Enzyme Commission
FDA	fluorescein diacetate
fw	fresh weight
G	growth index
kDa	kilodalton
k_La	volumetric oxygen transfer coefficient
L.	Linnaeus, Carolus
mM	millimol
mS	millisiemens
MS	Murashige & Skoog
NADP	nicotinamide adenine dinucleotide phosphate
OTR	oxygen transfer rate
PAL	phenylalanine ammonia lyase
L-Phe	L-phenylalanine
Pi	inorganic phosphate
ptox	podophyllotoxin
rpm	rotations per minute
t_d	mass doubling time
L-Trp	L-tryptophan
L-Tyr	L-tyrosine
vvm	aeration volume per culture volume
Δx	boundary layer
μ	specific growth rate
$Y_{x/s}$	growth yield

A. INTRODUCTION

A.1 General aspects of natural products and their application

Throughout the ages, humans have been exploiting plants for the production of foodstuff, clothing, flavours, fragrances and, last not least, medicines. For the treatment of human ailments elaborate healthcare systems evolved in countries like China, India and the tropical areas around the world. Up to now plant-derived drugs and remedies for primary health care have played an important role particularly in the rural parts of developing countries. It has been estimated by the World Health Organization (WHO) that approximately 80% of the world's population depends on traditional medicine and even half of the most-prescribed drugs in the US is linked directly to natural product research (NEWMAN, et al. 2000). During the last centuries highly sophisticated methods have been developed to find out the effective principle of a single phytopharmacon, known for its beneficial potential, or proved *in vitro* activity. In fact, many isolated natural products such as digoxin, morphine, reserpine and quinine have replaced the source from which they were derived. One of the most prominent examples is the discovery of salicylic acid in *Filipendula ulmaria* L. and in the bark of *Salix alba* L. – plants whose pain-relieving properties were described hundreds of years ago. As prolonged usage of salicylic acid causes stomach problems further chemical modification was needed to obtain aspirin in the middle of the 19th century (MANN & PLUMMER 1993). Apart from this simplistic view that a single constituent accounts for the pharmacological function of a drug, in many cases investigations failed to depict the active principle of one single plant extract. In this context PHILLIPSON (1999) stated that the antimalarial herb *Artemisia annua* L. contains many other compounds in addition to artemisinin which *in vitro* enhances the activity against *Plasmodium falciparum*. Another well-known example is ginseng (*Panax ginseng* C.A.Mey.), which has been used in traditional Chinese medicine for over 2000 years. Most of the pharmacological benefits of ginseng (stimulatory effects on the central nervous system, immunmodulatory properties) could have been attributed to the steroid-like group of ginsenosides. However, overall effects can be quite complex due to the variable composition of structurally diverse ginsenosides, numerous intrinsic modes of action and the occurrence of non-steroid constituents (ATTELE, et al. 1999).

Despite the fact that plant based complementary medicine has recently gained wide public recognition it has to be pointed out that natural products often contain toxic compounds and unpredictable side effects. Pregnant women, children and elderly patients, in particular, are mainly at risk because of false body weight dose estimation and because of the poor hepatic enzyme activity for biotransformation (CHAN 1994; CHANG, et al. 2000). Moreover phytochemists are concerned about inadequate quality control, lack of prove of efficiency and the non-critical attitude of the public towards herbal medicines (D`ARCY 1991; ROTBLATT 1999).

Though being highly toxic some natural products have long since been attractive for medical purposes or have been applied as molecular tools in basic research because of their unique structure and interesting biological activity. In fact, the most poisonous organic substances known have been found in nature or are derived from corresponding lead structures. The lectin ricin for example accumulates in seeds of the castor oil plant *Ricinus communis* L. and causes an inhibition of protein biosynthesis after the absorption into the blood system (LD_{50} 6.00 mg kg^{-1}). And even the most toxic poison occurring in nature, botulinum toxin (LD_{50} 0.00001 mg kg^{-1}) has recently entered therapeutic treatment. Botulinum toxin is a bacterial polypeptide, which is synthesised by *Clostridium botulinum* under anaerobic conditions, acts as an efficient neurotoxin and can also lead to food poisoning (botulism). After it had been standardised, it was licensed by the US Food and Drug Administration (FDA) for treating eye malfunctions in connection with abnormal muscle posture and tension.

A.2 Plant-derived antineoplastic agents – the case of podophyllotoxin

The following substances, quoted in this context, are good examples of how traditional knowledge about medical plants can draw attention to their phytochemical characteristics and further clinical use in cancer therapy. Currently, only a few drugs which are applied for the treatment of cancer were discovered on the basis of rational chemical design. Most other substances have been found as a result of large screening programs, serendipity or ethnopharmacological studies (WALL & WANI 1996). The problem arising from the latter possibility is based on insufficient definition of cancer in folklore and traditional medicine. Furthermore, a discrepancy exists between the prevailing illnesses in regions where the historical knowledge about phytomedication still remains a part of the common health care system and the orthodox medication of industrialised countries. This divergence is presented by BALICK & COX (1996), who claim that infections, cancer, cardiovascular- and nervous diseases account for most of the death rates in Western civilization. Indigenous people tend to suffer more from gastrointestinal diseases, inflammations and problems in connection with pregnancy and birth. It appears that, especially in the case of cancer therapy, finding a promising plant species for phytochemical analysis can be quite difficult or misleading.

A well-known example is the discovery of the two indole-dihydroindole alkaloids vinblastine and vincristine in the Madagascan periwinkle *Catharanthus roseus* G. Don. Both substances are used clinically for the treatment of a variety of human neoplasms like Hodgkin's disease, testicular cancer and small cell lung cancer. They have also proved to be excellent lead structures for further modifications on the increase of anti-cancer activity (RAMAWAT 1999). The parent plant was traditionally used in different countries for the treatment of conditions similar to diabetes. Therefore early studies were conducted to find the decisive principle of the plants supposed hypoglycaemic effect. Hence, the discovery of the two antineoplastic agents vinblastine and vincristine can be indirectly attributed to the observation of an unrelated medicinal use of the source plant. Another striking example of a plant-derived antitumor drug is paclitaxel, the effective ingredient of Taxol® which is already being used for the chemotherapeutic treatment of cancer (Bristol-Myers Squibb Company 1998; SOHN & OKOS 1998). The complex structure and unique mechanism (AMOS & LÖWE 1999) of the diterpenoid paclitaxel have made it an interesting pharmacophore in phytochemical screening programs. Paclitaxel along with its biosynthetic precursors, the baccatins, occurs in the bark as well as in the leaves of many yew species. It is only the attractively coloured arillus which does not show any toxic effects after ingestion. Initially, the group of taxanes were isolated from the bark of *Taxus brevifolia* Nutt. (Taxaceae), the Pacific yew, as a part of a random collection program by the US-American National Cancer Institute in the late 1960s (STULL, et al. 1995). In the following chapters paclitaxel will also serve as an illustrative example for sustained biotechnological production of natural products in order to meet the growing demand in clinical cancer therapy.

As mentioned earlier, the traditional use of a medical plant does neither necessarily need to reflect its phytochemical composition, nor the definite application in modern medicine. To some extent this also holds true for the two species *Podophyllum peltatum* L. (Figure A-1) and *Podophyllum hexandrum* Royle (Berberidaceae), native to North America and the Himalayan region, respectively (BOWEN & CUBBIN 1993). In ancient times, the podophyllum resin (podophyllin) was obtained by extraction of the plants rhizome and utilized in folklore medicine as a cathartic, cholagogue, as an anthelminthic and for skin disorders, thus indicating its cytotoxic activity (IMBERT 1998). In the middle of the last century, renewed interest in *Podophyllum* species arose from the observation that podophyllin could accomplish regression of condylomas when topically applied – this has also remained the only actual pharmaceutical indication of an alcoholic *Podophyllum hexandrum* extract (VON KROGH & LONGSTAFF 2001). The results led to a comprehensive chemical examination of the earlier knowledge about the constituents of podophyllin and its effect on tumour tissue.

Figure A-1

Specimen of *Podophyllum peltatum* L. (Berberidaceae) in flower. The photo was taken in the Bot. Garden of Düsseldorf at the end of may.

Figure A-2

Specimen of *Linum album* Ky. (Linaceae) in flower. Photo taken in the region of Teheran (Iran) by BAHRAM ZEHZAD.

Podophyllotoxin, the main toxic compound found in the resin had already been described by PODWYSSOTZKI in 1880 and was crystallised by KÜRSTEN in 1890 (BOHLIN & ROSÉN 1996). It belongs to the class of aryltetralin lactone lignans, a group of dimeric phenylpropanoids (C_6-C_3). This lignan type is widespread in nature and not exclusively restricted to the *Podophyllum* genus. Although present in lower amounts, podophyllotoxin and its derivatives have been mainly detected in e.g. *Thujopsis dolabrata* L. and *Juniperus chinensis* L. (Cupressaceae), *Daphne genkwa* (Thymelaeaceae) and different *Linum* species (MURANAKA, et al. 1998; OKUNISHI, et al. 2001; WEISS, et al. 1975; KUHLMANN, et al. 2002; MOHAGHEGHZADEH, et al. 2001). Figure A-2 shows a specimen of *Linum album* which has been used at our institute for *in vitro* production of podophyllotoxin by cell suspension cultures.

As it is still difficult to limit the antineoplastic activity of the therapeutic agent to only one specific target, the originally isolated compound frequently turns out to be too toxic. This was also the case for podophyllotoxin (CASSIDY, et al. 1982; KO 1999), which was tested in pharmacological studies by Sandoz, Ltd. in the early 1950s. Subsequent work on selective modification of the parent molecule resulted in more than 500 derivatives from which two, VM26 (teniposide) and VP16 (etoposide), gave excellent cytostatic potency coupled with a lowered overall toxicity (STÄHELIN & VON WARTBURG 1991). The following figure shows the lignan-type structure of podophyllotoxin, etoposide and teniposide.

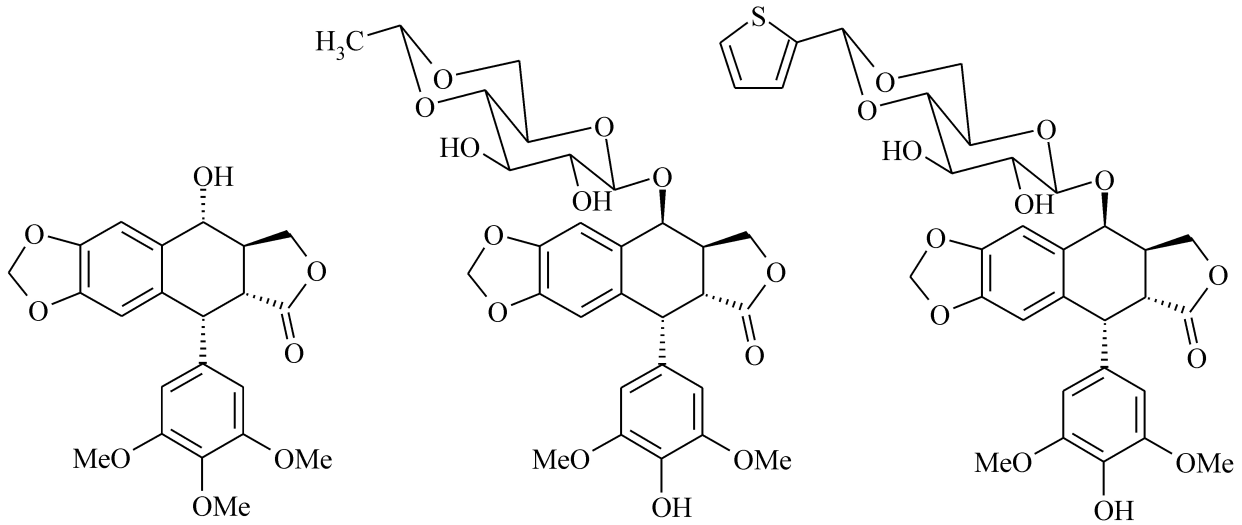

Figure A-3

Podophyllotoxin (a) and the two derivatives etoposide (b) and teniposide (c). Both represent semisynthetic aldehyde condensation products of demethylepipodophyllotoxin glucoside.

Clinical use of the epipodophyllotoxin derivatives comprises a wide spectrum of indications for the treatment of cancer types such as refractory childhood lymphoblastic leukemia and testicular germ-cell tumours (SQUIBB 1997; SQUIBB 1998). The major drawback of etoposide is the inherent poor water solubility which can cause allergic reactions and requires utilization of noxious solvents like polyethylene glycol or polysorbate (BUDMAN 1996). Further research yielded the water-soluble prodrug etoposide phosphate (Etopophos®, Bristol-Myers Squibb Company). In this case a phosphate moiety was added to the free phenolic hydroxygroup which is *in vivo* readily removed by endogenous phosphatases, thus releasing the active drug into the circulatory system (SCHACTER 1996).

Besides their distinctive structure and improved pharmacological activity the epipodophyllotoxins also exhibit a different mechanism of action in comparison to podophyllotoxin. For the latter it has already been shown in 1946 that on cellular level it can reduce cell proliferation by inhibiting the assembly of mitotic spindle (STÄHELIN & VON WARTBURG 1991). Thereby cells will still enter mitosis and will perform a normal prophase before they become arrested at the beginning of the metaphase, in which microtubules are needed for accurate chromosome dissection. Comparative functional studies of podophyllotoxin and colchicine, an alkaloid from the Autumn Crocus *Colchicum autumnale* L. (Colchicaceae), revealed overlapping binding sites on tubulin monomers for both competitive molecules (DESBÈNE & GIORGI-RENAULT 2002). Interestingly a similar mechanism for etoposide and teniposide were found at high concentrations, whereas lower, clinically relevant amounts gave rise to a different function in the depression of cell proliferation. It has been elucidated that even low levels of epipodophyllotoxins are capable to arrest cell division in the late S or early G_2 phase of the cell cycle (KAUFMANN 1998; OWA, et al. 2001). Thus, the treated cells will not enter mitosis but rather remain in the premitotic stage. Further studies described the underlying mechanism as mediated by stabilizing a complex formed between the topoisomerase II and the DNA, which is referred to as the cleavable complex. Topoisomerase II is a homo-dimeric enzyme that plays a vital part in the control of the DNA transcription, recombination, replication and the chromosome partitioning during the cell cycle. It functions by forming enzyme-bridged strand-breaks that act as transient gates for the passage of second duplex molecule prior to releasing. That's why the occurrence of double-strand breaks was attributed to the inhibition of topoisomerase II enzymatic strand-rejoining activity (BOTTA, et al. 2001; DAMAYANTHI & LOWN 1998).

A common feature of cytotoxic drugs, utilised in cancer chemotherapy, is their potency for repressing abnormal cell proliferation. On molecular levels this can be achieved by different approaches like inducing cell differentiation, necrosis or triggering for programmed cell death, termed apoptosis. The following list (Table A-1) will summarise some potent plant-derived compounds and their exerted cytotoxic effect.

Table A-1: Plant-derived compounds, their major source and mode of cell toxicity

compound **structure**	**plant species** **family**	**mode of action** **site**
colchicine tropolone alkaloid	*Colchicum autumnale* L. Colchicaceae	tubulin polymerisation inhibitor tubulin β-subunit
podophyllotoxin aryltetralin lactone lignan	*Podophyllum hexandrum* Royle Berberidaceae	tubulin polymerisation inhibitor tubulin β-subunit
vinblastine / vincristine indole alkaloid	*Catharanthus roseus* G.Don Apocynaceae	tubulin polymerisation inhibitor tubulin α-subunit
9-aminocamptothecin semisynth. quinoline alkaloid	*Mappia fretida* Miers Icacinaceae	topoisomerase I inhibitor binary DNA-topo I-complex
etoposide / teniposide semisynth. ptox derivative	*Podophyllum hexandrum* Royle Berberidaceae	topoisomerase II inhibitor binary DNA-topo II-complex
paclitaxel diterpenoid	*Taxus brevifolia* L. Taxaceae	microtubules stabilizing assembled microtubules

A.3 Occurrence and function of secondary metabolites

The term "secondary metabolite" implies that there exists something like a primary counterpart in nature. In fact, the distinction between both groups was drawn in 1891 by KOSSEL (HADACEK 2002) in order to designate secondary products by their proposed less significant function. Although over the last century the majority of pharmacognosists has adopted this classification, the term represents an unfortunate choice as it denotes an inferior role in plant metabolism. The following chapter intents to verify this attitude by informing about their manifold occurrences and functions in nature and characterize the lignans as unique products of the phenylpropanoid metabolism.

The modern chemistry of natural products generally defines secondary metabolites by a molecular weight of less than 1500 Da, thereby distinguishing them from high molecular weight polymers like proteins or polysaccharides for example in pectic compounds. Though the total number of structures designated as secondary metabolites exceeds 139000, nature only uses a few basic building blocks e.g. the acetate (C_2), isoprenoid (C_5) and the phenylpropanoid (C_9) unit (VERPOORTE 2000). Table A-2 should give an overview of the paramount structures found in natural sources (MACRAE & TOWERS 1984; RAMAWAT 1999).

Table A-2: Classes of secondary metabolites, their direct structural building blocks, characteristic / alternative source and biological importance - with lignins included for comparison.

class	building block	main source / alternative sources	biological importance
alkaloids	acetate amino acids terpenoids cholesterol	plants (dicotyle) / marine sponges, algae fungi (*Claviceps purpurea*) bacteria (*Streptomyces* sp.) amphibia (salamander, frog)	nitrogen reserve detoxification deterrent allelochemical poison
cyanogenic-glycosides	amino acids	plants / fungi, bacteria insects (only accumulation)	poison deterrent
flavonoids	malonylCoA + cinnamoylCoA	gymno.- angiosperms / mosses (*Mnium hornum*) marine coral (*Echinopora sp.*) ferns, algae, bacteria	spasmolytic antibiotic colouration development of fertility signalling (*Rhizobia*)
cardiac glycosides	squalene + oligosaccharide	plants (angiosperms) / amphibia (*Salamandra sp.*)	heart tonic poison
monoterpenoids	isoprene	plants / animal glands fungi, intestinal bacteria	antimicrobial neurotoxic repellent
lignans	phenylpropanoids	terrestrial gymno.- angiosperms / pteridophytes liverworts, hornworts	antimicrobial insecticide germiation inhibitor
lignins	phenylpropanoids	terrestrial gymno.- angiosperms / pteridophytes (bryophytes)	structural support protective barrier

Especially for the nitrogen-bearing alkaloids, proteins and non-protein amino acids, it becomes obvious that a clear distinction between primary and secondary metabolites cannot be drawn exclusively. An often cited example is the arginine analogue canavanine that accumulates in legume seeds up to 10% of dry weight. Both canavanine and its breakdown product, canaline (an analogue of ornithine), act as enzymatic substrates, leading to their false incorporation into proteins, a fact which explains their deleterious effects as defence compounds. The question whether the non-proteinous amino acid is a secondary metabolite or not arises from the fact, that during germination it is metabolised to make the nitrogen available for the growing seedling. Against this background canavanine seems to serve as a nutrition reservoir (HADACEK 2002). On the other hand there are always organisms coping with such defence mechanisms. Some insects for instance, feeding on canavanine-containing seeds, have developed a mechanism on enzymatic level to distinguish between the protein and non-protein amino acids.

Much interest has been focused on the ecological and toxic effects of lignans concerning their ability to interfere in mitosis. Apart from this, there is an increasing interest in plant-derived and non-toxic lignans like matairesinol, secoisolariciresinol and pinoresinol, which are found e.g. in flax seeds (*Linum usitatissimum* L.) (MEAGHER, et al. 1999). Several studies suggest that food, such as whole grain rye bread, soya or flaxseed meals have positive effects on the reduction of cancer incidences by altering the metabolism of steroid hormones. In fact, vegetarian or semi-vegetarian dietary gives rise to an implication of phytoestrogenes such as isoflavonoides and lignans. While the lignans do not have any intrinsic estrogenic activity, they are metabolised by the gut microflora to enterodiol and enterolactone, which are termed mammalian lignans (ADLERCREUTZ 2002; HEINONEN, et al. 2001). These estrogen-like substances have been proven to possess an antagonistic effect on endogenous estrogens, which have been attributed to an increased growth and proliferation of breast and prostate cancer (OTTO 2000). In this case, secondary metabolites like the non-toxic lignans in human dietary can be seen as prodrugs for further metabolization.

Regarding the great variety of biological functions exerted by secondary metabolites, it becomes obvious that most of them result from an evolutionary interaction among the organisms (WINK 2003). The ability of plants to synthesize pigments and low molecular volatiles has evolved to attract pollinators, whereas toxins can help to reduce herbivore injury (BENNETT & WALLSGROVE 1994; PICHERSKY & GANG 2000). As a consequence, such ecological interesting compounds have been found throughout the flora and fauna. Even in the case of the lichens over 800 compounds have been characterized – most of them unique in respect to those of higher plants (MÜLLER 2001). Other habitats of intensively interacting organisms are found in the species rich tropical regions and the marine coral reefs. They might serve as natural libraries for the discovery of novel lead structures with remarkable biological activities (STAFFORD 2002). Recently, much interest has been devoted to combinatorial chemistry and its potential against large screening programs of natural sources (DEFURIA 2002; VERPOORTE 2000). Although any such approaches like ethno-pharmacology or screening programs are viewed as time-consuming and uneconomic, alternative methods still lack proof of efficiency. Due to complex structures and high incidences of optical isomers in natural products it is thought that rational chemistry would have never created molecules such as paclitaxel (PEZZUTO 1997). A comparative study by HENKEL et al. (1999) concerning the structural characteristics of natural and synthetic compounds revealed that both sources are rather more complementary than uniform. It also became obvious that for instance bacteria and fungi have already been well characterized, whereas classes like the molluscs still represent a valuable field for future research.

A.4 Lignans and lignin as products of the phenylpropanoid metabolism

Although lignans and lignin rely on the phenylpropanoid metabolism as the central source of monomers for their biosynthesis they display some profound differences. For terrestrial plants lignins played a crucial role on the evolutionary process from the aquatic habitat to upright growth under varying conditions of humidity (BOUDET 2000). It has been estimated that approximately one-third of the organic plant biomass can be attributed to lignin which makes it the second most abundant polymer on earth – eclipsed only by cellulose. Lignin consists of a three-dimensional heteropolymeric structure, which is synthesized at so called initiating sites of the middle lamella and further extends into the secondary cell wall (DONALDSON 2001; WALLACE & FRY 1994). It is here where the oxidase and peroxidase are located too. They are needed for the H_2O_2 mediated free radical polymerisation process in the apoplast. Since the unstable and toxic monomers, mostly coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol (Figure A-4) are synthesized in the cytoplasm, they are transported or stored as glucosides.

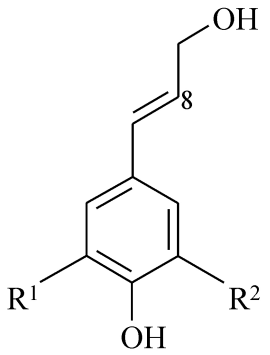

Figure A-4

All three monolignol units serve as building blocks for lignin biosynthesis. In the case of podophyllotoxin (lignan) biosynthesis two molecules of coniferyl alcohol (b) are stereoselectively joined at C_8 position.

p-coumaryl alcohol	(a)	R^1 = H,	R^2 = H
coniferyl alcohol	(b)	R^1 = OMe,	R^2 = OH
sinapyl alcohol	(c)	R^1 = OMe,	R^2 = OMe

The whole process leads to a complex structure with varying proportions of the monomeric phenolics depending on the taxa, stage of development, tissue or environmental conditions (BAUCHER, et al. 1998; WHETTEN & SEDEROFF 1995). As mentioned above, the closely-related lignans are generally regarded as dimeric or oligomeric phenylpropanoids, emerging from an enzymatically controlled coupling of hydroxycinnamoyl alcohol units. In contrast to lignins and any other bimolecular phenoxy radical coupling (insect cuticle sklerotization, algal cell wall polymers, fruiting body development in fungi) this stereospecific reaction gives in most cases rise to the formation of optically active compounds – the lignans (DAVIN & LEWIS 1992; ROUHI 2000). In order to confer regio- and stereospecificity in a 8,8`-linked lignan formation DAVIN and co-workers (DAVIN & LEWIS 2000; 1997) have found a 78-kDa glycoprotein being involved. Although this dirigent protein lacks any intrinsic oxidative properties, *in vitro* experiments containing the protein, coniferyl alcohol and an oxidant, resulted in optical pure (+)-pinoresinol. Subsequent cyclisation steps and the modifications of pinoresinol lead to the formation of podophyllotoxin or related lignans. Regarding the implication of a dirigent protein in lignan biosynthesis it has been argued whether a similar enzyme could be involved in the construction of lignin. However, so far, no optically active compound has been found in isolated lignins and the concept of a stereospecific lignin synthesis remains open to debate (LEWIS 1999; SEDEROFF, et al. 1999).

Major aspects of the essential phenylpropanoid metabolism are presented in Figure A-5 with respect to the associated shikimate pathway and other phenylpropanoid derivatives. According to Table A-2 the phenylpropanoids are mostly found in terrestrial vascular plants, which is consistent with the general view of their ecological function. The suberins, coumarins, flavonoids, lignins and lignans are, so to say, a prerequisite for plant live outside the aquatic habitat (LEWIS & DAVIN 1999). Moreover, the enzymatic deamination of L-phenylalanine to cinnamic acid (phenylalanine ammonia lyase, PAL) denotes the first committed step from primary to secondary metabolism.

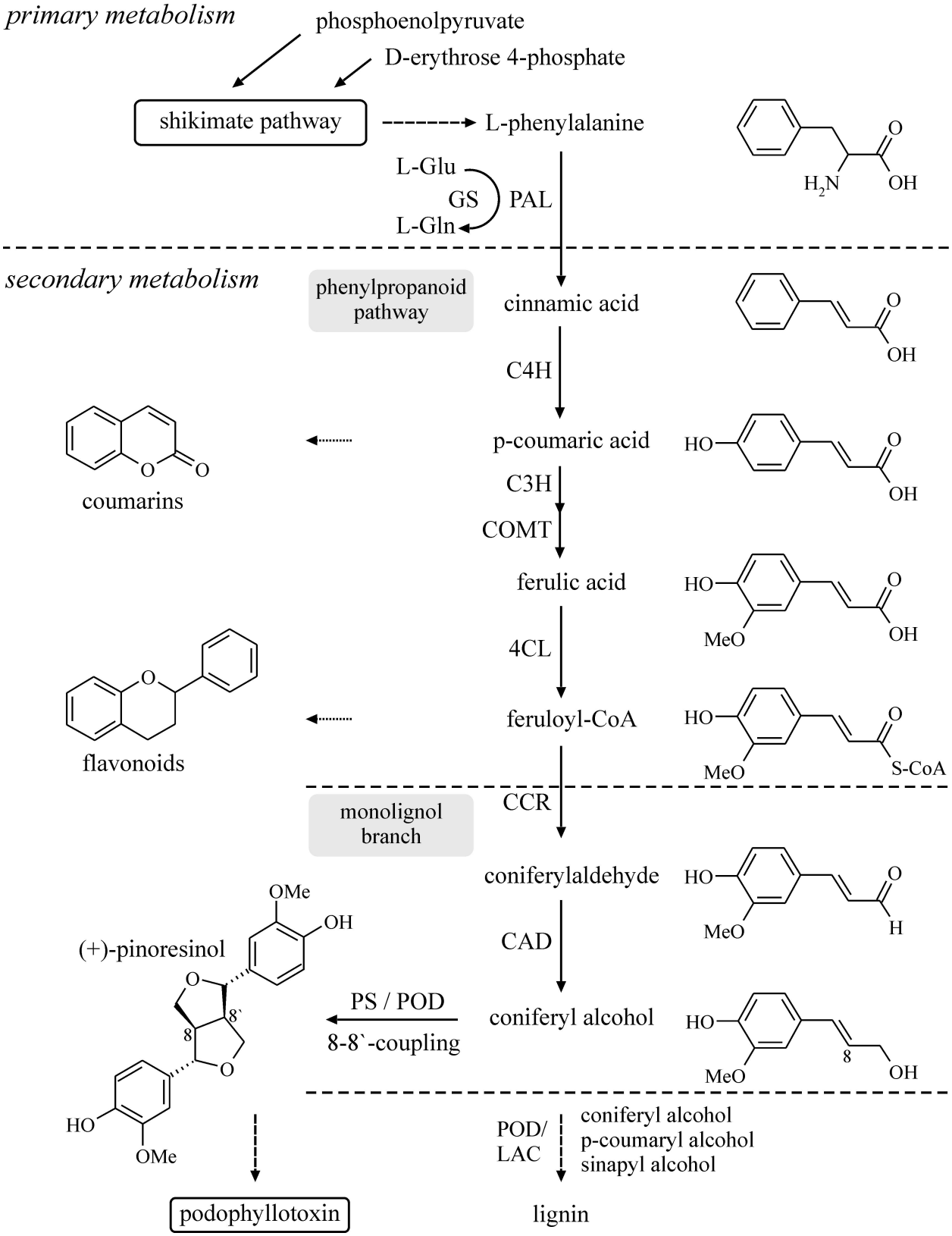

Figure A-5

General aromatic metabolism in plants. The phenylpropanoid metabolism has been restricted to the coniferyl alcohol synthesis. Abbreviations as follows: L-Glu (L-glutamate), L-Gln (L-glutamine), GS (glutamine synthetase), PAL (phenylalanine ammonia-lyase), C4H (cinnamic acid 4-hydroxylase), C3H (coumaric acid 3-hydroxylase), COMT (caffeic acid O-methyltransferase), 4-CL (4-coumarate:CoA ligase), CCR (cinnamoyl-CoA reductase), CAD (cinnamyl alcohol dehydrogenase), POD (peroxidase), LAC (laccase), PS (pinoresinol synthase).

Since animals and humans lack the shikimate pathway, L-phenylalanine represents an essential aromatic amino acid, which can only be synthesized by microorganisms and plants (DEWICK 1994). Starting with phosphoenolpyruvate and D-erythrose-4-phosphate this sequence ought to be considered in two parts: first it provides chorismic acid, which is subsequently converted into prephenic acid, leading to L-Phe, L-Tyr and L-Trp (HERRMANN 1995). Although the shikimate pathway features as a source of well-known substances such as the gallotannins and benzoic acid derivatives, the provision by aromatic amino acids seems to characterize its function best. The general phenylpropanoid metabolism links the shikimate pathway to the formation of lignins as well as to many other classes of phenolic secondary compounds. Initially the deamination of L-phenylalanine yields cinnamic acid. The latter can be transformed into different hydroxycinnamic acids and hydroxycinnamyl-CoA esters, varying in their degrees of hydroxylation and methylation. Regardless of the grid-like structure and the diverse cross reactions of the phenylpropanoid metabolism, in this context only the main steps concerning the synthesis of coniferyl alcohol are presented in Figure A-5. As indicated, the hydroxycinnamyl-CoA esters and free acids form precursors for the synthesis of the flavonoids and coumarins. In terms of the CoA-esters a two-step reduction to the hydroxycinnamoyl alcohols (Figure A-4) brings forth the so called monolignols. These two reactions are carried out by the cinnamoyl-CoA reductase (CCR) and cinnamoyl alcohol dehydrogenase (CAD), which are termed the monolignol branch (BARBER & MITCHELL 1997; BAUCHER, et al. 1998).

For years, the biochemistry and molecular biology of the phenylpropanoid metabolism has been under active investigation because of its relation to lignification. And this might have been one reason why the earliest identified enzymes of plant secondary metabolism were PAL, 4CL and chalcone synthase of the shikimate-pathway (KUTCHAN 2001). As far as the enhancement of lignan accumulation in plants is concerned, the characterization of enzymatic steps on molecular as well as on physiological basis represent a rational approach towards production improvement. Additionally, it has been shown by several authors that the enzymatic activity of definite steps in the phenylpropanoid and monolignol pathway responds to environmental and developmental conditions (BOUDET, et al. 1995; HAHLBROCK & SCHEEL 1989). The following three enzymes have already been described in detail for various plants in lignin biosynthesis and will serve as an indicator for overall phenylpropanoid activity in correspondence with the accumulation of podophyllotoxin (DAVIN & LEWIS 1992).

1. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5)

- L-Phe $\rightarrow$ cinnamic acid + NH_4^+
- L-Tyr as an alternative substrate in grasses
- kinetic inhibition by cinnamate
- not found in animals, sporadic in prokaryotes, widely distributed in fungi and plants
- probable location in the cytoplasm; membrane association has been shown
- high-molecular weight (240-330 kDa) protein, composed of very similar subunits
- multiple isoenzymes and gene families identified
- strong correlation of activity in response to environmental stimuli

2. 4-Coumarate:CoA ligase (4CL; EC 6.2.1.12)

- hydroxycinnamic acid + CoA + ATP $\rightarrow$ hydroxycinnamoyl-CoA + AMP + diphosphate
- requires magnesium as a co-factor
- detected in gymnosperms and angiosperms
- monomeric enzyme with a molecular weight between 45 and 60 kDa
- several isoenzymes with different biochemical properties detected in plants
- inducible activity upon infection by fungi, UV irradiation and wounding
- proposed involvement in metabolic channelling into different phenylpropanoid branches

3. Cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195)

- cinnamaldehyde + NADPH + H^+ → cinnamyl alcohol + $NADP^+$
- reversible reaction possible
- detected in gymnosperm (not polymorphic) and angiosperms (polymorphic)
- zinc-containing dimer (63-84 KDa)
- belongs to the family of long-chain alcohol dehydrogenases (also in prokaryotes)
- detected in the cytoplasm, mitochondria and plastids
- several isoenzymes with different biochemical properties (substrate specificity) proven
- high degree of activity correlation to areas of lignification and environmental stress

A.5 Application of plant cell culture in pharmaceutical biotechnology

A.5.1 Historical development

Long before microorganisms were discovered, they had been exploited for the needs and wishes of human being. Even 5000 years ago prominent examples of fermentation technology were invented for the use of wine in food preservation or the conversion of milk to lactic acid for yoghurt production. By 100 BC, the production of carbon dioxide, generated by the action of the brewer's yeast was able to leaven bread in ancient Rome. In more recent times the application of microorganisms or cell-free extracts has been coined fermentation, according to the basic ingredient needed for such processes, the ferment (*lat. fermentum, fervere / bubble up, dough raising*). L. PASTEUR was the first attributing the lactic acid production to bacteria which turned the beer fermentation broth sour and moreover proved the existence of strictly anaerobic life (DEMAIN & FANG 2000). However, it was not until 1929 that A. FLEMMING described the antagonistic effects of *Penicillium notatum* on cultures of the bacterium *Staphylococcus aureus* and thereby, he opened the door for the production of antibiotics with biotechnological methods. In fact, the antibiotic collection derived from the culture broth and further use of *P. chrysogenum* reflects various approaches such as downstream processing and cell line selection, leading to modern biotechnology (FIECHTER 2000; SCHÜGERL 2000). Since that time a large-scale production of new antibiotics, food additives and pharmaceuticals has emerged and has mainly been restricted to microbial organisms. After 1970, projects were initiated on the scope of the secondary metabolite production by plant- or insect-cell cultivation. Even though both systems have their drawbacks and industrial application has proved in many cases not to be economic, cell *in vitro* culture represents an essential element of recent pharmaceutical biotechnology.

Although the term pharmaceutical biotechnology has been brought to very recently, it comprises well-known research fields like microbiology, biochemistry, process technology, pharmaceutical biology and molecular biology (KREIS & STOLL 2000). However, the development of recombinant DNA technology and genetic engineering in the early 70s (COHEN, et al. 1973) provided the means to create, rather than traditionally to select, high productive microbial strains in biotechnology and pharmaceutical research (KAYSER & MÜLLER 1999). Microorganisms could be used as "biological factories" for the production of hormones, blood factors, immunoactive proteins and secondary metabolites. In the following years heterologic expression of human somatostatin and insulin paved the way for a large-scale formation of numerous therapeutic antibodies (diagnostics, passive immunisation, cancer therapy), amino acids (feed additives, sweeteners, pharmaceuticals), antibiotics (pesticides, growth stimulants, therapeutics), enzymes (genetic engineering, diagnostics, chemical synthesis) and therapeutic proteins (blood factors, digestive aids) (RATLEDGE & KRISTIANSEN 2001). In the middle of the 80s, eukaryotic cloning technology emerged and made plant transformation possible, thus opening a new field for plant *in vitro* culture.

Such a system, consisting of mostly undifferentiated plant cells, has gained much interest for secondary metabolite production, heterologic protein expression and as an alternative source of metabolites in organic chemistry. In general, foreign proteins or other vital compounds can be obtained from different expression systems, depending on viability, safety, cost and ethical considerations. It is believed that undifferentiated cells or even whole plants offer excellent opportunities for heterologic gene expression and large-scale cultivation. Even though this idea came up years ago, it today has conceived much interest because other systems such as prokaryotes or animals (molecular farming) proved not to be reasonable in terms of post-translation processing or safety issues (FISCHER, et al. 1999; JAMES & LEE 2001; SCHILLBERG, et al. 2002).

The basic research regarding *in vitro* culture of plant cells was already carried out 100 years ago by G. HABERLANDT, who cultivated isolated mesophyll cells of *Lamium purpureum* L. although cell division has never been observed in that time (ALFERMANN & PETERSEN 1995; DÖRNENBURG & KNORR 1995). In the 1930s, the first proliferating plant cell culture was established. From that time on, such systems were used as research tools in order to study the physiology and biochemistry of plants. Initial attempts in 1952 by Pfizer & Co, to exploit plant cells on a large-scale failed until FUJITA (1982) published the production of shikonin (naphthaquinone, bright red colour) on a large-scale by *Lithospermum erythrorhizon* L. Shikonin is a traditional medicine in Japan and has been used for its anti-bacterial, anti-inflammatory effects as well as a dye in cosmetics. Because the plant cannot be grown in commercial quantities to meet the total demand of 10 tons a year for several Asian countries, the establishment of a high yielding cell line for commercialisation in a 750 L bioreactor became an attractive opportunity (CURTIN 1983). Unfortunately, other attempts in Japan, Germany and the US to produce high-value products like vanilla flavour or the alkaloid sanguinarine failed. It was due to a lack of experience, to small market demands or to unique problems, presented in the following chapter (DÖRNENBURG & KNORR 1995; SCRAGG 1995).

A.5.2 Problems associated with large-scale plant *in vitro* culture

In spite of the great advances which have been made in plant cell culture, it has to be pointed out that up to now only a few systems have launched an industrial large-scale production. This lack of success can be blamed on biochemical bottlenecks and characteristics of plant cells (Table A-3).

- **Process technology:** First of all it is obvious that industrial processes were started at a time when basic knowledge was lacking about both plant *in vitro* culture and secondary compounds. Most of the processes were developed to meet the requirements of microbial mass production (shear insensitive, only semisterile conditions needed) and did not take into consideration the unique nature of plant cells (WILSON & HILTON 1995). From an engineering point of view, the bioreactor design and process technology has to be adjusted to the inherent requirements of plant cells: large size associated shear sensitivity, high settling rate, aggregate or even organ formation, slow growth and long culture runs. To take this into account, suspension cultures have been applied to different bioreactor systems, which led to the development of low shear impellers and optimised cyclisation systems in airlift bioreactors (BÖHME, et al. 1997; FISCHER, et al. 1994; SINGH & CURTIS 1994).
- **Economics:** Economic considerations have long been ignored for commercialisation of plant cell culture. It is evident, that such a costly process can only be competitive with traditional agriculture or microbial productions, if the desired product is of high value and the market big enough. As a rule of thumb, it has been estimated that for an assumed productivity of 0.3 g L^{-1} per 2 weeks (40 g dw L^{-1}) an average product price of 1500 US $ per kg might meet the costs for media and energy (VERPOORTE, et al. 2002).

Table A-3: Characteristics of biocatalysts used in liquid media (KIERAN, et al. 1997; SCRAGG 1991; TATICEK, et al. 1991) – t_d for the average mass doubling time.

biocatalyst	shape	size (μm)	cell wall	shear sens. / t_d (h)	inoculation density	oxygen cons. (mmol L^{-1} h^{-1})
plant cells	spherical cylindrical	50-100	yes	moderate 20-100	high	10^{-1}
animal cells	spherical	10-20	no	sensitive 20	high	10^{-1}
bacteria	spherical cylindrical	2-10	yes	insensitive 0.5-10	low	10^{3}
yeasts	spherical	5-10	yes	insensitive 10	low	10^{2}

- **Synthetic capacity:** It is a well-known phenomenon that plant cells can lose their ability to synthesize the desired compound found in the intact plant. This has also been frequently observed upon transfer from an Erlenmeyer flask to the bioreactor system – it is called the scale-up process. The literature provides several explanations, so that the individual behaviour of the cell cannot be predicted: (a) enzymes for the biosynthetic pathway are not being produced, (b) a lack of precursors and hormones due to dedifferentiation, (c) incorrect air composition, (d) hydrodynamic stress, (e) genetic instability (CHIN & PEDERSON 1992). Fortunately, the growth conditions of a bioreactor system can be adapted to the distinct requirements by improving the culture medium and the quality of the inlet-air (BUITELAAR & TRAMPER 1992; COLLIN 2001; RAO & RAVISHANKAR 2002).
- **Sensitivity:** Further problems arise from shear forces associated with the bioreactor power input and increased cell size during the culture period (CHISTI 2000; TANAKA 2000). Foam production and a high viscosity of the culture broth at an elevated cell density may also lead to oxygen deprivation. Though plant cell growth is generally characterised as being tolerant with regard to low concentrations of oxygen, an inhibition of secondary metabolite production has often been observed (SNAPE & THOMAS 1989; WONGSAMUTH & DORAN 1994).

A.5.3 Potential of plant cell culture for the production of secondary metabolites

During the last 20 years a great deal of information has been gathered about plant cell culture for the production of fine chemicals. This effort has resulted into some examples in which high production rates of the *in vitro* system can compete with the agricultural cultivation of medicinal plants – some of them even surpass the values found in the intact plant.

- **Yield:** Of all aspects which play a part in biologically based process, the output is the most important aspect. It is reasonable to choose high yielding parent plants for culture initiation and concurrent cell line selection in terms of improved compound accumulation and growth rates. Even though there are problems of low *in vitro* productivity, such as in the case of camptothecine (2.5×10^{-4} % dw) and vincristine (5×10^{-4} % dw), there are also examples of higher values in contrary to the entire plant (given in % dw): diosgenin (*Dioscorea deltoidea*, 3.5 culture / 2 plant), ajmalicine (*Catharanthus roseus*, 2.2 culture / 0.3 plant), rosmarinic acid (*Coleus blumei*, 27 culture / 3 plant), shikonin (*Lithospermum erythrorhizon*, 14 culture / 1.5 plant), paclitaxel (*Taxus brevifolia*, 0.6 culture / 0.007 plant), according to ZHONG (2001) and FOWLER (1986).

- **Metabolic engineering:** Major advances in the field of plant transformation and sophisticated modern biochemical methods have established the exploration of metabolic engineering as a potential method in pharmaceutical biotechnology (CHARTRAIN, et al. 2000). In many cases this approach relies on the identification of limiting enzyme activities, after a successful pathway elucidation and metabolic mapping (metabolomics). However, so far, research has been predominantly focused on improving traits concerning agricultural and nutritional aspects of plants (GRUSAK 1999; UZOGARA 2000). This is partly due to the complex metabolic pathways, compartimentation and low extractable enzyme concentrations in secondary metabolism. The manipulation of one single enzyme which has been recognized for its putative regulative function, rarely led to a raise in efficiency. It was rather apprehended that in some cases an overexpression had its limitations in feedback control whereas in other examples an increased carbon flux was physiologically compensated to branch pathways. For that reason it is of prime interest that multiple gene-encoding-enzymes, functioning at different control points of the metabolic pathway, are manipulated (LEECH, et al. 1998; VERPOORTE, et al. 1999). Such approaches were put into practice in the following examples: the provitamin A production in rice (HALPIN, et al. 2001), an improved flavonoid composition in tomato (COLLIVER, et al. 2002), the minimization of an undesired monoterpene formation in the peppermint plant to maximize menthol production (MAHMOUD & CROTEAU 2002) and the scopolamine accumulation in transgenic *Atropa belladonna* (YUN, et al. 1992).
- **Synthetic chemistry:** Often the structural complexity, characteristic of many biological active compounds like podophyllotoxin, demands a stereospecific multistep synthesis, which renders such routes impractical for synthetic organic chemistry (SIERRA & TORRE 2000). Plant cell culture and fermentation technology can offer one possible solution for a renewable source of enzymatic activity, namely the acquisition of biosynthetically elaborate molecules. Moreover, the biotransformation of available precursors by plant cells or isolated enzymes has already been successfully applied and created a broad spectrum for further research (ALFERMANN, et al. 1980; KUTNEY 1999; RAO & RAVISHANKAR 2002).
- **Basic research:** It has to be stressed, however, that apart from the commercial benefits, plant cell culture has attracted attention as a reliable system in basic research. Cell growth, structure, compartimentation, physiology and metabolic pathways can be studied under definite conditions (KUTNEY 1993).
- **Plantation:** Apart from chemical synthesis, secondary metabolites have long been obtained from traditional agriculture. Yet, the plants have adopted to the individual environmental situations of their habitat and do not tolerate any change of climate or mass plantation. For that reason cell culture reduces the incidence of pesticides or linear gene transfer and offers independence from political and environmental situations, thereby ensuring a stable quality of raw materials throughout the year (COLLIN 2001; DEFURIA 2002). The latter argument is of pronounced importance, since secondary compounds may show tremendous fluctuations, according to their stage of development, the population and the growth season. PUROHIT et al. (1999), for instance, examined different *Podophyllum* populations and proved great differences regarding to the podophyllotoxin content. Moreover, a metabolite found in the intact plant might only occur after years of growth, rendering the traditional plantation a time consuming endeavour: (a) paclitaxel (extremely slow growing trees of *Taxus brevifolia*, (b) ginsengosides (after 5-7 years first harvest of *Panax ginseng*), (c) shikonin (after 5-7 years first harvest of *Lithospermum erythrorhizon*), (d) podophyllotoxin (poor germination and long juvenile phase for *Podophyllum* species).

- **Conservation:** As an alternative source of secondary metabolites plant cell *in vitro* culture may reduce genetic erosion of medicinal plants and the risk of getting extinct from their natural habitat. It has already been discussed that some plants are difficult to cultivate, so that they still need to be collected from the wild. Thus, given a growing popularity of phytomedicine in Europe and the US, pressure might be increased on wild populations. At the moment the total demand for podophyllotoxin is still being met by collecting *Podophyllum hexandrum* at its biotope in the Himalayan. It can be estimated, that for 1 kg of podophyllotoxin approximately 5000 plant rhizomes have to be collected and extracted by percolation (12% resin on dw basis, containing 40% podophyllotoxin; 50 rhizomes yielding 1 kg biomass) (RAI, et al. 2000). Recently, the substantial decline of its population forced India to propose this species as endangered (FORSTER 1993; NADEEM, et al. 2000). Since there is still a great interest for podophyllotoxin as a starting compound for the production of semisynthetic derivatives, alternatives for a reliable supply are needed (MORAES, et al. 2000). Although the plantation of *Podophyllum* species has its obstacles in terms of slow growth and low biomass yield, different attempts have been made for *in vitro* propagation and the agricultural utilization of both the Indian and American *Podophyllum* species (HYNUM 2000; MORAES, et al. 2002; MORAES, et al. 1998). In the mid 1970s a random screening program yielded *Linum album* and its major lignan ingredient podophyllotoxin. At our institute *Linum album* cell suspension cultures were successfully initiated, tested for podophyllotoxin biosynthesis and have been used for the elucidation of lignan biosynthesis (ARROO, et al. 2002; PETERSEN & ALFERMANN 2001). In the field of secondary metabolism, intensive research on plant *in vitro* culture has brought forth several cases of a successful scale-up of phytochemically interesting plant species (Table A-4). The recent example of paclitaxel production on a large-scale by Phyton Inc. (facility located near Hamburg, Germany) confirms the feasibility of any such approaches (HECKENMÜLLER 2002).

Table A-4: Examples for scale-up of secondary metabolite production with plant cell culture, according to MÜHLBACH (1998)

product	**cell source**	**process**	**culture volume** (L)
shikonin	*Lithospermum erythrorhizon*	two-stage culture	750
berberine	*Thalictrum minus*	batch culture in stirred-tank bioreactor	4000
ginseng saponins	*Panax ginseng*	cell and root culture	20000
paclitaxel	*Taxus spp*	stirred-tank bioreactor	75000
sanguinarine	*Papaver somniferum*	airlift bioreactor	300

B. SCOPE OF THE THESIS

Plant cell *in vitro* culture has long been recognized in basic and applied research as a tool in order to study the physiology and biochemistry of plants. Further development, concerning the process-technology and growth conditions, have enabled researchers for the application of plant *in vitro* culture on a large-scale. Though there are many factors in favour of plant-based systems, such as a low risk of product contamination, the correct fold of proteins and the avoidance of ethical concerns, however, only in a few cases such systems have been successfully used for biotransformation, micropropagation and secondary metabolite production. This is partly due to a lack of understanding of the unique physiology and requirements of dedifferentiated plant cells. Nevertheless, since there is a growing demand for therapeutic proteins and natural products as lead structures in the development of new pharmaceuticals, plant cell culture represents a reliable expression system and viable source of secondary metabolites.

It can be expected that a growing demand for podophyllotoxin might cause overharvesting of *Podophyllum* species, resulting in supply problems of podophyllotoxin as an essential precursor in semisynthetic production of anti-cancer therapeutics. Because of the slow growth and the poor reproduction efficiency of *Podophyllum* plants, concomitant with the difficult downstream processing, biotechnological approaches in terms of plant cell culture represent an attractive alternative.

The foremost aim of this study was to establish and improve a bioreactor system for podophyllotoxin production by suspension cultures of *Linum album* Ky. (Linaceae). Moreover, the system could serve as a model for further application to other medicinal plants. For a detailed analysis of the culture requirements and production characteristics concerning the Erlenmeyer flask and the bioreactor system, the following issues have been taken into consideration:

(1) Elaboration of reproducible working practice in terms of media preparation, culture sterility, inoculation procedure and bioreactor-setup.

(2) Determination of the system's inherent mass-transfer characteristics (oxygen transfer) at different working conditions.

(3) Characterisation of *Linum album* cell cultures in Erlenmeyer flasks and in an airlift bioreactor system with different analytical and biochemical approaches.

(4) Elucidation and quantification of key-factors for podophyllotoxin production – results have been used for further culture improvements.

(5) Correlation of enhanced podophyllotoxin biosynthetic capacity to the enzymatic activity of the phenylpropanoid metabolism.

(6) Cell line evaluation and biochemical characterisation with respect to the productivity of podophyllotoxin formation.

C. MATERIAL AND METHODS

C.1 Plant material and culture conditions

Cell suspension cultures of *Linum album* were initiated by SMOLLNY (1993) from seeds and a subsequent shoot and callus passage under sterile conditions. The seeds were collected in 1988 in the vicinity of Teheran (Iran) by Dr. Abbas Shahsavari (Research Institute of Forests and Rangelands, Teheran). Suspension cultures were grown for maintenance as follows: cultivation in a 50-mL modified MS-culture medium (Table C-1) in 300-mL Erlenmeyer flasks, on a horizontal rotary shaker (5 cm elongation, 120 rpm), serially propagated every 7 days with an inoculation density of 100 g L^{-1}, kept at 26 °C room-temperature and dark conditions. For the experiments different cell lines (X4, X4F, A) had been chosen for their capacity of podophyllotoxin accumulation.

Table C-1: Composition of modified Murashige & Skoog medium (MURASHIGE & SKOOG 1962)

macro-nutrients (mg L^{-1})		**micro-nutrients** (mg L^{-1})		**organic supplements** (mg L^{-1})	
KNO_3	1900.00	Na_2-EDTA	37.30	nicotinic acid	0.50
NH_4NO_3	1650.00	$FeSO_4 \times 7\ H_2O$	27.80	pyridoxine-HCl	0.50
$CaCl_2 \times 2\ H_2O$	440.00	$MnSO_4 \times H_2O$	16.90	thiamine-HCl	0.10
KH_2PO_4	170.00	$ZnSO_4 \times 7\ H_2O$	10.60	naphthylacetic acid	0.40
		H_3BO_3	6.20	inositol	100.00
		KJ	0.83	glycine	2.00
		$Na_2Mo_4 \times 5\ H_2O$	0.25		
		$CuSO_4 \times 5\ H_2O$	0.025		
		$CoCl_2 \times 6\ H_2O$	0.025		

To avoid iron-phosphate precipitation, a concentrated iron-chelate stock solution was prepared by dissolving the amount of $FeSO_4 \times 7\ H_2O$ in 1 L of Na_2EDTA solution. This was incubated in an autoclave for 20 min at 121 °C. Prior to the sterilization of the complete medium, sucrose was added (30 g L^{-1}) and the medium pH was adjusted to 5.6 with 0.5 N KOH. To establish aseptic conditions, the flasks had been sterilized in an autoclave for 20 min at 118 °C. For all the media and the antifoam solution deionised water had been used.

C.2 The bioreactor system

Airlift bioreactors have been widely used for different purposes in fermentation and are generally described as suitable for plant cell cultures due to their low power-input, moderate shear effects and their overall simplicity. As the name implies, cyclisation and mixing is achieved by continual air supply. In contrast to a bubble column, the basic pneumatic reactor-type, an airlift bioreactor consists of a circulation system for the culture broth. In the case of an internal-loop bioreactor this is realised by a concentric tube, where the upward motion of the gas phase is utilized for the movement of the liquid. It has been shown, that because of limited oxygen transfer in the downcomer, the oxygen supply characteristics of the airlift bioreactor are generally inferior to those of the bubble column. On the other hand the circulation ensured an improved flow of the culture broth, which is essential under the constraints of a high-density culture to avoid sedimentation (PAYNE, et al. 1987).

C.2.1 Design of the airlift bioreactor

The characteristics of the airlift bioreactor, which had been used for the experiments, are shown in Figure C-1. It was based on a model designed by WAHL (1977) and consisted of a simple concentric tube bioreactor with a working volume of 18 L. The height to diameter ratio measured 2.9 and it had a ratio of 0.25 for the cross-sectional area of the riser to the cross-sectional area of the downcomer. In order to achieve aseptic working conditions, the cylindrical glass vessel (not shown in the figure) had been closed on both sides by metal plates and sealed with silicone rings. The bioreactor lid contained different connections for mass exchange, the temperature control (j) and the oxygen electrode (a), which was located at the downcomer region of the internal circulation system. Air was provided by the inlet tube and released at the sparger ring (e) through 15 openings, each of them were 0.8 mm in diameter. An internal glass tube (f) was affixed by holding devices of the temperation system and it separated the rising region from the downcomer. To avoid cell sedimentation a concave teflon-ring was set down at the bottom at the inner side of the main glass vessel (not shown in the figure). Samples were taken under aseptic conditions by the sample tube (b) from the bottom of the bioreactor (c). Additional ports of the lid permitted the inoculation, the addition of water or medium and foam control during the operation. Each of these ports were sealed by a silicone septum, which had to be penetrated by a sterile needle in order to add water or antifoam.

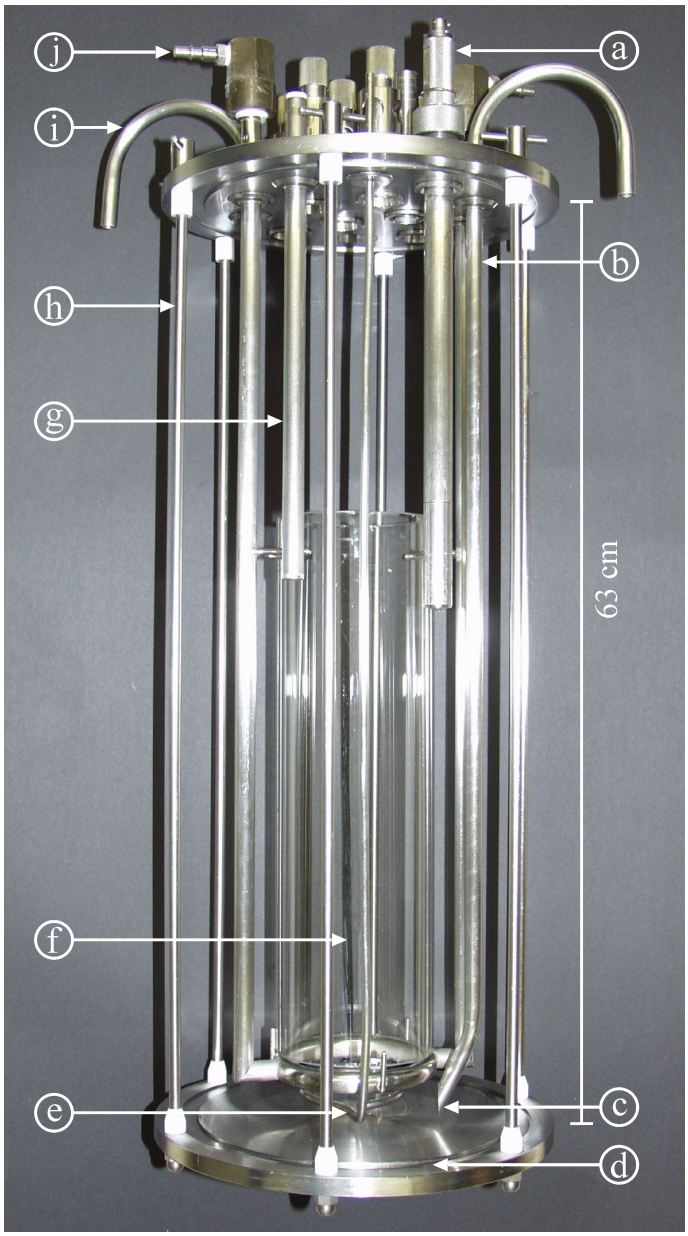

Figure C-1

Design of the airlift bioreactor:

(a) oxygen electrode
(b) sampling tube
(c) entrance of the sampling tube
(d) notch of the bottom plate
(e) sparger-ring of air-inlet
(f) draught-tube
(g) water-filled pipe
(h) outermost stabilization-rod
(i) air-outlet
(j) water temperation system

A closed tube (g) reaching into the culture broth was used for temperature control by an external thermometer. The air-outlet (i) ensured an ambient pressure and was separated from the culture surface by a 15 cm headspace. Thus, generally, foam or cell debris could not enter the outlet tube. All metal parts of the bioreactor system had been manufactured of stainless steel (V2A) and both glass cylinders were made of Duran glass. Six metal rods (h) were put around the bioreactor to affix the head and bottom plate to the main glass vessel.

C.2.2 Setup and operation of the bioreactor system

A general overview of the airlift bioreactor and additional parts such as the aeration system or the stock solutions is provided by Figure C-3. The internal circulation system of the bioreactor (1) is indicated by black arrows – the riser is placed in the middle, surrounded by the downcomer. The fluid flow was pneumatically driven by a density gradient between the air injection on the bottom and the surface of the culture. The hydrodynamic characteristics of an airlift bioreactor can be described in terms of the relationship between the gas throughput, the gas hold-up and the circulation velocity. For a given configuration, the aeration rate is the only controllable variable, whereas other factors such as the liquid velocity or the oxygen transfer will always depend on flow conditions and the reactor geometry. The regulation of the aeration rate was carried out by mass-flow meters (10), which were connected to the main control device (3). The inlet air pressure (university in-house system, 7) had to be reduced to 2.5 bar and cleaned off a possible oil contamination by an oil-separator (8). The pressure of the incoming air was adjusted to 1.0 bar by further pressure-reducers (5) and was ceaselessly sterilized by sterile filter devices (24). Some experiments required an elevated oxygen saturation of the culture, which was achieved by the addition of pure oxygen (4) into the air supply (11). An analogue flow-meter (6), at a pressure of 1.4 bar, guaranteed a constant injection of pure oxygen at branching-point (9). By the integration of a ceramic-sparger (Figure C-2) directly beneath the internal tube, pure oxygen could be added separately from the inlet air (12, 13). This was of great advantage because it allowed mixing of the suspension independently from oxygen provision. Since in the former construction (9, 11) pure oxygen was used uneconomically by its addition into the inlet air, a 10-times-enhanced efficiency was achieved by the ceramic-sparger at high oxygen cultivation.

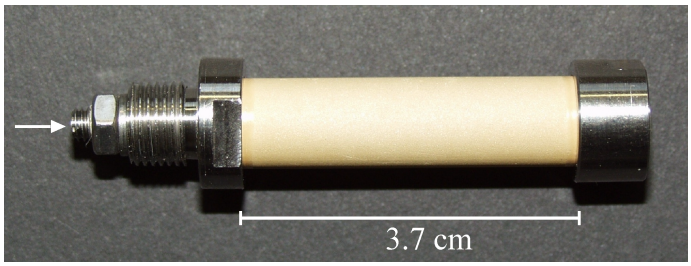

Figure C-2

Arrow indicates the air-inlet of the porous ceramic-sparger for small bubble generation.

Consequently, the more economical system was used for the experiments at elevated oxygen concentrations. The overall air flow rate, as well as the amount of injected oxygen, were just for the control of the oxygen saturation, which was analysed by an oxygen probe (14) and displayed by the amplifier (3). Superfluous gas could escape the bioreactor through the air-outlet and was dipped into a copper sulfate solution (2) to avoid contamination. For temperature adjustment of the culture (26 °C, by a water-bath, 20) water circulated through an internal tubing system. Storage tanks for the addition of water (23) or antifoam (polypropylene glycol or silicone emulsion, 22) were connected to the bioreactor. As for the antifoam, the system was equipped by a pump (21) for precise foam control. Samples of the culture were withdrawn for analysis, using a sampling system (15) after temporary closing of the air-outlet. The black bars (19), indicated at different positions in the diagram, represent the clasps of the silicone tubings. A branching point (18) was needed to clear the sampling tube from residual cells and the culture medium by nitrogen-gas injection. Because of cell aggregate formation, a wide silicone tube was used for the inoculation (17). On the one side it was fixed to the bioreactor and offered a sterile coupling on the flexible side for the connection to the inoculation flask. Both couplings, the one at the bioreactor and the corresponding one at the inoculation flask, were wrapped in cotton and covered with aluminium foil. Before each cultivation, the bioreactor assembly (containing 18 L water), the antifoam and the water storage had been sterilized separately at 121°C, in a Webeco-type autoclave for 20 min. For the medium storage and the inoculation flask a reduced temperature of 118 °C (20 min) was chosen to minimize high-temperature-related alterations of the medium constituents.

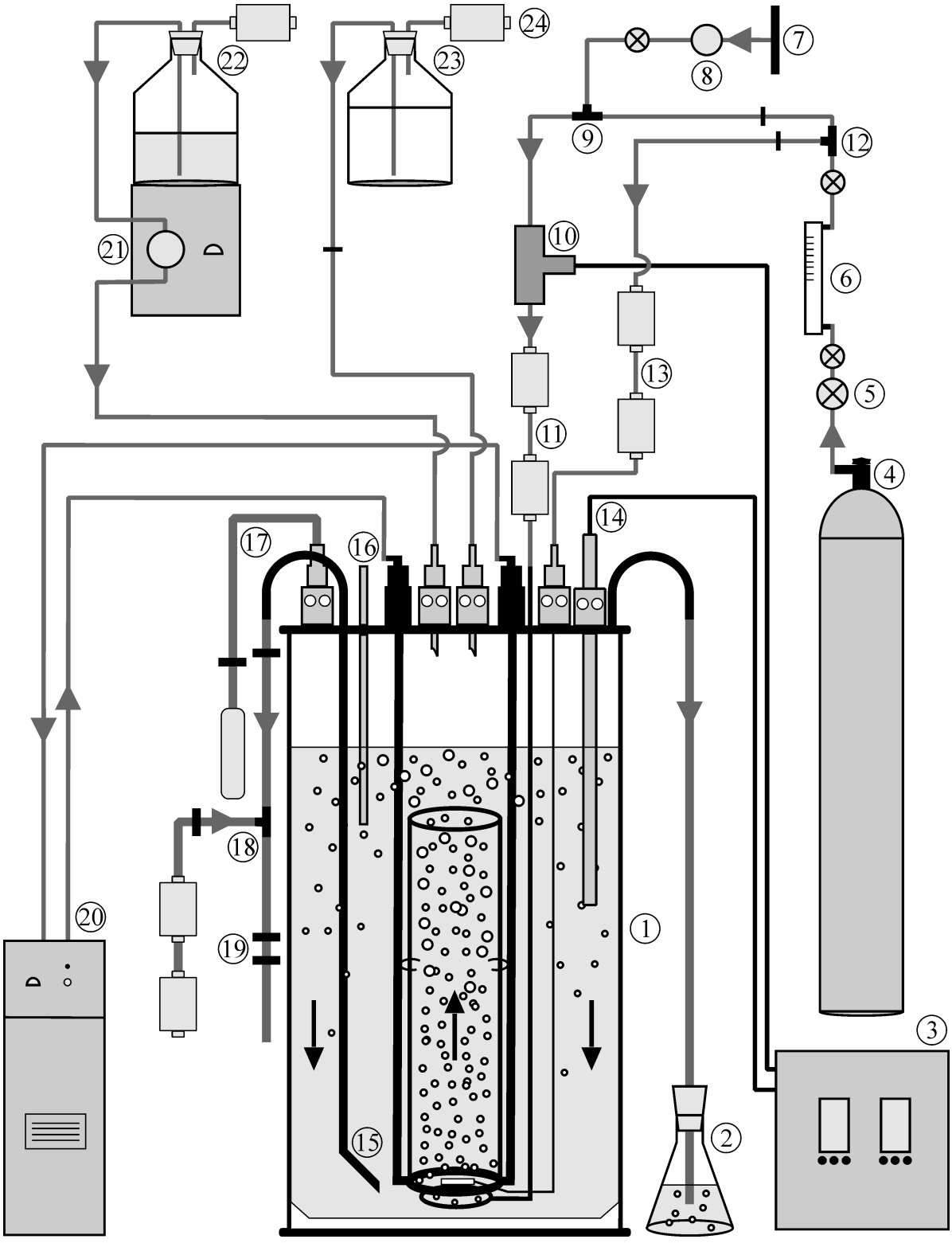

Figure C-3

Components of the bioreactor system: airlift bioreactor (1), copper sulfate solution for air-outlet (2), control device (3), oxygen pressure-flask (4), pressure-reducer (5), analogue flow-meter (6), in-house air-supply (7), oil-separator (8), connection (9), mass-flow-meter (10), main inlet air-stream (11), connection (12), oxygen tube (13), oxygen probe (14), internal sampling tube (15), water-filled pipe (16), inoculation tube (17), cleaning bypass (18), sampling tube (19), water bath (20), pump (21), antifoam storage (22), water storage (23), sterile filter (24).

Prior to inoculation, the dissolved oxygen electrode was calibrated to 100 % of the full scale after the water had sufficiently been sparged at a constant temperature of 26 °C. Subsequently, the DO zero point was established after nitrogen saturation. A similar maximum oxygen saturation was presumed for water and the culture medium to reduce fermentation costs. Before inoculation, the bioreactor was empty and connected to the inoculation flask by the silicone tube (17) in a laminar flow hood under sterile conditions. The cell material consisted of 7-days-old 1000-mL flask cultures (approx. 200 mL internal volume), which were poured into the 10-L inoculation flask. The total bioreactor volume was adjusted to 18 L by fresh culture medium. Generally, at 1-day intervals, samples were withdrawn from the bioreactor under sterile conditions. For each sample (approx. 200 mL suspension culture) an adequate volume of sterile water (23) was added to readjust the bioreactor working volume of 18 L.

C.3 Analytical procedures

C.3.1 Characterization of the oxygen-transfer capacity - k_La determination

Physically, the diffusion of oxygen from the gas liquid interface (boundary layer) of an air bubble into the culture medium represents the slowest step in oxygen-transfer. The total flux between both phases (j_{O2}) is dependent on the oxygen-concentration gradient (ΔC), the thickness of the boundary layer (Δx) and on an oxygen-specific diffusion coefficient (D_{O2}): $j_{O2} = D_{O2} (\Delta C / \Delta x)$. According to this simplistic model, the k_L-value (oxygen transfer coefficient) is defined as: $k_L = D_{O2} / \Delta x$. The combination of both equations relates the k_L-value to the oxygen-flux: $j_{O2} = k_L \Delta C$. If the area of all bubbles on a unit volume of liquid is also taken into consideration (a, in cm^2 per litre of liquid), then the oxygen-flux can be defined as the volumetric oxygen transfer rate (OTR): $OTR = k_L a \Delta C$. In the literature the factor "a" is termed the volume-specific gas-liquid interfacial area. Because it is difficult to determine both the k_L- and a-value independently, in cell culture the k_La-value (volumetric oxygen transfer coefficient) in units of h^{-1} is used as a combination to describe the oxygen transfer in bioreactors.

The k_La-values of the Erlenmeyer and the bioreactor system were established by the so called dynamic gassing-out method (GOGATE & PANDIT 1999; PAYNE, et al. 1987). Therefore, the oxygen of the liquid medium was first stripped off with nitrogen (dissolved oxygen concentration zero, maximum ΔC). With a defined aeration rate the sparged gas was then changed into ambient air and the increasing dissolved oxygen concentration was being recorded by an oxygen probe. If ΔC ($\Delta C = \hat{c}O_2 - cO_2$, $\hat{c}O_2$ is the maximum oxygen concentration in equilibrium and cO_2 the actual concentration of the liquid) was plotted semilogarithmically versus the time (t), the k_La-value resulted from the slope of a linear regression: $k_La\ t = \ln(\hat{c}O2 / (\hat{c}O_2 - cO_2))$. However, after some time, due to possible reabsorption of nitrogen from the headspace of the bioreactor, the regression line did not fit to the slope of the semilogarithmic plot (MENZEL, et al. 1998). Thus, the k_La-values presented were obtained only until a divergence was observed. In the case of the Erlenmeyer flasks it was possible to rapidly degass the headspace by ambient air before the increase of the oxygen concentration was recorded, so that the data-plot and the regression line were in good agreement.

The experiments were carried out with two different oxygen probes of a short response time, such as a standard Clark-type oxygen electrode and a fiber-optical microsensor (micro-optode, Figure C-4). If aseptically conditions were required for the bioreactor-culture or continuous measurement inside a shake flask, the electrochemical probe (Clark-type) had to be used since the micro-optode was too fragile. Nevertheless, the small scale of the micro-optode permitted a high resolution measurement inside the thin liquid-film of a shaken flask with only minor changes in fluid dynamics. Micro-optodes have already been applied to various biological systems for almost non-invasive quantification of carbon dioxide, oxygen, ammonia or temperature measurement.

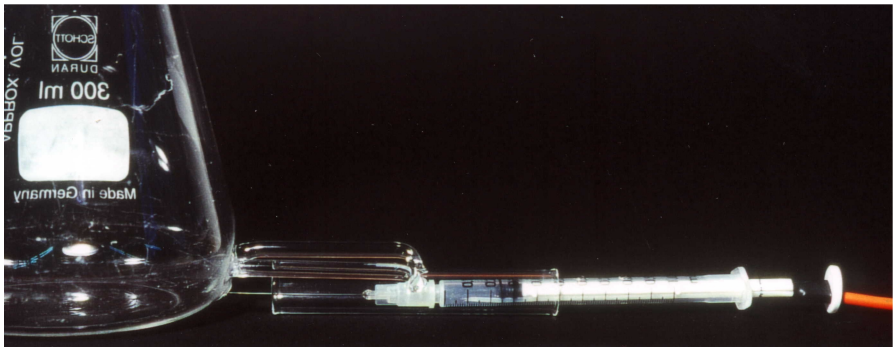

Figure C-4

A fiber-optical microsensor whose oxygen-sensitive tip was directed into an Erlenmeyer flask.

The underlying mechanism of a micro-optode based dissolved oxygen measurement consists of a laser-light-excitable indicator, whose luminescence is quenched by triplet molecular oxygen. For quantification, the relationship between the luminescence intensity as well as the luminescence lifetime and the oxygen concentration in the sample is described by the STERN-VOLMER equation. Since the luminophore is embedded in an ion-impermeable matrix, interferences by the medium-pH, other solutes or the fluid velocity can be neglected and no oxygen is consumed during the measurement. The coated tip of the glass-fiber (30 µm in diameter) was inserted at the base of a shake flask and stabilised by a glass side-arm. A frequency-modulated laser light was directed through an optical fibre to illuminate the sensor, which was suited in a plastic syringe. The time delay between the excitation of the luminophore and the emitted signal was recorded by a photodetector.

In order to determine the bioreactor-specific k_La-values the micro-optode was placed next to the Clark-type electrode in the downcomer. For both oxygen-probes similar results had been obtained in the bioreactor at low aeration rates whereas values above 0.2 vvm (4 L min^{-1}) led to the accumulation of small air-bubbles on the membrane-surface of the Clark-type electrode. Such conditions resulted into an unstable signal, but had not been observed in the presence of a cell suspension, presumably because of a reduced air-bubble flotation in the downcomer and increased medium viscosity. Hence, at elevated aeration rates measurements had been performed by the micro-optode for k_La determination (Table D-1).

C.3.2 Measurement and quantification of culture-growth

To describe the dynamics of the culture growth, the accumulation characteristics of podophyllotoxin and in order to analyse the medium constituents, samples were taken in certain intervals. The whole content of one or more flasks was used for the Erlenmeyer system, while from the bioreactor a sample had been withdrawn from each culture. After vacuum filtration using Miracloth-filters the cell fresh weight was determined and a definite aliquot of the cells was kept in petri-dishes for further freeze drying (lyophilization) at -18 °C. The cell-free culture medium was directly used for taking the measurements of the conductivity (conductometer, mS cm^{-1}), the pH (pH-meter), the carbohydrate contents (refractometer, %) and aliquots were stored at -18 °C for further analysis of inorganic constituents. It is worth mentioning, that, according to the literature, due to the high temperature and high pressure sterilization process, the saccharose became to some extend hydrolysed and chemically modified (BALL 1953). In addition, it is a common feature of a cell culture that secreted or cell-wall-bound invertases account for the hydrolysation of saccharose into its monomer glucose and fructose (GOA & LEE 1992). Although both sugars are utilized by plant cells with different preferences, in most cases they are completely taken up from the medium during the culture period. Consequently, determination of the percentage of residual carbohydrates in the culture medium was thought to sufficiently depict the overall carbohydrate provision. For the characterization of the culture-growth the parameters presented in Table C-2 were also considered.

Table C-2: Quantification of the culture-growth and growth-related processes

cell parameter	symbol	unit	calculation	commentary
fresh weight	fw	g	by weight	weigh of wet cell mass
dry weight	dw	g	by weight	cell weight after freeze-drying
specific growth rate	μ	d^{-1}	$\mu = \frac{\ln\left(\frac{C_{x,k+1}}{C_{x,k}}\right)}{t_{k+1} - t_k}$	$C_{x,k}$ (cell mass at the start of exponential growth, time t_k) $C_{x,k+1}$ (cell mass at the end of exponential growth, time t_{k+1})
mass doubling time	t_d	d	$t_d = \frac{\ln 2}{\mu}$	
growth index	G	-	$G = \frac{X}{X_0}$	X (final max. cell mass) X_0 (initial cell mass)
growth yield	$Y_{x/s}$	-	$Y_{x/s} = \frac{\Delta X}{S_0 - S_f}$	ΔX(max. final - initial cell mass) S_0 (initial carbohydrate content) S_f (final carbohydrate content)

All calculations were based on the cell dry weight. The specific growth rate (μ) and the mass doubling time (t_d) were calculated for the time interval of exponential growth (PAYNE 1991).

C.3.3 Analytical procedures for NH_4^+, NO_3^- and PO_4^{3-} quantification

Prior to analysis all samples of the culture medium were thawed, incubated for one minute in an ultra-sonic bath and centrifuged at 4000 rpm in an Eppendorf-centrifuge (5804 R) for 5 minutes. The supernatant was used for further photometrical analysis of the major nutrient components NH_4^+, NO_3^- and PO_4^{3-}. All solutions were made up of deionised water.

NH_4^+-determination:

The quantification of the ammonium-content was carried out under alkali conditions to convert the ammonium into ammonia, which reacted with hypochlorite to monochloramine. With phenoxide the latter yielded a blue coloured indophenol with a characteristic absorption at 570 nm. (quantified with a spectro-photometer). The method is known as the "indophenol blue method".

Table C-3: Components and procedure for ammonium-quantification

solutions			**procedure**
phenol-reagent	10.0	g phenol	0.1 mL (standard, test or water)
	0.051	g nitroprusside	+ 2.5 mL phenol-solution
	0.2	g NaCl	+ 2.5 mL hypochlorite-solution
		ad 1000 mL	30 min incubation in a water bath (36°C)
hypochlorite-reagent	0.6	mL Na-hypochlorite	15 min incubation at room temperature
	80.0	mL 8 N NaOH	measurement at 570 nm
		ad 1000 mL	

Hypochlorite acts as a strong oxidizing agent whereas nitroprusside ($Na_2[Fe(CN)_5NO]$) serves as a metal-containing catalyst. A calibration curve was established for quantification (1, 2, 4, 6, 8, 10, 12, 14, 16 mM ammonium nitrate). Each step followed in a quick succession and finished by mixing on a vortex.

NO_3^--determination:

This method employed a procedure, where nitrate is reduced to nitrite by a copper / hydrazine reductor. The nitrite ion reacted with sulfanilamide under acidic conditions to form a diazo compound which coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride, to yield a red-purple azo-dye (BRATON-MARSHAL reaction). This was measured on the basis of its absorbance at 540 nm.

Table C-4: Components and procedure for nitrate-quantification

solutions			**procedure**
$CuSO_4$ $5H_2O$	3.94	mM	4.0 mL (standard, test or water)
NaOH	0.1	M	+ 0.2 mL $CuSO_4$-solution
hydrazine sulfate	0.21	%	+ 1.0 mL NaOH-solution
acetone	10.0	%	+ 0.2 mL hydrazine sulfate-solution
diazo-reagent	10.0 %	phosphoric acid	30 min incubation in a water bath (65°C)
	4.0 %	sulfanilamide	5 min cooling on ice
	0.2 %	naphthyldiamine	+ 0.4 mL acetone-solution
			+ 1.2 mL diazo-solution (mix!)
			+ 3.0 mL deionised water
			15 min incubation at room temperature
			measurement at 540 nm

A calibration curve was prepared for quantification (1, 5, 10, 15, 25, 30 mM KNO_3). All samples were diluted at 1:300 with water before analysis. To avoid excessive water evaporation during the 65 °C-incubation, the tubes were covered with glass marbles.

PO_4^{3-}-determination:

According to the method of GOMORRI (1942) phosphate reacted with ammonium-molybdate at low pH to the yellowish acid $H_3P(Mo_3O_{10})_4$. After 4-methyl-amino-phenol-sulfate (Photorex) and sodium-bisulphite had been added, the acid was reduced to the bluish molybdenum-blue ($MoO_{3-x}(OH)_x$), which was detected at 660 nm.

Table C-5: Components and procedure for phosphate-quantification

solutions			**procedure**		
ammonium-molybdate-reagent				2.25 mL	water
25.0	mL	10 N H_2SO_4	+	2.0 mL	ammonium-molybdate-reg.
50.0	mL	2.5 % $(NH_4)_6MoO_{24}$	+	0.25 mL	reduction-reagent, mix
	ad	400 mL water	+	0.5 mL	(standard, test or water), mix
reduction-reagent			30 min incubation at room temperature		
40.0	g	Na_2SO_3	measurement at 660 nm		
1.0	g	Photorex			
	ad	100 mL water			

A calibration curve was prepared for quantification (0.1, 0.2, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.0 mM NaH_2PO_4). Since even trace amounts of phosphate gave a positive reaction, the glassware had to be cleaned carefully with deionised water.

C.3.4 Atomic-absorption spectroscopy

The potassium, iron, manganese and calcium content of culture media was measured by atomic absorption spectroscopy. Samples of the medium were collected and kept in PE-flasks at -20 °C until the analysis was performed by a flame-atomic-absorption spectrometer. Here the ions and salts of the solution were decomposed into gaseous atoms (atomised) in an acetylene-air flame. Because under such conditions, most of the atoms did not become exited to a higher energy level but rather remained in the ground state, they showed specific light absorption characteristics. For the quantification the remainder of a distinct radiation wavelength, emitted by a hollow-cathode lamp, was detected and correlated to a calibration curve of the individual element. The samples had to be mixed and centrifuged after being thawed, to prevent any disturbance by insoluble proteins or carbohydrates. Furthermore, samples and standards were diluted with deionised water before measurement: potassium (1:100), iron (no dilution), manganese (1:1), calcium (1:20).

C.3.5 Extraction and quantification of podophyllotoxin

Podophyllotoxin was extracted from freeze-dried cell material and quantified by means of high-performance liquid chromatography (HPLC). For the extraction 0.2 g of powdered cells was incubated in an ultra-sonic bath with approximately 2 mL methanol for 1 minute. Subsequently 6 mL of deionised water was added and the pH was adjusted to 5 by diluted phosphoric acid. Hydrolysation of the podophyllotoxin-glucosides was achieved by supplementation of 0.1 mL of a β-glucosidase solution (10 mg mL^{-1}) to each sample followed by the incubation at 35 °C in a water bath for 60 minutes. 12 mL methanol were added before further storage in an ultra-sonic bath at 70 °C for 10 minutes. The methanolic extracts were centrifuged at 4000 rpm in an Eppendorf centrifuge for 5 minutes. The volume of the cell-free supernatant was determined by decantation, an aliquot was transferred into an Eppendorf tube, centrifuged at 10000 rpm for 5 minutes (Eppendorf centrifuge) and stored at -20 °C for further quantification of the podophyllotoxin content. HPLC analysis was performed by a C_{18}-column of a Waters-HPLC system with a two-solvent gradient procedure (Table C-6).

Table C-6: HPLC gradient program

time (min)	water (%)	acetonitrile (%)	flowrate (mL min^{-1})
0	60	40	0.8
13	40	60	1.0
15	33	67	1.0
16	60	40	0.8
20	60	40	0.8

The solvent system consisted of filtered, acidified deionised water (0.01% phosphoric acid) and HPLC-grade acetonitrile.

Podophyllotoxin was detected at 290 nm by an UV-detector and the identity was confirmed by a reference spectrum and co-chromatography. Commercially available podophyllotoxin was used as a standard for the calculation of the podophyllotoxin content in relation to the total peak-area (relative units). Results are presented on dry-weight basis (mg podophyllotoxin per g dw).

C.3.6 Crude protein extraction and measurement of enzyme activity

In order to determine the enzymatic activity of the phenylalanine ammonia-lyase (PAL), the 4-coumarate-CoA ligase (4CL) and the cinnamyl alcohol dehydrogenase (CAD) a crude protein extract was obtained from fresh cell material after vacuum filtration. Extractions were done at 4 °C. Before homogenisation, each sample consisted of 5 g cells, 1 g polyvinylpyrolidone (Polyclar), 50 µL dithiothreithol (DTT, 1 mM) and 5 mL chilled KPi buffer (8.0 pH, 0.1 M). The samples were homogenized in an "Ultra-Turrax" three times for 30 seconds and cooled on ice in between. After centrifugation at 20000 rpm (Sorvall, SS34 rotor) for 20 minutes the supernatant was filtered through glass wool. It had been used for spectrophotometric enzyme-assays as well as for protein determination by the BRADFORD-method (1976). All enzymes had been measured according to standard methods (SCHÖNELL 1996):

Table C-7: Assays for enzyme activities

enzyme	test-components	procedure
PAL	650 µL KPi buffer 0.1 M, pH 8.0 150 µL crude extract 200 µL 0.1 M L-Phe in KPi buffer*	After incubation at 36 °C for 10 min the accumulation of cinnamic acid was measured as an increase of absorbency at 290 nm. ε(cinnamic acid) = 10 $mM^{-1}cm^{-1}$
4CL	780 µL KPi buffer 0.1 M, pH 7.5 50 µL p-coumaric acid 20 mM in 50% methanol 20 µL ATP 50 mM 20 µL $MgCl_2$ 125 mM 10 µL DTT 0.1 M 100 µL crude extract	After incubation at 30 °C for 2 min the reaction was started by addition of 20 µL of CoA* (5 mM) dissolved in water. Subsequently the increase of absorption was recorded at 333 nm for 10 min. ε(p-coumaryl-CoA) = 21.1 $mM^{-1}cm^{-1}$
CAD	900 µL Tris/HCl buffer 0.1 M, pH 8.8 50 µL $NADP^+$ 2 mM 25 µL crude extract	After incubation at 30 °C for 5 min the reaction was triggered by addition of 25 µL coniferyl alcohol* (2 mM, in 5 % methanol) and recorded at 390 nm for 10 min. ε(coniferylaldehyde) = 21 $mM^{-1}cm^{-1}$

Enzymes were assayed in disposable UV-cuvettes (1.5 mL volume) with a spectro-photometer. A blanc-test was prepared for simultaneous measurement by the substitution of one indicated test-component*: PAL (200 µL KPi buffer pH 8.0), 4CL (20 µL water), CAD (25 µL methanol 5%). Relative enzyme activities were expressed on basis of the cell fresh weight (enzyme activity, pkat g^{-1}) or protein concentration of the crude extract (specific enzyme activity, µkat kg^{-1}).

D. RESULTS & COMMENTS

D.1 Preliminary studies

D.1.1 Cell growth and production dynamics of *Linum album* cell suspensions

Prior to any approach for an optimisation of the culture or even an application to the bioreactor system, basic knowledge was needed concerning the nutrient requirements and podophyllotoxin accumulation dynamics. In this context, the objective of this study was to characterize the growth, nutrient uptake and production patterns of *Linum album* suspension cultures. Cells of line-X4 grew in 1000-mL shake flasks under the conditions described in Chapter C.1. During a culture period of 21 days samples were harvested as indicated in Figure D-1. Since, in this preliminary study each data-point denotes the culture characteristics of only one single flask, the absolute values should be considered with caution. However, the data obtained proved to be consistent with previous results (PETERSEN & ALFERMANN 2001; SMOLLNY 1993) and was later verified by further experiments. The uniform production and nutrient uptake pattern featured the shake flasks as a reliable system for more comprehensive studies.

Starting with an initial fresh weight concentration of approximately 50 g L^{-1} (inoculation density of 100 g L^{-1}, including the adherent culture medium) the culture did not exhibit any lag-phase. As shown in Figure D-1a the biomass increase rather showed exponential growth characteristics until the 4th day of culture on a fresh and dry weight basis. Followed by a linear increase of biomass, the dry weight curve already levelled off at the 6th day and declined after a maximum dry weight of 14.6 g L^{-1} was reached at the 8th day of the batch. It has often been observed for plant cell suspension cultures that the fresh weight increased further in later culture phase. This was also true for the *Linum album* (X4) suspension. Hence, the highest fresh weight concentration of about 400 g L^{-1} was reached at the 10th day. Figure D-1b depicts the course of the medium pH and the nutrient parameter over the time. Concomitant with the culture growth, a substantial decline of the carbohydrate content and conductivity could be observed. It is true for both factors that the culture became fully depleted between the 8th and 10th day, coinciding with the point at which the cell dry weight reached its upper limit. During the latter half of the culture period the refractometric index, as well as the conductivity, rose up to a certain value. Because, during that time, the macroscopic appearance of the cell suspension also implied reduced culture performance, the increase observed could have been attributed to the onset of the cell lysis.

The podophyllotoxin accumulation is expressed on basis of the cell dry weight (mg podophyllotoxin g^{-1}) and in relation to the culture growth (mg podophyllotoxin L^{-1}). A plot of the podophyllotoxin production dynamics is provided by Figure D-1c, which displays a distinct lag-phase during the first days of culture. Due to the early increase of biomass, the lag-phase was shorter in the case of the volumetric podophyllotoxin production. After a certain cell density had been reached, podophyllotoxin accumulation was discerned between the 5th and 10th day with a maximum content of 1.8 mg g^{-1}. Although in plant cell culture it is generally believed that most secondary metabolites are produced non-growth-associated with an emphasis on a stationary phase (KAKEGAWA, et al. 1995; VILLARREAL, et al. 1997), compounds like betalains or carotenoids proved to be accumulated during a phase of active growth (BOURGAUD, et al. 2001; YEOMAN & YEOMAN 1996). Moreover, production dynamics have been shown to be influenced by culture conditions and inhomogeneity of the cell population, so that differences even appeared between shake flask cultures and bioreactor ones (KNOBLOCH & BERLIN 1980). Consequently, intermediate secondary metabolite production characteristics were also observed, which were designated partly-growth associated (CHATTOPADHYAY, et al. 2001; Lee & SHULER 2000). A growth related podophyllotoxin production by *Linum album* suspension cultures required further confirmation.

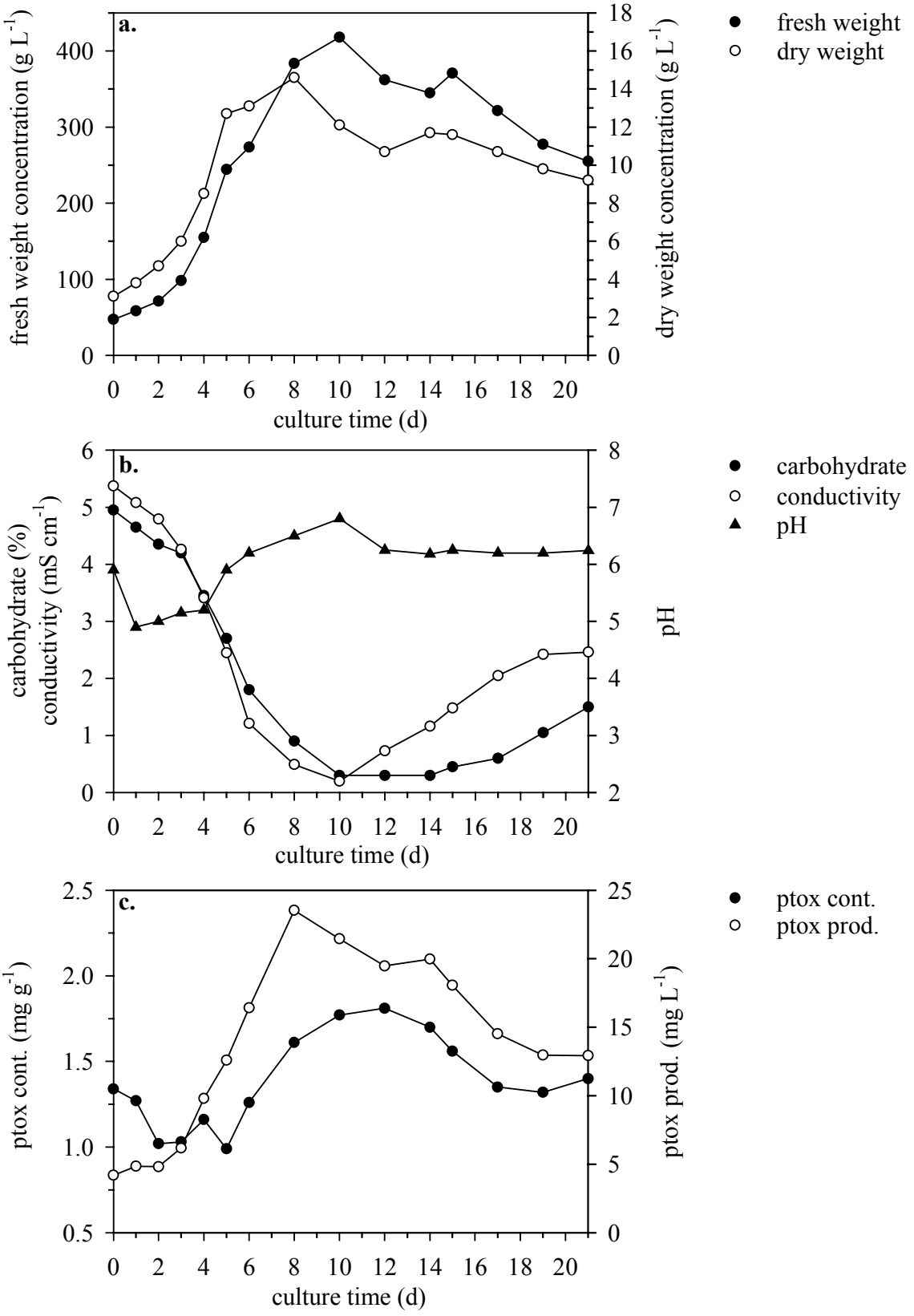

Figure D-1

Biomass accumulation, medium parameter, podophyllotoxin content and podophyllotoxin production (on dry weight basis) by *Linum album* suspensions. Experimental setup: 1000-mL shake flasks, 200 mL culture medium, 120 rpm. Each symbol represents the data of one sample.

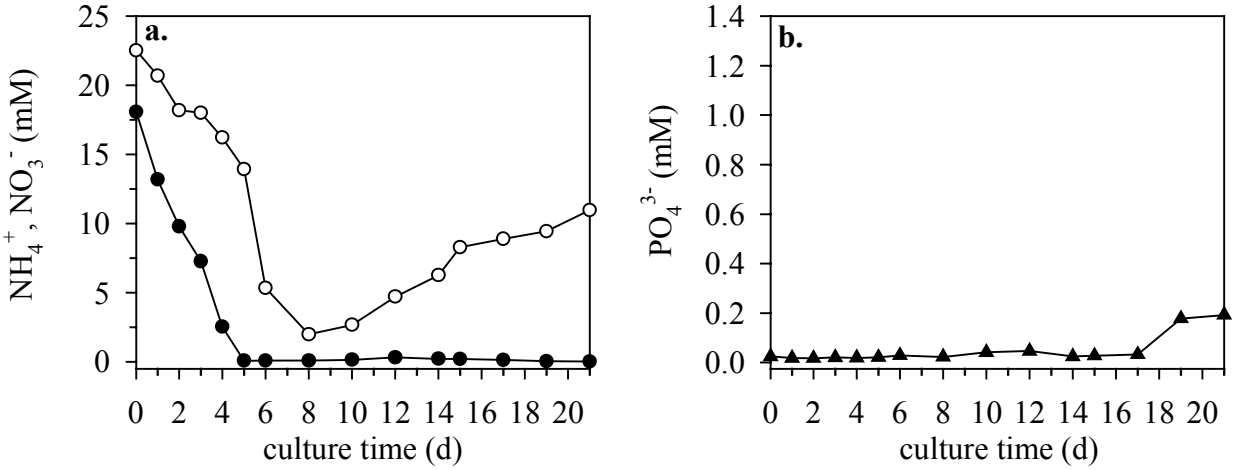

Figure D-2

The remaining ammonium (●), nitrate (○) and phosphate (▲) of the culture medium was being analysed during the growth period of *Linum album* (experimental setup as in Figure D-1).

Apart from the carbohydrates, further nutrients in plant culture media are represented by ammonium, nitrate and phosphate, which are taken up by the cells at different kinetics (ARCHAMBAULT, et al. 1996; SCHLATMANN, et al. 1995; SHIMOGAWARA & USUDA 1995). The residual nitrogen containing mineral salts of the culture medium are shown in Figure D-2a. It is obvious, that ammonium was taken up at a faster but more constant rate in comparison to nitrate. The culture medium already became depleted by ammonium at the 5th day of culture. In contrast, a rather biphasic nitrate uptake has been described by several authors (DO & CORMIER 1991; JACKSON, et al. 1973). For the *Linum album* culture a relatively slow initial phase was followed by an accelerated nitrate consumption between the 4th and 6th day. No depletion was observed until day 8 of culture. Coinciding with the increase of conductivity in the second half of culture, a raise of nitrate was detected. As shown in Figure D-2b, the reduction of the phosphate concentration was exceptionally swift compared to the other ions. Even two hours after the inoculation no phosphate was found in the medium (initial phosphate concentration of 1.2 mM). Similar results have been published by other authors, who proposed a refilling of an intracellular phosphate reservoir (BÖHME, et al. 1997; SANO, et al. 1999). However, the formation of specific storage compounds (e.g. phytates) was disapproved by showing that intracellular phosphate was readily incorporated into nucleic acids (45% of Pi), phospholipids (30% of Pi), nucleotides (3% of Pi) or into sugar phosphates and proteins (3% of Pi) (ASHIHARA & TOKORO 1984).

D.1.2 Initial obstacles of the bioreactor culture

Plant cells have been grown in a variety of bioreactor types, such as stirred-tank reactors, rotating drum reactors, bubble columns and airlift bioreactors. In most cases problems arose in connection with the scale-up process, which rendered the bioreactor culture a rather difficult challenge (NAMDEV & DUNLOP 1995). Although first attempts to cultivate a fast-growing but low-podophyllotoxin-yielding *Linum album* cell line (X4F) in an airlift bioreactor were successful in terms of biomass production (data not presented), similar experiments with the X4-cell line failed. These cells, which were also routinely subcultivated and characterised in shake flasks, exhibited a distinct sensitivity in correlation to shear-stresses and oxygen fluctuations. Even at low aeration rates of 0.05 vvm (1 L min^{-1}) the culture medium, not the cells, turned subsequently red or brownish upon a rapid increase of the culture oxygen concentration (Figure D-3). Such effects were observed predominantly after samples had been taken from the bioreactor by the establishment of an elevated internal pressure.

Attendant with this operation method, a peak oxygen concentration was monitored by polarographic-probes. Pronounced stress reactions arose especially after a period of oxygen-limited-growth in the bioreactor. Usually, at the onset of the discolouration, the culture medium turned yellow or orange, followed by a dark-red and resulted in a brownish colour. The red-dye could be extracted by chloroform and showed a pH-dependent maximum absorbance at 480 nm with a shoulder at 520 nm (pH above 7). Upon acidification the red-colour receded at a pH lower than 7 and was recovered after alkalisation with KOH. The discolouration of the medium was accompanied by an increase of the culture-oxygen saturation, which was probably due to an interrupted cell growth – later on the cell aggregates appeared necrotic and did not recover even after their transfer to fresh media. It has to be noted that no microbial infection was detected and aliquots of the culture medium did not induce any such reaction in healthy shake flask cultures.

In Figure D-4 simple bubble columns are shown, which served as a model for parallel experimental approaches, regarding the impact of shear-stress and oxygen-fluctuation on the culture performance. After a gentle aeration of the flask in the middle with ambient air for 4 days, the flow-rate was increased and a red colour developed during the ensuing twelve hours. For the flask on the left side nitrogen had been used for the bubble generation and a switch to ambient air after 5 days resulted into a lesser colouration compared to the air-bubbled flask (photo taken at the end of the nitrogen incubation). Though the cells were depleted by oxygen for 5 days, cell viability was confirmed by a standard FDA-test. The flask on the far right served as a control and was not gassed, so that the oxygen exchange only occurred by diffusion. Aeration of this flask at the 5^{th} day did not result in any equivalent colouration.

Since even the head-space aeration of an oxygen limited culture in a stirred 5-L applikon-type bioreactor (Facultad de Farmacia, Universidad de Barcelona, Barcelona, Spain) revealed similar problems, the main factors for the "oxygen-stress" were defined as follows: if metabolic active cells were exposed to shear stress for a prolonged period of oxygen-limited-growth, a rapid increase of the oxygen partial pressure in the culture medium led to the described adverse effects. Consequently, a sampling-system of the bioreactor, which was independent of the main air supply, concurrent with the utilization of nitrogen to clean the sampling tube, reduced the risk for medium discolouration and cell death. In addition, the aeration of the cells inside the inoculation flask, prior to transfer into the bioreactor, aided the performance of the whole process.

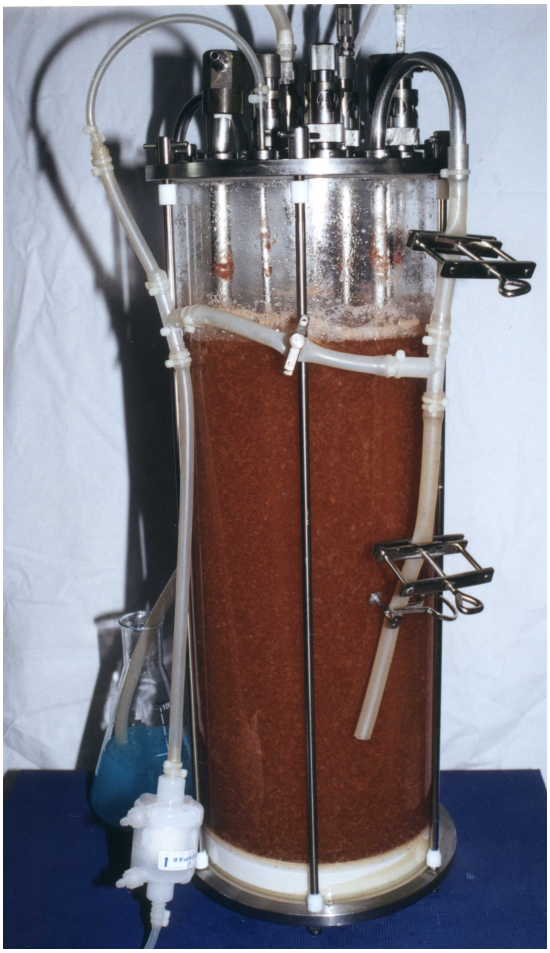

Figure D-3

Preliminary bioreactor-setup with a "stressed" *Linum album* (X4) suspension. The photo was taken at the 3rd day of culture, twelve hours after sample withdrawal.

Figure D-4

Erlenmeyer flasks equipped with a silicon-tube for gas-bubbling into a *Linum album* (X4) culture. The outlet gas escaped through a sterile filter fitted in a silicone plug. The left and middle flask were gently gassed with nitrogen or, respectively, ambient air. The right flask was not aerated and gas exchange only occurred by diffusion through a sterile filter device (description in the text).

D.1.3 Determination of the oxygen transfer rates

For aerobic bioprocesses oxygen transfer represents a vital factor since any shortage of oxygen drastically affects the metabolic activity of the cells and the process performance. A considerable amount of publications deals with the implication of oxygen delivery in biological systems (GOA & LEE 1992; HOHE, et al. 1999; SAHOO & AGARWAL 2002), as well as the different measurement techniques for the determination of gas-liquid-transfer capacities (GOGATE & PANDIT 1999; MAIER & BÜCHS 2001). The k_La-values presented in Table D-1 were needed for an interpretation of the podophyllotoxin accumulation characteristics in different culture systems.

Table D-1: k_La-values for the bioreactor and the shake flask system measured by a polarographic probe or a micro-optode (dynamic gassing-out method)

system	liquid volume	vvm rpm	polarographic probe $k_La \pm$ stand. deviations (h^{-1})	micro-optode $k_La \pm$ stand. deviations (h^{-1})
airlift bioreactor				
	18 L	0.11	5.2 ± 0.4	-
		0.22	8.9 ± 0.3	7.96 ± 0.13
		0.33	11.7 ± 0.9	10.86 ± 0.03
		0.44	-	13.23 ± 0.06
		0.55	-	14.11 ± 0.12
shake flask				
300 mL flask	50 mL	120	-	33.1 ± 0.8
		180	-	58.0 ± 0.9
1000 mL flask	200 mL	120	-	29.7 ± 1.5
		180	-	49.1 ± 0.2

All k_La-values represent an average of three measurements for each experimental setup. Deionised water was used in all cases with different filling volumes.

At an aeration rate of 0.22 and 0.33 vvm, both probes, the polarographic as well as the micro-optode, gave similar results. As expected, the highest values, found in the bioreactor system, remained lower than the oxygen transfer capacities of the shake flasks. Though the results were in good alignment with the data published elsewhere (KOBAYASHI, et al. 1989; LECKIE & SCRAGG 1991; WILLIAMS, et al. 1996), it has often been pointed out that the addition of antifoam, the increase of medium-viscosity during the culture period or flask closures influence the k_La-values (MORAO, et al. 1999; TANAKA 2000). However, it can be deduced from Table D-1 that a higher aeration rate of the bioreactor, as well as a reduced liquid volume or an increased rotation rate of the shake flasks, improved the k_La-values. Regarding the shake flask system, its has to be pointed out, that the tip of the micro-optode was positioned approximately 0.5 cm below the liquid surface. Additional oxygen-transfer-experiments deeper inside the 50-mL liquid-volume revealed drastically reduced k_La-values of about 15 h^{-1}. This observation will be of importance for further discussion concerning the performance of the shake flask system.

D.2 Effect of the oxygen supply on podophyllotoxin production – the shake flask system

D.2.1 Monitoring the culture oxygen saturation during the growth period

Culture medium characteristics and product formation of a *Linum album* (X4) cell suspension in correlation to the oxygen saturation are shown in Figure D-5 & Figure D-6. Investigations on the residual oxygen saturation in shake flask cultures provided basic information about the dynamics of oxygen consumption and possible involvement of the nutrient supply. Both oxygen probes gave similar results and indicated a decreasing oxygen saturation after inoculation. At day 5 of culture the lowest value (40% of saturation) was reached, followed by an increase of the oxygen contents between the 7th day until the end of culture. With respect to the nutrient supply, the culture medium became depleted by the carbon-source at the 8th day, paralleled by a decline in the medium conductivity. As explained in Chapter D.1.1, ammonium was consumed with a faster kinetic than nitrate, which exhibited a biphasic uptake-rate. After a moderate consumption until the 5th day of culture, about 50% of the total nitrate was taken up between the 5th and 7th day (Figure D-6b). Phosphate could not be detected in the medium four hours after the inoculation (data not shown). Since nitrate significantly contributes to the overall conductivity of culture media, a pronounced increase of conductivity could be attributed to the extracellular accumulation of nitrate in the second phase of culture. Typical growth characteristics were obtained (data not shown), which gave a maximum dry weight of 14 g L^{-1} at day 8 of culture. Apparently, the external carbon-source and inorganic-nutrient supply were consumed for the maintenance of the cell mass and represented growth-limiting factors. Figure D-6a describes the formation of podophyllotoxin during the culture period. The podophyllotoxin accumulation started after a short lag-phase of two days and reached its maximum at about the 12th day. It is apparent, that the podophyllotoxin synthesis had already taken place at a time when the cells were still metabolically active, which was indicated by a low oxygen concentration of the culture medium.

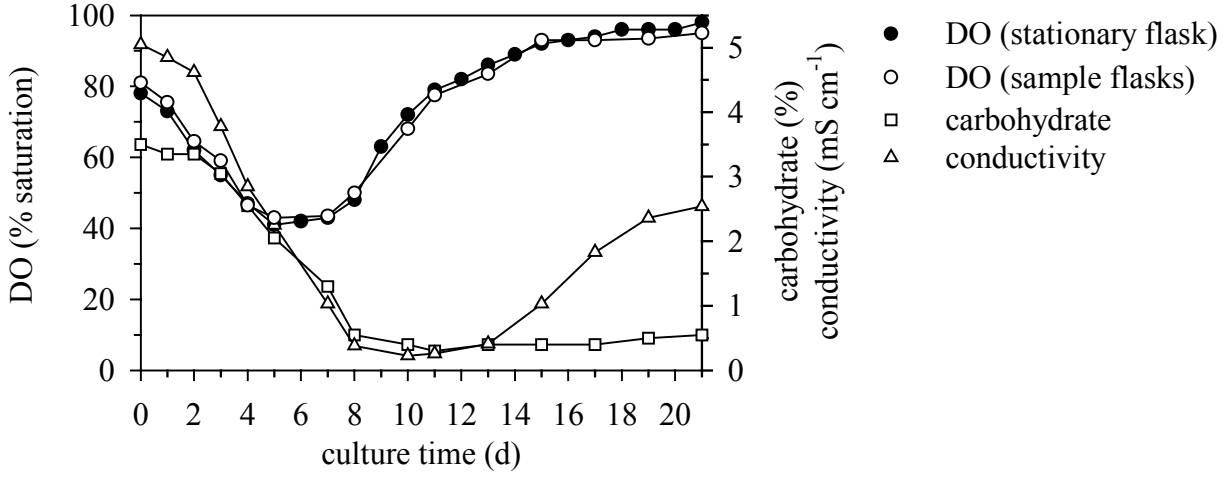

Figure D-5

Culture characteristics of *Linum album* (X4) in 1000-mL Erlenmeyer flasks. Oxygen measurements were performed by two polarographic electrodes, which were inserted through the main flask-neck and positioned in the middle of the culture broth. Gas exchange was ensured by a second opening (closed by a cotton plug). A sterile probe (stationary, closed symbols) was fixed in one flask for continuous monitoring of the oxygen saturation, the flexible probe (non-sterile, open symbols) was used for each individual sample flask. The culture medium characteristics and oxygen data of the samples (flexible probe) taken from the rotary shaker represent mean values of two flasks.

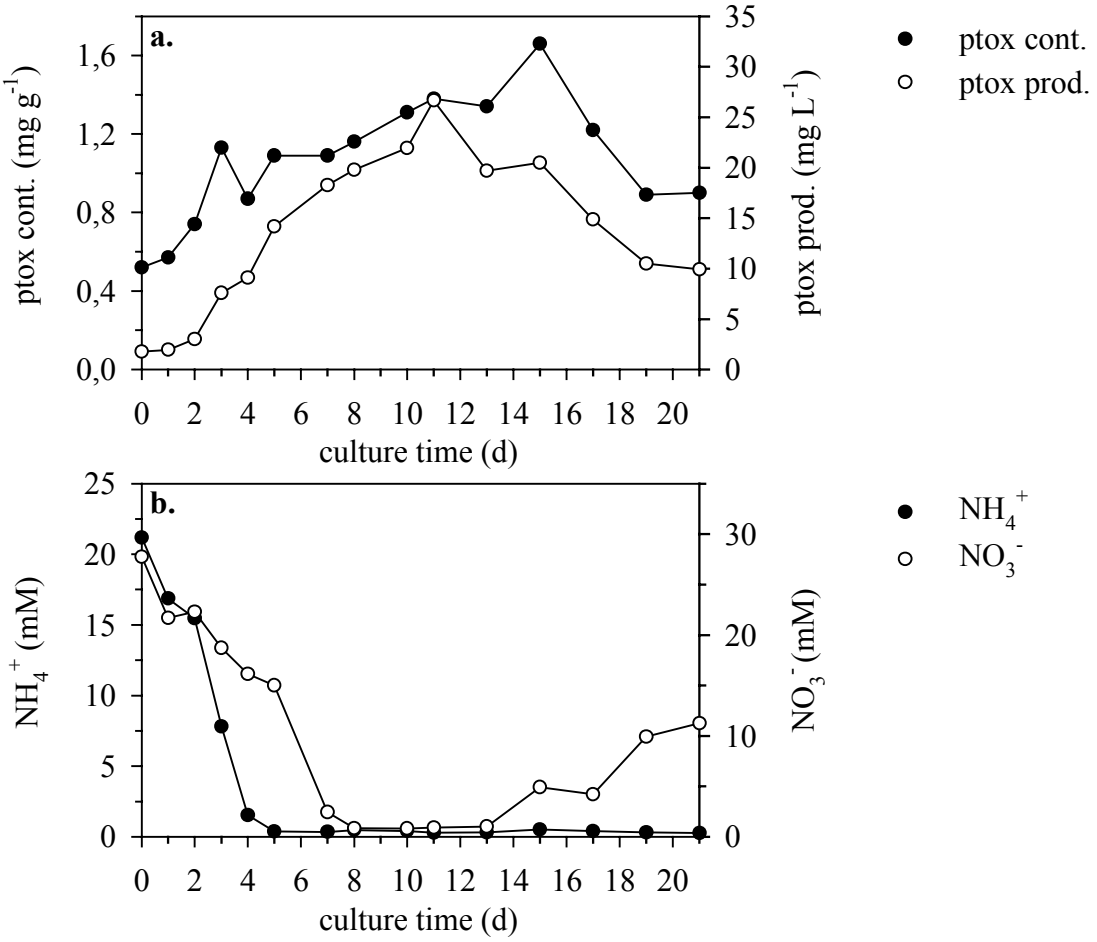

Figure D-6

Podophyllotoxin (on dry weight basis) and nutrient profiles of a *Linum album* (X4) culture in 1000-mL shake flasks for investigation on the oxygen consumption rates during the culture period.

D.2.2 Influence of different gyratory shaker speeds on podophyllotoxin production

It has long been known that secondary metabolite production by plant cells is affected by growth conditions, such as hydrodynamic effects, the nutrient source or the gaseous composition inside the culture flask (HUANG & CHOU 2000; LINDEN, et al. 2001). Due to the relative ease way of carrying out large numbers of replicate tests, preliminary experiments were done in small-scale shaken cultures before bioreactor studies were initiated. Although many authors have concerned themselves with problems of the oxygen supply in bioreactors (KOBAYASHI, et al. 1989; LAI, et al. 2001), the shake flask system has been of major interest in terms of mass transfer characteristics of various closures types or the shape of the flasks (MROTZEK, et al. 2001 ; TUNAC 1989). Especially in the case of large liquid volumes at reduced rotation speeds the oxygen demand may exceed the supply capacity, which renders the oxygen a limiting nutrient.

The study reported here was carried out for the purpose of elucidating the effect of oxygen supply rates on the growth and podophyllotoxin accumulation of a *Linum album* (X4) suspension culture. Since it is widely accepted that even at increased shaker speeds Erlenmeyer flasks exhibit only minor shear rates in comparison to the bioreactor culture (SAJC, et al. 2000), an enhanced oxygen supply was achieved without any additional stress factors. Parallel samples of the cells had been adapted to the increased shaker speeds 6 weeks before the characterization was started. It has to be stated that if such a pre-incubation was omitted, similar results on basis of the podophyllotoxin accumulation were obtained in a separate experiment (data not presented).

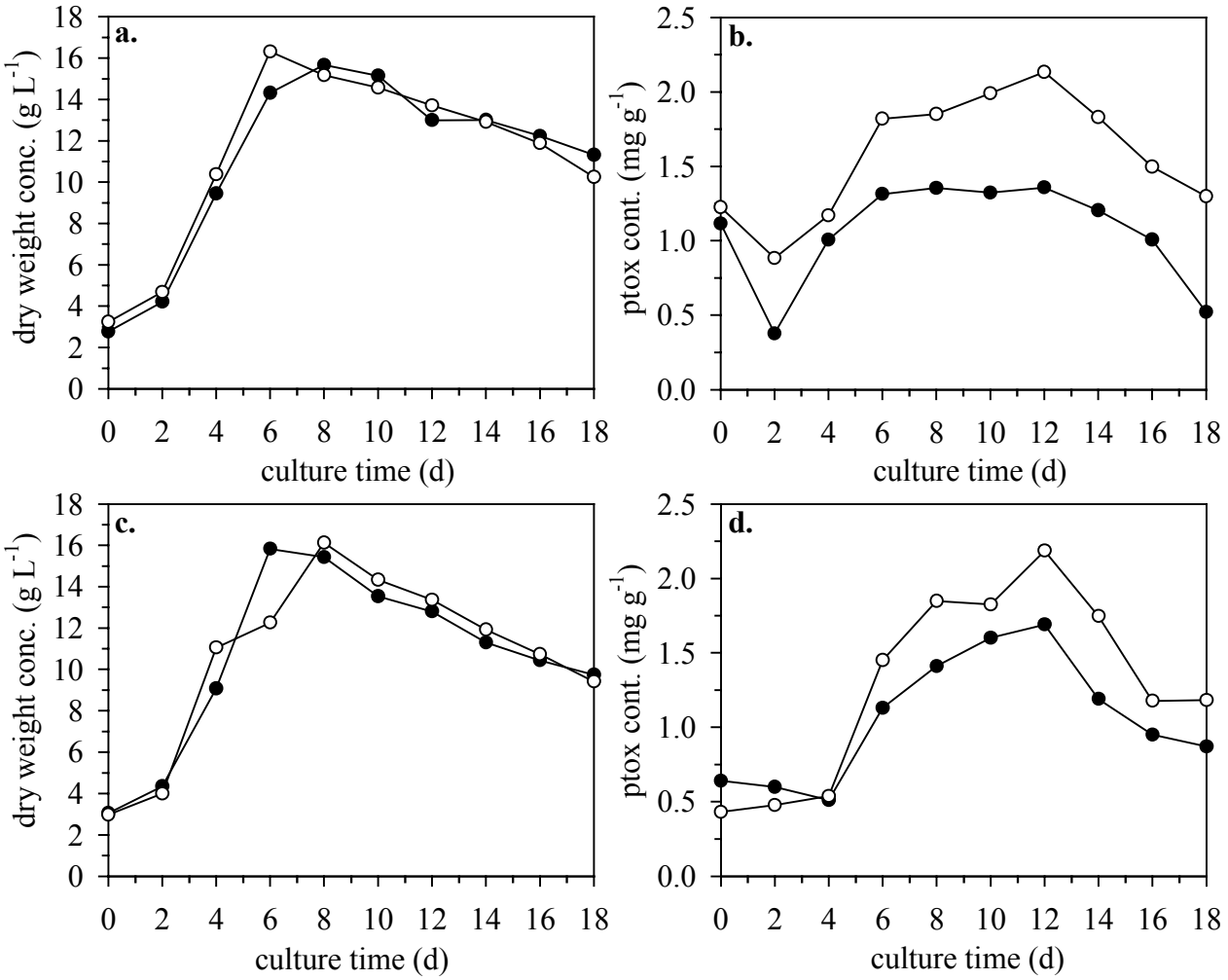

Figure D-7

Effects of the shaker speed (120 rpm / ●, 180 rpm / ○) on biomass production and podophyllotoxin accumulation (on dry weight basis) in 1000-mL flasks with 200 mL of liquid volume (a, b) and 300-mL flasks with 50 mL of liquid volume (c, d). Each symbol represents the mean value of duplicate flasks.

Growth curves and podophyllotoxin accumulation rates obtained with different shaker speeds are presented in Figure D-7. No effect of the increased frequency on the dry weight production can be seen in both culture volumes. A characteristic profile of the cell growth was obtained with a maximum dry weight concentration of 16 g L^{-1}. In contrast to this, higher values were reached for the podophyllotoxin accumulation at elevated stirring rates. For almost the whole culture period, there was an improved podophyllotoxin content detected at 180 rpm, compared with the normal culture condition of 120 rpm for both flask sizes. Moreover, the pattern of the curves seems to be essentially the same for each culture volume (1000 mL and 300 mL) and it is worth noting, that both cultures exhibited roughly the same capacity for podophyllotoxin accumulation. This will be of importance as far as further discussions in relation to the k_La-values presented in Table D-1 are concerned.

D.2.3 Physiological responses to different culture volumes

Besides the variation of the shaker speed the oxygen supply of a suspension culture is influenced by the liquid volume inside the flask. Although Erlenmeyer flasks have often been chosen for basic research as they provide an essentially uniform environment and enable replication, the culture volume has its limitations, due to inferior oxygen transfer capacities (ANDERLEI & BÜCHS 2000; BÜCHS 2001). Taking into consideration a probable oxygen-dependency of the podophyllotoxin biosynthesis, the objective of this study was to investigate the influence of larger culture volumes on podophyllotoxin accumulation.

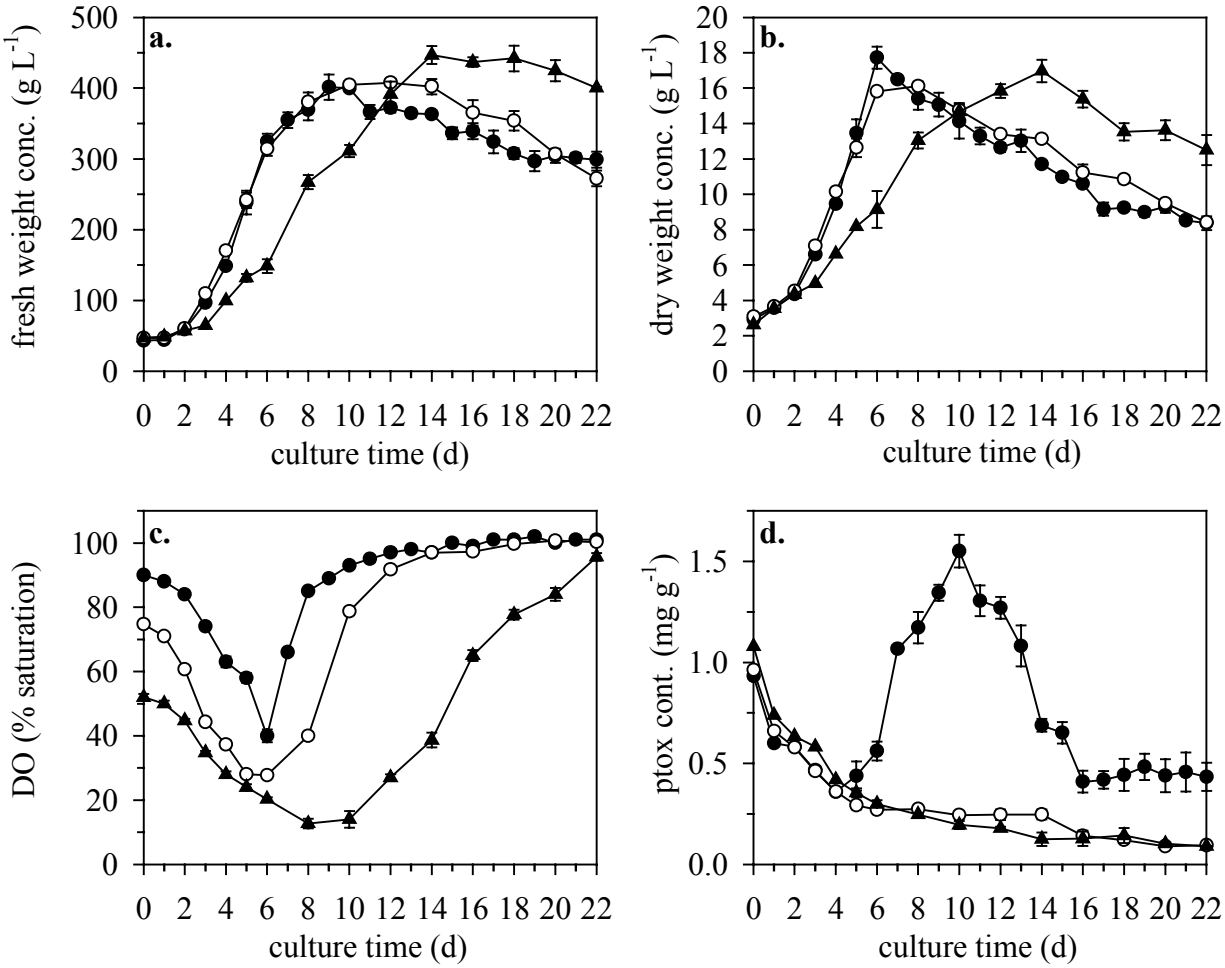

Figure D-8

Effects of the culture medium volume on the biomass production, dissolved oxygen saturation and podophyllotoxin accumulation (on dry weight basis) of a *Linum album* (X4) suspension culture. Erlenmeyer flasks (300 mL) were used at a shaker speed of 120 rpm with different liquid volumes: 50 mL (●), 100 mL (○), 200 mL (▲). The residual dissolved oxygen, as a percentage of saturation, was determined 5 mm under the surface for each individual sample by a micro-optode. Between day 0 and day 4 the contents of 3 flasks were combined for further analysis. Error bars indicate standard deviations (n = 3) of samples taken between day 5 and the end of the culture period.

Linum album (X4) suspensions, which have been cultivated in various liquid volumes marked differences in cell growth, nutrient consumption and podophyllotoxin accumulation. The characterisation was started with the same inoculation density for each experimental setup at the 7th day of culture without any time of incubation. Figure D-8a & -8b describe a similar growth pattern for the standard culture (50 mL) and the 100-mL culture. In both cases, any difference was detected, neither for the fresh weight, nor for the cell dry weight concentration. However, a reduced growth

rate was observed in the 200-mL culture. It showed, respectively, a delay of 4 and 8 days for the fresh weight and dry weight production. In spite of this, the highest biomass concentrations reached, were almost the same for all experimental setups. Microscopic inspections during the initial phase of the culture proved that most of the cells grew in clumps and accumulated starch granules (Figure D-9), which gradually disappeared after the 7th day of culture. Carbohydrate-storage in the form of starch has also been observed for the batch culture of *Catharanthus roseus* (SAKANO, et al. 1995; Van GULIK, et al. 1989) but not in the case of *Papaver* species (PÉPIN, et al. 1995). In view of the fact that the rising number of single and small cells (presumably the younger cells) in the first culture period did not show any starch accumulation, interpretation of the biomass dynamics has to take the inhomogeneity of the culture into account.

In Figure D-8c the effects of an increased culture volume on the oxygen saturation is clearly shown. Even three hours after the inoculation a marked divergence was detected, followed by a further decline, until an individual minimum of the oxygen saturation was reached for each culture. As a result of the increased medium volume in the flasks, lower values of oxygen saturations were achieved concomitant with a rise of the cell concentration during the culture time. Distinctive of the 50-mL culture, which showed an oxygen concentration above 60% of saturation throughout the culture period (except of day 6, 40% of saturation), a higher liquid volume led to a reduced oxygen availability for the cells. Hence, the 100-mL culture exhibited oxygen saturations below 60% between the 2nd and approximately the 9th day with a minimum DO of 28%. In the case of the 200-mL culture, even lower values were detected. The oxygen saturation dropped to 50% right after the inoculation and did not recover before the 16th day of culture. Additionally, very low values of residual oxygen in the culture broth were measured (12% of saturation) between the 8th and 10th day, reflecting the low k_La-values and the delayed growth. Consistent with the inferior oxygen-transfer-rates deeper inside the culture broth (k_La-value of 15 h^{-1}) a 30% lower residual oxygen saturation was measured within the 50-mL filling volume if compared to the 5 mm surface measurement (data not presented). Thus, a gradual reduction of the oxygen supply may be assumed from the culture-surface to the bottom of the flask.

Even though the final biomass concentration reached was not affected by an increased filling volume, a reduced podophyllotoxin accumulation was evident. A plot of the podophyllotoxin accumulation behaviour is shown in Figure D-8d. At standard conditions (50-mL culture), typical results of a maximum podophyllotoxin content (1.6 mg g^{-1} dw) were obtained at day 10 of culture. As already described in Chapter D.1.1, the highest podophyllotoxin accumulation rates paralleled the biomass increase during the late linear-growth-phase, prolonged into the stationary-phase. In contrast, no increase of the podophyllotoxin was recognized if the cells were grown in a larger volume. The 100-mL culture, as well as the 200-mL culture, showed a progressive reduction of the podophyllotoxin content during the culture period. Similar results have been published by other authors, supporting the fact that the biomass production is less vulnerable to oxygen limited growth than the secondary metabolism (Ten HOOPEN ten, et al. 1994; SCHIEL & BERLIN 1987).

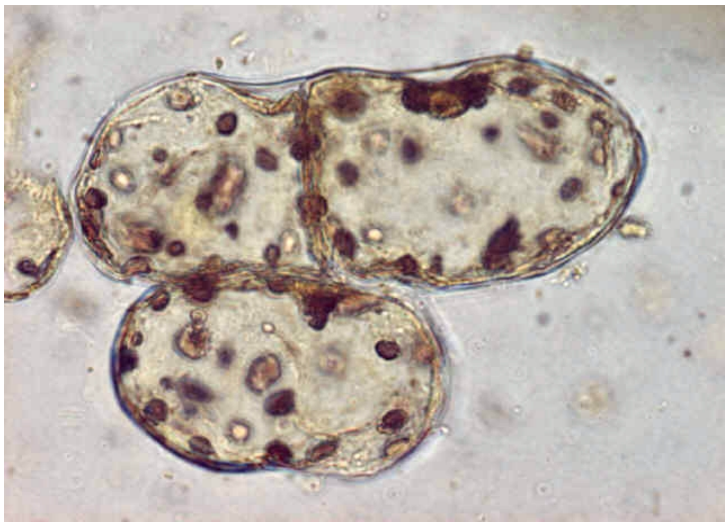

Figure D-9

Microscopic photo of *Linum album* (X4) cells at the 6th day of culture. A standard J_2/KI-solution was used to stain the starch granules (brown colour).

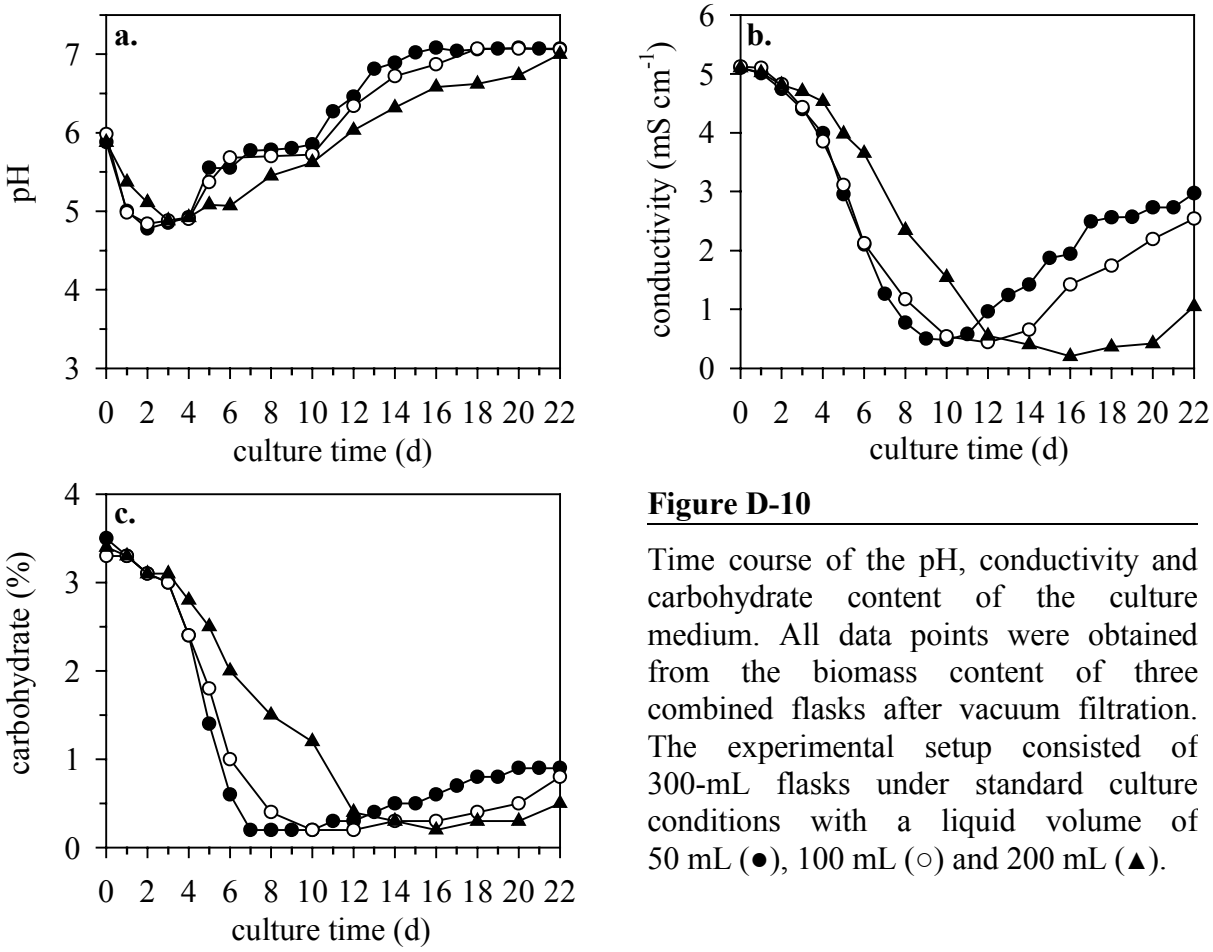

Figure D-10

Time course of the pH, conductivity and carbohydrate content of the culture medium. All data points were obtained from the biomass content of three combined flasks after vacuum filtration. The experimental setup consisted of 300-mL flasks under standard culture conditions with a liquid volume of 50 mL (●), 100 mL (○) and 200 mL (▲).

Typical curves of the main culture parameters for the 50-mL culture are presented in Figure D-10. As described in previous experiments, the day of the highest dry weight concentration coincided with the time when the culture medium became depleted by the carbohydrate source at day 7. Similar results were obtained from the 100-mL culture, although a slight delay in the later growth phase became apparent. A more pronounced delay of the dry-weight production for the 200-mL culture (Figure D-8b) is reflected by a slower decline of the culture medium conductivity and the carbohydrate content (Figure D-10b & -10c). Besides this difference, during the second phase of culture, an increase of both parameters was perceived in relation to the liquid volume. The earlier the culture medium became depleted by the carbon source, the more evident the increase of the medium conductivity was. It has to be stressed that the characteristic sinking of the medium-pH during the first days of culture did not differ in the experimental setups, though later on, a slower kinetic was noticed for the 200-mL culture (Figure D-10a).

The nutrient curves in Figure D-11 show mean values of the residual ammonium, phosphate and nitrate concentrations during the culture period. When the cells were transferred to a fresh culture medium, ammonium was consumed rapidly, within 5 days. No difference was detected between the three culture volumes. The same held true for phosphate, which was completely consumed by all cultures within the initial three hours and which increased again at the end of the culture period. This corresponded to an increase, dependent on the culture-volume, of the medium conductivity (Figure D-10b) and was also obvious in the case of the nitrate-anion (Figure D-11c). The higher nitrate concentrations of the 50-mL reference culture might be explained by a volume-dependent shorter incubation time during the sterilization process of the medium inside the autoclave. If this is neglected, a comparable nitrate uptake behaviour throughout the first 6 days of culture could be assumed for the three liquid volumes.

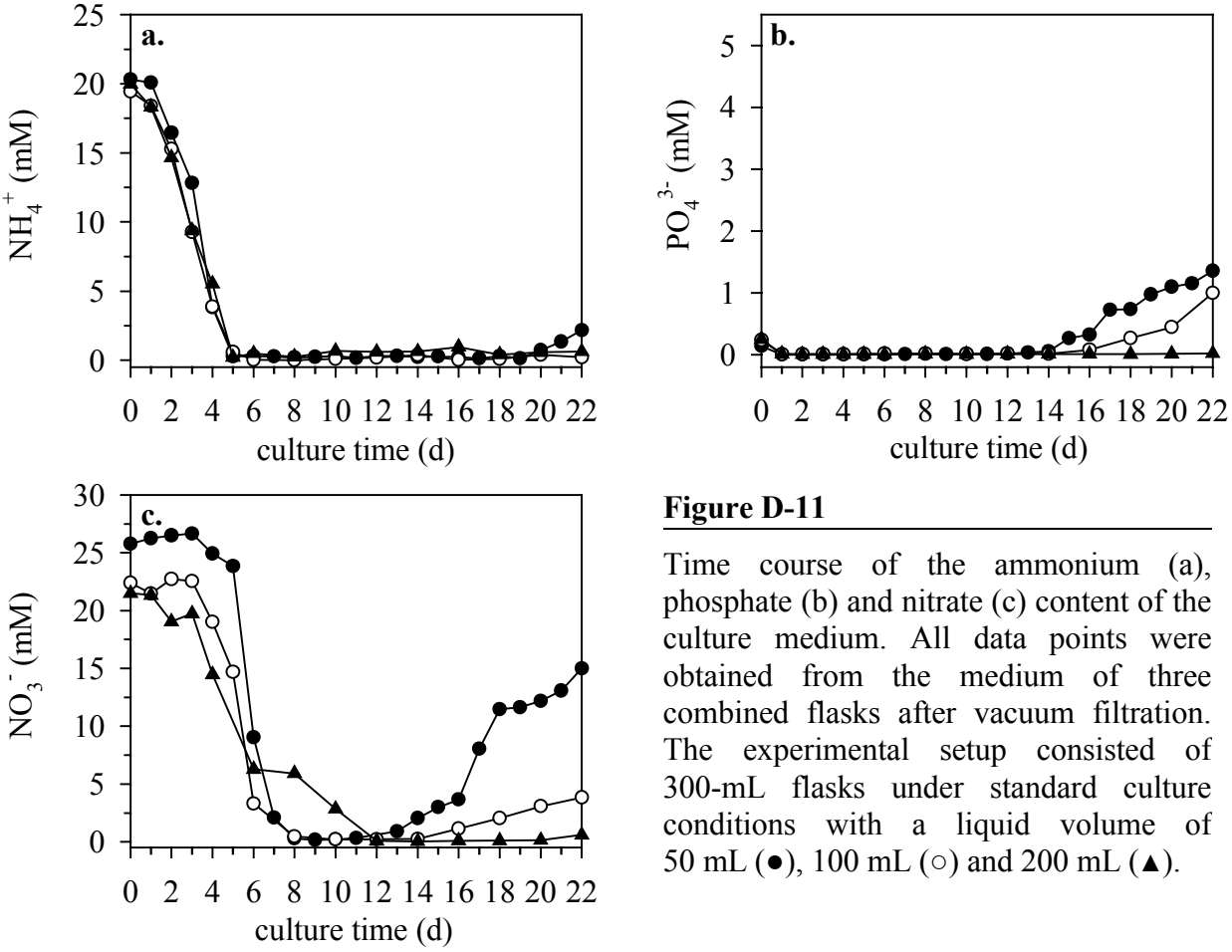

Figure D-11

Time course of the ammonium (a), phosphate (b) and nitrate (c) content of the culture medium. All data points were obtained from the medium of three combined flasks after vacuum filtration. The experimental setup consisted of 300-mL flasks under standard culture conditions with a liquid volume of 50 mL (●), 100 mL (○) and 200 mL (▲).

The following study was based on the dynamics and physiological responses of the podophyllotoxin production by *Linum album* suspension cultures. Its objective was to acquire a detailed description of the induction of phenylpropanoid and monolignol-related enzymes, according to the distinct oxygen-supply characteristics, which became apparent in different culture volumes. For various cell-culture systems a concomitant increase of the phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL) and cinnamyl alcohol dehydrogenase (CAD) activity has been reported upon elicitation, exposure to abiotic factors or transfer to new culture media (HAHLBROCK, et al. 1976; MESSNER & BOLL 1993; SCHLATMANN, et al. 1995). Similar results are shown in Figure D-12, which indicate a simultaneous increase of enzymatic activity and extractable protein concentration during the first days of culture. After the transfer of 7-day-old cells into different volumes of culture medium, the highest PAL-activities were measured one day after the inoculation, followed by a subsequent decline. However, the extent of the increase apparently depended on the liquid level inside the shake flask. Smaller PAL-activities were described for the volumes of 100 mL and 200 mL in contrast to the standard culture. In addition, a PAL-specific second rise during the overall decline of activity was only observed in the case of the 50-mL culture. In contrast, the induction of the 4CL was less rapid, but displayed a prolonged activity until the 14th day of culture (Figure D-12b). Volume-dependent differences of the 4CL were rather subtle in comparison with the CAD-activity presented in Figure D-12c. A reduced activity was detected in the high-volume culture. All enzyme activities were expressed on fresh weight basis. Because the total enzyme activity, which was extracted from a definite cell mass, had been used for the assays, the amount protein needs also to be considered for further discussions. The protein concentration in the crude cell extracts revealed an enhanced accumulation until day 3 in all experimental setups. A peak concentration of 3.5 mg mL^{-1} for the standard culture and 2.9 mg mL^{-1} for the 200-mL culture was found (Figure D-12d).

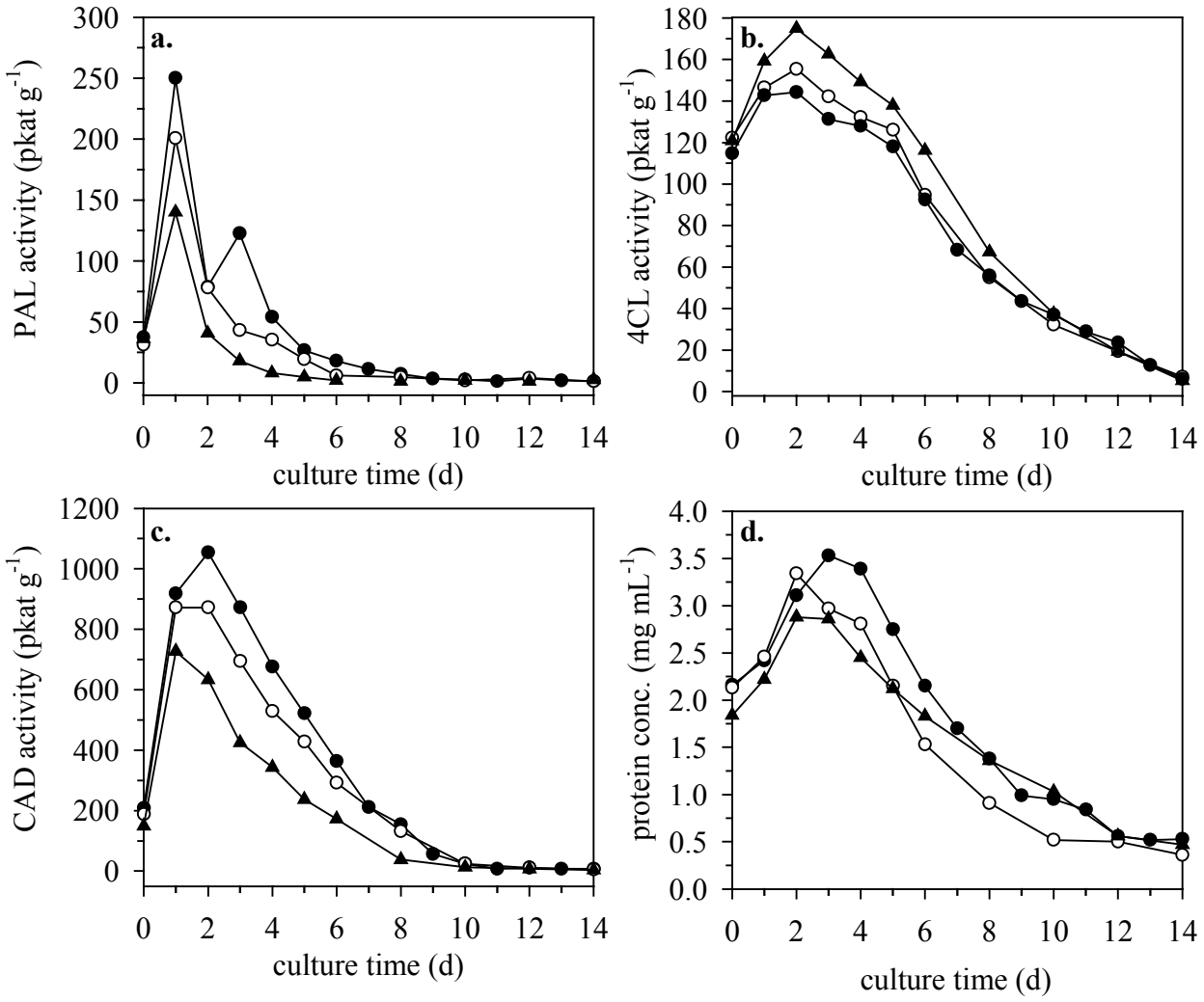

Figure D-12

Time course of changes in phenylalanine ammonia-lyase (PAL, a), 4-coumarate-CoA ligase (4CL, b) and cinnamyl alcohol dehydrogenase (CAD, c) activity and protein concentration of the crude cell extract. 7-day-old cells of *Linum album* (X4) were inoculated into different volumes of culture media. Relative enzyme activities were calculated on basis of the cell fresh weight (pkat g^{-1}). All data points were obtained from the combined biomass of three flasks after vacuum filtration. The experimental setup consisted of 300-mL flasks under standard culture conditions with a liquid volume of 50 mL (●), 100 mL (○) and 200 mL (▲).

Figure D-13 depicts the results obtained from additional experiments pertaining to the impact of oxygen deprivation on the enzymatic activity. Similar to the effects, which have been previously described, a reduction of the peak activity followed by a lowered overall enzymatic performance can be seen. Although the use of a silicon-closure led to conditions, which were not observed under standard culture conditions, it served as a good example for the response of the cells to anaerobic conditions. Due to a possible diffusion of air through the cotton-closure, even an oxygen saturation low as 10% of the maximum was not equivalent to absolute oxygen deficiency. In such a case, 90% of the oxygen available was taken up by the cells, leading to an oxygen-limited growth. On the contrary, in this experiment no further oxygen-delivery was possible after the initial reservoir inside the culture medium and the small head-space became depleted (despite very small amounts of oxygen passing through the silicon stopper by diffusion). Such conditions resulted into a marked reduction of the PAL, 4CL and CAD activity, paralleled by decreased protein concentrations.

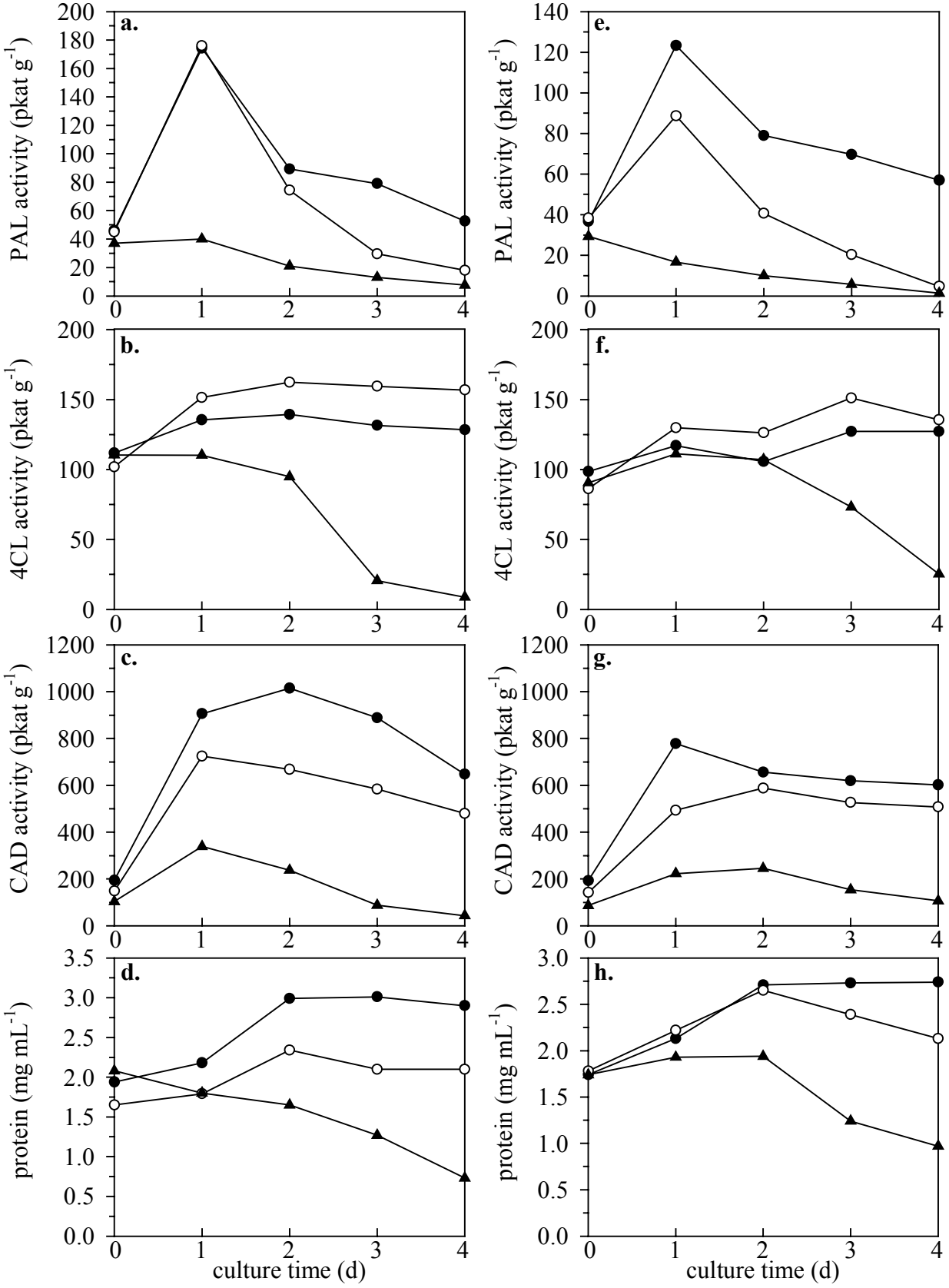

Figure D-13

Time course of PAL, 4CL, and CAD activity and protein concentration of the crude cell extract. Enzyme activities on basis of cell fresh weight (pkat g^{-1}). Each symbol represents the mean value of duplicate flasks. Two distinct experiments (a-d, e-h): 300-mL flasks with 50 mL (●), 100 mL (○) and 200 mL medium (▲). The first two with cotton-, the latter one equipped with a silicon-closure.

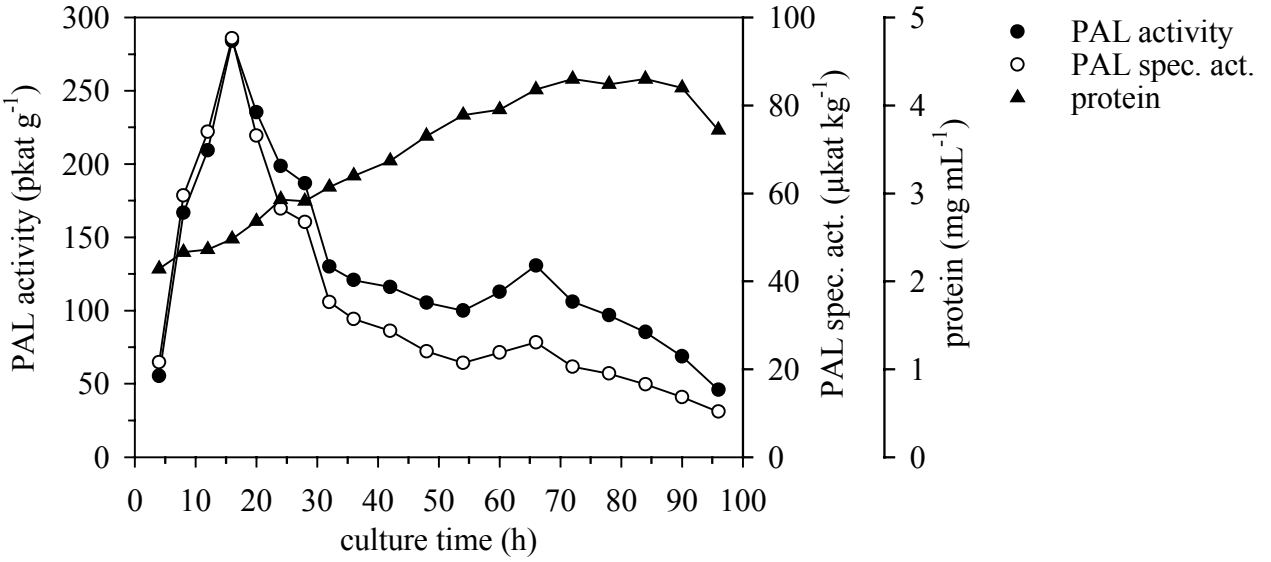

Figure D-14

Time course of phenylalanine ammonia-lyase (PAL) activity on basis of cell fresh weight and protein concentrations of the crude cell extract. Standard inoculation procedure was followed (300-mL shake flasks, 50-mL culture volume) and sample flasks were taken from the gyratory shaker, as indicated during the initial 96 h of culture. All data points were obtained from the biomass content of three combined flasks after vacuum filtration.

As a consequence of the fast kinetic of the PAL-induction, a more detailed analysis was needed to gain an insight into the quantitative changes upon inoculation. Figure D-14 shows a fast increase of enzymatic activity during the first sixteen hours on basis of both, the cell fresh weight, as well as the protein content. Comparable results have already been published by ZUCKER (1965) about potato tuber tissue, although a second rise after sixty hours of induction was not observed in the potato-system. Due to the increasing protein content of the crude cell extract, an earlier decrease of the specific PAL-activity was noticed after thirty hours of culture. Even though an induction or repression of the PAL-activity has been described by various authors (BERNARDS, et al. 2000; BOLWELL, et al. 1986; MORENO, et al. 1996), the underlying functional mechanism of elicitors, irradiation, nutritional factors or cell dilution is still a controversial issue. In the following investigations, the PAL-, 4CL- and CAD-activities were used as indicators of the phenylpropanoid and monolignol metabolism in relation to various *in vitro* conditions, which affect the podophyllotoxin biosynthesis.

D.3 Effects of the oxygen supply on podophyllotoxin production – the bioreactor system

In airlift bioreactors, pneumatic agitation and oxygen delivery are both put into practice by air injection at a definite flow rate. Therefore, in the absence of detrimental effects, the needs for suspended plant cells should be met easily. Unfortunately, this is not the case, since the rising and bursting air bubbles exert shear forces, generate foam at the culture surface and do not withstand the mixing and the oxygen requirements of a high-density culture. Such adverse effects have been described elsewhere for plant and mammalian cell cultures (CHISTI 2000; HUA, et al. 1993; SHIBASAKI, et al. 1995). In order to establish a bioreactor system for *Linum* suspensions and to confirm the results of previous studies, batch cultures were carried out in an 18-L airlift bioreactor at different growth conditions.

D.3.1 Influence of the aeration rate on podophyllotoxin accumulation

The present study addressed the behaviour of culture growth and the podophyllotoxin production under various aeration regimes. The effect of this operational parameter on the performance of the bioreactor culture was investigated at aeration rates between 0.22 vvm (4 L min^{-1}, BR_A & BR_B) and 0.55 vvm (10 L min^{-1}, BR_G & BR_H). Therefore, initial two bioreactors were established in a batch-mode (no further addition of culture-medium nutrients beside water) to reveal the growth characteristics and dynamics of the oxygen saturation during the culture period. As a consequence of the inoculation procedure, the final medium inside the bioreactor was more diluted than in conventional shake flaks cultures (approximately 14% less concentrated). Furthermore, a higher inoculation-density and low aeration rates had been applied to avoid any adverse reaction of the cells (compare Chapter D.1.2) and to ensure a sustained growth in the initial culture period. However, the changes proved to have an impact on the podophyllotoxin accumulation performance and were investigated in additional studies.

Total growth as a function of time is presented in Figure D-15a and shows a good reproducibility of both reactors (BR_A & BR_B). The development of the cell fresh weight, as well as the dry weight, shows a relatively slow and extended growth pattern, though, as already described for *Linum album* shake-flask cultures, the dry weight levelled off prior to the fresh weight. Figure D-15b reflects the prolonged growth and metabolic activity in a high residual carbohydrate concentration and a low oxygen saturation (DO 10%) over an extended period. After the drop of the pH from 5.8 to 5.1 during the first 2 days, the pH of the culture medium afterwards increased until day 13 (pH 6.8), and thus coinciding with the lowest conductivity values.

As expected from the low oxygen saturation of the culture broth, only a minor podophyllotoxin content was found for both reactors. Similar to the high-volume culture described in Chapter D.2.3, a steady decline of the podophyllotoxin content was observed until the end of the culture period. So the production dynamics mostly depend on the less affected biomass accumulation (Figure D-16). The results gathered by these initial bioreactors resemble those of the oxygen-limited 100- and 200-mL shake flask culture. A reduced culture performance was evident in terms of a slow increase of biomass and minor podophyllotoxin accumulation. In order to clarify the reliance of podophyllotoxin biosynthesis on the oxygen saturation of the bioreactor culture, additional information was essential. Therefore, further bioreactors were established with higher aeration rates (0.33 vvm – BR_C & BR_D, 0.44 vvm – BR_E & BR_F and 0.55 vvm – BR_G & BR_H), which improved the oxygen transfer capacities (see Table D-1). Since consistent changes between the experiments had been observed, the setup of BR_E & BR_F (0.44 vvm) served as a model for the impact of an increased aeration rate on the bioreactor performance.

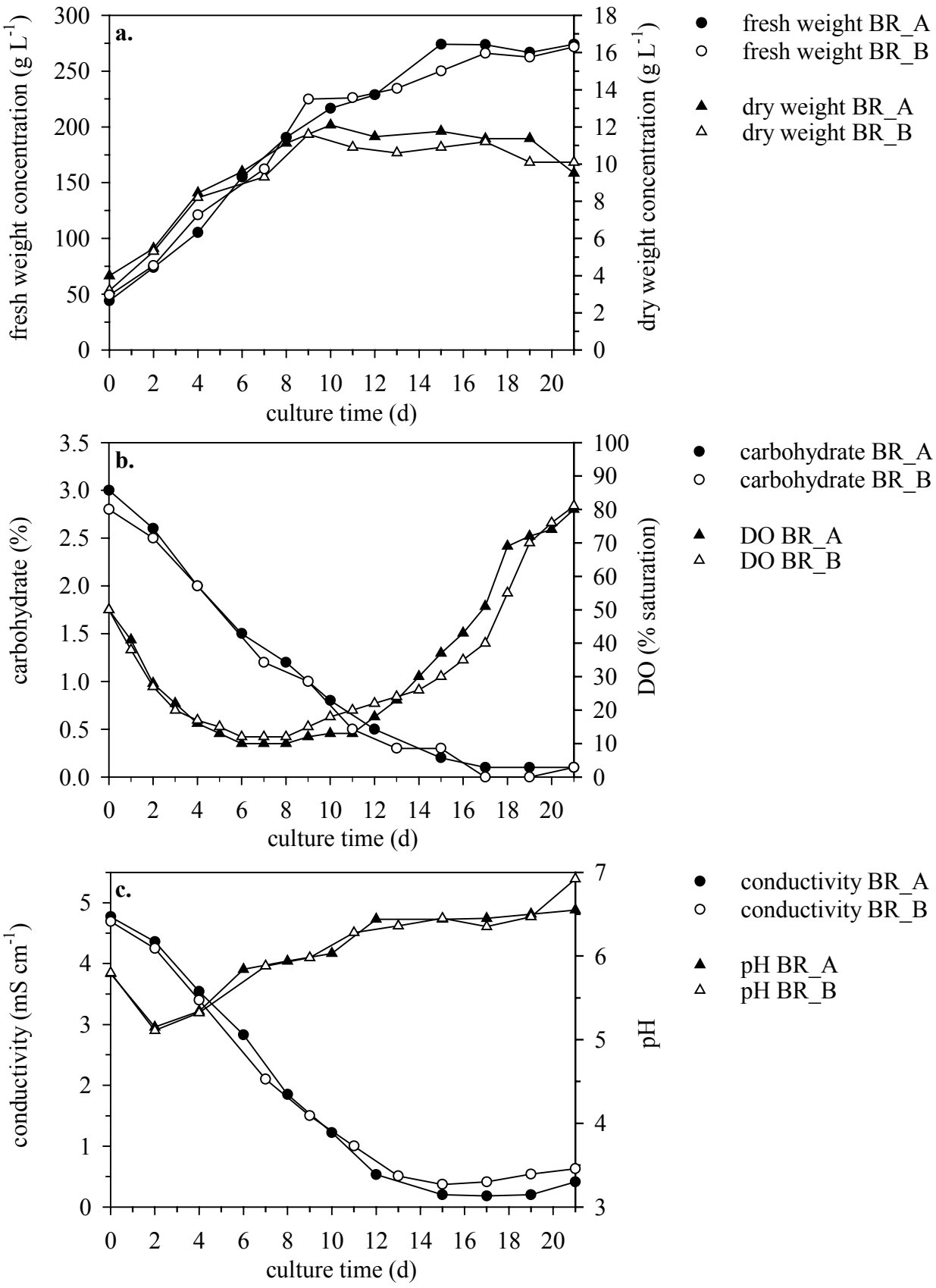

Figure D-15

Biomass concentrations, dissolved oxygen saturations and culture medium parameter as a function of time for two bioreactors (BR_A & BR_B). Experimental setup: 0.22 vvm, batch culture, no active DO-regulation, high inoculation density, diluted culture medium, PPG-antifoam.

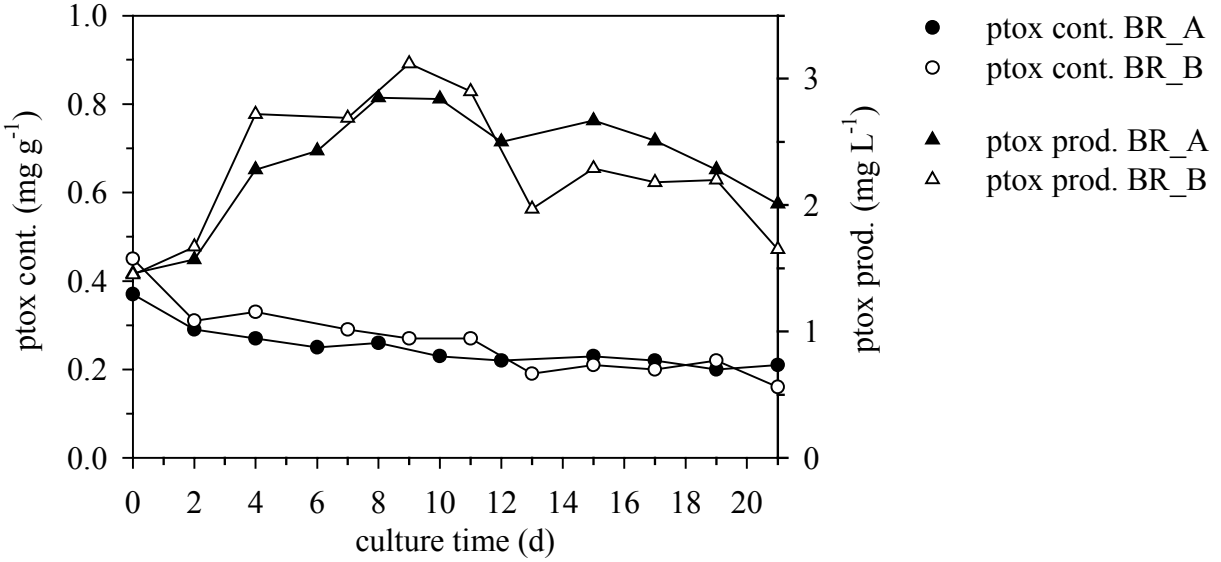

Figure D-16

Podophyllotoxin content and production (both on a dry weight basis) by *Linum album* (X4) suspension cultures in duplicate bioreactors (BR_A & BR_B) at an aeration rate of 0.22 vvm.

Concomitant with the elevated aeration rates in the presented experiment, the addition of larger amounts of antifoam was needed to prevent excessive foam formation. Especially in the case of pneumatic bioreactors, foam destruction has been of major concern in biotechnological engineering. A foaming culture broth is prone to infections and adherent wall growth, hence influencing both the product formation and biomass determination (PIEHL, et al. 1988; WILSON & HILTON 1995). Different approaches for foam prevention and its destruction, such as mechanical foam breakers, ultrasound, electrical treatment or the addition of surface-active-substances have been laid out in the literature (VARDAR-SUKAN 1998). Although PPG had been successfully used for foam control at lower aeration rates, detrimental effects were evident at values above 0.22 vvm. Besides the well-known negative effect of antifoaming agents on the oxygen transfer capacity (LIU, et al. 1994; ONODERA, et al. 1993), bioreactor cultures (BR_I & BR_J, data not shown), with an aeration rate of 0.44 vvm, displayed a reduced peak biomass on the 5th day of culture (12 g dw L^{-1}). In other cases cell lysis occurred, paralleled by a steep increase of the oxygen saturation at about the same time. Interestingly enough, these cultures recovered after the aeration rate was reduced for several days, thus indicating that a microbial infection was not the reason for the abnormal behaviour. Various effects of polypropylene glycol (PPG), ethylene glycol or silicone based antifoams have been investigated elsewhere (ABDULLAH, et al. 2000; Van POL, et al. 1993). Diverse explanations are given for the growth retardation and the cell lysis of plant suspension cultures in bioreactors. Similar to HASHIMOTO (1988), the intensified foaming of the *Linum album* (X4) culture, throughout the phase of active growth, might have resulted from extracellular polysaccharides and was effectively controlled by an silicone emulsion (O`LENICK 2000). Even at aeration rates of 0.55 vvm, no negative consequences were perceived. As the hydrophobic and water-insoluble silicone adsorbs not only at the gas-liquid interfaces but also onto surfaces of the cells or the culture vessel, which has been described by ZHANG et al. (1992), its final concentration in the liquid phase of the culture broth can not be estimated. Generally, at an aeration rate of 0.44 vvm, 3 L of antifoam (5% aqueous emulsion of commercially available Antifoam-A) were sufficient to control foam formation. This operative procedure was adopted for the following bioreactors.

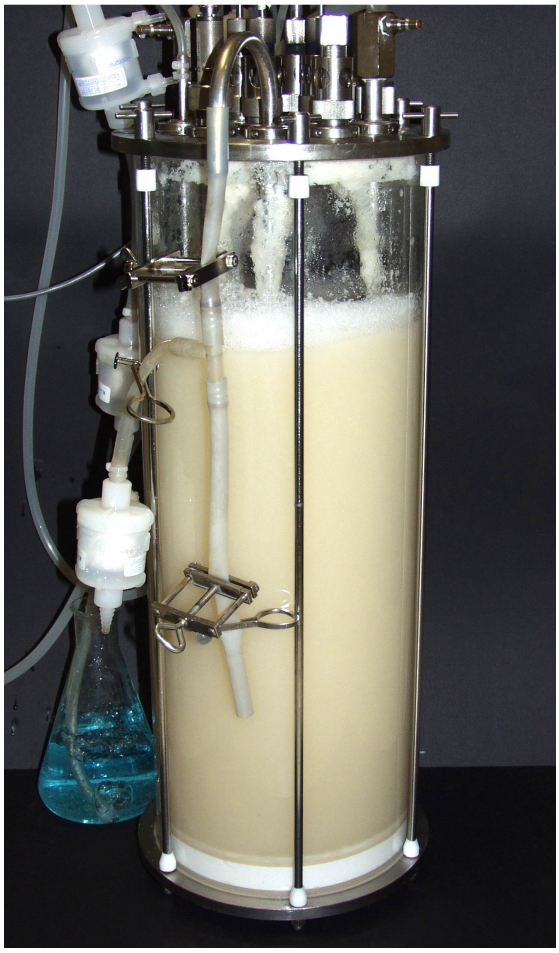

Figure D-17

Final bioreactor-setup with a 7-day-old *Linum album* (X4) cell suspension, grown under standard conditions at an aeration rate of 0.44 vvm.

The results above indicate that *Linum album* (X4) cells can be cultivated at increased aeration rates in airlift bioreactors by choosing the right antifoaming agent. It was expected that such improved conditions of the oxygen supply would lead to a higher yield of podophyllotoxin. Results obtained under these conditions for BR_E & BR_F are presented in Figure D-18. The podophyllotoxin content, as well as the podophyllotoxin production were higher than in the case of the lower airflow rate of 0.22 vvm (BR_A & BR_B). A plateau-like value of 0.6 mg g^{-1} podophyllotoxin had already been reached at the 3rd day of culture. It can be deduced that on the one hand, a higher k_La-value of the 0.44 vvm culture may enable the onset of podophyllotoxin biosynthesis, whereas on the other hand the plateau-like pattern implies further limitations in comparison with the shake flask culture. Figure D-19 describes the course of the enzyme activity at elevated aeration rates. The obvious induction of the phenylpropanoid- and monolignol-pathway-related enzymes (PAL, 4CL and CAD) upon inoculation to fresh culture media was also apparent in the case of the shake flask culture. Except for the 4CL, all enzymes showed a typical increase of activity on fresh weight as well as on protein basis. During the first twenty-four hours the PAL activity was rapidly induced (6-fold increase on fresh weight basis), followed by a sudden decline – activity was not regained until the end of the enzyme characterisation. The early increase of the CAD activity was about 8-fold on the fresh weight basis and exhibited a slower decline in comparison to the PAL. Such results are consistent with data published by others (BERNARDS, et al. 2000; HOTTER 1997; MESSNER & BOLL 1993). Although both enzymes displayed a transient pattern of activation, no clear time-dependent order was observed, concerning to the consecutive position in the phenylpropanoid and monolignol metabolism.

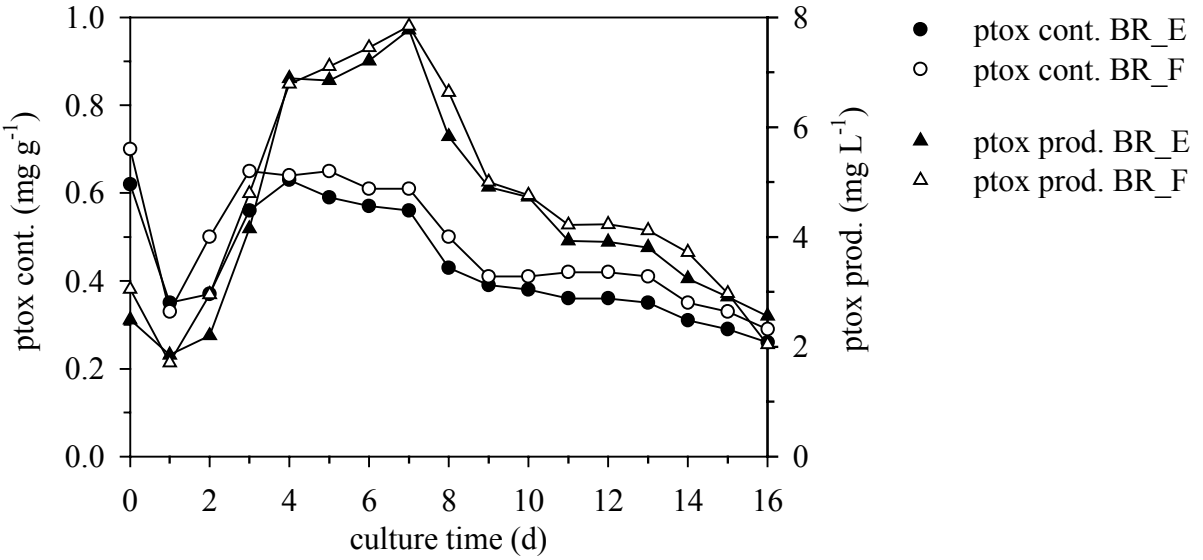

Figure D-18

Podophyllotoxin content and production (both on dry weight basis) by *Linum album* (X4) suspension cultures in duplicate bioreactors (BR_E & BR_F) during the culture period. Experimental setup: 0.44 vvm, batch culture, no active DO-regulation, high inoculation density, diluted culture medium, silicone-antifoam.

To study the growth characteristics and nutritional requirements of suspension cultures at elevated aeration rates in the bioreactor, the cell-mass, culture medium parameter (carbohydrate content, pH, conductivity) and dissolved oxygen saturation were measured at intervals during batch culture of BR_E & BR_F. Both bioreactors yielded a higher fresh and dry weight (Figure D-20) if compared to BR_A & BR_B (Figure D-15). At an aeration rate of 0.44 vvm the cells showed a faster increase of the fresh weight with a peak value of 300 g L^{-1} at day 8 of culture. In opposition to the cell dry weight, which reached its highest concentration on the 7th day (13.5 g L^{-1}) and levelled off afterwards, the fresh weight exhibited a typical plateau phase until the end of experiment. As usual, the biomass increase stopped, coinciding with the time when the culture medium was almost exhausted. This was indicated by a low carbohydrate content and minimal conductivity roughly on about the 8th day of culture. A direct comparison of the culture characteristics after variation of the distinct parameter, such as the aeration rate or active oxygen-saturation control is presented in Chapter D.7. Besides a sufficient oxygen supply, the medium formulation is of prior importance among the factors which influence the secondary metabolism, since the growth and the production of metabolites in a bioprocess are the result of direct interactions between suspended cells and intra- and extracellular effectors (ERTOLA, et al. 1995). Even though the impact of the mineral composition of culture media on plant cells has been investigated in various examples (FULCHERI, et al. 1998; MORARD & HENRY 1998; ZHANG, et al. 1998) the individual physiological response of the cell line is not predictable. Consequently, information about the fade of inorganic nutrients during the culture period was vital in order to clarify whether any component may become rate-limiting or not. The residual concentrations of four microelements were determined. With the exception of manganese, all other ions were taken up completely from the medium with different kinetics. Since calcium and iron were both present throughout the growth phase and did not disappear before the 11th day of culture, it was assumed that only the potassium ion could have exerted a growth limiting effect (ZHONG 2001). However, though the addition of KH_2PO_4 naturally brings an increase in the potassium concentration, it is suggested that cellular growth was attributed to the phosphate anion (Ten HOOPEN, et al. 1994; KANDARAKOV, et al. 2000). Regarding to the podophyllotoxin content, neither the potassium nor the other microelements show any involvement into the observed plateau of podophyllotoxin accumulation between the 3rd and 6th day of culture.

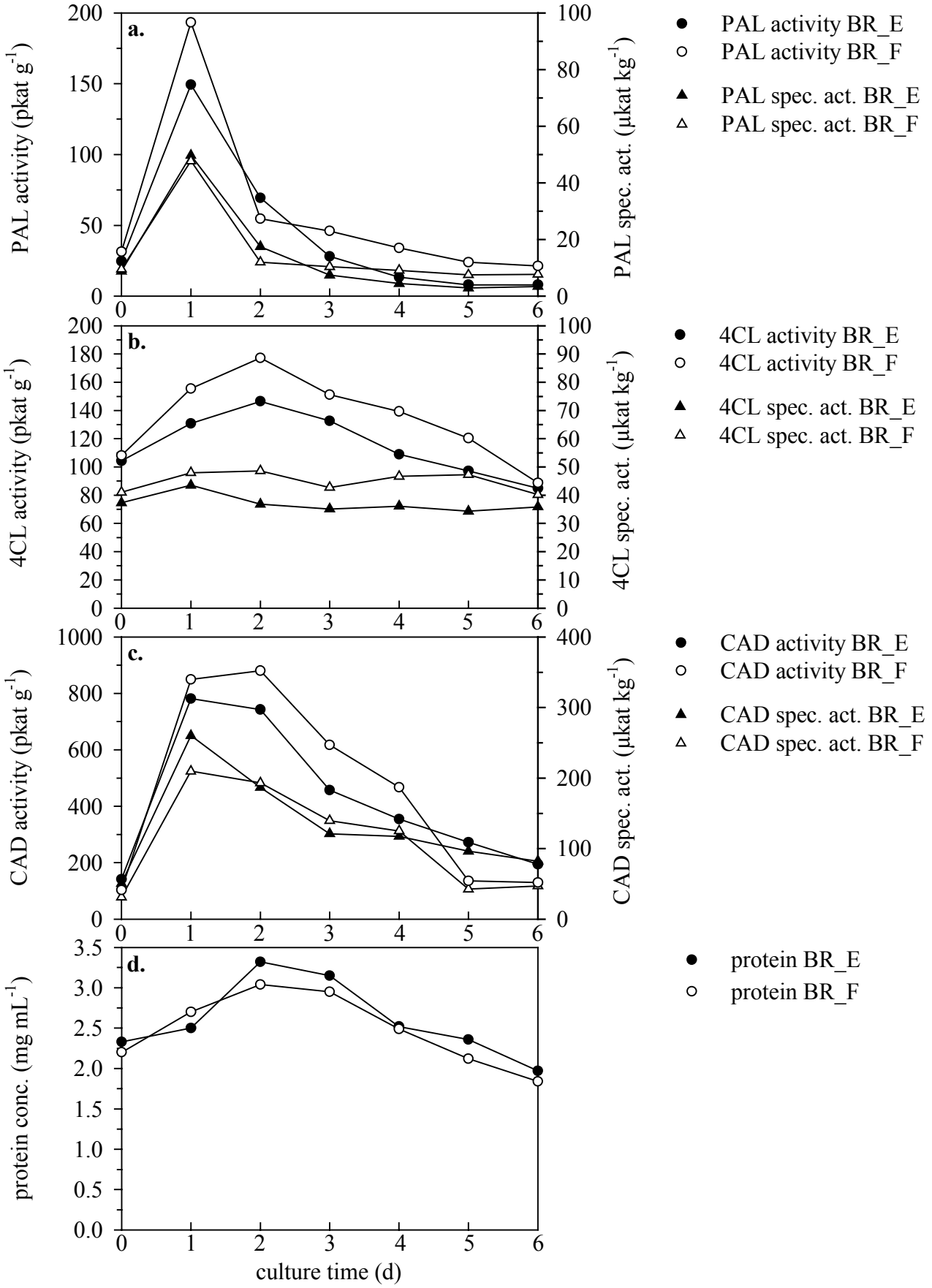

Figure D-19

Enzyme activity on the basis of cell fresh weight (pkat g^{-1}) or protein concentration (µkat kg^{-1}) of the crude cell extract (BR_E & BR_F). Samples were taken during the first 6 days of culture.

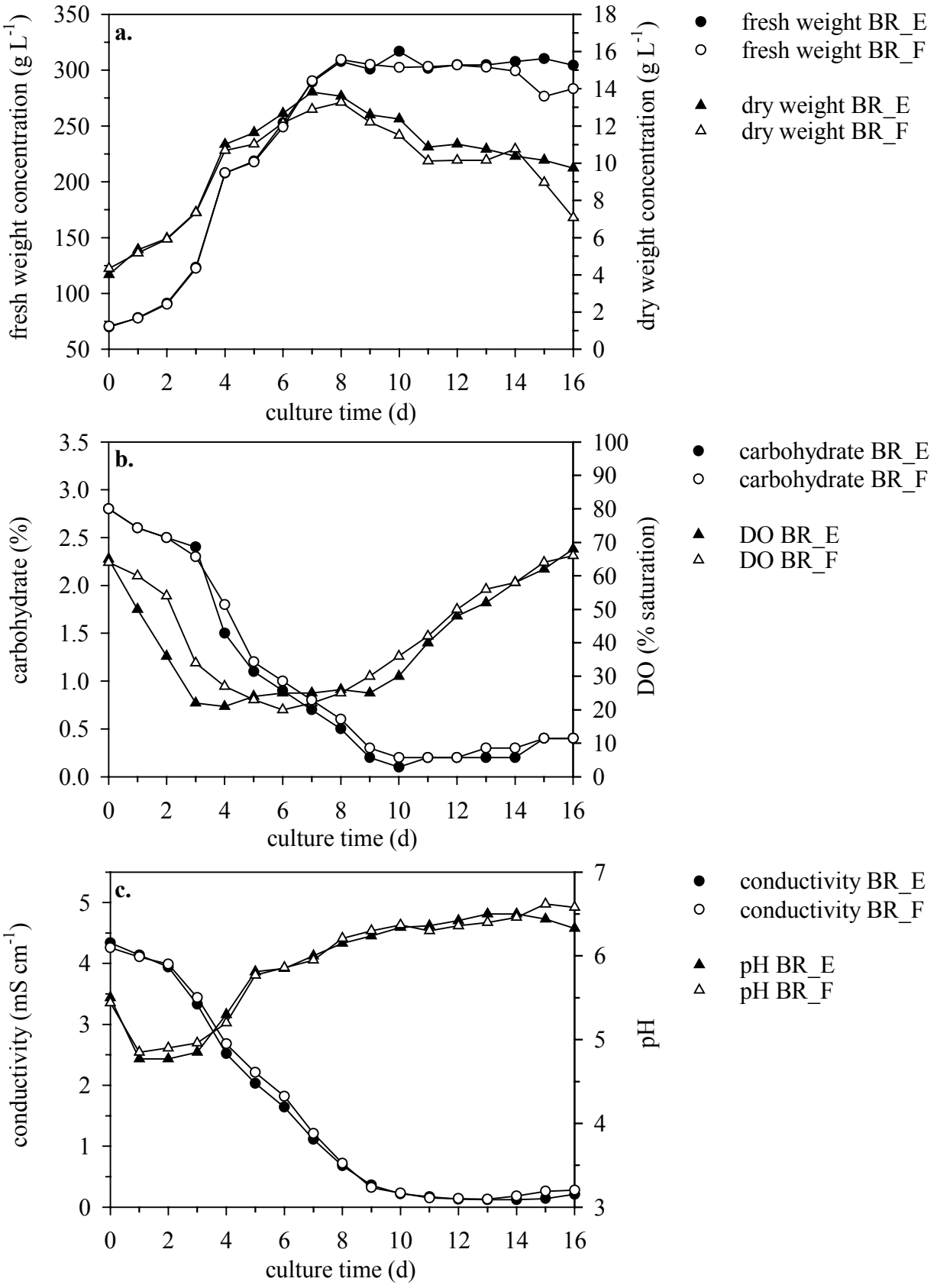

Figure D-20

Biomass concentrations, dissolved oxygen saturations and culture medium parameter as a function of time for two bioreactors (BR_E & BR_F). Experimental setup: 0.44 vvm, batch culture, no active DO-regulation, high inoculation density, diluted culture medium, silicone-antifoam.

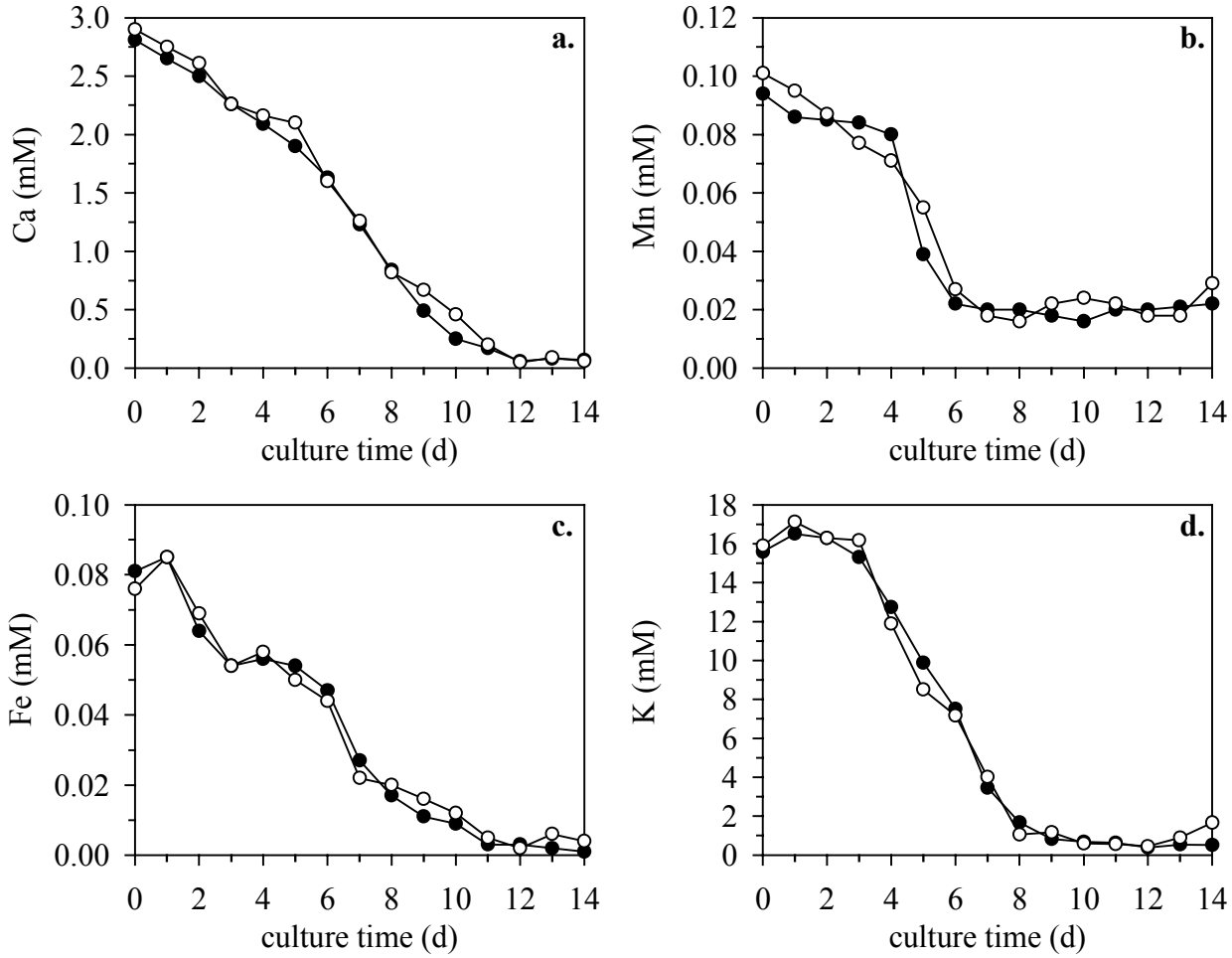

Figure D-21

Time course of residual calcium (a), manganese (b), iron (c) and potassium (d) concentrations of the culture medium. The symbols represent BR_E (●) & BR_F (○). Experimental setup: 0.44 vvm, batch culture, no active DO-regulation, high inoculation density, diluted culture medium, silicone-antifoam.

D.3.2 Studies on the bioreactor culture at an elevated oxygen saturation

The major drawback of an airlift bioreactor is the link between the agitation characteristics of the system and the oxygen mass transfer capacity. The results presented in the previous chapter proved the feasibility and the oxygen-dependency of a *Linum album* bioreactor culture, but revealed an upper limitation of the aeration rate. If, for example, the culture was aerated with 0.55 vvm (10 L min^{-1}), cells were stripped off the surface and excessive wall growth in the headspace of BR_G & BR_H occurred (data not presented). Although a slight increase of the podophyllotoxin content was reached for these bioreactors (0.8 mg g^{-1} dry weight) and the DO had never been below 25%, a k_La-value of 14.11 (Table D-1), seemed to be insufficient. For that reason, it was the aim of this study to uncouple the need for bulk mixing and oxygen supply. This was achieved by an injection of pure oxygen into the riser of the bioreactor system. Since the rate of oxygen mass transfer within the system correlates not only with the volume of air introduced but also with the interfacial area and partial oxygen pressure of the bubbles, the integration of a ceramic sparger (Figure C-2) helped to compensate possible oxygen limitations. Thereby, it became possible to maintain the oxygen saturation of the culture broth at a certain value. For this study a minimum DO of 60% had been chosen to ensure non-oxygen-limited growth and podophyllotoxin biosynthesis.

This decision was based on results of the shake flask system (minimum DO 40%) and on published data reviewed by PAYNE (1987) and SINGH & CURTIS (1994). A critical oxygen concentration of approximately 50% or 60% was estimated for *Catharanthus roseus* and for the anthraquinone production by *Morinda citrifolia*, respectively. Under such non-oxygen-limited condition of BR_K & BR_L (Figure D-22), the maximum content of podophyllotoxin (plateau-value) increased by 66% in comparison to the data presented in Figure D-18. Neglecting the rise in the later culture phase, a plateau-value of approximately 1 mg g^{-1} dry weight was reached at the 6th day. The pronounced increase of podophyllotoxin accumulation was preceded by a lag-phase of 2 days after inoculation. For further discussion it must be apprehended that, in spite of the elevated oxygen saturation of the culture broth, the podophyllotoxin content was still substantially lower in contrast to the values found in the shake flask system. If the stationary phase of the podophyllotoxin accumulation was also taken into consideration, a limitation by other factors than oxygen could have been postulated.

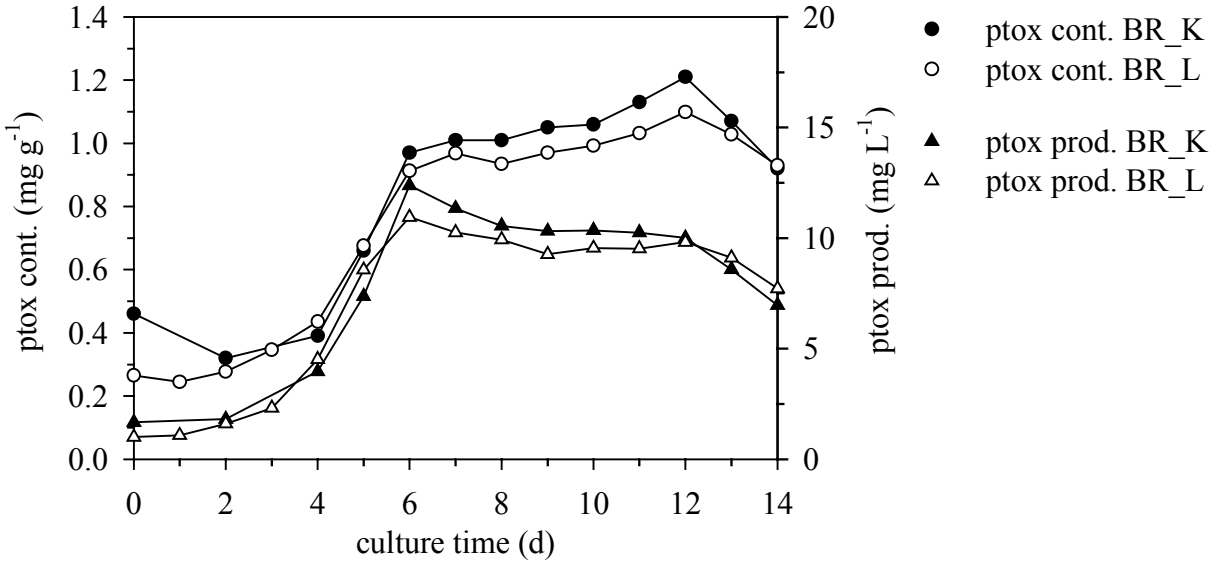

Figure D-22

Podophyllotoxin content and production (both on dry weight basis) by *Linum album* (X4) suspension cultures in duplicate bioreactors (BR_K & BR_L) during the culture period. Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), high inoculation density, diluted culture medium, silicone-antifoam.

An induction of the phenylpropanoid and monolignol metabolism upon inoculation was examined by assaying PAL, 4CL and CAD activities under non-oxygen-limited conditions of BR_K & BR_L. A characteristic transient pattern of PAL and CAD induction is shown in Figure D-23. Though an unusual decline of 4CL activity was observed for BR_K, no effect of the elevated oxygen tension was observed and the results were consistent with the bioreactors, without an active oxygen regulation (Figure D-19). Since conventional aeration (0.44 vvm) was sufficient to meet the oxygen demand of BR_E & BR_F until the 4th day of culture, enzyme activities were not affected. By summarising the outcome of the shake flask and bioreactor experiments concerning the implication of oxygen in enzyme activity, it can be deduced that: (1) oxygen limitations only affected the activity during the first days after the inoculation, (2) PAL and CAD activities were lower in the bioreactor than in the shake flask system, (3) an elevated oxygen saturation after day 4 of culture did not improve or extend the overall enzymatic performance.

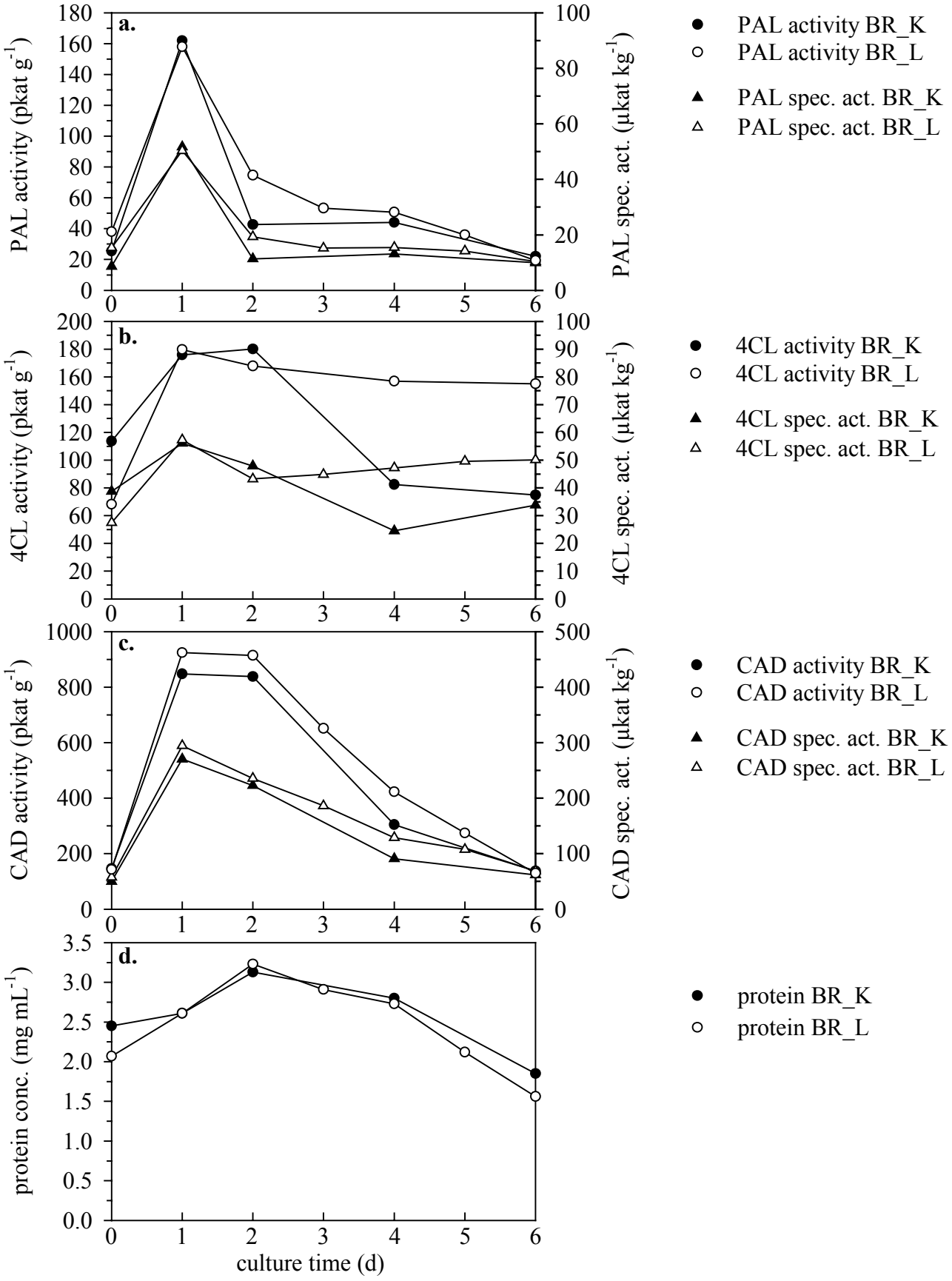

Figure D-23

Enzyme activity on basis of cell fresh weight (pkat g^{-1}) or protein concentration (µkat kg^{-1}) of the crude cell extract (BR_K & BR_L). Samples were taken during the first 6 days of culture.

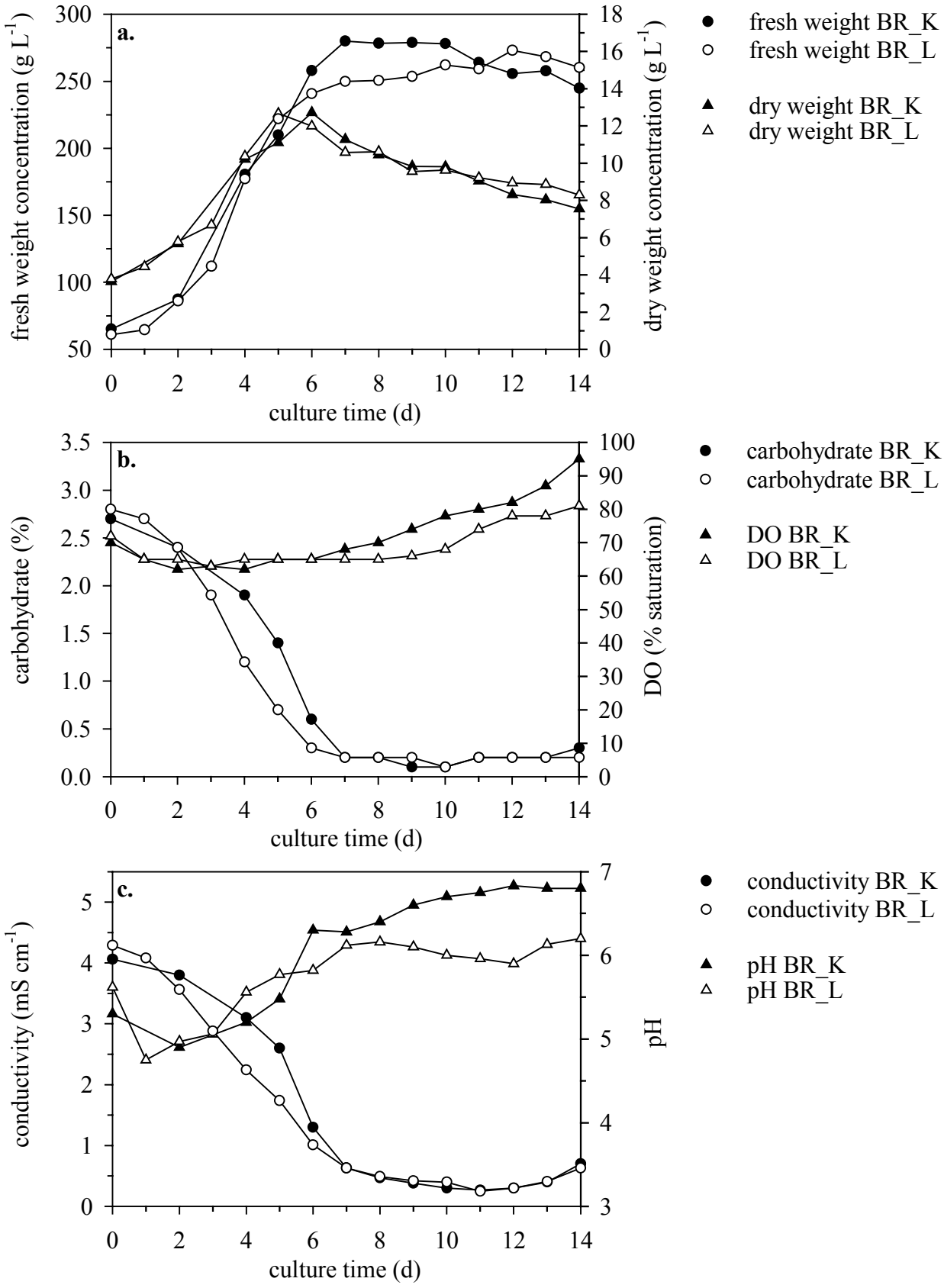

Figure D-24

Biomass concentrations, dissolved oxygen saturations and culture medium parameter as a function of time for two bioreactors (BR_K & BR_L). Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), high inoculation density, diluted culture medium, silicone-antifoam.

Unlike BR_E & BR_F, which were devoid of pure oxygen supplementation, a lower peak dry weight concentration of 12 g L^{-1} had already been reached at day 5 of culture in the case of BR_K & BR_L. In Figure D-24 a probable stimulation of the metabolic activity is also reflected by a faster decline of the carbohydrate content and the medium conductivity. As a consequence of the changing oxygen demand of the cells and presumably lower k_La-values in the later batch culture phase, a 10% variation of the oxygen setpoint was noted. Nevertheless, oxygen regulation by the ceramic-sparger proved to be sensitive enough to achieve an approximate DO of 60% of saturation (Figure D-24b). Coinciding with the extracellular depletion by the carbohydrate source at day 6, the addition of supplementary oxygen was reduced, due to an increasing oxygen saturation of the culture broth. In Figure D-25 the remaining concentrations of microelements (calcium, manganese, iron and potassium) at elevated oxygen concentrations are presented. Except for the potassium ion, which showed a slightly faster decline between the 4th and 8th day of culture (BR_K & BR_L), the results were comparable to those shown in Figure D-21. Obviously, non-oxygen-limited conditions in a bioreactor yielded a faster growth but a lower final concentration of the biomass. Because of the accelerated nutrient removal from the medium and a higher accumulation of podophyllotoxin, it can be assumed that: (1) the biomass accumulation is limited by the carbohydrate supply or another medium constituent and (2) increased metabolic activity of the cells is preferably utilized for an alternative sink.

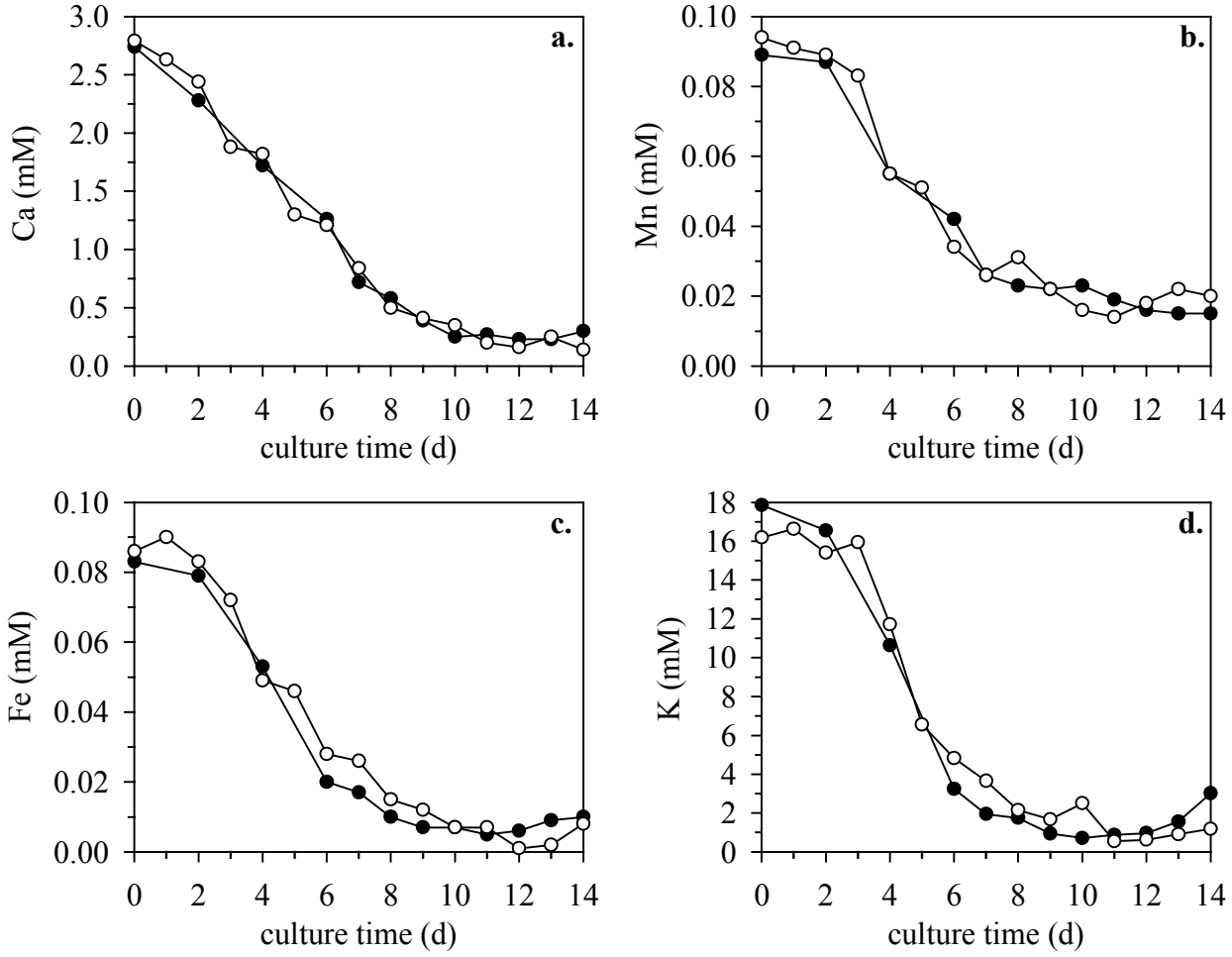

Figure D-25

Time course of residual calcium (a), manganese (b), iron (c) and potassium (d) concentrations of the culture medium. The symbols represent BR_K (●) & BR_L (○). Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), high inoculation density, diluted culture medium, silicone-antifoam.

D.4 Influence of the inoculum on enzyme activity and ptox accumulation

Little attention has been paid to the effect of the inoculum density on the performance of podophyllotoxin production by *Linum album* suspension cultures. As far as the inoculation cell mass is concerned, research at our institute has been mainly focused on an optimised culture growth rather than on an increased phenylpropanoid activity and podophyllotoxin accumulation. Even though the underlying mechanism is still not fully understood, it has been shown in plant, as well as in animal cell cultures, that highly diluted suspended cells did show a prolonged lag-phase or even failed to proliferate (DUTTON, et al. 1999; Van GULIK, et al. 1994). A possible explanation has been given by SAKANO (1995), who claims that excessive phosphate uptake on a per-cell-basis induces starch phosphorolysis. This is followed by an increase of cellular osmolarity concurrent with an excessive water uptake which gives rise to cell swelling and rupture. If the effect of the inoculum density is assessed in terms of secondary metabolite production, factors, such as the per cell nutrient level, the percentage of conditioned (used) medium or the oxygen saturation during the initial culture phase, have to be taken into account. These parameters may contribute, but not entirely, to the diverse results described in connection with optimised inoculum cell densities. In this chapter the effects of different initial cell concentrations on phenylpropanoid- and monolignol-related enzymes, as well as the podophyllotoxin accumulation, are reported.

D.4.1 Effects of different inoculum densities on shake flask culture

Contrary to the bioreactor system, in shake flasks, oxygen delivery and saturation of the culture broth is solely dependent on diffusion through the closure and liquid surface (MROTZEK, et al. 2001) – no active DO regulation possible. As a consequence, oxygen limitations may arise especially under the constrains of a high-density-culture, leading to a low yield of podophyllotoxin. The establishment of an optimised podophyllotoxin production necessitates basic information about the yield of biomass and the enzymatic capacity at different inoculation sizes. Under standard conditions (300-mL shake flask, 50 mL liquid volume, 120 rpm) *Linum album* (X4) suspension cultures were inoculated at a density of 3 g, 5 g and 7 g per 50 mL liquid volume (fresh weight including approximately 50% adherent culture medium).

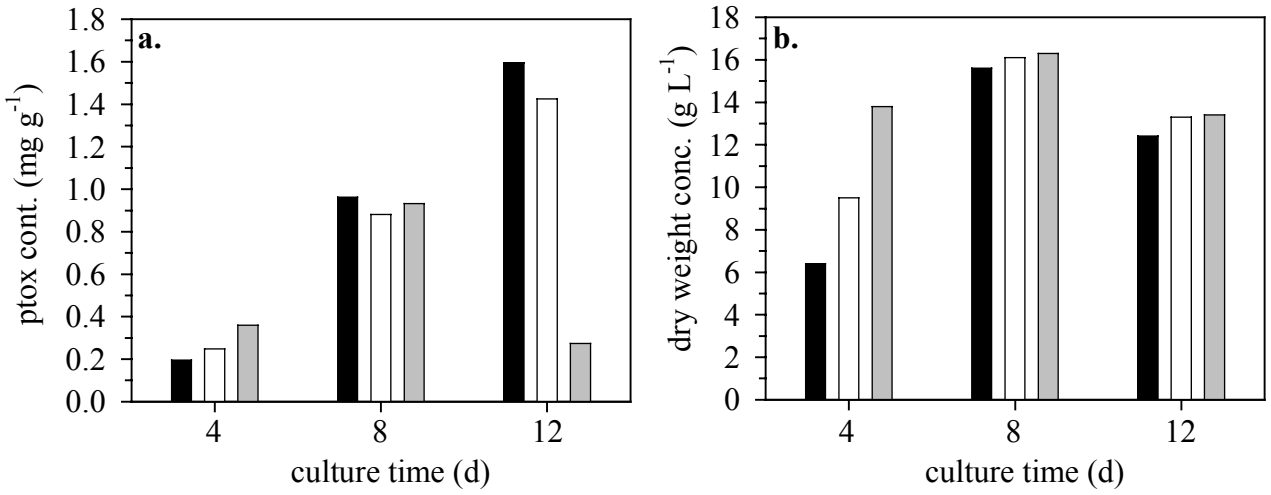

Figure D-26

Comparison of the effect of different inoculum densities on the podophyllotoxin content per cell dry weight (a) and of the biomass concentration (b). Inoculum densities (g wet-fresh weight per 50 mL liquid volume) in 300-mL shake flasks: 3 g (black bar), 5 g (white bar, standard density), 7 g (grey bar). Mean values of two distinct experiments with two flasks per sampling day.

The effect of inoculum density on the podophyllotoxin content is shown in Figure D-26a. On the first two sampling days (day 4 & day 8 of culture) a similar podophyllotoxin content was found for the three experimental setups, whereas a distinctive reduction was evident for the high-inoculation-density culture on day 12 (0.3 mg g^{-1}, grey bar). The low- and standard-inoculation-density cultures showed comparable values of approximately 1.5 mg g^{-1}. For all three setups a dry weight concentration of nearly 13 g L^{-1} was reached on day 12, although different initial culture densities were still reflected by graduated dry weight concentrations at the 4th day of culture (see Figure D-26b). The early research on PAL activation upon the dilution of cells into fresh media dates back to the 1970s (HAHLBROCK & SCHRÖDER 1975; JONES 1984). Similar to these studies, a more pronounced induction of PAL and CAD was also observed at lower inoculation densities for the *Linum album* culture (Figure D-27). Though the 4CL activity did not show any difference between the experimental setups, distinctive higher CAD and PAL activities had been reached for the 3-g-culture in comparison to the 7-g-culture throughout the first days of characterisation.

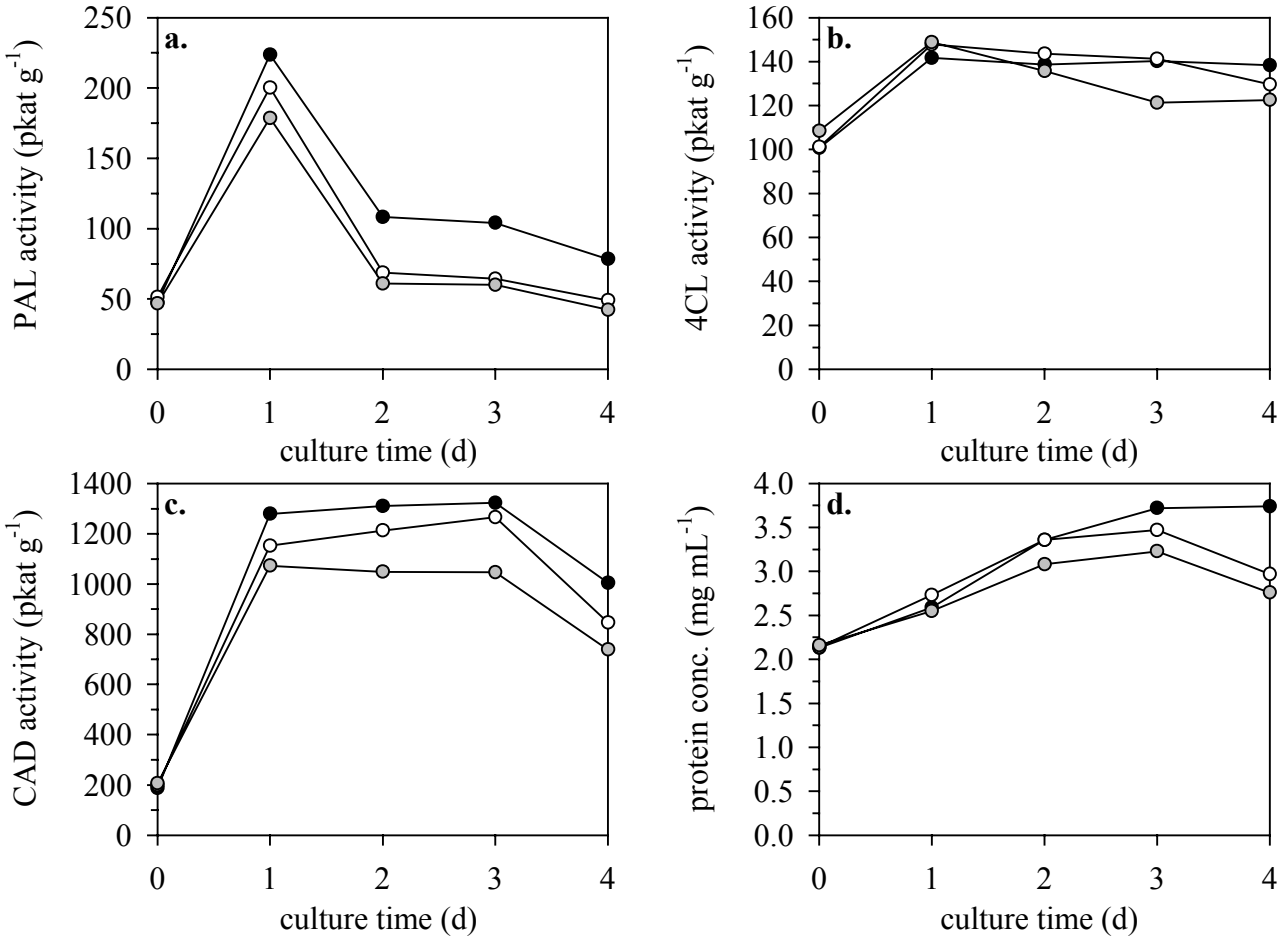

Figure D-27

The time course of changes in phenylalanine ammonia-lyase (PAL, a), 4-coumarate-CoA ligase (4CL, b) and cinnamyl alcohol dehydrogenase (CAD, c) activity and protein concentration (d) of the crude cell extract. Relative enzyme activities were calculated on the basis of cell fresh weight (pkat g^{-1}). All data points were obtained from the combined biomass of two flasks after vacuum filtration. Inoculum densities (g wet-fresh weight per 50 mL liquid volume) in 300-mL shake flasks: 3 g (black symbol), 5 g (white symbol, standard density), 7 g (grey symbol). Mean values of two distinct experimental sequences with two flasks per sampling day.

D.4.2 Effects of the inoculum density on bioreactor culture

In view of the fact that oxygen limitations inside the shake flask cultures could have obscured the effects of different initial cell densities, the bioreactor system with an active oxygen adjustment on 60% of saturation was to be preferred (VEGLIO, et al. 1998). Although inherent dissimilarities between both systems prevent a direct comparison (NAMDEV & DUNLOP 1995), the results obtained from shake flask cultures proved to serve as an indicator for further studies. In order to find out the rate limiting factors of podophyllotoxin biosynthesis – plateau-values obtained in BR_K & BR_L were still lower than in shake flasks – a reduced inoculum density of the bioreactor culture (40 g fw L^{-1}), at elevated oxygen saturations of 60%, was applied.

Figure D-28 shows a typical pattern of podophyllotoxin accumulation during the culture time. After a lag-phase of 3 days, a steep increase of the podophyllotoxin content can be seen for a duration of only 2 days, which is being followed by a plateau of approximately 1.6 mg g^{-1} dry weight. Though a slower increase of some kind had been observed in the case of BR_M, comparable values were finally reached for both reactors. For the first time, the podophyllotoxin content, as well as production values of bioreactor cultures, were comparable to those obtained from the shake flask system. However, the reason for the lag-phase was still not known and the prevailing plateau phase indicated the existence of additional limiting factors.

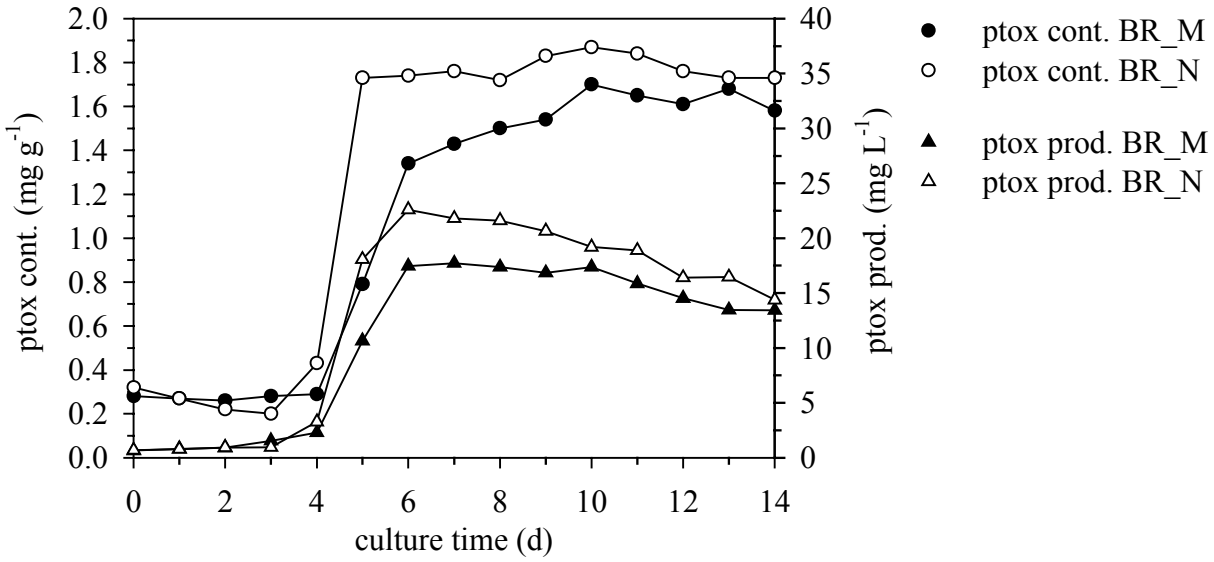

Figure D-28

The podophyllotoxin content and production (both on dry weight basis) by *Linum album* (X4) suspension cultures in duplicate bioreactors (BR_M & BR_N) during the culture period. Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), low inoculation density, diluted culture medium, silicone-antifoam.

Although the second rise of PAL activity, which has been a characteristic feature of shake flask cultures was still not observed under the conditions of a lower inoculation density, a high PAL induction (280 pkat g^{-1} fw), during the first twenty-four hours after the inoculation, is shown in Figure D-29. It is of major importance that the increased PAL activity was evident for a prolonged period of time until the 4th day of culture contrasting with BR_K & BR_L, which were inoculated at a high cell density of 60 g fw L^{-1} (Figure D-23). Activities of CAD and to some extent values of 4CL were also found to be increased for several days. No difference was observed as to the protein concentration of the crude cell extract if compared to Figure D-23d. In addition, the improved enzymatic activities of PAL and CAD at lower initial cell densities seem to correlate with an enhanced podophyllotoxin accumulation capacity.

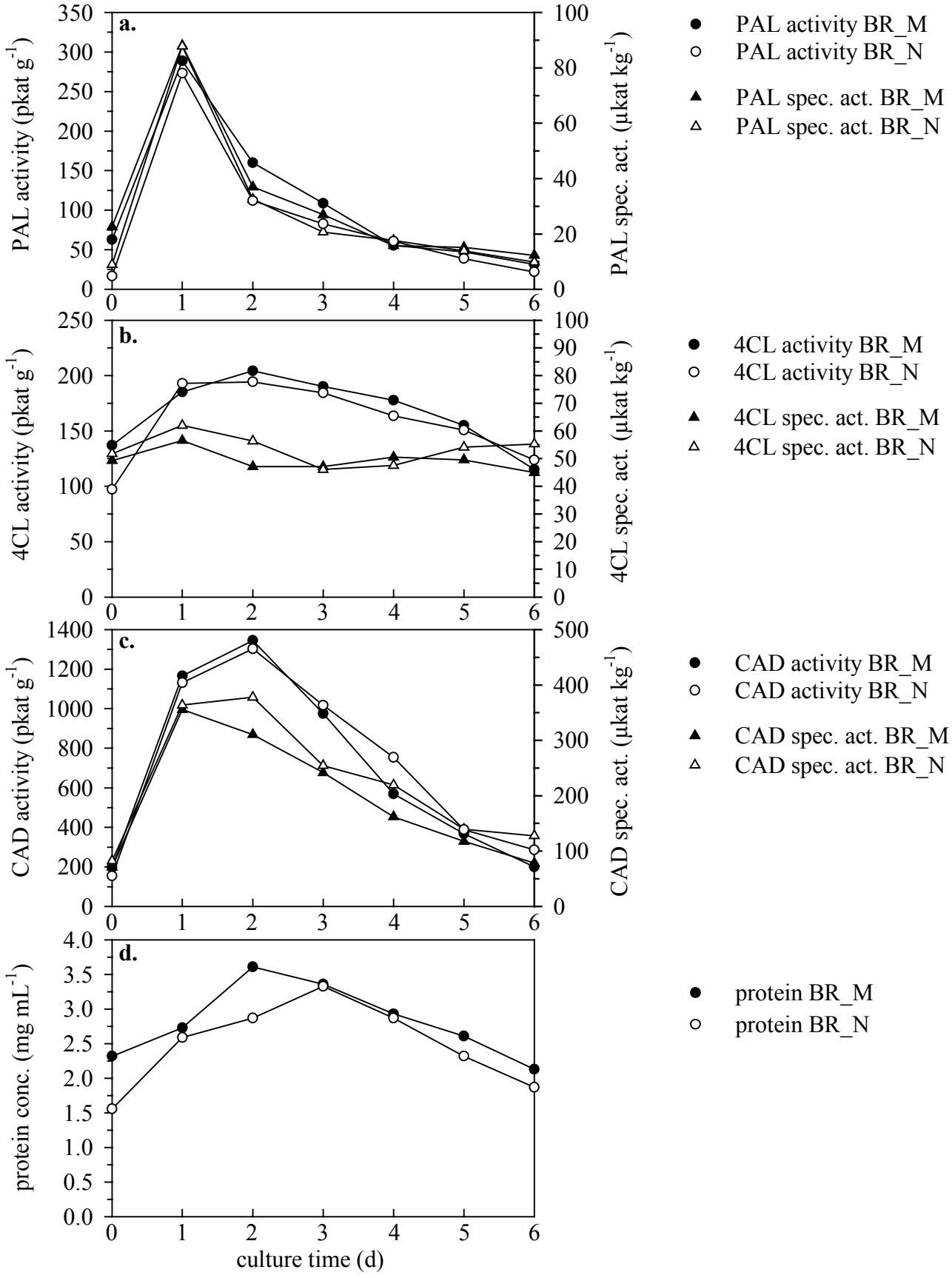

Figure D-29

Enzyme activities on basis of cell fresh weight (pkat g^{-1}) or protein concentration (µkat kg^{-1}) of the crude cell extract (BR_M & BR_N). Samples were taken during the first 6 days of culture.

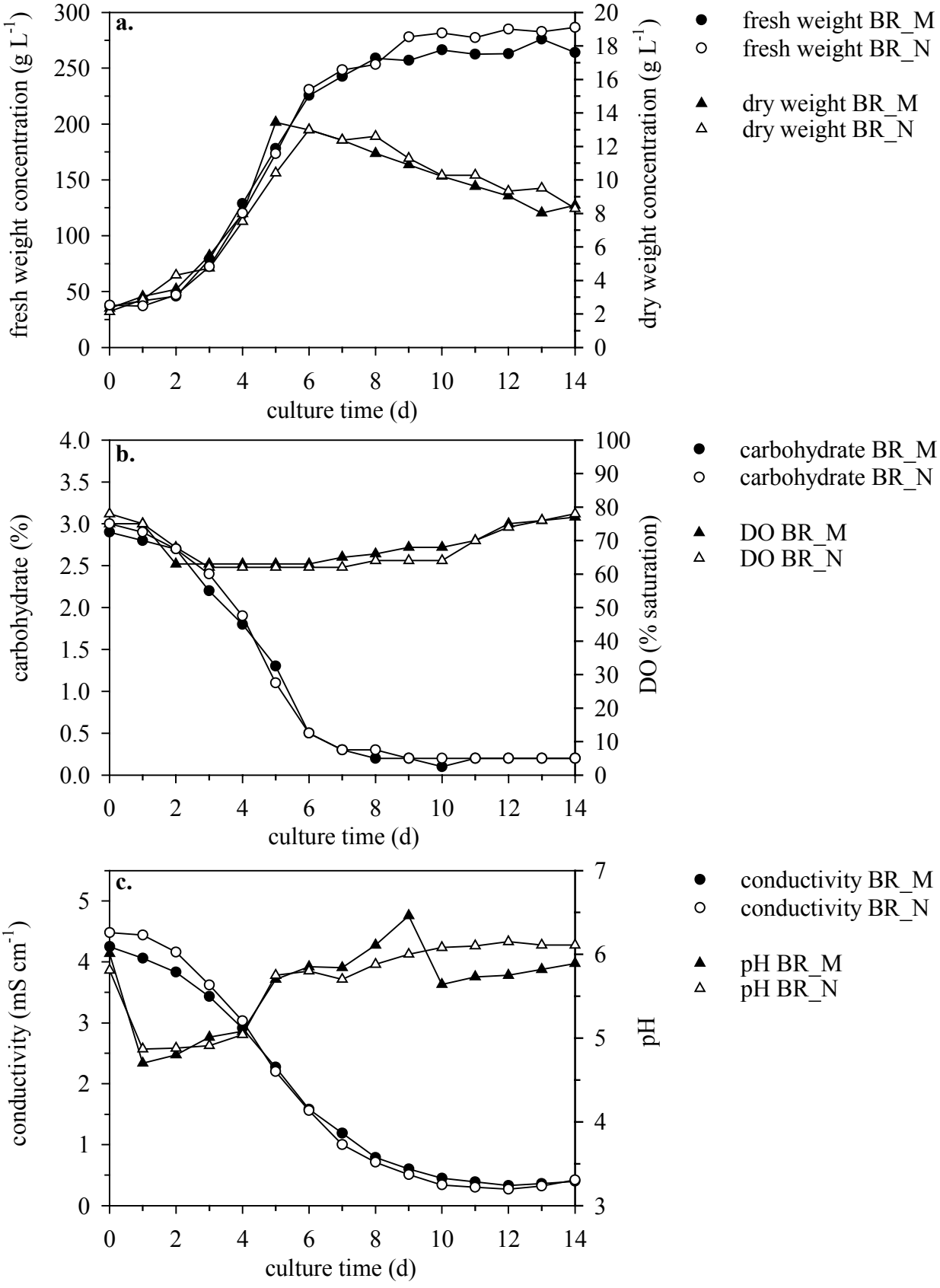

Figure D-30

Biomass concentrations, dissolved oxygen saturations and culture medium parameter as a function of time for two bioreactors (BR_M & BR_N). Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), low inoculation density, diluted culture medium, silicone-antifoam.

Figure D-30a reveals the characteristic dynamics of biomass accumulation during the culture time. A final dry weight concentration of 13.2 g L^{-1} was found on day 6, which was similar to those bioreactors of a higher inoculation density (12.7 g L^{-1}). Consequently, BR_M & BR_N exhibited a higher specific growth rate (see Table D-5). If the carbohydrate fading and the reduction of medium conductivity is also taken into consideration (Figure D-30b & -30c), the maximum cell mass attained coincided with the time, when almost all the major nutrients were exhausted. It is interesting to note, that the relatively higher per cell nutrient supply enabled the *Linum album* suspension culture for a faster cell growth to achieve a normal maximum biomass density. This is in contrary to various investigations by other authors, who found a lower final biomass at reduced inoculation densities (CONTIN, et al. 1998; LEE & SHULER 2000; VILLARREAL, et al. 1997).

It can be summarised that a lower initial cell density under non-oxygen limited conditions of a bioreactor culture brings about (1) an improved enzymatic activity of PAL and CAD, expressed as a higher maximum induction value, concomitant with a prolonged activity, (2) a greater podophyllotoxin accumulation capacity and (3) retained final biomass concentrations.

D.5 Optimisation of the culture medium

Bioprocesses can be characterized by complex interactions among physical, chemical and biochemical aspects, which influence the cell physiology in form of intra- or extracellular effectors. Advances reported in the past years about the role of some medium constituents have provided evidence that nutrient deficiencies or inappropriate medium composition rather than metabolic constrains represent limiting factors for secondary metabolite production. Unfortunately, medium design is a complex subject, since the primary and secondary metabolism may be inversely effected. KNOBLOCH & BERLIN (1980) reported a suppression of ajmalicine accumulation concomitant with an improved growth in the presence of phosphate. Similar results have been provided in relation to the taxol and anthocyanin production by plant cell cultures (KAKEGAWA, et al. 1995; MEI, et al. 1996). Thus, in some cases two-stage processes with different nutrient regimes in the growth and production medium were proposed to be in favour for the special requirements of an active primary and secondary metabolism (HIRASUNA, et al. 1991; SCHLATMANN, et al. 1995).

Preliminary experiments concerning an improved podophyllotoxin production by the induction of nutrient stress (increased copper, boron, nitrogen or phosphate concentration) failed, although such factors have been proven to be positively correlated with the accumulation of phenolics in plants (DICOSMO & MISAWA 1995; GERSHENZON 1984). According to the sensitivity of *Linum album* suspensions to any stress-parameter, an optimisation of the culture medium was performed in two steps: first, the final concentration of the standard medium inside the bioreactor was adjusted to the conditions, which were found in the shake flasks. Second, the response of PAL, 4CL and CAD activities in correlation to the culture medium composition was investigated. The results were related to the capacity of podophyllotoxin accumulation and had been used for further medium improvement.

D.5.1 Adjustment of the culture medium concentration

As a consequence of the inoculation procedure, the final culture medium inside the bioreactor exhibited a lower concentration (diluted culture medium) in comparison to the standard medium used in the shake flasks. This was due to fact that not only the cells, but the whole content of the 7-day-old culture flasks had been used for the inoculation of the bioreactor. It had to be tested whether the smaller availability of nutrients could have been the reason for the plateau-shaped accumulation of podophyllotoxin. In this experiment a 14% higher concentrated culture medium was prepared for BR_O & BR_P, which gave a final nutrient content inside the bioreactor comparable to the standard MS-medium.

Under such conditions an improved podophyllotoxin accumulation was observed (Figure D-31). A value of approximately 2.5 mg g^{-1} dry weight surpassed even the highest amounts found in BR_M & BR_N (1.6 mg g^{-1} dw). This effect was attributed to a better nutrient supply of the cells, without a differentiation of the medium composition. Nevertheless, the incidence of the two-phased podophyllotoxin accumulation denoted a possible implication of additional limiting factors. With the exception of the PAL, all enzyme activities were comparable to those of the bioreactors with a lower concentrated medium. Although three hours after inoculation no difference could be observed for the peak values of PAL induction, a higher activity was apparent between the 3rd and 6th day of culture. Considering the dynamics of PAL activity during the first days of the culture period (Figure D-14), the enhanced values should be viewed with caution. Besides the increased podophyllotoxin content, both bioreactors showed the highest productivity so far. A peak value of 30 mg L^{-1} was reached at the 6th or 7th day of culture and declined thereafter until the characterization was stopped at day 16. This decline could have been attributed to a reduction of the dry weight concentration, which is shown in Figure D-33.

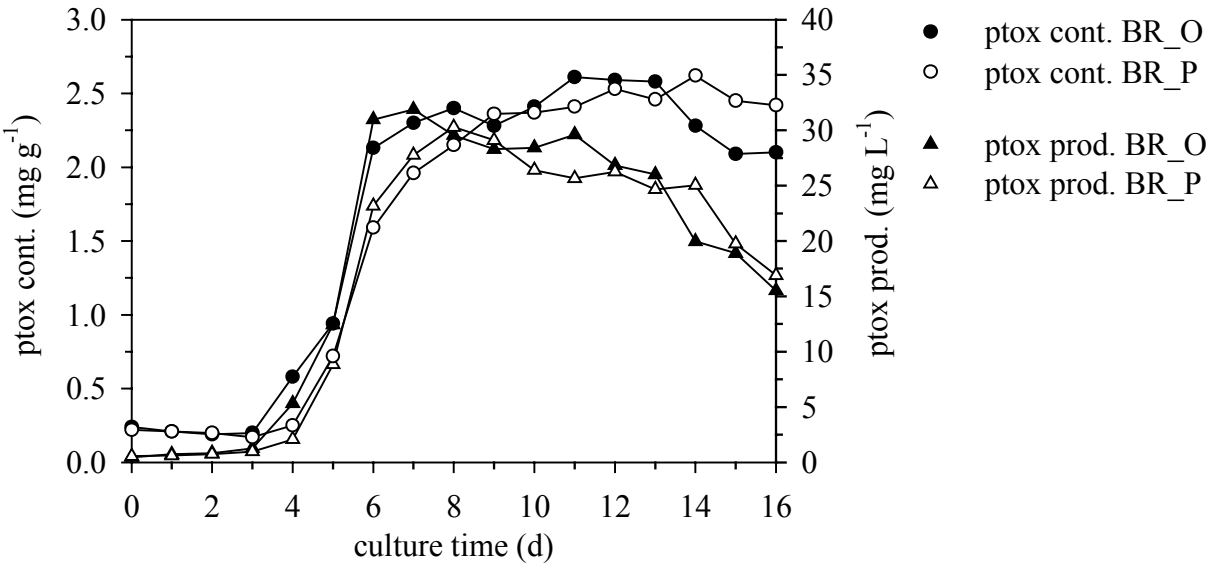

Figure D-31

The podophyllotoxin content and production (both on dry weight basis) by *Linum album* (X4) suspension cultures in duplicate bioreactors (BR_O & BR_P) during the culture period. Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), low inoculation density, MS culture medium, silicone-antifoam. Samples were taken during a culture period of 16 days.

According to Figure D-33a the highest dry weight concentration of 14.5 g L^{-1} (day 6) coincided with the medium depletion by its main carbon source between the 6th and 7th day of culture. Apparently, the higher initial carbohydrate content and increased inorganic-nutrient concentration of the culture medium were consumed at a faster rate in comparison to former bioreactors (diluted culture medium). Starting with a sucrose content of 3.4% (2.9%, BR_M & BR_N) and a conductivity of 4.9 mS cm^{-1} (4.3 mS cm^{-1}, BR_M & BR_N), which was equivalent to an approximate 14% increase of the nutrient concentration, the culture medium became exhausted after the same time. Since the inoculation density was the same (40 g fw L^{-1}), a higher nutrient supply on a per cell basis has to be considered for discussion of the enhanced podophyllotoxin accumulation. A literature survey concerning the influence of the initial nutrient supply on biomass production revealed similarities with respect to the *Linum album* suspension culture. Most of the authors focused on the variation of the initial sucrose, phosphate and nitrogen content of the culture medium, since these parameters proved to be of major importance for a sustained growth and cell proliferation. Generally, the results indicated an optimal concentration of phosphate (KANDARAKOV, et al. 2000; KATO, et al. 1977; ZHANG & ZHONG 1997), which led to a faster growth, increased final culture density and accelerated sucrose and inorganic-nitrogen consumption (WEN & ZHONG 1997). Comparable effects have been reported for the initial sucrose content of the medium (AKALEZI, et al. 1999), which also exhibited an influence on the formation of various secondary metabolites in plant cell culture (AKALEZI, et al. 1999; LUX-ENDRICH, et al. 2000; SHIBASAKI, et al. 1995). As a consequence of the dependency on C-skeletons, which are provided by the shikimate pathway (Figure A-5), an interrelation with the carbohydrate supply can be assumed for the phenylpropanoid metabolism (RÜHMANN, et al. 2001). Analysis of the specific growth rate and biomass yield in Chapter D.7 will provide detailed information about the influence of a higher concentrated medium on culture growth and carbon utilization. If compared to BR_M & BR_N (Figure D-30c), no effect of the increased per-cell-availability of inorganic-nitrogen (ammonium and nitrate) on the medium pH can be seen in Figure D-33c. As in the case of the low concentrated medium, a decreased pH was observed during the first 4 days of culture, paralleled by a concurrent decline of the ammonium concentration (Figure D-34a).

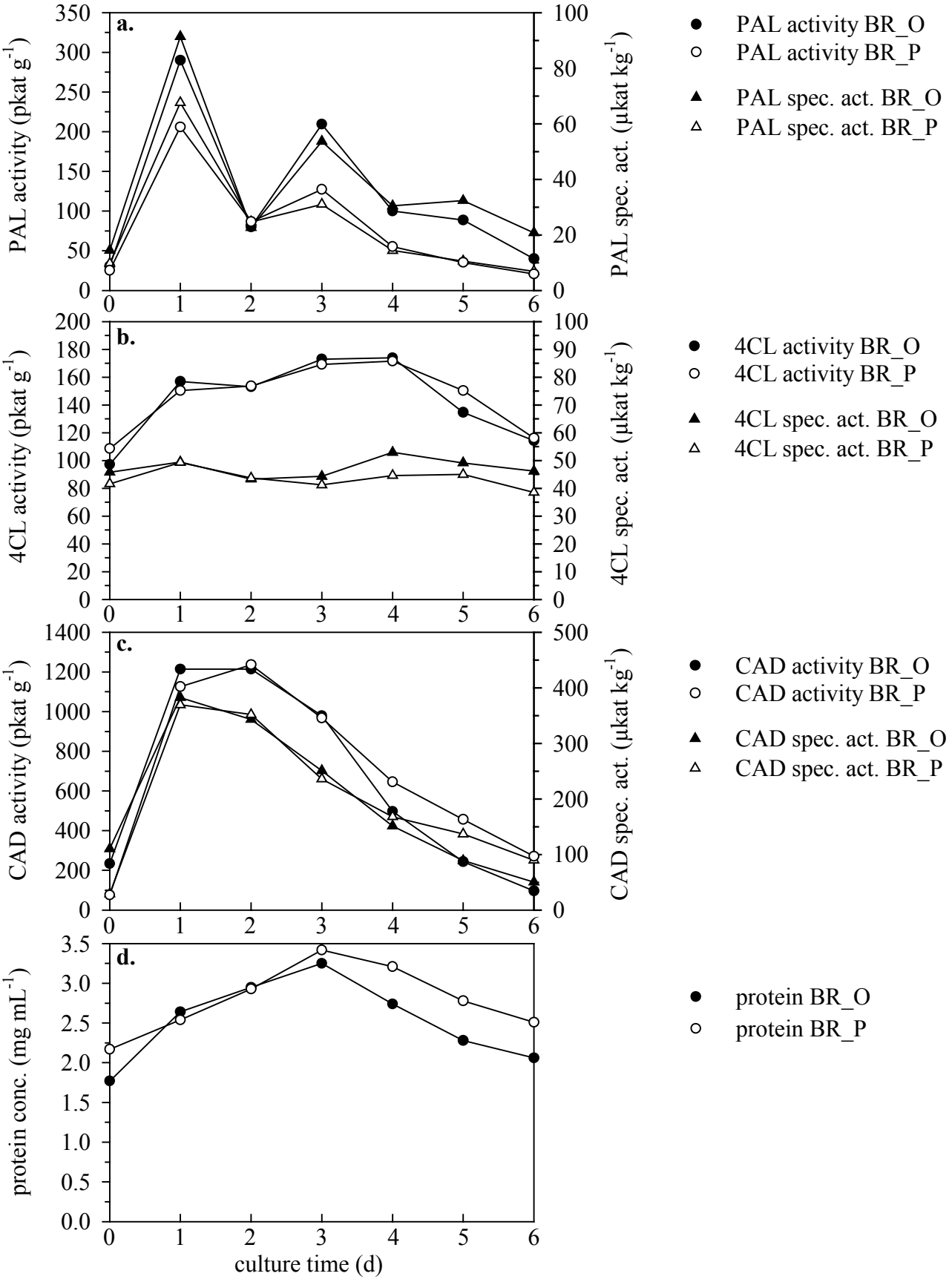

Figure D-32

Enzyme activities on basis of cell fresh weight (pkat g^{-1}) or protein concentration (µkat kg^{-1}) of the crude cell extract (BR_O & BR_P). Experimental setup described in Figure D-31. Samples were taken during the first 6 days of the culture period.

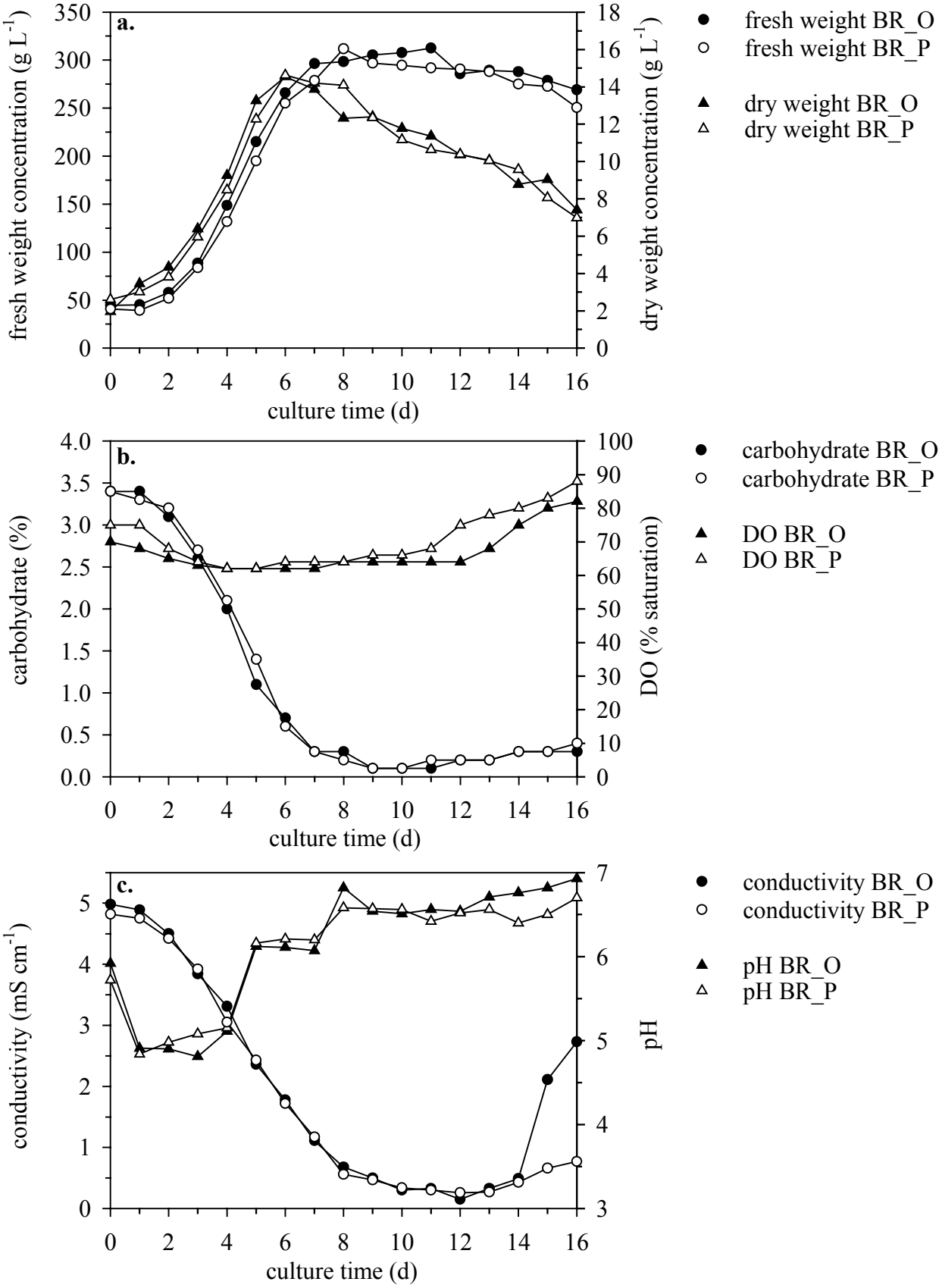

Figure D-33

Biomass concentrations, dissolved oxygen saturations and culture medium parameter as a function of time for two bioreactors (BR_O & BR_P). Experimental setup described in Figure D-31. Samples were taken during a culture period of 16 days.

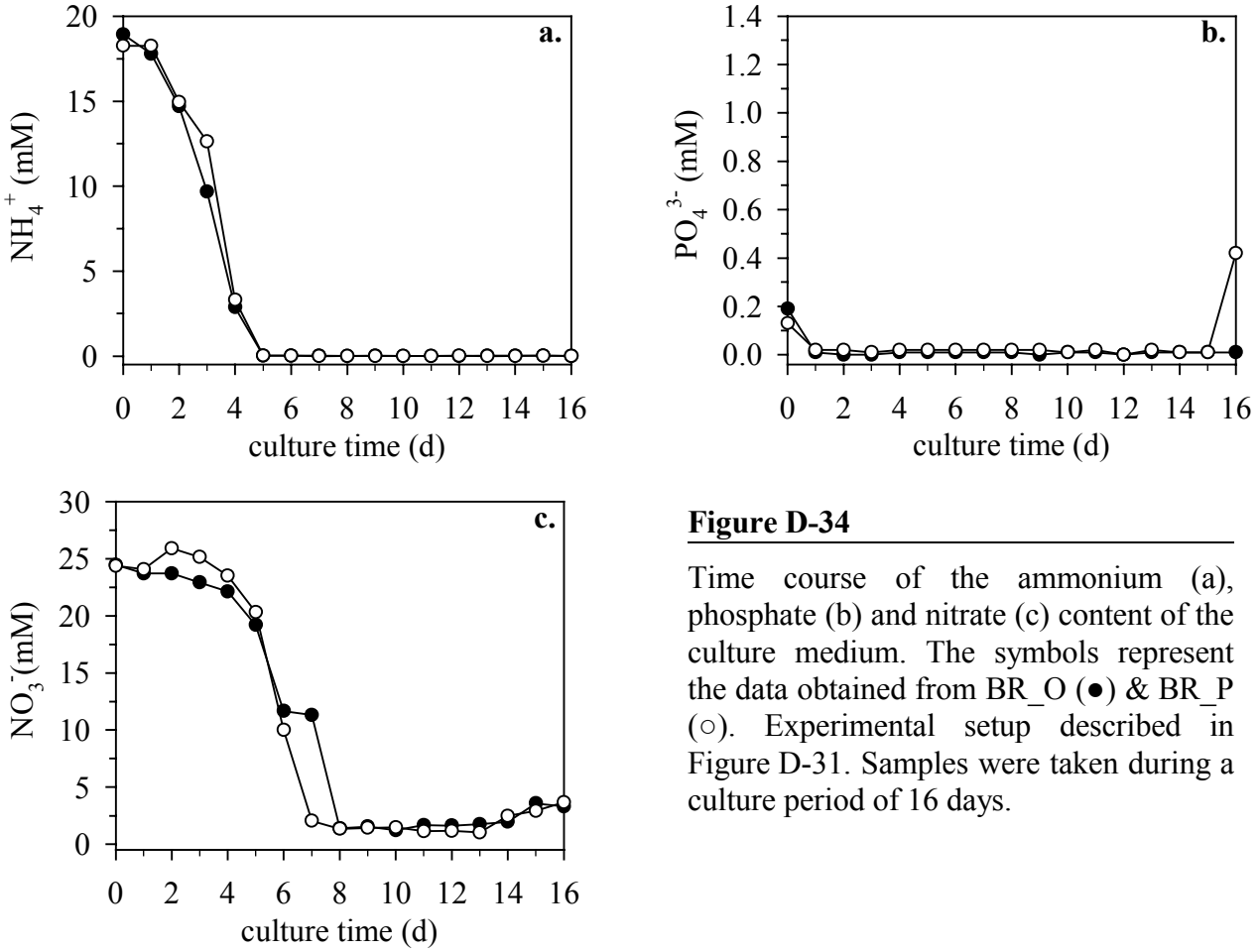

Figure D-34

Time course of the ammonium (a), phosphate (b) and nitrate (c) content of the culture medium. The symbols represent the data obtained from BR_O (●) & BR_P (○). Experimental setup described in Figure D-31. Samples were taken during a culture period of 16 days.

Regarding the experiments, carried out on a small scale with shake flasks, similar initial ammonium and nitrate concentrations were found in BR_O & BR_P. Also, comparable dynamics of the nutrient consumption can be seen in Figure D-34. The medium became depleted by ammonium at the 5th day of culture, whereas in the case of nitrate an accelerated uptake was recognized not before the 4th day – when the ammonium content of the culture medium reached its lower limit. As expected, the conductivity of the medium followed the nitrate concentration after an initial early decrease, which could be attributed to the ammonium uptake. In addition to the effects on the culture medium conductivity, the nitrate uptake between day 4 and day 8 of culture is also reflected by an increase of the medium pH (Figure D-33c). The incidence of a pH decrease during the initial culture phase, followed by a trend toward alkalisation had already been described elsewhere (BLIGNY, et al. 1997; OGAWA, et al. 1996). It was attributed to a primary ammonium uptake in exchange of H^+ (medium acidification) and simultaneous nitrate- and H^+-pumping (medium alkalisation) after an initial lag-phase (CRAWFORD 1995; ZHANG, et al. 1998). The influence of the nitrogen source on the medium pH was taken into consideration for further medium improvements, since a high concentration of either ion may not only affect the secondary metabolism but can also exert toxic effects on the cell physiology (BUTZ & JACKSON 1977; LANG & KAISER 1994). Nevertheless, a reduced nitrate concentration of the culture medium on the day of inoculation gave rise to an influence of the sterilization process on the final medium composition. Since phosphate has been known to be taken up by the cells immediately after transfer and the ammonium concentration showed typical values of approximately 20 mM, it only was the nitrate concentration, which was lower than would have been expected from the medium preparation.

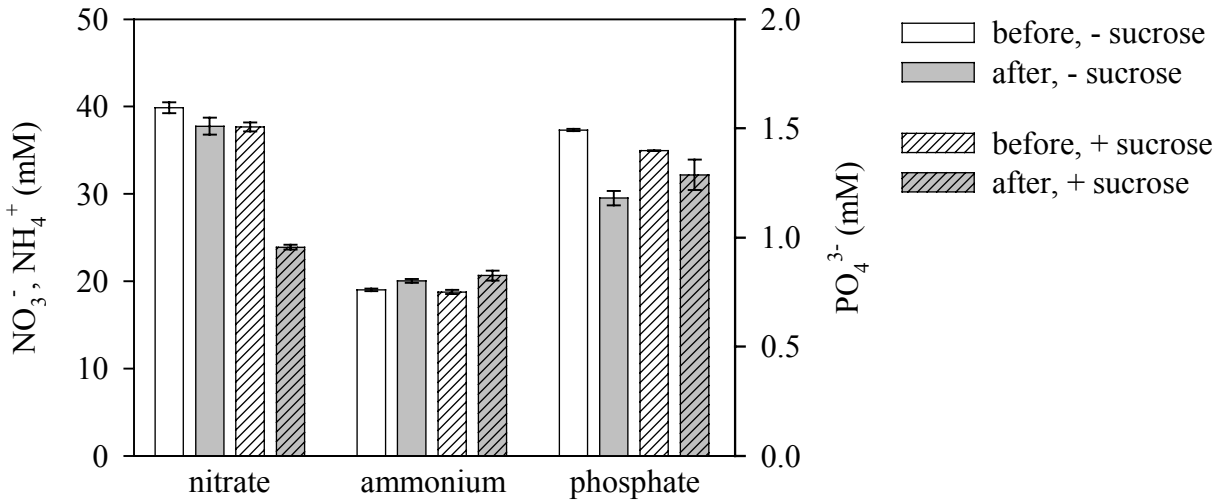

Figure D-35

Influence of sucrose on the nitrate, ammonium and phosphate concentration in autoclaved culture media. Standard MS-medium was prepared with (+) or without (-) the addition of sucrose in 300-mL standard shake flasks. Samples for analysis were taken before (open bar) and after (grey bar) sterilization at 118 °C for 20 minutes. Error bars were calculated for three sample flasks.

An alteration of the culture medium constituents by high-temperature-autoclaving or medium storage has already been shown for sucrose (BALL 1953), iron and phosphate (DALTON, et al. 1983) and auxins (DUNLAP, et al. 1986). Therefore, it had to be tested whether there was a significant loss of nitrate during the sterilization process and if this could be attributed to the occurrence of sucrose. As shown in Figure D-35, standard MS-medium was prepared with or without the addition of sucrose and samples were analysed for the nitrate, ammonium and phosphate concentration. Interestingly, only in the presence of sucrose a reduction of the nitrate concentration was observed. According to Table C-1, the MS-medium contains 1600 mg L^{-1} ammonium nitrate (20.61 mM) and 1900 mg L^{-1} potassium nitrate (18.79 mM). Whereas the final nitrate concentration in the presence of sucrose was reduced by 40% after autoclaving to a value of 24 mM. No such effect was observed in the case of the ammonium or phosphate. The results of this experiment imply an effect of sucrose or its hydrolysation products (glucose and fructose) on the nitrate concentration, which was also apparent in previous studies (Figure D-6, Figure D-11).

D.5.2 Effects of fed-batch culture and high oxygen saturation

The above results clearly indicate a dependency of the podophyllotoxin production on an elevated oxygen saturation concomitant with the need for a sufficient nutrient supply. In the case of non-oxygen-limited growth, apparently, the steep increase of the podophyllotoxin content was stopped after the medium became exhausted by its main carbon source and the dry weight production ceased. In relation to the podophyllotoxin content, the ensuing period could be characterized as a plateau-phase with only a small increase of podophyllotoxin. Hence, it was tested whether the supplementation of sucrose during the phase of active growth could enable a sustained biomass increase and an enduring podophyllotoxin accumulation.

Provided a partly-growth associated biosynthesis of podophyllotoxin, then a fed-batch operation, where the growth-limiting nutrient is carefully added can be used to control the growth rate and productivity of the culture. The results obtained from the supplementation by a concentrated sucrose solution at the 4th and 5th day are presented in Figure D-36 & Figure D-37. Though the overall shape of the podophyllotoxin accumulation curves resemble the data of earlier bioreactors, the maximum podophyllotoxin content was repressed to a value of 1.1 mg g^{-1}. If compared to BR_K & BR_L, which showed a similar content of the cells, the high production of podophyllotoxin in the present study was attributed to an elevated biomass yield. Apparently, the addition of sucrose at day 4 and 5 led to an enhancement of dry and fresh weight formation (19.0 g L^{-1}, day 7 and 370 g L^{-1}, day 12, respectively), instead of an improved podophyllotoxin accumulation. It should be noted that also in this case a drastic increase of the podophyllotoxin content was recognized between the 3rd and 6th day of culture. As typical, it was preceded by a lag-phase after inoculation and ensued by a minor increase during the second half of culture, which was designated the plateau-phase in previous chapters. At day 6, neither the carbohydrate concentration nor the medium conductivity indicated a limited nutrient supply.

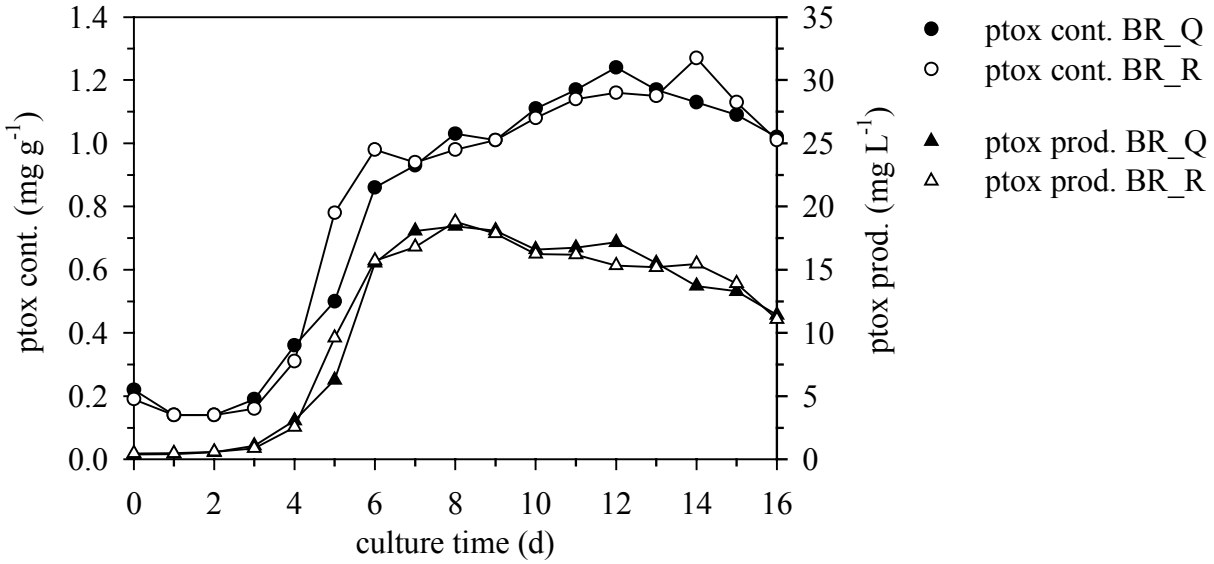

Figure D-36

The podophyllotoxin content and production (both on dry weight basis) by *Linum album* (X4) suspension cultures in duplicate bioreactors (BR_Q & BR_R) during the culture period. Experimental setup: 0.44 vvm, fed-batch culture, active DO-regulation (minimum DO 60%), low inoculation density, MS culture medium, silicone-antifoam. Approximately 200 mL of an autoclaved sucrose-solution (450 g L^{-1}) was added at day 4 and 5 after a sample of an equivalent volume was taken.

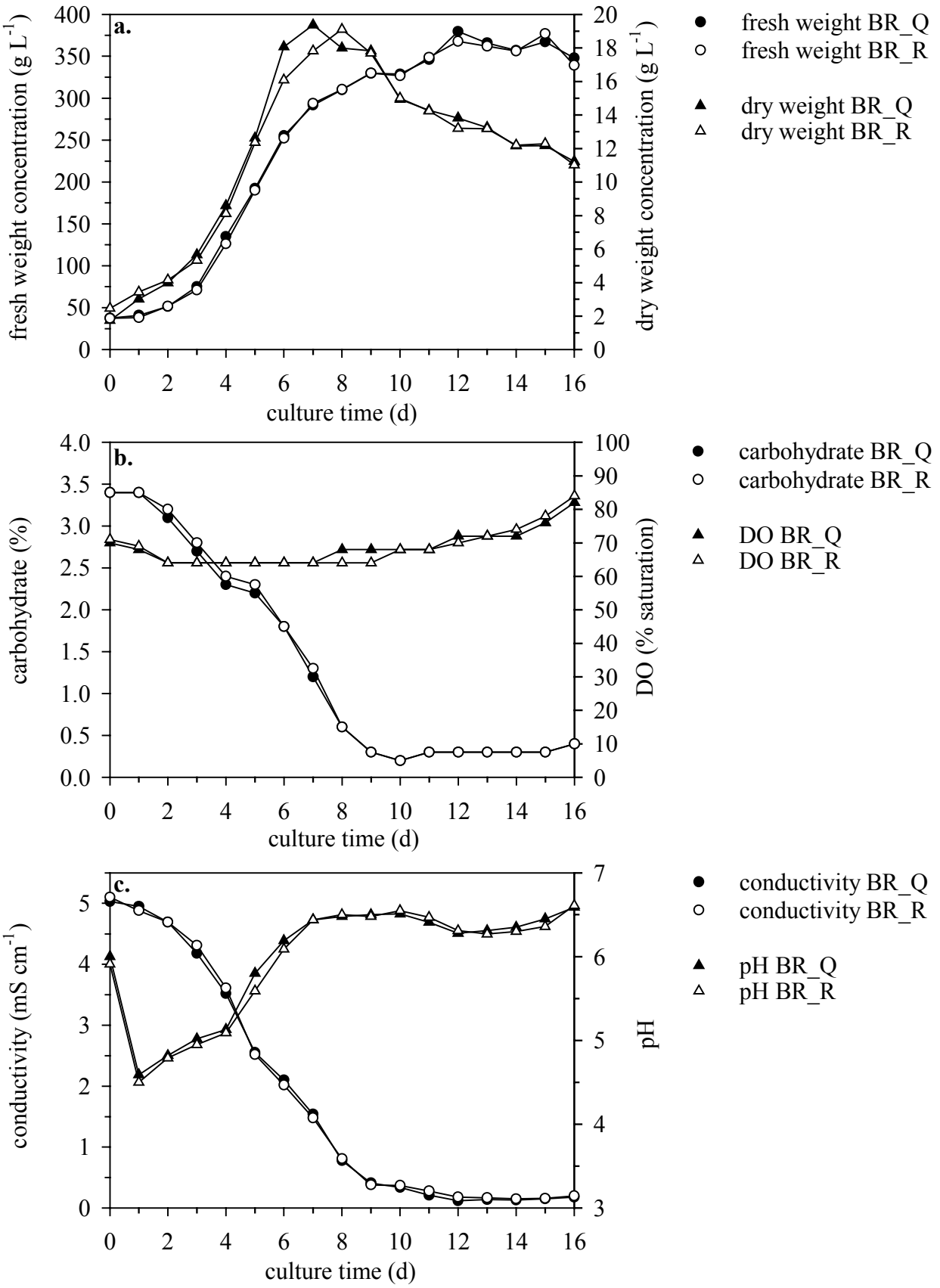

Figure D-37

Biomass concentrations, dissolved oxygen saturations and culture medium parameter as a function of time for two bioreactors (BR_Q & BR_R). Experimental setup described in Figure D-36. Samples were taken during a culture period of 16 days.

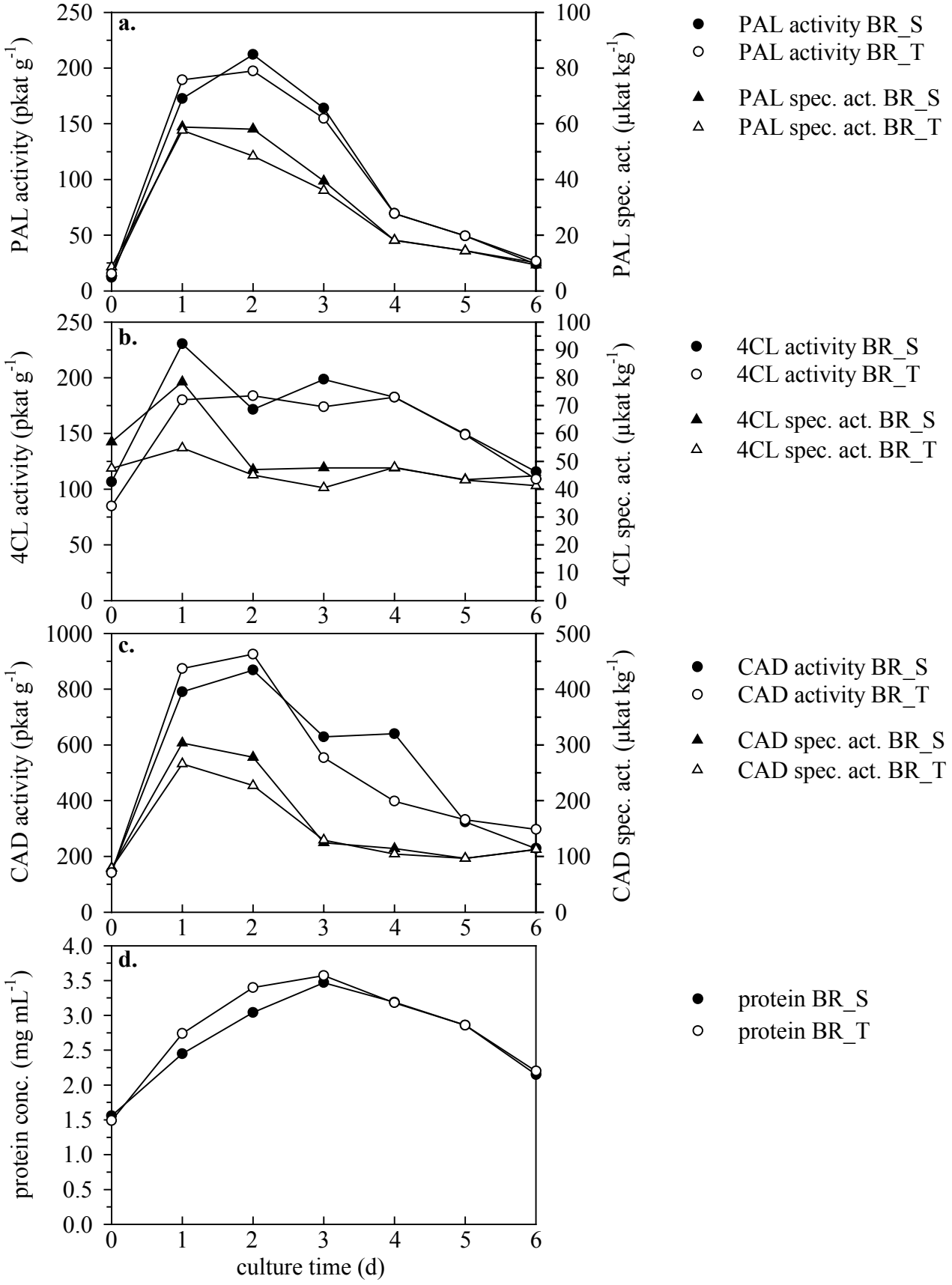

Figure D-38

Enzyme activities on basis of cell fresh weight (pkat g^{-1}) or protein concentration (µkat kg^{-1}) of the crude cell extract (BR_S & BR_T). Experimental setup: 0.44 vvm, batch culture, active DO-regulation (DO 100%), low inoculation density, MS culture medium, silicone-antifoam.

The results of BR_Q & BR_R show that the accumulation of podophyllotoxin must not be inevitably connected to the culture growth, since a slow but evident increase can be observed after the 7th day of culture. It appears as if the culture dynamics in terms of growth and product formation are not an inherent and invariable characteristic of the *Linum album* suspension, but proved to be influenced by the culture system (shake flask or bioreactor) and mode of operation (batch or fed-batch culture). This notion is supported by PAYNE et al. (1987) who reviewed the information concerning various kinetics of secondary metabolite formation by plant cells.

The second part of this chapter deals with the results of BR_S & BR_T, which were performed at high oxygen saturation. An elevated oxygen saturation is not only essential secondary metabolite production, it may also enhance the formation of the reactive superoxide radicals, peroxides and hydroxyl radicals in complex culture media (GRZELAK, et al. 2000; SULLIVAN, et al. 1991). Though organisms have evolved efficient defence systems, such as the tripeptide glutathione, the catalase and the superoxide dismutase (BERGLUND & OHLSSON 1995; SCANDALIOS 1993), reactive oxygen species can exert various cytotoxic effects on the cells (EMERY, et al. 1995). A literature survey concerning hyper-oxygenated systems revealed that most of the experiments were conducted under extreme culture condition, such as the use of air pressure (PINHEIRO, et al. 2000), a high aeration rate (HEGARTY, et al. 1986) or a dissolved oxygen level of 478% of air saturation (EMERY, et al. 1995). Nevertheless, HUANG et al. (2000) reported a reduced growth and product formation at an oxygen level of 40%. Whereas the growth of *Catharanthus roseus* cell suspensions in shake flasks was suppressed at an even higher saturation of 70% - no such effects were observed in the case of a *Daucus carota* culture (TATE & PAYNE 1991). On the other hand, cell aggregates of submerged aerobic cultures can benefit from oxygen-enriched conditions, due to an improved oxygen diffusion into the core of the callus clumps (VAN DER PLAS & WAGNER 1986).

The results of this study (100% oxygen saturation, BR_S & BR_T) did not reveal any repression of the biomass formation. Typical growth curves (not presented) were obtained, which show a peak value of 14.6 g dw L^{-1} at the 7th day of the culture period. However, the accumulation of podophyllotoxin was affected (plateau-value of 1.0 mg g^{-1} dw), contrasting with BR_O & BR_P (60% oxygen saturation), where a maximum podophyllotoxin content of 2.5 mg g^{-1} dw was found. In addition, Figure D-38 shows how the enzyme activities responded to a 100% oxygen saturation. During the first days, the PAL revealed a different pattern of activation than described for any culture before. For both bioreactors the PAL did not exhibit the typical biphasic activation, but rather showed a relatively high activity during the first 3 days, which could have been attributed to an oxygenation-stress. Such observation are supported by the fact that an induction of PAL and phenylpropanoid metabolism has been described as a response to environmental factors (DIXON & PAVIA 1995; JONES 1984). On the other hand, a reduced CAD activity was found during the first days of culture (Figure D-38c), corresponding to the decreased podophyllotoxin content of the cells. Given that the CAD reflects the activity of the monolignol pathway, its down-regulation could be a hint for a stress-induced carbon channelling to other phenylpropanoid-related pathways.

D.5.3 Influence of macronutrients on enzyme activity and podophyllotoxin accumulation

Preliminary screenings on the influence of the main medium constituents revealed no uniform pattern in terms of podophyllotoxin production. It became obvious that the podophyllotoxin accumulation was affected by most of the medium variations. Thus, the objective of this study was to examine whether changes of the medium composition are reflected in the PAL, 4CL and CAD activity. Furthermore, the results had been used for the development of an improved culture medium and correlated to changes in the podophyllotoxin content.

Based on a standard MS-medium (Table C-1), the concentrations of the main macronutrients were modified as described in Table D-2. In the case of med._b and med._e the added nitrate or ammonium concentration corresponded to the molarity of NH_4NO_3 as in the standard MS-medium (20.6 mM). Since the potassium-ion is relatively highly concentrated in the basic medium and it is also added by KOH for pH-adjustment, the standard potassium content was not restored. To ensure an equivalent osmotic potential of med._d, the final mannitol concentration was calculated on the basis of a 3% sucrose solution (87.6 mM). A disparity of the osmotic potential, which arose from a possible sucrose hydrolysis during sterilization, was neglected (BALL 1953; SAWYER & HSIAO 1992).

Table D-2 : Variation of the culture medium constituents

culture medium characteristics	**altered composition**
med._a no inorganic nitrogen source	- NH_4NO_3, - KNO_3
med._b no ammonium	- NH_4NO_3, + KNO_3 (2.08 g L^{-1})
med._c no phosphate	- KH_2PO_4
med._d no sucrose	- sucrose, + mannitol (16 g L^{-1})
med._e no nitrate	- NH_4NO_3, - KNO_3, + NH_4Cl (1.1 g L^{-1})

The different culture media were prepared and autoclaved under standard conditions (autoclaved at 118°C for 20 minutes). Components were omitted (-) or added (+) to the MS-culture medium at the concentrations given in brackets.

In Figure D-39 the PAL and CAD activities of the cells, which had been cultivated in the different media, are compared to standard conditions (MS-medium). Removing the inorganic nitrogen source (med._a) had no important impact on the PAL activity, but the CAD activity was drastically repressed during the time of characterization. The same was true for med._b, in which the ammonium-ion was omitted. Judging by the fast kinetic of PAL-activation during the first days after inoculation, the higher activity of the reference culture (day 1) might be of minor importance. If, contrary to med._b, the nitrate-ion is omitted from the medium (med._e), then only a few cultures could survive (data not presented). Growth of the cells was drastically reduced, which was attributed to a pH of approximately 3.5. A similarly low pH has been encountered in various culture systems, whenever ammonium was used as the main nitrogen source (MATSUMOTO, et al. 1971; SCHNEIDER, et al. 1996). Due to possible ammonium-toxicity, its use in culture media has its limitations, depending on the growth characteristics of the cells, the buffer-capacity and the nitrate content in the medium (LIU & ZHONG 1997). As a consequence, it has been generally assumed that for each individual culture-system the total content and ratio of NH_4^+/NO_3^- needs to be optimised (FULCHERI, et al. 1998; OGAWA 2000).

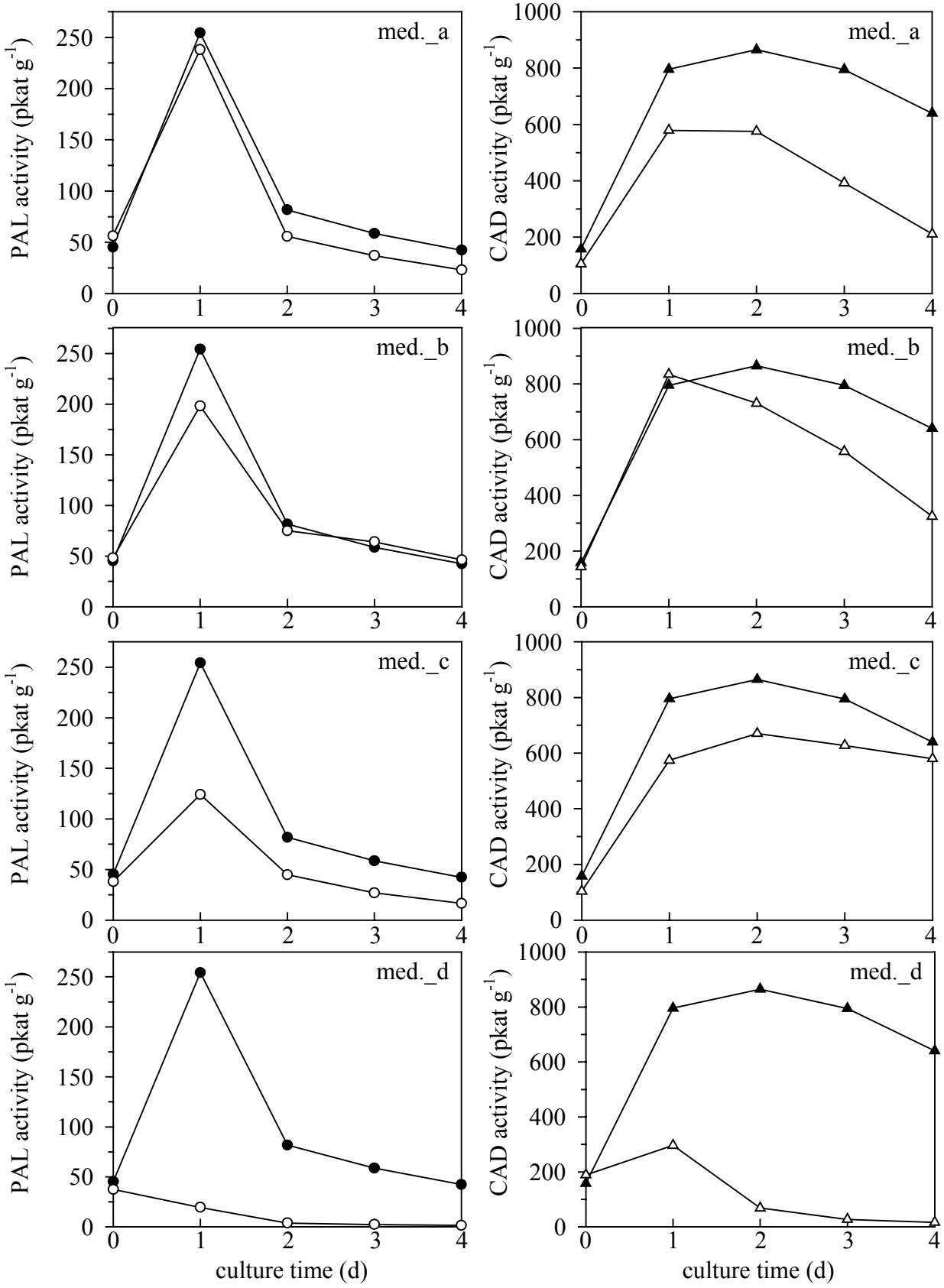

Figure D-39

Comparison of the enzyme activities on basis of cell fresh weight (pkat g^{-1}). Experimental setup: 300-mL shake flasks, 50-mL liquid volume, 120 rpm rotation speed. Standard MS-medium (closed symbol), medium variation (open symbol). Mean values of two distinct experiments are presented. At each sampling day the content of three flasks was combined and has been used for analysis.

Though deleterious effects of ammonium as the main nitrogen source concomitant with the lack of nitrate were observed for most of the sample flasks, some cultures regained growth and showed a typical pH after 6 days of culture. In addition, the growth-limited cells of med._e revealed a high and prolonged enzymatic activity, which prompted the development of a revised culture medium in terms of the ammonium and nitrate composition (Chapter D.5.4).

The importance of phosphate for the induction of the PAL and CAD activity is depicted by med._c in Figure D-39. Here, no phosphate was added to the MS-medium, resulting in a pronounced repression of both enzymes. According to the literature, phosphate represents an important trigger for cell-division (PÉPIN, et al. 1995; SANO, et al. 1999) and influences the secondary metabolism. Hence, lower product amounts (anthocyanins) were found in the presence of phosphate for a given negative correlation between cell division and PAL activity (KAKEGAWA, et al. 1995). Contrary to these results, EKNAMKUL et al. (1985) proved that a lower phosphate concentration restrained not only the culture growth but also the formation of rosmarinic acid. In this case cell growth and secondary metabolite biosynthesis were coupled to one another and proved to be affected by nutritional constrains. However, both cited references denote rather extreme examples of product formation. And, as implied in previous chapters, the podophyllotoxin accumulation is not inevitably connected to the culture growth in the case of *Linum album*. Thus, it has to be considered whether the primary metabolism (cell growth) and podophyllotoxin biosynthesis are both dependent on similar nutritional factors or on intermediates, which are built up during growth or directly provided by the culture medium. In the case of med._d, no PAL induction and only a small increase of CAD activity was obtained. These observations conflict with earlier findings by HAHLBROCK & SCHRÖDER (1975), who described a PAL induction upon dilution of *Petroselinum* cells in media, lacking certain components or even in distilled water. Although it is tempting to attribute the absence of any PAL or CAD activity to a severe lack of energy (no carbohydrate source), an influence of mannitol itself cannot be excluded (ZHAO, et al. 2001).

For discussion of the extractable enzyme activity the protein concentrations of crude cell extracts are presented in Figure D-40. The cells, which were cultivated in a standard MS-medium (r), showed a typical peak concentration of 3.5 mg mL^{-1} on the 3rd day of culture. In all the other setups minor amounts were found.

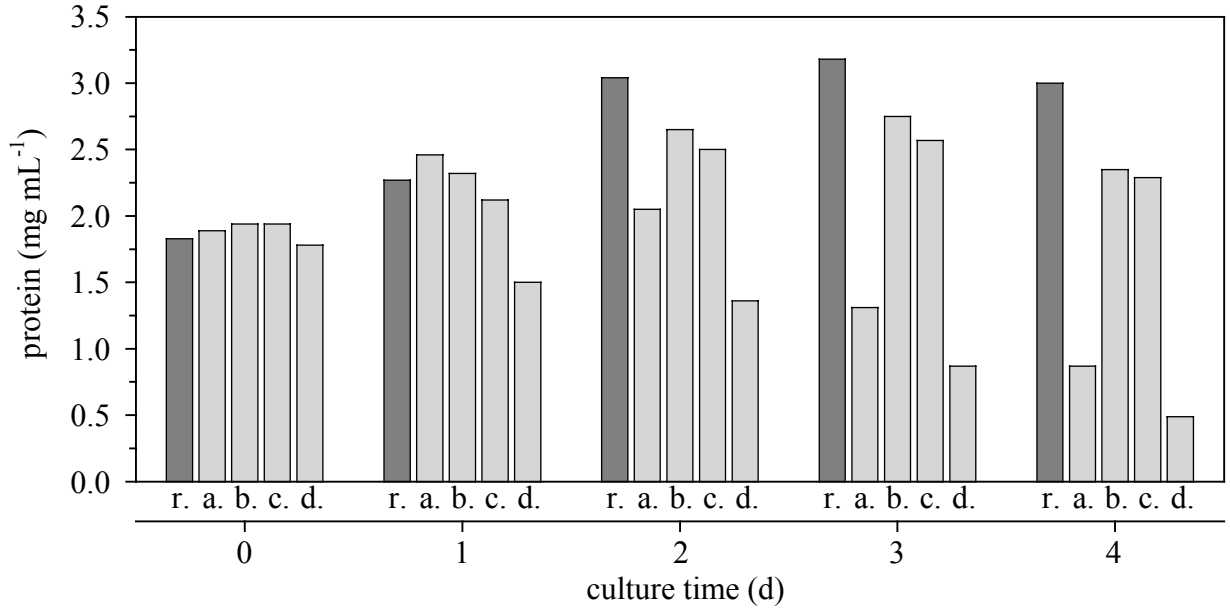

Figure D-40

Protein concentrations of the crude cell extracts used for measurement of enzyme activities. Experimental setup as in Figure D-39. MS-medium (r) and media a-d according to Table D-2.

Having started with the same protein concentrations, all systems, except med._d, showed a higher protein concentration on the first day after inoculation. During the following days the protein content of the cells, cultivated in med._a, was drastically reduced from 2.5 mg mL^{-1} (day 1) to a value of 0.9 mg mL^{-1} at day 4 of culture. This was reflected by a drop in CAD activity (Figure D-39) whereas the PAL activity was obviously not affected. According to the protein concentrations of med._b, the cells were able to utilize nitrate for protein synthesis. Though the absolute values (2.8 mg mL^{-1} on day 3) were lower than in the reference culture (r), it seems that the PAL activity does not correlate with the overall protein content of the extract. Apparently, these results are not consistent with the general view of PAL as being activated by a *de novo* synthesis (DIXON, et al. 1998; SARMA, et al. 1998). However, the effects of med._a and med._b on PAL activity have to be viewed with caution since the absolute PAL concentrations are low and may not be reflected by the gross protein metabolism (JONES 1984). Similar protein concentrations were found in the case of med._c, in which phosphate was not part of the medium. As expected, the cell dry weight concentration was reduced to 3.8 g L^{-1} when compared to the reference medium (16.4 g L^{-1}) at the 8th day of culture.

In Figure D-41 the podophyllotoxin content is presented with respect to the different culture media. Corresponding to the affected PAL or CAD activities, the podophyllotoxin accumulation by the cells was restrained in all medium variations (med._a - med._d). Despite a normal PAL activity of med._a and med._b, a low product formation was observed in these cultures. It has to be pointed out, that, regardless of the negative effects on podophyllotoxin formation, a reasonable dry weight concentration of approximately 12.5 g L^{-1} was reached for med._a and med._b at the 8th day of culture.

According to this study, the content and composition of the inorganic nitrogen form is of great importance for secondary metabolite production by the *Linum album* cell culture. Furthermore, both ions, the nitrate as well as the ammonium, proved to be essential for the establishment of CAD activity. Since high enzyme activities concomitant with an elevated podophyllotoxin content were found in some cultures of med._e (data not presented), the nitrogen composition of the standard MS-medium was assumed not to be optimal.

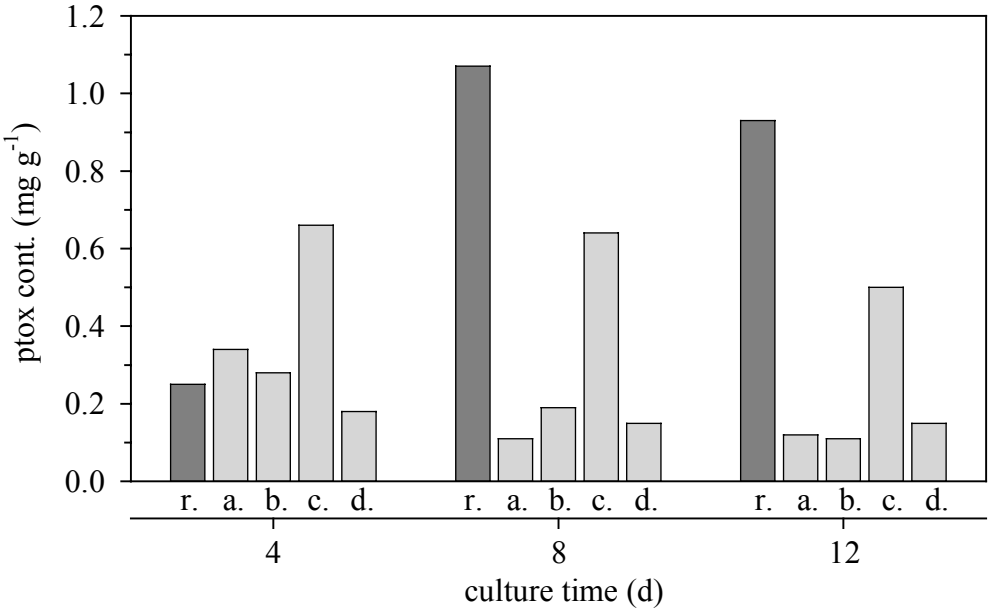

Figure D-41

Podophyllotoxin content on dry weight basis of *Linum album* suspension cultures in different media. Experimental setup as in Figure D-39. Standard MS-medium (r) and media a-d according to Table D-2. The content of three flasks was combined after vacuum filtration on the 4th, 8th and 12th day. Mean values of two distinct experiments.

D.5.4 Improved podophyllotoxin production in a revised culture medium

As presented in Chapter D.5.3, the macronutrients of the culture medium play an important role in the activation of PAL, 4CL and CAD and for podophyllotoxin production. Although only a few cultures survived in the case of ammonium as the sole inorganic-nitrogen source (med._e, Table D-2), this medium directed to a further improvement of the nitrogen source. Thus, various media compositions were tested regarding their ammonium and nitrate content. This led to an overall lower inorganic-nitrogen concentration, concurrent with an increased ammonium to nitrate ratio of the improved culture medium. Physiological responses of the cells in terms of the nutrient-uptake rates and pH-dynamics were similar to those as described by ARCHAMBAULT (1996) and ZHONG (1998). An elevated podophyllotoxin production, due to the use of the improved culture medium, is described in this chapter.

According to Chapter D.5.1, the influence of the sterilization process on the inorganic nitrogen composition of the culture medium has to be taken into consideration. Therefore, the data has been summarized in Table D-3, including the composition of the improved medium. If compared to the autoclaved MS-medium, a higher ammonium to nitrate ratio was established concomitant with an overall lower total inorganic nitrogen content: KNO_3 (omitted), NH_4NO_3 (10 mM), NH_4Cl (5 mM). It has to be emphasized, that, in terms of the nutrient concentrations, no differences were observed for the improved medium before and after the sterilization process. This could be attributed to the low nitrate content of the medium.

Table D-3 : Inorganic nitrogen composition of culture media

		MS-medium before sterilization	MS-medium after sterilization	improved medium
NH_4^+	(mM)	20.0	18.0	15.0
NO_3^-	(mM)	40.0	24.0	10.0
$NH_4^+ + NO_3^-$	(mM)	60.0	42.0	25.0
$NH_4^+ : NO_3^-$	ratio	1 : 2	3 : 4	3 : 2

Approximate values of the ammonium and nitrate concentrations.

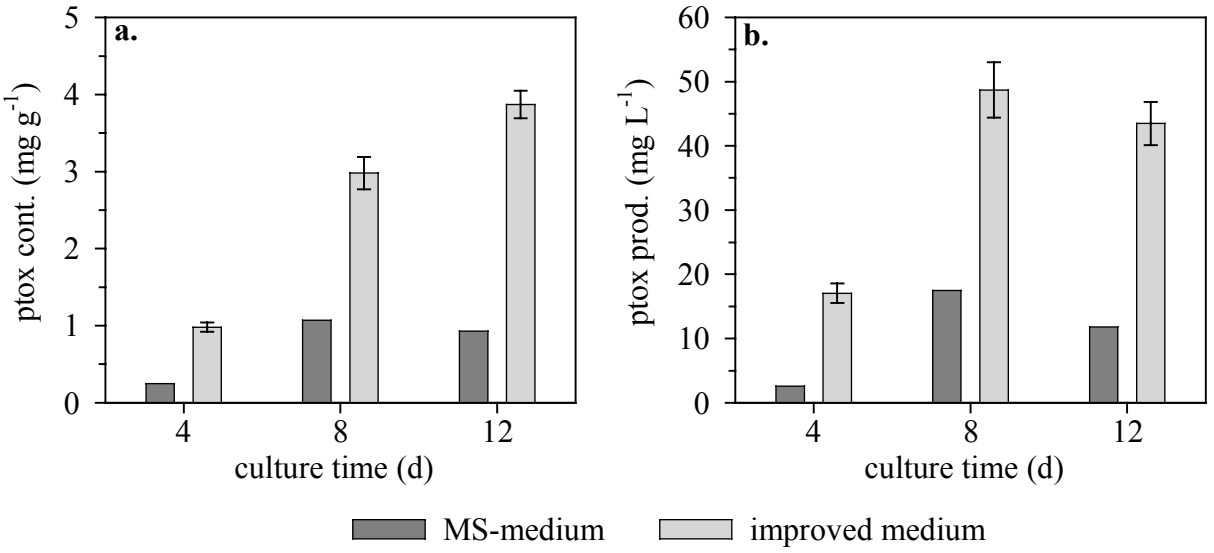

Figure D-42

Comparison of the podophyllotoxin content and production (both on dry weight basis) by *Linum album* (X4) suspensions in standard MS-medium (data taken from Figure D-41 for comparison) and in the improved medium (mean values of two distinct experiments, error bars were calculated for six flasks, three per experiment).

The results of a preliminary experiment, using the improved medium in shake flasks are presented in Figure D-42 & Figure D-43. A minimum pH of the culture medium had stabilized at 4.8 until the 6th day and increased afterwards (data not shown), no cell-lysis was observed. In the present study, the highest podophyllotoxin content (3.9 mg g^{-1}) and production (48 mg L^{-1}) was attained by the cultivation of line-X4 in the improved medium. The increase of podophyllotoxin production capacity was paralleled by a prolonged PAL and CAD activity if compared to the standard MS-medium. No effect was observed for the 4CL activity and, obviously, the reduced nitrogen content did not affect the protein concentration of the cells (Figure D-43c).

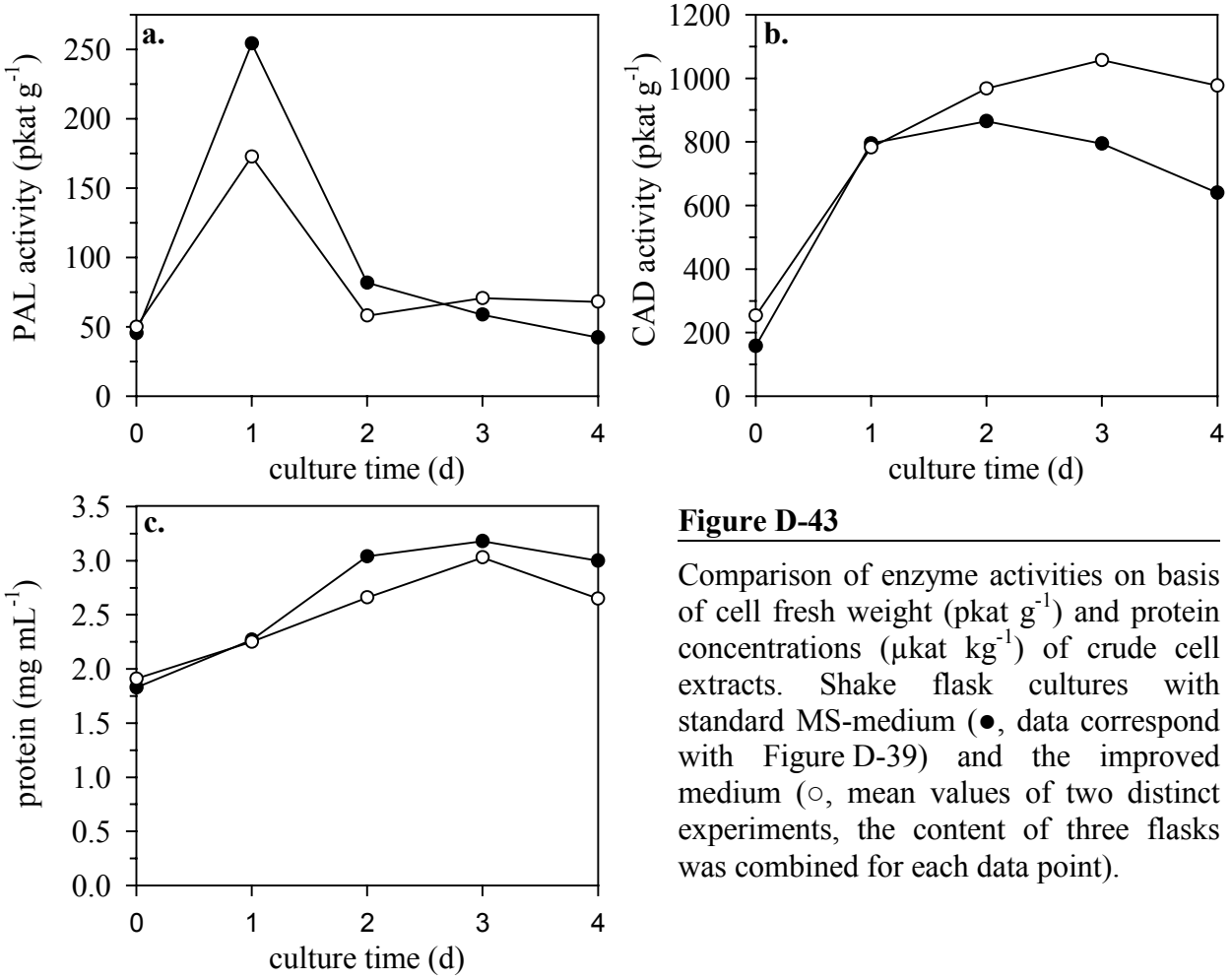

Figure D-43

Comparison of enzyme activities on basis of cell fresh weight (pkat g^{-1}) and protein concentrations (µkat kg^{-1}) of crude cell extracts. Shake flask cultures with standard MS-medium (●, data correspond with Figure D-39) and the improved medium (○, mean values of two distinct experiments, the content of three flasks was combined for each data point).

For verification of the observed effects, the improved culture medium was applied to the bioreactor system under non-oxygen limited conditions. The following figures describe the performance of a *Linum album* cell suspension (line X-4) at a lower inorganic-nitrogen concentration and a higher ammonium to nitrate ratio in BR_W & BR_X. First of all, it has to be stated that the increased podophyllotoxin content was confirmed by both bioreactor runs (Figure D-44). A plateau-value of 4.2 mg g^{-1} dw, which had been reached at the 9th day of culture, resulted in a podophyllotoxin production of 53 mg L^{-1}. Although this was 40% higher than at the use of a standard MS-medium (BR_O & BR_P), the podophyllotoxin accumulation was not faster but seemed rather to occur at the same rate. As for the shake-flask-study, the induction of PAL and CAD showed a prolonged activity of both enzymes. In Figure D-45 it can also be seen that comparable protein concentrations were attained even in the improved medium, which can be characterized by a low nitrogen content of 25 mM (see Table D-3).

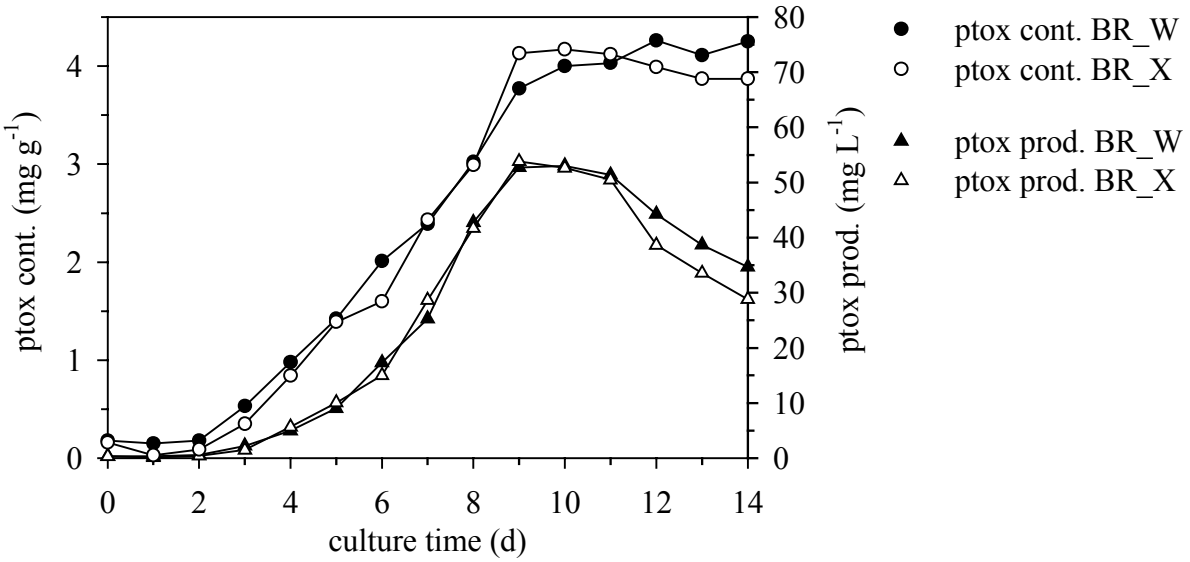

Figure D-44

The podophyllotoxin content and production (both on dry weight basis) by *Linum album* (X4) suspension cultures in duplicate bioreactors (BR_W & BR_X) during the culture period. Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), low inoculation density, improved medium, silicone-antifoam.

Since an efficient production of secondary metabolites is not only a matter of the absolute metabolite content but also necessitates a sufficiently high biomass accumulation, attention has to be paid to cell growth dynamics. Therefore, the values of the fresh and dry weight production by BR_W & BR_X are presented. Although the peak biomasses (336.5 g L^{-1} fw, 14.2 g L^{-1} dw) were similar to those obtained by the use of a standard MS-medium (BR_O & BR_P, Figure D-33), it is evident that the highest dry weight concentration was reached with a delay of 2 days. Hence, a slower growth rate by the improved medium can be assumed (Table D-5). As far as the late culture phase is concerned, a stable fresh weight concentration was established, whereas the onset of dry weight reduction coincided with a time when the culture medium became depleted by its main carbon-source (Figure D-46b). Moreover, the lower initial ammonium and nitrate concentration gave a decreased conductivity of the improved medium (Figure D-46c). When being compared to the standard MS-medium, the conductivity was approximately 50% lower (2.5 mS cm^{-1}). In this context, a variation of the inorganic nitrogen composition of the improved medium led to a prolonged time of growth at a low medium-pH during the initial culture phase. Subsequently, after inoculation, the pH dropped to a value of 4.8 during the first 2 days of culture and did not recover before the 5th day. Interestingly, pH-control of the medium by ARCHAMBAULT et al. (1996) did not eliminate a possible growth-retarding effect of a low pH. As shown by Figure D-47, the pH started to increase again at the same time when the ammonium content of the medium was almost spent and may have resulted from an accelerated nitrate uptake. About 60% of the initial nitrate concentration was presumably used up or stored by the cells between the 5th and 6th day. As usual, no phosphate was detected after the first day of culture.

For a better definition of culture protocols to improve the productivity of the *Linum album* (X4) suspension, the residual macronutrients of a standard MS-medium and the improved medium were detected and modified. A low inorganic nitrogen content and an increased ratio of ammonium to nitrate made a similar improvement of the culture performance possible, as being published by MATSUMOTO et al. (1971). Furthermore, the results obtained are to some extent consistent with MEI et al. (1996). They achieved a higher paclitaxel production, concurrent with a slower culture growth, by reducing the inorganic nitrogen content of a standard MS-medium.

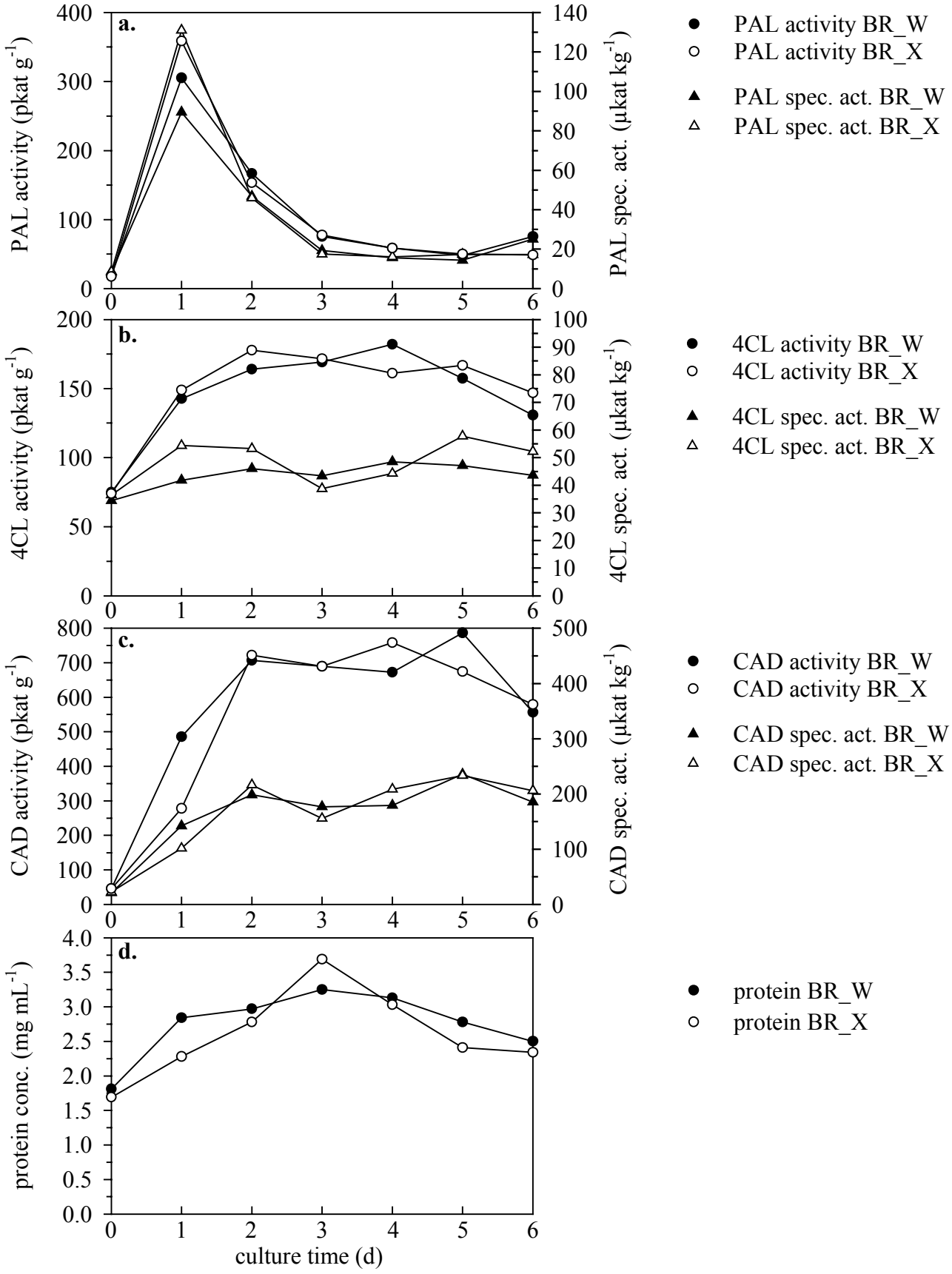

Figure D-45

Enzyme activities on basis of cell fresh weight (pkat g^{-1}) or protein concentration (µkat kg^{-1}) of the crude cell extract (BR_W & BR_X). Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), low inoculation density, improved medium, silicone-antifoam.

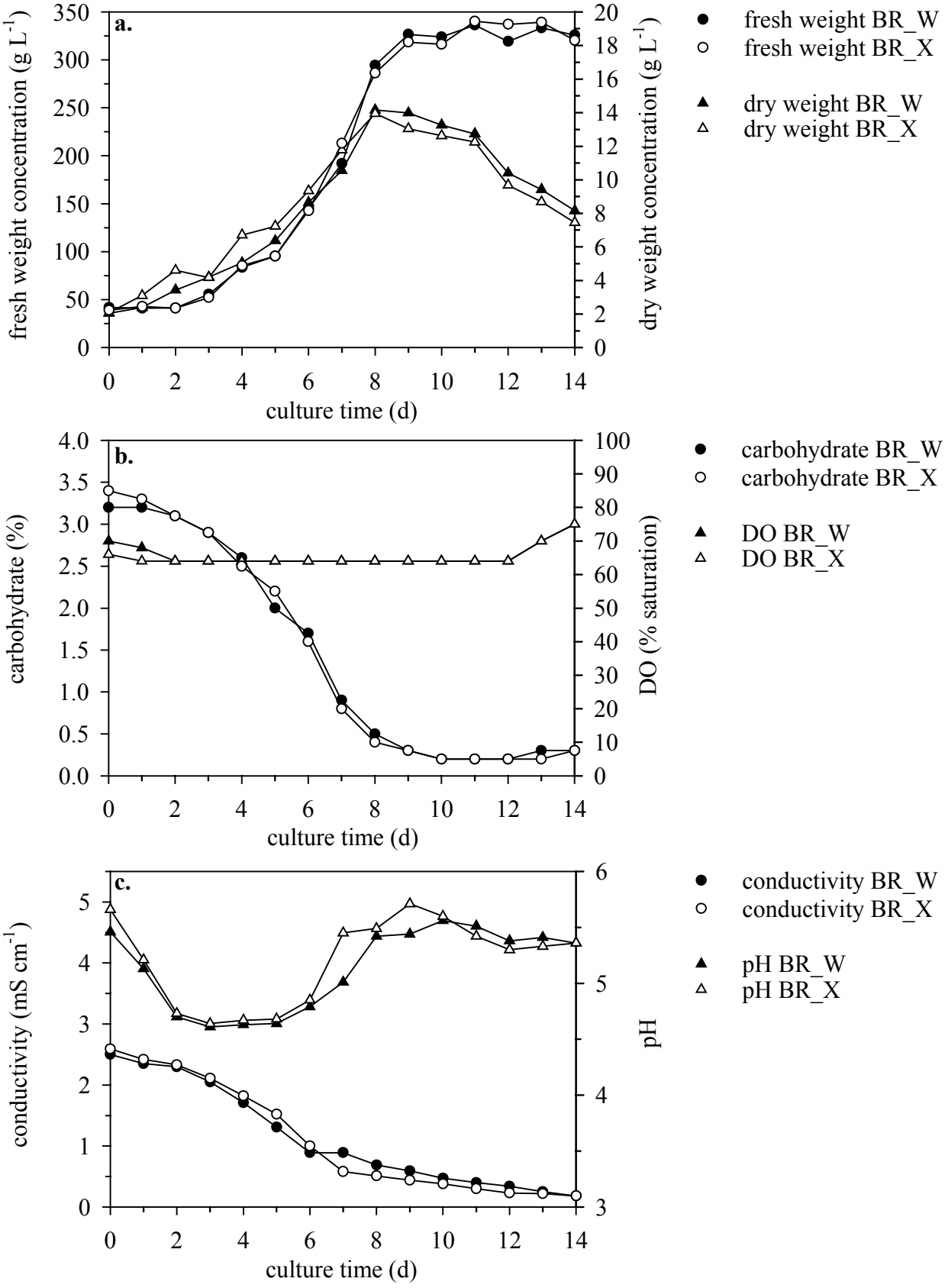

Figure D-46

Biomass concentrations, dissolved oxygen saturations and culture medium parameter as a function of time for two bioreactors (BR_W & BR_X). Experimental setup described in Figure D-45. Samples were taken during a culture period of 14 days.

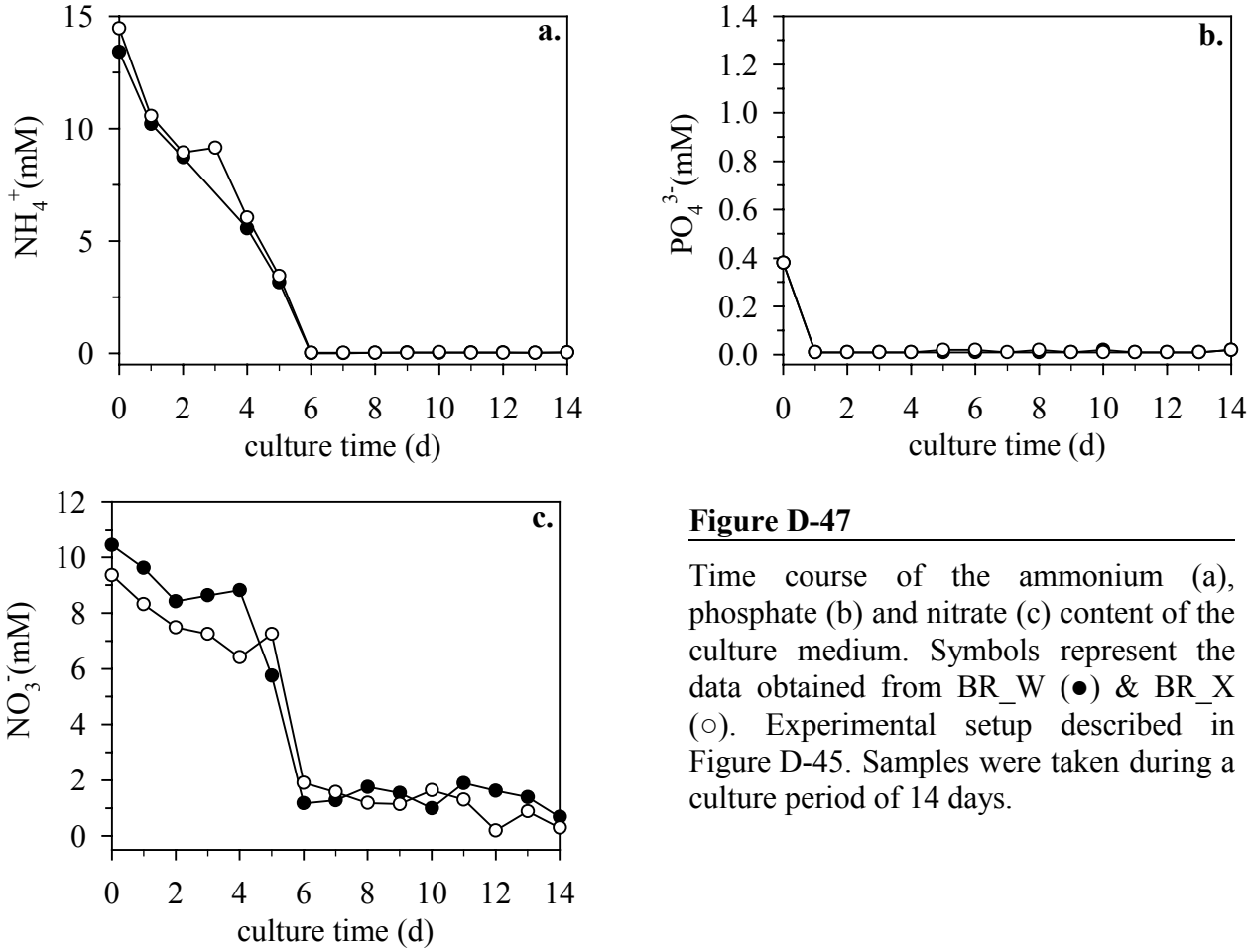

Figure D-47

Time course of the ammonium (a), phosphate (b) and nitrate (c) content of the culture medium. Symbols represent the data obtained from BR_W (●) & BR_X (○). Experimental setup described in Figure D-45. Samples were taken during a culture period of 14 days.

D.6 Selection of a high-producing cell line

The selection of plants according to their inherent pest-resistance, superior growth characteristics or their adaptation to environmental conditions has a long tradition in agriculture. Although various forms of genetic engineering have been indirectly practiced to emphasize certain attributes by cross-fertilizing different species to create new varieties or gathering the seeds of plants that exhibit the most desirable characteristics, such traditional procedures are time-consuming and imprecise (UZOGARA 2000). More elaborate manipulation of the genome became possible by genetic engineering techniques for the development of improved plant varieties or *in vitro* cultures at the end of the 1960s. In this context, some of the most prominent examples to be mentioned are the Flavr Savr tomato (THAYER 1994), various vitamin E and beta-carotene-enriched seeds (HIRSCHBERG 1999; VAN WIJK 2002), high value polymeric carbohydrates (DUNWELL 1999) or recombinant proteins (DIERYCK, et al. 1997; DORAN 2000). However, only a few models are available pertaining to transgenic engineering of secondary metabolites and other highly regulated pathways: improvement of terpene (MAHMOUD & CROTEAU 2002) and alkaloid (VERPOORTE, et al. 2002; YUN, et al. 1992) composition or tissue-specific flavonoid (COLLIVER, et al. 2002; MEMELINK, et al. 2001) and amino acid accumulation (DAWAELE, et al. 2002). Moreover, most of these cases merely a limited number of enzymes had to be manipulated (HALPIN, et al. 2001), whereas it is generally believed, that for the multi-directed phenylpropanoid metabolism only a coordinated flux control and simultaneous modification of several genes would guarantee a successful adjustment of product formation (DIXON, et al. 2002; MEMELINK, et al. 2001).

Due to the obstacles mentioned, the risk of genetic instability and gene silencing (COLLIN 2001; JAMES & LEE 2001), cell line selection and biochemical studies still offer attractive alternatives to enhance the metabolite accumulation of *in vitro* cultures. Similar to a possible genetic variability of plant species, which has been shown for *Podophyllum* and *Taxus* (PARC, et al. 2002; PUROHIT, et al. 1999), the heterogeneity of the biochemical activity can be exploited for obtaining a highly productive cell line. Despite the encountered genetic instabilities of high-producing cell lines (VERPOORTE, et al. 1999; YEOMAN & YEOMAN 1996), the empirical selection of a *Linum album* suspension culture with superior biosynthetic capacities seemed to offer a feasible approach. It was believed that such cell suspensions, which have been cultivated successfully for years, would already possess a stable and up-regulated podophyllotoxin production.

D.6.1 Preliminary shake flask studies on different cell lines

Screening of various *Linum album* cell lines for their podophyllotoxin accumulation capacity yielded the high-producing line-A culture. Initial inspection of the cell suspension revealed that the cells were slow proliferating, growing in fairly solid clumps and showed a positive phloroglucinol/HCl staining (Figure D-48). The latter observation gave rise to a pronounced accumulation of lignin-like material or phenolic aldehydes (DAVIDSON, et al. 1995). Fortunately, mechanical disaggregation of cell clumps gave a fast growing suspension, whose potential for podophyllotoxin accumulation had been retained, whereas the positive phloroglucinol / HCl reaction was diminished.

Inherent culture characteristics are contrasted in Figure D-49 & Figure D-50 with the standard line-X4 (utilised in the previous experiments) and the low accumulating culture (line-X4F) in which only trace amounts of podophyllotoxin were found. On the 10^{th} day of culture line-A exhibited a six-times higher podophyllotoxin content compared to line-X4. The value of 9.0 mg g^{-1} dw of the line-A culture represents the highest podophyllotoxin accumulation which has been so far detected in *Linum album* suspensions. Moreover, the elevated product formation was preceded by a high and prolonged PAL and CAD activity during the first days of culture. For the first time, a distinctively higher 4CL activity correlated with an increase of the podophyllotoxin accumulation capacity, whereas, in the case of the non-producing line-X4F, only minor activities could be detected. Unfortunately, the line-A suspension displayed a slower initial growth after its transfer to new media and proved to be sensitive in relation to the inorganic nitrogen composition of the improved medium. Hence, line-A was applied to the bioreactor system in a standard MS-medium and served as a model for the biochemical potential of different cell lines.

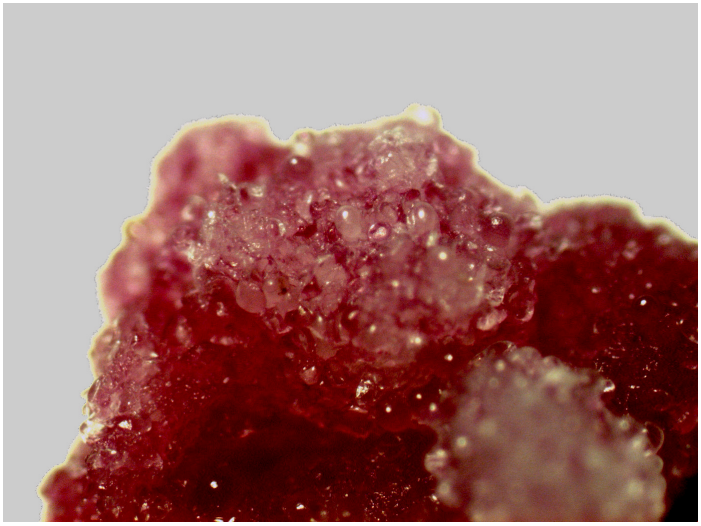

Figure D-48

Positive phloroglucinol/HCl (CLIFFORD 1974) staining of line-A cell clumps.

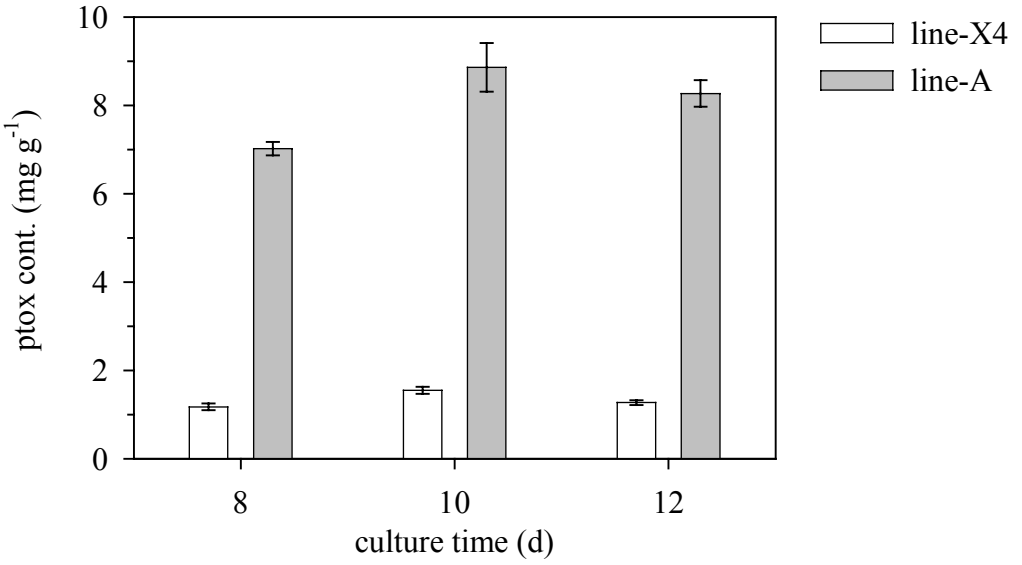

Figure D-49

Comparison of the podophyllotoxin content (on dry weight basis) of two *Linum album* cell lines in MS-medium at different days of culture: a standard (line-X4) and the selected (line-A) culture, data of line-X4F not shown. Mean values and error bars calculated for three individual flasks.

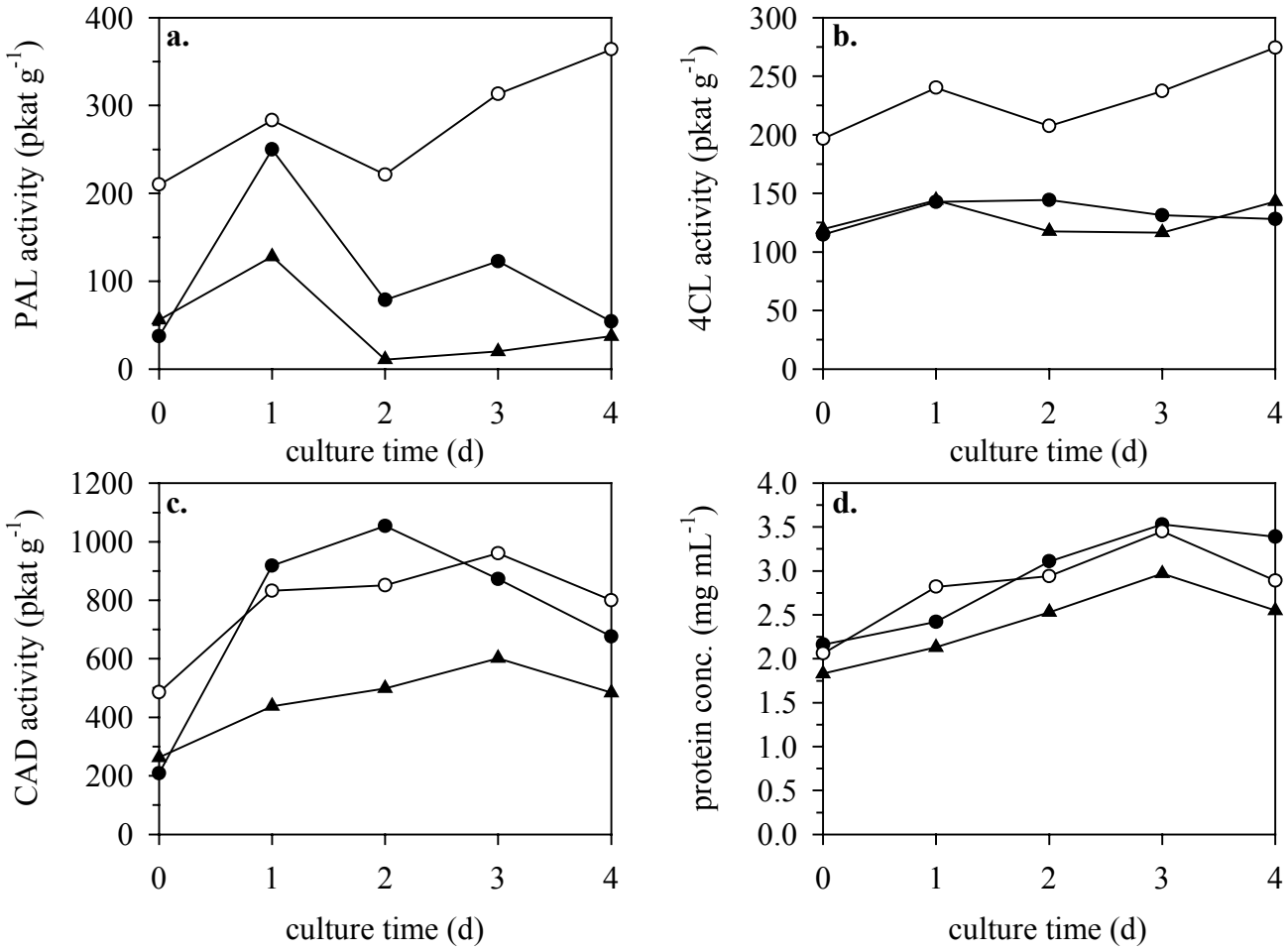

Figure D-50

Enzyme activities on basis of cell fresh weight (pkat g^{-1}) and protein concentrations (mg mL^{-1}) of the crude cell extract: line-X4 (●), line-A (○) and line-X4F (▲).

D.6.2 Characterization of a high-producing cell line in the bioreactor system

Application of the *Linum album* (line-A) cell suspension to the airlift bioreactor proved to be successful in terms of culture growth and product yield. As illustrated by the curves in Figure D-51, the podophyllotoxin accumulation started after a lag-phase of 4 days and reached a maximum content of 10.5 mg g^{-1} dw on the 10th day of culture. Even though the absolute values of line-A were distinctively higher than in the case of line-X4 (standard MS-medium), the overall accumulation dynamics seemed to be comparable in both cultures. In addition, a plateau-shaped curve of the podophyllotoxin content was also established in the present study and a peak-production of 130 mg L^{-1} indicated a reasonable biomass formation in BR_U & BR_V.

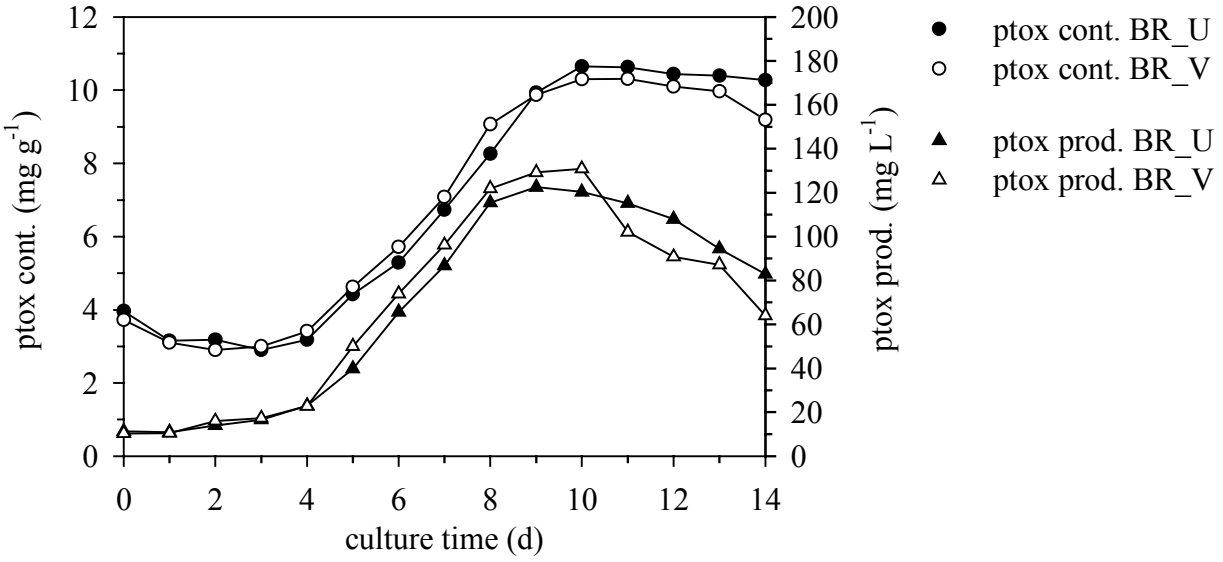

Figure D-51

The podophyllotoxin content and production (both on dry weight basis) by *Linum album* (line-A) suspension cultures in duplicate bioreactors (BR_U & BR_V) during the culture period. Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), low inoculation density, MS-medium, silicone-antifoam.

In Figure D-52 it can be seen that, unlike in previous experiments, the induction of the PAL after the inoculation (day 1) was relatively lower in relation to a pronounced second peak of activity at the 5th day. The height of the first peak (approx. 225 pkat g^{-1} fw) was comparable to the averaged PAL-curve of line-X4 (Figure D-32). Similar to the preliminary shake flask studies, a high and prolonged CAD activity was achieved by line-A in BR_U & BR_V. It should be noted that in this case the increase of protein concentration ensued until the 4th day (3.8 mg mL^{-1}), whereas line-X4 reached the maximum one day earlier. As expected from the elevated podophyllotoxin production shown in Figure D-51, a reasonable dry weight accumulation of 13.7 g L^{-1} was found between the 6th and 8th day of culture. Nonetheless, the cell fresh weight concentration was reduced for the high-producing cell line. After the second day of culture, line-A exhibited a lower fresh to dry weight ratio in comparison to line-X4. A ratio of 14.5 (fw/dw) was reached by line-A at the 8th day of culture, whereas on the same time BR_O & BR_P displayed a value of 23.5 (fw/dw). This may be due to a lesser vacuolisation of line-A, which, according to SHIBASAKI et al. (1995), reduces the risk of cell rupture and may contribute to the culture performance in a bioreactor. Concurrent with these observations, in relation to the lower fresh weight accumulation, a high residual medium conductivity was apparent in the case of line-A even in the later culture phase. This, vice versa, may indicate a connection between the high fresh weight formation of line-X4 and a marked reduction of the medium conductivity (possible storage of inorganic ions inside a larger vacuole).

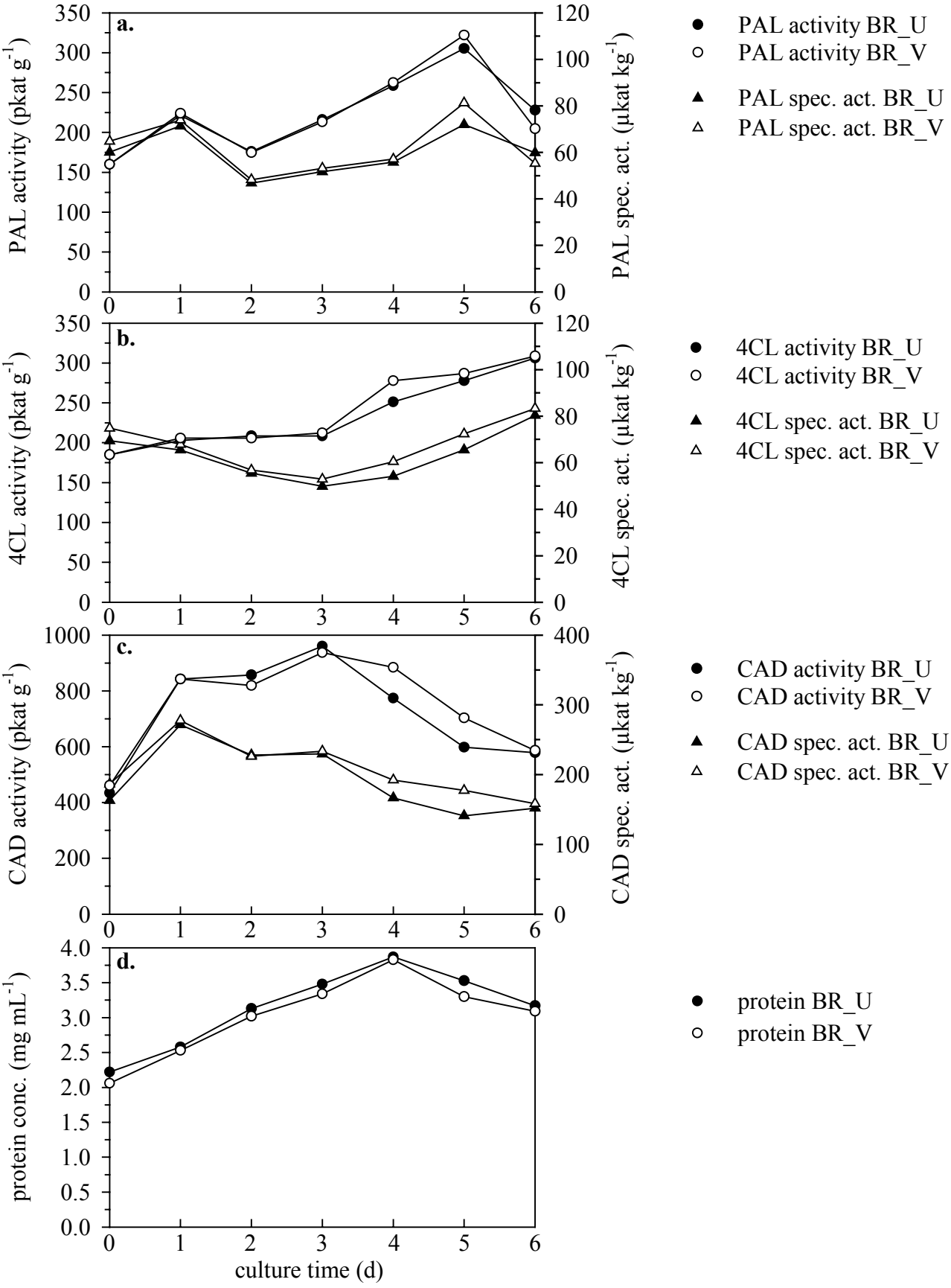

Figure D-52

Enzyme activities on basis of cell fresh weight (pkat g^{-1}) or protein concentration (µkat kg^{-1}) of the crude cell extract (BR_U & BR_V). Experimental setup: 0.44 vvm, batch culture, active DO-regulation (minimum DO 60%), low inoculation density, MS-medium, silicone-antifoam.

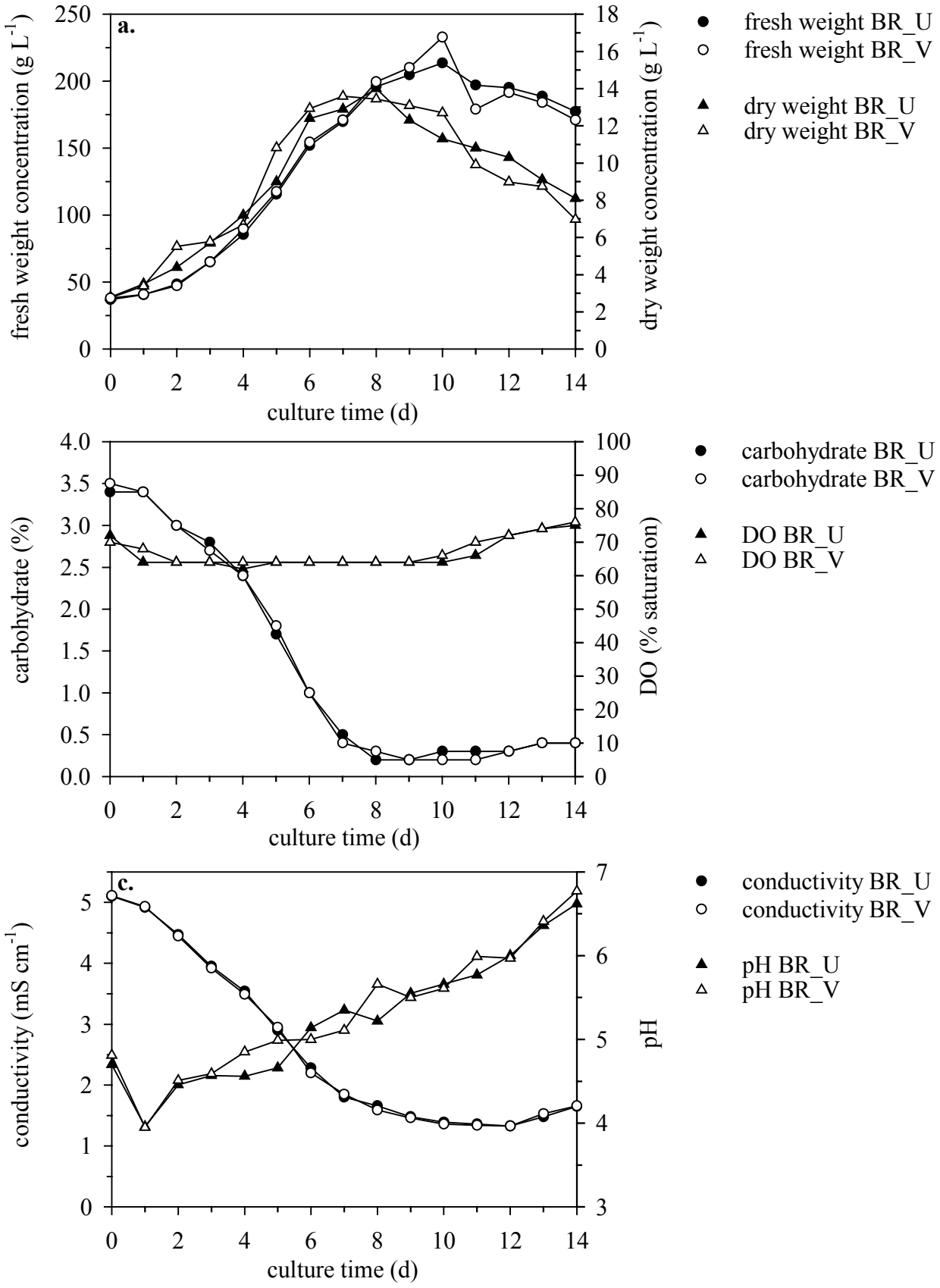

Figure D-53

Biomass concentrations, dissolved oxygen saturations and culture medium parameter as a function of time for two bioreactors (BR_U & BR_V). Experimental setup described in Figure D-52. Samples were taken during a culture period of 14 days.

D.7 Summery of results

This chapter represents a summary of the major results obtained from the bioreactor experiments, which have been described in previous sections. Though the following tables and figures are not commented, they will be referred to throughout the discussion regarding to the different experimental setups:

Table D-4: Comparison of the various bioreactor-setups

bioreactor (BR_)	Chapter	aeration rate	minimum DO (%)	inoculation density	medium concentr.	cell line	anti-foam	culture mode
A & B	D.3.1	4 L min^{-1}	10	high	low	X4	PPG	batch
C & D	D.3.1	6 L min^{-1}	16	high	low	X4	PPG	batch
E & F	D.3.1	8 L min^{-1}	22	high	low	X4	silicone	batch
G & H	D.3.1	10 L min^{-1}	28	high	low	X4	silicone	batch
I & J	D.3.1	8 L min^{-1}	22	high	low	X4	PPG	batch
K & L	D.3.2	8 L min^{-1}	60	high	low	X4	silicone	batch
M & N	D.4.2	8 L min^{-1}	60	low	low	X4	silicone	batch
O & P	D.5.1	8 L min^{-1}	60	low	standard	X4	silicone	batch
Q & R	D.5.2	8 L min^{-1}	60	low	standard	X4	silicone	fed-batch
S & T	D.5.2	8 L min^{-1}	100	low	standard	X4	silicone	batch
U & V	D.6.2	8 L min^{-1}	60	low	standard	A	silicone	batch
W & X	D.5.4	8 L min^{-1}	60	low	improved	X4	silicone	batch

Oxygen-saturation values as a percentage of maximum ambient-air saturation. Only for BR_W & BR_X the improved medium had been used instead of a low or standard concentrated MS-medium.

Table D-5: Growth and yield parameters of selected bioreactor-setups

bioreactor BR_	specific growth rate (d^{-1})	mass doubling time (d)	growth index	growth yield
A & B	0.21	3.35	3.18	0.35
E & F	0.24	2.87	3.23	0.41
K & L	0.26	2.64	3.43	0.43
M & N	0.32	2.16	5.90	0.45
O & P	0.34	2.06	6.51	0.46
S & T	0.30	2.34	5.49	0.39
U & V	0.26	2.66	4.93	0.34
W & X	0.24	2.91	7.03	0.44

Calculation according to Table C-2 for the mean values of relevant bioreactor pairs.

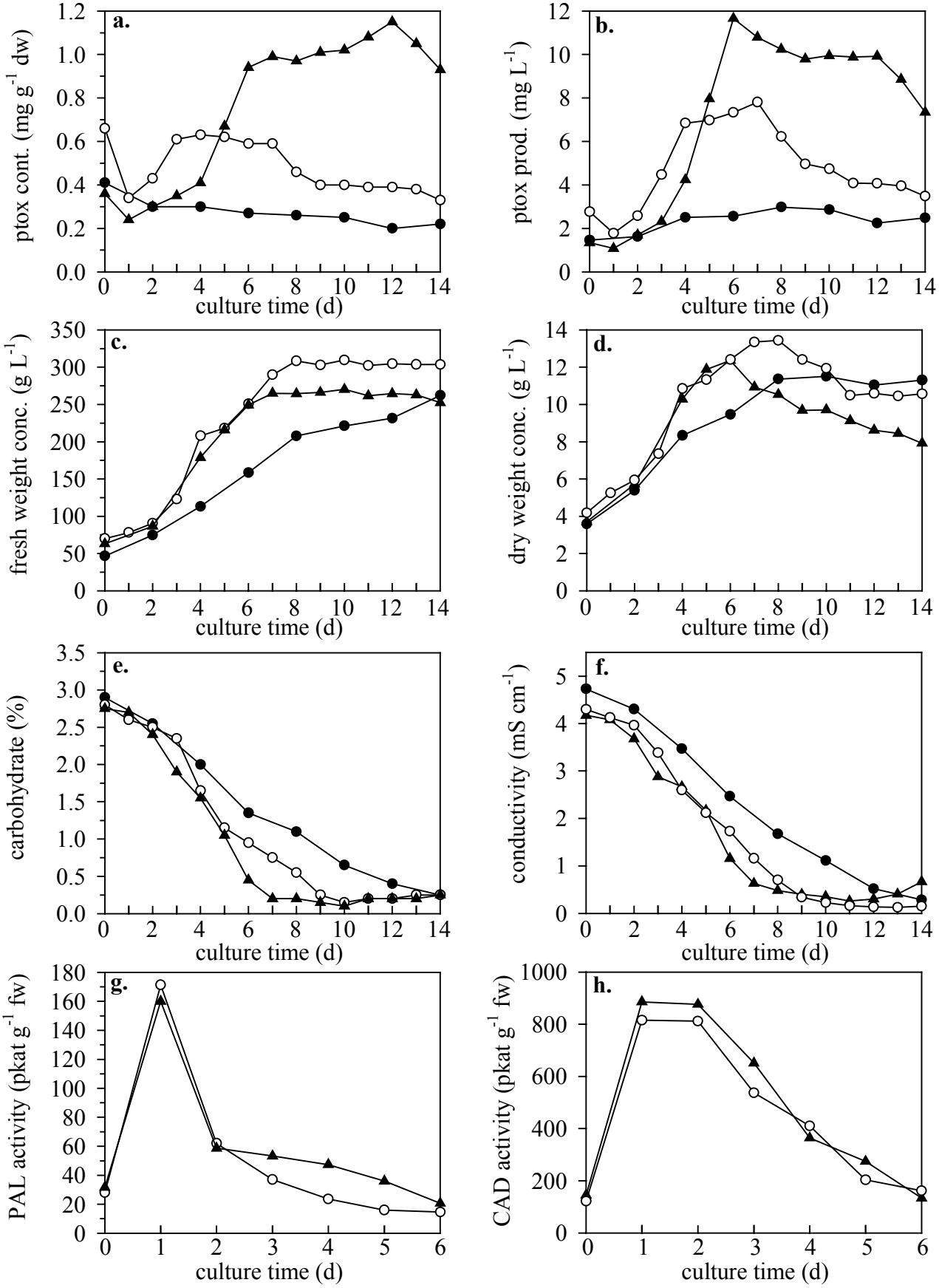

Figure D-54

Comparison of the mean values of BR_A & BR_B (●, 0.22 vvm), BR_E & BR_F (○, 0.44 vvm) and BR_K & BR_L (▲, 0.44 vvm and oxygen-supplementation).

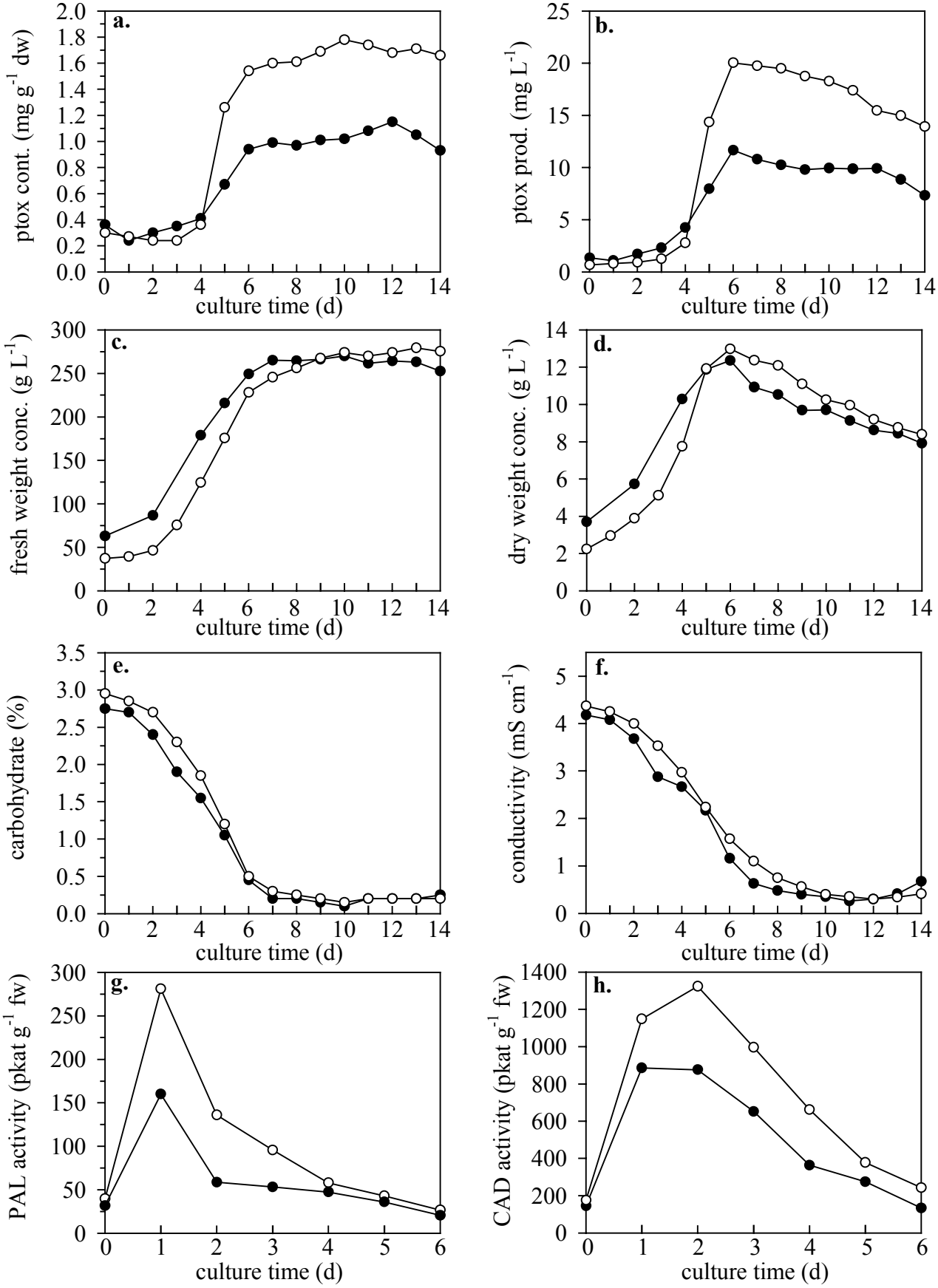

Figure D-55

Comparison of the mean values of BR_K & BR_L (●, high inoculation density) and BR_M & BR_N (○, low inoculation density).

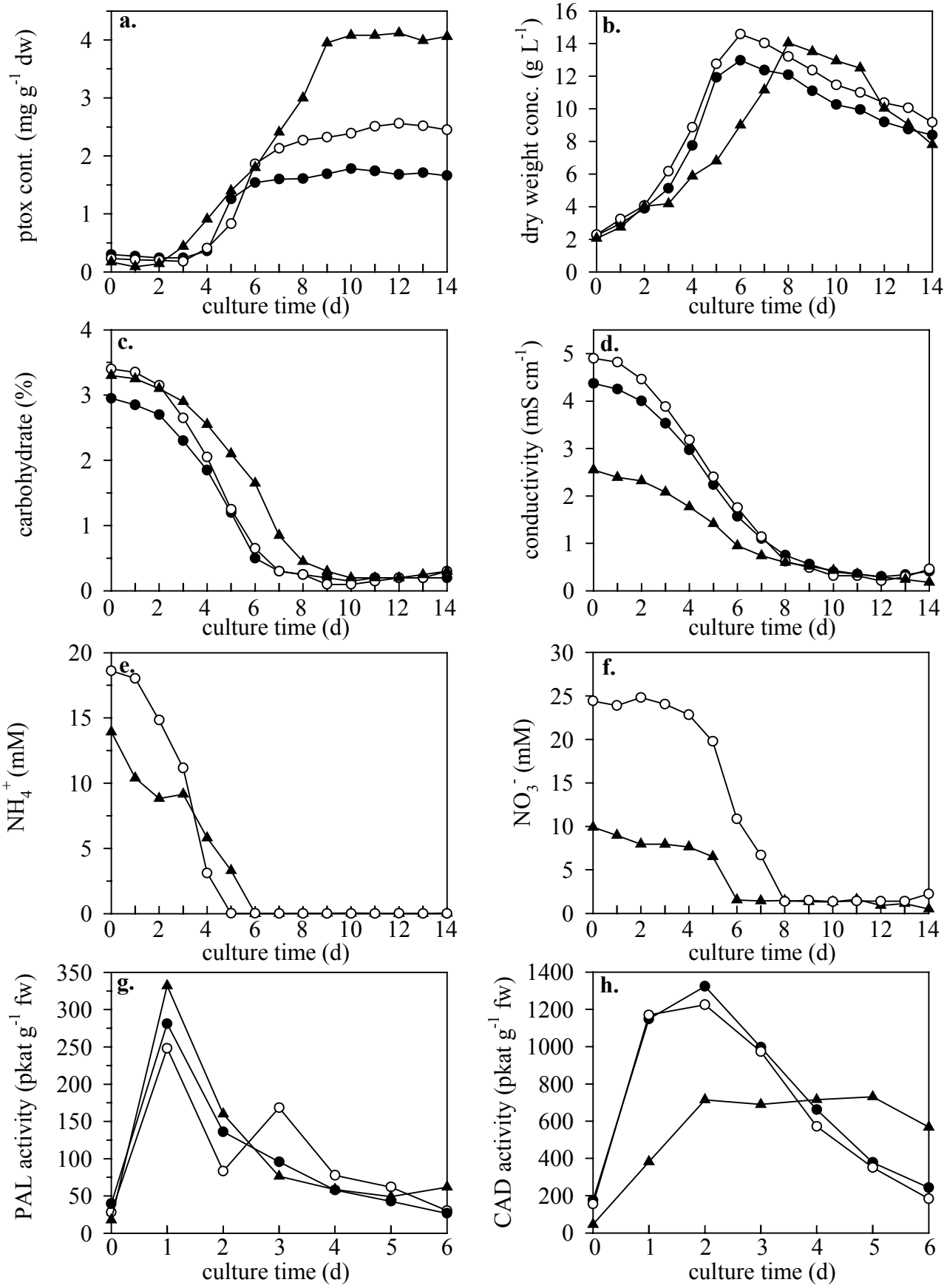

Figure D-56

Comparison of the mean values of BR_M & BR_N (●, low concentrated MS-medium), BR_O & BR_P (○, standard concentrated MS-medium) and BR_X & BR_W (▲, improved medium).

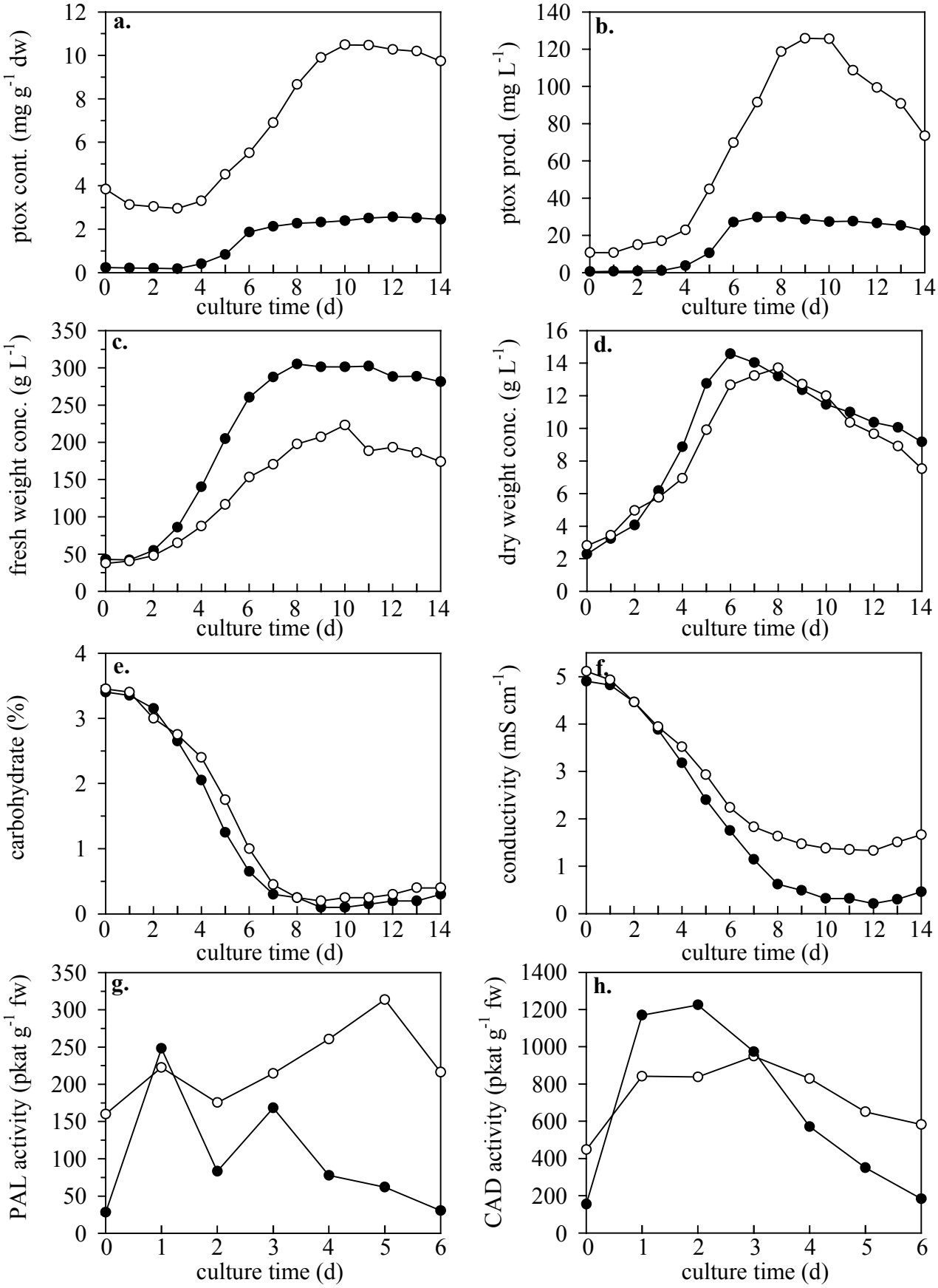

Figure D-57

Comparison of the mean values of BR_O & BR_P (●, line-X4), BR_U & BR_V (○, line-A) in a standard MS-medium.

E. DISCUSSION

In the previous chapters, the development of an airlift bioreactor system for the production of podophyllotoxin by *Linum album* suspension cultures has been presented. Data, descriptive of the shake flask and the bioreactor system as well as inherent cell characteristics, is given in relation to various culture conditions. The studies aimed for a process-optimisation regarding to an enhanced podophyllotoxin production. Hence, the following discussion considers the influence of certain factors on the process performance and reveals an interrelation of the culture conditions, enzyme activities and production dynamics.

E.1 Basic studies on culture characteristics - the shake flask system

Shake flasks represent a system widely used for studying the features and requirements of cell suspension cultures, which is indispensable for a further upscale. Despite inadequate mass-transfer capacities of Erlenmeyer flasks, especially in the case of a high-filling volume or dense closures, the use of shake flasks obviously has a number of advantages in comparison to a bioreactor system (FREEDMAN 1970; VEGLIO, et al. 1998). Those advantages are amongst others: little material expense, high reproducibility, low shear environment and the ease for manipulation. As indicated by the data obtained, intrinsic characteristics of the shake flask regime explain the apparent differences between both systems in terms of the biomass production and podophyllotoxin accumulation.

E.1.1 Dynamics of biomass formation during the growth period

An important factor in biotechnological processes is the dynamic of the culture, since the demands of the cells change rapidly as they mature from inoculation, through the growth and the production phase. Moreover, especially in the case of a batch culture, growth conditions, such as the medium composition, oxygen availability and the accumulation of waste products vary drastically throughout the culture period. Therefore, the process-optimisation for podophyllotoxin production by *Linum album* suspensions requires a detailed analysis of both parameters, the biomass formation and the podophyllotoxin content of the cells. Apparently, growth curves of plant suspension cultures resemble those of microorganisms (MUTTZALL 1993). In many cases it has been inferred that plant suspensions reveal an initial lag-phase, which is followed by an exponential and linear growth before the establishment of a rather short stationary phase and the onset of culture decay. A similar structure is depicted by the curves of dry weight formation in this study. However, comparable to data described elsewhere (CONTIN, et al. 1998; SAHOO & AGARWAL 2002), almost no lag-phase was evident in the shake flask system with respect to the biomass increase. Instead, on the one hand, the dry weight concentration increased after transfer to fresh media without any delay, which can be attributed either to an induction of cell proliferation or an initial carbohydrate accumulation (ROSE, et al. 1972). On the other hand, both factors might have simultaneously contributed to an increase of biomass since macroscopic inspections of the cultures revealed an elevated cell density on the 3rd day. In addition, formation of starch granules became apparent at the 2nd day after inoculation (Figure D-9). The storage of carbohydrates has also been shown elsewhere (PÉPIN, et al. 1995; SCHLATMANN, et al. 1995) whereas no starch granules were detected by IBRAHIM & PHAN (1978) in light grown *Linum usitatissimum* L. callus or *Vitis vinifera* L. suspensions (PÉPIN, et al. 1995). For an estimation of the contribution of stored carbohydrates to dry weight formation, KEßLER et al. (1999) reported a considerable accumulation of starch in aggregates of *Catharanthus roseus*. It accounted for up to 90% of dry-cell-mass and might therefore have

effected biomass estimations (Ten HOOPEN, et al. 1994). Thus, a more accurate analysis of the culture dry weight dynamics would render a calculation on basis of the cell number or an estimation of the mitotic-active cells necessary. Relevant experiments by Van der PLAS (1995), HENSHAW (1966) and PÉPIN, et al. (1995) gave rise to a growth-condition-dependent and time-limited cell proliferation in the case of *Vitis vinifera* L. and *Linum usitatissimum* L. cultures. The investigations showed a peak proliferation activity of *Linum usitatissimum* L. suspensions between the 3rd and 6th day of culture while the cell dry weight increase reached its maximum not before day 16. Data descriptive of the progress of growth during cultivation of the *Linum album* (X4) culture also demonstrated a prolonged dry weight increase up to the 6th or 16th day, depending on the growth conditions. Unfortunately, due to a pronounced cell aggregation by line-X4, the total cell number could not be determined. However, despite a possible influence of the starch granules on the biomass calculation during the initial growth phase, the highest podophyllotoxin content of the cells coincided with the time when no starch granules were detected in the cells. Consequently, one ought to be careful when using the data on dry weight increase as a measure of growth. Nevertheless, the peak values of podophyllotoxin accumulation can be assumed to be less affected.

Ensuing a period of linear dry weight increase, a short phase of negative acceleration preceded the achievement of the peak dry weight concentration, which was drastically retarded in the case of the oxygen-limited high-liquid-volume culture (Figure D-8). In general, the cessation of dry weight formation was parallel to the depletion of the culture medium by its main carbon source, indicated by a diminished refractometric index. Contrary to the cell fresh weight, a subsequent decline was observed for the dry weight concentration, concurrent with the fading of the starch granules. Such findings point to overlapping dynamics of carbohydrate storage and an increase of cell density. Apparently, the formation of biomass (dry weight conc.) was restrained by the initial sucrose concentration of the culture medium, which was probably hydrolysed to its monomers, glucose and fructose (KATO & NAGAI 1979; SHIMON-KERNER, et al. 2000). However, it cannot be excluded that the influence of cell proliferation on the total dry weight formation was superimposed by starch granules, a fact that has also been discussed by SCHLATMANN et al. (1995). Thus, other factors than the initial sucrose concentration could have limited a further increase of culture cell density. In this context, HENSHAW et al. (1966) provided evidence for a growth-restraining function of the nitrate supply rather than any other medium constituent. A similar reduction of growth at low nitrate concentrations has been described for tomato plants (MATSUKI 1996) or suspension cultures of *Panax ginseng* Wall. (LIU & ZHONG 1997), whereas, in the case of *Vitis vinifera* suspensions, cell division had already ceased before nitrate depletion (PÉPIN, et al. 1995). In addition, the cessation of cell division has been attributed to a fast reduction of the ammonium and phosphate concentration in the medium. Comparable results were obtained from a quantification of residual medium constituents (ammonium, nitrate and phosphate) in shake flask cultures of *Linum album* suspensions. Not only because of the slow extracellular nitrate consumption, but also for its reappearance during the late culture phase, nitrate has been assumed not to be growth limiting in these studies.

As mentioned before, the cell fresh weight concentration remained stable even after a decline of the dry weight and showed a fairly slow decrease until the end of culture characterization. According to literature, the increase of the fresh to dry weight ratio during the late culture phase represents a common feature of a submersed plant cell culture. It has been ascribed to intra- and extracellular osmotic changes (SAKANO, et al. 1995; SINGH & CURTIS 1994), leading to an increased cell size because of a swelling of the vacuole. Generally, this is of major importance for the cell physiology and its sensitivity to culture conditions. A high fresh to dry weight proportion brings about an enhanced vacuolar storage capacity for inorganic nutrients and glycosides (MIMURA 1999; SANO, et al. 1999; WHETTEN & SEDEROFF 1995) as well as an increased susceptibility to shear stress and cell lysis (BAILEY & NICHOLSON 1989; TATICEK, et al. 1991).

Besides the possible influence of the stored carbohydrates on the determination of the biomass production, it is likely that the granules have been utilized by the *Linum album* culture as an energy source after medium exhaustion (TATICEK, et al. 1991; Van GULIK, et al. 1989). In this context, the fading of the extracellular carbon source is reflected by the residual dissolved oxygen saturation of the culture broth (Figure D-5 & Figure D-8). It can be seen that subsequently after the refractometric index had reached a value of 0.5% (low residual carbohydrates), the oxygen saturation gradually increased until the end of culture. This observation is in accordance with a concept proposed by ROSE et al. (1972), who claim that during batch culture the metabolic rates and culture conditions are never constant. Thus, the oxygen demand and presumably the primary metabolism as the main carbon sink were closely interrelated. Furthermore, the use of intracellular stored carbohydrates might have accounted for a low but prevailing oxygen demand of the cells even after the culture medium became depleted by its main carbon-source (TATICEK, et al. 1990). Finally, at the end of culture, cell lysis was indicated by cell debris and a discolouration of the broth. During the culture decay an elevated medium conductivity, associated with an increased nitrate concentration, was observed and an oxygen saturation of almost 100% had been reached. This confirmed the reliability of both oxygen probes over a culture time of 21 days.

E.1.2 Podophyllotoxin accumulation by *Linum album* (X4) cell suspensions

For a discussion of the podophyllotoxin accumulation kinetics under standard conditions in shake flasks it is essential to refer to the detailed characterization presented in Chapter D.2.3. In depths analysis of parameters influencing the podophyllotoxin accumulation such as the oxygen supply, the inoculation density or culture medium constituents will be the subject of the following chapters. According to VERPOORTE et al. (2002) the expression of biosynthetic pathways may be affected by a variation of *in vitro* conditions. Besides, in case of a non-producing callus or suspension culture, hardly any optimisation of the growth conditions will be successful. Thus, even at this preliminary stage, much can be gained from a description of product formation before the cells are applied to the bioreactor.

As depicted by the curves in Figure D-8d, a maximum podophyllotoxin content of 1.6 mg g^{-1} dw was found on the 10^{th} day of culture, which was in agreement with data published by SMOLLNY (1993). Hence, it can be concluded that with some exceptions the *Linum album* cultures show a stable podophyllotoxin accumulation, even over several years after their initiation. Moreover, an approximate podophyllotoxin production of 25 mg L^{-1} surpassed the highest values of a *Podophyllum hexandrum* shake flask culture in a basal MS-medium (CHATTOPADHYAY, et al. 2001). Due to an extended lag-phase in the case of the *Podophyllum* culture, it took one month until a peak production of 4 mg L^{-1} was reached. Although data obtained from shake flask studies has to be viewed with caution, a comparison of the production rate (0.13 mg L^{-1} d^{-1}, *Podophyllum*-culture and 2.8 mg L^{-1} d^{-1}, *Linum*-culture) featured the *Linum album* suspension as a promising system for biotechnological application.

Besides the importance of a high peak content of the cells, biotechnological approaches also have to take product-formation dynamics during the growth period into consideration. Contrasting with the bioreactor studies, the podophyllotoxin content revealed a sudden decrease after the highest value had been reached in the shake flask system. This could be due to an accumulation of waste products or endogenous hormones in the culture medium and head space of the flask. It has been controversially discussed that gaseous substances such as carbon dioxide or ethylene may exert effects on cell physiology and secondary metabolite production (NEUMANN 1995; SCHLATMANN, et al. 1993). Indeed, both compounds were detected in culture systems and are known to play an important role in plant development (LINCOLN 2000; LINDEN, et al. 2001). It is possible that the restricted gas-transfer capacities through the sterile cotton-plug (MROTZEK, et al. 2001) led to an accumulation of such volatiles in the head space, which could have been one reason for the accelerated fading of podophyllotoxin compared to the airlift bioreactor system.

Moreover, a pronounced increase in the medium conductivity and inorganic nutrient reappearance (Figure D-10 & Figure D-11) indicated the onset of cell lysis after medium exhaustion. In all experiments, after transfer to fresh medium, the podophyllotoxin content was considerably lower than it would have been expected from a 7 day old culture. Even three hours after inoculation a decline was encountered which further ensued until the onset of the podophyllotoxin accumulation on day 4. As a matter of fact, in undifferentiated suspensions cultures the fading of any secondary metabolite cannot be attributed to transport and storage in definite plant tissues (HADACEK 2002). It has been proved for the anthocyanins, monolignols and for podophyllotoxin itself that the rather lipophilic, unstable and cytotoxic compounds are stored as glycosides inside the vacuole (CANEL, et al. 2001; WHETTEN & SEDEROFF 1995). Although it is generally believed that thereby the metabolites are protected from catabolic activities, a turnover by enzymatic degradation or autocatalytic mechanisms cannot be excluded. According to ZHANG et al. (2002) half live-times of secondary metabolites range between several hours (morphine) or even 2 days (coniferin). However, degradation processes generated by the polyphenol oxidase (PPO) or peroxidases may be accelerated by a loss of cell integrity or other stress reactions. Since no podophyllotoxin was found in the medium throughout the culture period, it was assumed to be readily degradated. In addition, a marked PPO activity was detected in the culture medium of the *Linum album* suspensions (data not presented), which could have had an impact on the loss of podophyllotoxin. Since so far no quantitative data is available concerning the stability of podophyllotoxin in a plant cell system, no assumptions can be made regarding the underlying mechanism of the initial loss of content. In order to the rate of podophyllotoxin production, it would be of importance to know whether the molecule or its glycoside is built up and decomposed simultaneously or if it is synthesised during a definite time of culture. Obviously, any approach to inhibit the catabolic processes may enhance the final yield of podophyllotoxin.

E.2 Annotations on culture stress and medium discolouration

As explained in Chapter D.1.2, the observed stress reaction by the cells was dependent on several factors and represented a severe problem for the establishment of a bioreactor system for podophyllotoxin production. Moreover, it became evident from various experiments that none of the factors alone, such as the oxygen saturation, shear stress, culture-age and the capability for podophyllotoxin production would lead to the described medium discolouration. Although the types of compounds developed during the stress reaction were not determined in this study, a literature survey revealed similar problems encountered in plant cell culture. Thus, in the current chapter different aspects of naturally occurring or *in vitro* described browning reactions are presented and correlated to the observations described for the *Linum album* (X4) culture.

Involvement of phenolics: A well-known discolouration of organic material represents the enzyme and oxygen mediated post-harvest browning of fruits and vegetables. As illustrated by MARTINEZ & WHITAKER (1995), a wide range of o-hydroxyphenols are prone to oxidation leading to the formation of brownish polymers (melanins). Furthermore, it was stated that the rate of browning is determined by a sufficient oxygen availability, and of cause, adequate substrate supply. The latter is of special interest, since it was shown that the browning intensity of post-harvest reactions concurred with an increase of PAL activity, suggesting a biosynthesis of phenolics at the site of injury. In addition, the establishment of a low oxygen atmosphere (use of nitrogen or carbon dioxide) slowed down or even prevented browning. This observation corresponds to the induction of culture medium discolouration by peak oxygen concentrations in the case of the *Linum album* (X4) system, whereas the low producing line-X4F revealed only minor PAL activities and trace amounts of podophyllotoxin. Thus, it is tempting to speculate whether an active phenylpropanoid metabolism might be a prerequisite for a sustained substrate supply.

In this context the following observations gave rise to an implication of special groups of phenylpropanoids in colour formation: yellowing of lignin-containing paper by coniferylaldehyde (DAVIDSON, et al. 1995), p-coumaric acid converted to brown products in rice (CHISHAKI & HORIGUCHI 1997) and oxidation of matairesinol derivatives as the reason for colouration of hemlock (*Tsuga heterophylla* Sarg.) sapwood (KAWAMURA, et al. 1998).

Browning of *in vitro* cultures: The culture vigour was not affected during its exposure to high oxygen concentrations *per se*, as has been proved by the elevated oxygen saturations of BR_K & BR_L (DO 60%) and BR_S & BR_T (DO 90%). It rather appeared that a prolonged growth at low oxygen saturations concomitant with shear effects, generated by the raising air bubbles, represented prerequisites for the medium discolouration upon rapid changes of the oxygen level. Though being only brief, such peak concentrations seemed to trigger the non-reversible formation of a red pigment. The accumulation of overshoot products, which can not be utilized under adverse or growth-limiting conditions, must also be taken into consideration. Hence, the observed browning of *Catharanthus roseus* cultures when transferred to a growth-limiting production medium could be attributed to a rapid change between physiological stages of the cells (SCHLATMANN, et al. 1995). As for the *Linum album* (X4) bioreactor system, one can deduce from the literature that external stimuli were involved in the browning reaction and subsequent decay of *Catharanthus roseus* cultures. Ten HOOPEN et al. (1994) suggested, that elevated shear stress may represent one factor since no adverse reactions were observed in the shake flask system. SCHLATMANN et al. (1993) further implied that these reactions are dependent on the cell line and appeared after changes of the oxygen concentration. Consistent with the *Linum*-system, comparable responses of *Catharanthus roseus* cell lines were encountered if exposed to high oxygen saturations after a prolonged period of oxygen-limited growth at a DO of 15%. Since it is known that plants can cope with complete oxygen deprivation for a period of time, it could be assumed that reactive oxygen species (e.g. the hydroxyl radical) are the cause for cell stress or the induction of any culture medium alteration after the re-increase of the oxygen concentration (SCANDALIOS 1993).

Extracellular compound accumulation: The question arises whether there is any hint for a release of phenolics into the culture medium, which may serve as substrates for the described process. Microscopic observations throughout the stress reaction revealed that pigment formation was restricted to the extracellular medium and no colouration of the cells was seen. Thus, considering the citations quoted, it could be supposed that during shear stress at low oxygen saturations distinct compounds had been released into the medium by the podophyllotoxin-producing cell line: BRUNOW, et al. (1990) - lignins; BRUYANT, et al. (1996) - proteins; FRY (1982) - peroxidase and low molecular phenolics. Though speculative, it seems possible that a putative phenolic compound accumulated during the phase of growth at low oxygen saturations in a shear intense environment of the airlift bioreactor. In fact, GODOY-HERNÁNDEZ et al. (2000) reported elevated PAL activity and an accelerated secretion of phenolics, associated with mechanical wounding. This, in turn, was also in agreement with the tolerance of line-X4F. Low enzymatic activities and reduced podophyllotoxin accumulation characteristics of this cell line (Figure D-50) suggested an active phenylpropanoid metabolism as a prerequisite for the proposed mechanism. Moreover, a directed alteration of the phenylpropanoid and monolignol-related enzymes was provided by RALPH et al. (2001) and LAPIERRE et al. (2000). They showed that plants downregulated by CAD activity resulted in the formation of unusual lignins, characterized by an elevated incorporation of hydroxycinnamyl aldehyde monolignol precursors. With respect to the red pigment, a pronounced utilization of the CAD-substrate for lignin synthesis led to a red-brown appearance of the cell wall in transgenic plants (BOUDET, et al. 1995). As in the case of the medium colouration of stressed *Linum album* cells, the authors showed a colour shift from wine-red to dark-brown during the course of reaction. Similar phenotypes have been found in naturally occurring mutants of corn and sorghum (HALPIN, et al. 1998; JORGENSON 1931), designated as “brown midrib”, which also possess an altered lignin composition.

Conclusion: Evidence has been provided for an implication of phenylpropanoids in the formation of a red or brownish compound as a response to sudden peak oxygen concentrations after growth at low oxygen concentrations in a shear intense environment. Furthermore, such conditions proved not to be restricted to the bioreactor system and could be confirmed in bubble-column-like Erlenmeyer flasks (Figure D-3 & Figure D-4). In avoidance of drastic oxygen fluctuations by the following techniques, a reliable system for the cultivation of podophyllotoxin-producing *Linum album* suspensions was established: (1) uncoupling the sampling system from the main air supply, (2) use of nitrogen instead of air for cleaning the sampling tube, (3) application of elevated aeration rates (0.44 vvm) to avoid insufficient culture mixture and the formation of stagnation zones, deprived of oxygen (SCHLATMANN, et al. 1995), (4) gradual addition of sterile deionised water for adjustment of the culture volume after sample withdrawal and (5) aeration of the 10-L flask during the inoculation process to avoid any oxygen deprivation.

E.3 Dependency of the podophyllotoxin accumulation on oxygen supply

Carbohydrates, such as sucrose and its hydrolysation products glucose and fructose, are commonly used as a carbon and energy source in plant cell *in vitro* culture. It is generally believed, that sugar catabolism by suspended plant cells under aerobic conditions is similar to the pathways used by other heterotrophic cells. Thereby, carbohydrates are taken up from the culture medium, stored as starch granules or channelled into glycolysis and following pathways for the production of energy and reduction equivalents. Unlike photosynthetically-active green plants (photolitho-autotrophic), submersed plant cells adapted to darkness, can be classified as chemoorgano-heterotrophic, which refers to their respiratory use of carbohydrates as both an electron and carbon source (instead of water and carbon dioxide). Nevertheless, under standard conditions green plants, as well as plant suspension cultures, are dependent on an adequate oxygen supply for efficient ATP production. Hence, a shortage of oxygen may affect the cells by lessening their ability to obtain metabolic energy under heterotrophic conditions (TATE & PAYNE 1991; Van der PLAS & WAGNER 1986).

Apart from the primary metabolism as an energy and carbon sink, which comprises the maintenance of cell integrity, cell growth and protein turnover (OOSTDAM & PLAS 1996), there is evidence for a dependency of plant secondary pathways on oxygen availability. Various examples have been provided where the product yield was affected by a restrained oxygen supply in the shake flask, as well as in the bioreactor system (KOBAYASHI, et al. 1989; PFEFFERLE, et al. 2000; ZHEN-GUI, et al. 1996). Although it is generally accepted that the scale-up of a suspension culture can be negatively influenced by a decreased k_La-value of the bioreactor-setup (SCHIEL & BERLIN 1987; TATE & PAYNE 1991; Ten HOOPEN, et al. 1994), there are also indications for an inferior culture performance of shake flasks regarding the oxygen transfer capacities (MCDANIEL, et al. 1965; SCRAGG 1995; VEGLIO, et al. 1998). As stated by TUNAC (1989), the oxygen supply of a shake flask is essentially dependent on a simple gas-liquid contact of a fairly small culture surface and on a limited permeability through the traditionally used cotton closures. Consequently, the presumable growth and secondary metabolite accumulation characteristics of a given culture in an optimised medium or at different inoculation densities could be obscured by oxygen limitations (VEGLIO, et al. 1998). Therefore, much can be gained from a description of the culture oxygen demand during the growth period and rather simplistic variations of the oxygen supply by increasing the shaker speed or by utilizing a higher filling volume.

E.3.1 Increase of the shaker speed

As described above, the disadvantages of shake flasks in cell culture are mainly attributed to their low mass-transfer capacities, resulting in oxygen-limited conditions, particularly during active cell growth and high culture viscosity (DRAPEAU, et al. 1986; LEITAO, et al. 1997; PÉPIN, et al. 1995). In the case of the *Linum album* suspension, initial investigations on the residual oxygen concentration in the culture broth of a 1000-mL Erlenmeyer flask (Chapter D.2.1) have revealed an interrelation of the medium-nutrient availability and oxygen concentration. Obviously, the latter parameter paralleled the culture growth until an equilibrium saturation of 40% was reached and did not re-increase before the main carbon source was spent. Although, if compared to literature, a minimum DO of 40% seemed to be adequate for the induction of secondary metabolite accumulation (SINGH & CURTIS 1994), the curves, in turn shown in Figure D-5, resemble the oxygen-transfer-rates of a oxygen-limited culture described by ANDERLEI & BÜCHS (2000). Since, in the present study, an active metabolism was still supported by a sufficient nutrient supply, it was concluded that oxygen delivery through the cotton plug or the transfer into the medium was inadequate. Therefore, it was expected that an increase of the gyratory shaker speed would result in an improved oxygen delivery and a possibly enhanced podophyllotoxin accumulation. In support of this assumption, the *Linum album* cells were incubated at 180 rpm contrary to the standard conditions of 120 rpm. Such an increase of the shaker speed had a positive effect on the k_La-values of the 1000-mL as well as on the 300-mL Erlenmeyer flasks (Table D-1) and led to a higher podophyllotoxin accumulation. It was deduced that at least part of the limited oxygen availability was attributed to a low surface to volume ratio and that the standard culture conditions seemed to be insufficient for optimal podophyllotoxin production. Although different techniques were applied by various authors for k_La-value determination, in all the investigations, an increased shaker speed aided the performance of the system in terms of mass-transfer (BAKER, et al. 1997; FREEDMAN 1970; VEGLIO, et al. 1998). Furthermore, the growth dynamics of *Linum album* cells were neither changed by an increased shaker speed, nor by the higher oxygen availability. It is presumed, that the biomass increase and final cell concentration are less vulnerable to low oxygen saturations and only extreme conditions are supposed to have a negative impact on culture growth (KATO & NAGAI 1979; SCHIEL & BERLIN 1987).

E.3.2 Variation of the culture volume inside the shake flasks

Bearing in mind that the oxygen supply in shake flasks may become a limiting factor, it was the objective of this study to provide quantitative data on the impact of an increased culture volume on the residual oxygen saturation during the culture phase. This was of major importance since a higher filling volume of the shake flasks, utilized as a pre-culture for each bioreactor run, would reduce the working effort and would speed up the whole inoculation procedure.

According to Van SUIJDAM et al. (1978) and MROTZEK et al. (2001) the mass-transfer resistance of a shake flask is mainly affected by a dense closure and the gas-liquid interface. It was demonstrated that by the use of baffled flasks the culture oxygen supply can be improved, since mixing of the culture broth in standard shake flasks is assumed to be low (ARCHAMBAULT, et al. 1996). Unfortunately, baffled flasks were not applicable to the *Linum album* culture due to excessive foam production. The data presented in Chapter D.2.3 clearly show a dependency of both the biomass formation and the podophyllotoxin accumulation on the culture volume in 300-mL Erlenmeyer flasks. Even three hours after inoculation the oxygen saturation was much lower in the case of a higher liquid volume. Most likely, this was due to a lowering of k_La-values (KOBAYASHI, et al. 1989; WILLIAMS, et al. 1996), which prevented the accumulation of podophyllotoxin in the 100 and 200-mL volume. Similar results were obtained by GOA & LEE (1992), who reported a reduced phenolics production by tobacco cells in different culture volumes. Thus, a higher oxygen supply could promote the metabolic activities in plant *in vitro* cultures and therefore enhanced the

pathways for secondary metabolite production. It is worth noting that, even though the intermediate volume of 100 mL prevented any increase of the podophyllotoxin content, the dry weight production was obviously not affected. In accordance with the previous investigation on an increased shaker speed, biomass formation proved to be less sensitive to oxygen supply. Only in the case of a 200-mL filling volume the biomass increase was distinctively slower whereas the final dry weight concentration was retained. A presumably slower primary metabolism of the 200-mL culture was also reflected by a reduced nutrient uptake from the culture medium, while the 50- and 100-mL culture showed almost no difference (Figure D-10). In conclusion, it appears that the critically DO for maintenance of an effective podophyllotoxin production should be above 40% of maximum air-saturation – a value which cannot be established in conventional Erlenmeyer flasks at higher filling volumes.

In order to gain insight into the mechanism behind the observed block of podophyllotoxin accumulation under the constrains of an oxygen deprived culture, the PAL, 4CL and CAD activities were measured during the culture time. The data presented in Figure D-12 clearly point to a reduced PAL and CAD activity in the case of a lower oxygen availability, which has also been confirmed by the results shown in Figure D-13. In view of the fact that the podophyllotoxin accumulation was drastically reduced in the high volume cultures, the question arises whether or not this could be ascribed to any alteration of the phenylpropanoid or monolignol pathway.

Even though the literature referred to a possible negative effect of hypoxia on the potential of PAL, 4CL and CAD induction (BURGESS, et al. 1999) or an implication of a greater oxygen availability after its transfer to fresh media (JONES 1984), there is still no prove for a direct enzyme and oxygen interrelation regarding to the phenylpropanoid biosynthesis. A possible explanation is given by SCHLATMANN et al. (1995) who proposed an allosteric enzyme regulation by the dissolved oxygen concentration in ajmalicine biosynthesis. Similar to the induction of podophyllotoxin accumulation at a minimum DO of approximately 40%, the data presented by SCHLATMANN et al. provided evidence for a sharp increase in ajmalicine production between 29% and 43%. Moreover, the authors found a sigmoid relationship between the DO and enzyme activity in support of the proposed allosteric regulation. In the particular case of PAL regulation SHIRSAT et al. (1981) could show an indirect control of enzyme activity by the oxygen availability. Since oxygen is a substrate for cinnamic acid-4-hydroxylase, the further metabolism of cinnamate will be blocked under oxygen-limited conditions (CREASY 1971). Consequently, the accumulation of cinnamate, which is known for its feedback regulation on PAL (BOLWELL, et al. 1986; BRINCAT, et al. 2002; GODOY-HERNÁNDEZ, et al. 2000; SARMA, et al. 1998) can lead to a delay of PAL synthesis as well as a direct inhibition of enzyme activity. These studies suggest that apart from the lack of ATP or any other compound from the primary metabolism, depletion of oxygen may exert other effects on the phenylpropanoid metabolism without necessarily affecting total protein biosynthesis.

Although an influence of the factors mentioned above can not be excluded, the data obtained in this experiment rather imply a competition between the primary and secondary metabolism in terms of carbon flux and energy charge of the cell depending on the oxygen saturation (RASI-CALDOGNO & MICHELIS 1978). Contrasting with relevant experimental data published by MORELAND (1974) and NAIR (1971), protein synthesis by *Linum album* suspensions was impaired at low oxygen saturations, which is in accordance to the notion of an overall downregulation of cell metabolic activity. In addition, it seems likely, that the carbon channelling from the shikimate towards the phenylpropanoid pathway (Figure A-5) could be lowered concurrent with a block of podophyllotoxin biosynthesis (MATSUKI 1996). Such a competition for precursors, utilized in different metabolic pathways, might also be true for the amino acid L-phenylalanine. According to CROES et al. (1995), the deficiency of a substrate (e.g. ATP or an amino acid) leads to preferential channelling of the limiting compound into primary metabolism at the expense of the secondary routes involved.

Thus, although the PAL and CAD activities were lower in the case of a restricted podophyllotoxin accumulation, it is reasonable to expect a more effective control site for its biosynthesis. The phenylpropanoid metabolism seems to be affected by the accessibility of precursors, by an overall lower metabolism and a reduced protein synthesis at low oxygen concentrations. From a purely formal point of view, in this case the distinction into a primary and secondary pathway was less pronounced and the conclusion can be drawn that a sufficiently high oxygen saturation is a prerequisite for an active phenylpropanoid metabolism and for podophyllotoxin accumulation.

E.3.3 Impact of the aeration rate

The cell transfer from a well-defined environment of an Erlenmeyer flask to the bioreactor represents the first and critical step for scaling-up to larger culture volumes. Due to distinct differences between both systems with regard to a higher shear rate, generally lower mass-transfer characteristics and an altered composition of the gas phase, the biosynthetic capacity of the cells is frequently diminished or even completely lost (GODOY-HERNÁNDEZ, et al. 2000; ZHEN-GUI, et al. 1996). This phenomenon has been exceptionally studied for the scale-up of *Catharanthus roseus* suspensions, whose ajmalicine production was drastically reduced after transfer to conventional bioreactors (SCHLATMANN, et al. 1993; TATE & PAYNE 1991). Moreover, Ten HOOPEN et al. (1994) showed a biosynthetical switch from ajmalicine to tryptamine production at low dissolved oxygen concentrations, as a hint of the intricate control of secondary metabolite biosynthesis. Since the formation of other compounds by plant cells or microorganisms such as berberine (BREULING, et al. 1985), actinorhodin (ELIBOL & MAVITUNA 1999) and glycerol (SAHOO & AGARWAL 2002) proved also to be negatively influenced by an inadequate oxygen supply inside the bioreactor, an optimisation of the culture system represents a crucial factor in aerobic biotechnological production. Contrary to the effects on secondary metabolite formation after transfer to a bioreactor, uncertainty exists about the susceptibility of the biomass accumulation (SCHIEL & BERLIN 1987; ZHEN-GUI, et al. 1996). As described by KIERAN et al. (2000) cell growth, viability and metabolic activity have, in some cases, been negatively influenced by shear forces in plant cell culture. Apart from a reduction of culture growth due to the loss of cell integrity, non-lethal effects of the rising air bubbles, such as a lower ATP production (TAKEDA, et al. 1998) or the formation of smaller aggregates (SINGH & CURTIS 1994), should also be taken into consideration. Since no information was available as to the behaviour of the *Linum album* cell suspension during a culture phase of two or three weeks inside an airlift bioreactor, a detailed study on the system and its optimisation had become necessary. Additionally, as described in the previous chapters, cultivation of these cells at decreased k_La-values signalised a dependency of the podophyllotoxin accumulation on an adequate oxygen supply. Therefore, the work reported in the present chapter was undertaken to provide data on the podophyllotoxin production characteristics and cell-physiological responses to different aeration rates. For a direct comparison of the various bioreactor-setups it will be referred to Chapter D.7, in which the mean values of the bioreactor pairs are contrasted.

In accordance with GAO & LEE (1992), an increase of the aeration rate from 0.22 vvm to 0.55 vvm (volume of air per unit volume of liquid per minute) resulted in an almost linear increase of the corresponding k_La-values (Table D-1). Although the aeration rate of 10 L min^{-1} (0.55 vvm, BR_G & BR_H) offered a maximum k_La-value of 14.11 h^{-1}, excessive foaming and wall-growth was observed under such conditions. Thus, all the other experiments were conducted at a moderate air supply of 0.44 vvm and compared to the results obtained from a 0.22 vvm value in Figure D-54. First of all, it has to be noted that neither an aeration of 0.44 vvm nor of 0.55 vvm indicated any detrimental effect on cell growth. Standard FDA-tests on the cell viability and an undisturbed biomass increase indicated a resistance of the *Linum album* cells to elevated shear stress. This was of major importance since any high-density culture, at a sufficient oxygen concentration inside the bioreactor, necessitated the application of elevated aeration rates. The results corresponded to KIERAN et al. (1997) and SCRAGG (1995), who stated that plant cells often proved to be more robust

than it was generally assumed. Moreover, besides a possible reduction of culture growth at high airflow rates (HEGARTY, et al. 1986; SMART & FOWLER 1981), a negative effect on secondary metabolite formation has been reported by LECKIE & SCRAGG (1991) and ZHONG et al. (1994). However, in the case of the *Linum album* culture no such reduction of product formation was observed. It rather appeared that the podophyllotoxin accumulation was positively correlated to an improved k_La-value concomitant with an increased aeration rate of BR_E & BR_F (0.44 vvm). Although a stimulating effect of the shear rates on the overall cell metabolism and culture growth can not be excluded (HUA, et al. 1993), it seems likely that the higher oxygen saturation has supported the respiratory pathways. This assumption is reflected by an earlier deprivation of the culture medium in terms of the carbon and macronutrient source and corresponds to the results obtained from the previously described shake flask studies.

For comparison to the experimental setups, quantification of the corresponding data is given in Table D-5. The specific growth rate provides information on how growth occurs during the exponential phase and shows only a small difference between the low (0.21 d^{-1} at 0.22 vvm) and high (0.24 d^{-1} at 0.44 vvm) aerated bioreactors. On the one hand, this might be deduced from a relatively low critical oxygen saturation for biomass production and, on the other hand, it could be a result from the time-interval of calculation. Since both bioreactor-setups revealed similar oxygen saturations during the first stage of culture (time of calculation), the biomass accumulation was less affected by the low and high aeration rate and, thus, comparable values for the specific growth rate were obtained. However, a further increase of culture viscosity and the addition of antifoam might have both contributed to the minor performance of BR_A & BR_B, as far as the growth yield value as an approximate measure of carbohydrate conversion is concerned. For this parameter, the clear difference between the bioreactor pairs could be traced back to a higher residual carbohydrate content of the culture medium after the maximum dry weight had been reached. Possibly, the low oxygen supply at an aeration rate of 0.22 vvm, between the 4^{th} and 10^{th} day of culture, might have restrained the cell metabolism, which is expressed by an extended mass doubling time.

As a result of this study, it can be concluded that the best culture conditions were reached by an ventilation rate of 0.44 vvm, whereas lower values gave improper mixing and even higher rates led to excessive foam formation and wall-growth. Unlike cell suspensions of *Catharanthus roseus*, which proved to be sensitive with respect to high aeration rates (HEGARTY, et al. 1986), the *Linum album* culture did not seem to be affected. Nevertheless, even if the podophyllotoxin accumulation was induced by an increase of the aeration rate, the maximum content of 0.6 mg g^{-1} dw was still small in comparison to the shake flask culture. Therefore, the following chapters are dealing with a further optimisation of the bioreactor culture.

E.3.4 Enhanced podophyllotoxin production by supplementation of pure oxygen

In the previous Chapter D.3.3 the feasibility of improving the oxygen saturation inside the bioreactor system by an increase of the aeration rate was discussed. The results clearly demonstrate that further alterations of the system were needed to enhance the oxygen supply for a sustained podophyllotoxin production. As to the facts given in Chapter C.2.2 & C.3.1, the utilization of a ceramic sparger for the injection of pure oxygen into the culture broth enabled non-oxygen-limited growth at high cell densities. Thereby, oxygen, as a sparingly water-soluble volatile, could be efficiently provided, owing to its importance for aerobic heterotrophic metabolism (PAYNE 1991). It has already been argued that an elevated oxygen tension inside the culture medium brings about higher levels of ATP (PRADET & RAYMOND 1983; SAGLIO, et al. 1984), which are needed for e.g. protein synthesis (RASI-CALDOGNO & MICHELIS 1978). Oxygen also provides the molecular basis for enzymatic reactions accomplished by oxygenases, which are involved in diverse secondary metabolic pathways (BUTT & LAMB 1981; HADACEK 2002). Consequently, it has been of major concern to ensure a continuous supply of adequate amounts of oxygen throughout the culture period before any other attempt was made for an increase of the podophyllotoxin production.

To meet the oxygen requirements of the culture, pure oxygen has been applied in form of small bubbles, which, according to Chapter C.3.1, gives rise to a simultaneous increase of the oxygen-concentration gradient (ΔC) and of the volume-specific gas-liquid interfacial area (a). In conclusion, it can be claimed that the main aeration system of the bioreactor was used for bulk mixing and the further incorporation of a ceramic-sparger made the adjustment of the DO at 60% possible without any additional foam formation.

Based on the considerations outlined above, the podophyllotoxin production was positively influenced when growth was performed at elevated oxygen concentrations. As shown in Figure D-54 the oxygen-supplementation in BR_K & BR_L (DO 60%, Table D-4) led to a distinct increase of podophyllotoxin accumulation. This is in agreement with previous studies pertaining to the positive correlation between an improved podophyllotoxin content of the cells and an enhanced oxygen delivery. Thus, the data presented in this study confirmed the findings by CHAO et al. (2001), LAI et al. (2001) or BREULING et al. (1985), who, respectively, found a higher bacterial cellulose, lovastatin or alkaloid yield in different kinds of bioreactors at elevated oxygen concentrations. Unfortunately, the establishment of a plateau-like maximum podophyllotoxin content, which was also apparent in BR_E & BR_F (no oxygen supplementation), could not be prevented. It rather seems as if any further factor, such as the nutrient composition of the culture medium, may determine the upper limit of podophyllotoxin accumulation.

With respect to the overall culture growth and nutrient uptake characteristics presented in Figure D-54, it can be seen that the development of the biomass concentration at a DO of 60% was similar to the non-oxygen-enriched culture. The presumably low critical oxygen saturation of a sustained cell dry weight increase proved to be met at much lower oxygen concentrations than in the case of the podophyllotoxin accumulation (see Chapter D.2.3). This fact has been discussed by PAYNE (1991), who proposed a greater oxygen affinity of cytochrome oxidases, involved in oxidative phosphorylation. In consequence, the critical DO for the provision of ATP by respiration for growth and maintenance can be assumed to be considerably lower than for secondary metabolism. Hence, in concordant with BREULING et al. (1985) and GAO & LEE (1992) only small differences can be seen between both bioreactor-setups in Table D-5 and the higher oxygen availability, mainly influenced the secondary metabolism concurrent with a faster nutrient uptake from the medium. The latter fact might be of importance in connection with the upper limit of podophyllotoxin production by the *Linum album* culture, since, apparently, the cell dry weight and podophyllotoxin accumulation ceased after the medium became depleted by its main carbon source. Therefore, further experiments were carried out to elucidate the potential of sucrose supplementation during a fed-batch culture (Chapter D.5.2). Regarding the obvious stimulation of podophyllotoxin biosynthesis at a higher oxygen tension, one may argue that the surplus of energy, provided by a triggered respiration (Van der PLAS & WAGNER 1986), could be channelled into secondary pathways such as the phenylpropanoid metabolism. However, as in the case of ajmalicine biosynthesis (SCHLATMANN, et al. 1995) the observed increase of podophyllotoxin accumulation could not be imputed to a higher PAL or CAD activity. Both enzymes (Figure D-54g & -54h) revealed a similar kinetic of activation upon inoculation of both bioreactor-setups. Only in the case of the PAL, if any, a slightly higher activity could be observed in the presence of 60% oxygen saturation. This would be in agreement with the previous argumentation concerning the results obtained by the shake flask studies (Chapter D.2.3 & E.3.2), which led to the assumption of a more effective control point for the podophyllotoxin biosynthesis with respect to the oxygen availability. Nevertheless, the following chapters will provide evidence for an implication of further culture parameter in the establishment of an increased PAL and CAD activity concomitant with a distinct improvement of podophyllotoxin production.

E.4 Adjustment of the inoculum density

Due to the prevailing discrepancy between the shake flask and the bioreactor system, in terms of the podophyllotoxin accumulation characteristics and enzyme activities, the present work was undertaken to provide evidence for the impact of the inoculation density on the *Linum album* culture performance. According to literature, the initial cell concentration has been known for its miscellaneous effects on cell growth and secondary metabolite production. That is, in case of a high initial density culture, a lower oxygen availability, a reduction of the nutrient supply on a per cell basis or the influence of a presumably shorter lag-phase. Thus, a lower inoculation density was anticipated to refine the podophyllotoxin production of the bioreactor system, in which an elevated initial cell concentration had been used (BR_A – BR_L) to diminish possible stress reactions.

Although the influence of the inoculation density has been of major concern in plant cell *in vitro* culture no definite conclusion can be drawn concerning the behaviour of each individual cell line (VILLARREAL, et al. 1997). This also holds true for the secondary metabolism, which appeared to be restricted at high initial cell densities in the case of saponin accumulation by *Panax ginseng* (AKALEZI, et al. 1999), or which proved not to be affected with regard to the ajmalicine accumulation by *Catharanthus roseus* (LEE & SHULER 2000). Furthermore, CONTIN et al. (1998) were able to prove that a higher inoculum size of the latter species favoured terpenoid production whereas lower initial cell densities led to a pronounced accumulation of indole alkaloids. Besides the effects associated with the secondary metabolism, for any biotechnological approach the final biomass yield has to be considered, which proved to be influenced by the initial cell density. Since, in general, a high biomass accumulation concomitant with an optimised secondary metabolite content, has been aimed for in plant and animal cell culture (DUTTON, et al. 1999), one could have expected the production rate to be reduced at a lower inoculation density which might have given rise to a restricted mass accumulation. Even though this assumption has been verified by some authors who encountered a decreased final dry weight concentration (AKALEZI, et al. 1999; CONTIN, et al. 1998), the absolute cell mass could be retained at a low-density inoculation of the *Linum album* (X4) suspension in this study. Thus, to clarify the distinctive characteristics of the X4 cell line, a detailed investigation, designed to the requirements of the *Linum album* bioreactor culture, was performed.

Data, descriptive of the inoculum effect, is presented in Chapter D.4, which reveals a negative correlation between a high initial cell concentration (7 g instead of 5 g per 50 mL – cell mass including adherent culture medium) and the podophyllotoxin content in shake flasks. In contrast to this fact, in the case of a low inoculum size (3 g per 50 mL), the podophyllotoxin content of the cells was comparable to the standard protocol. As a result, the presented shake flask studies confirmed the routinely used inoculation procedure of 5 g L^{-1} and, in turn, disapproved a higher initiation cell density. This observation is of importance for both systems, the shake flask as an oxygen-limited pre-culture as well as the bioreactor system, which can be characterized by a low k_La-value at an elevated culture viscosity. In accordance with the negative effects of an increased filling volume of the 300-mL flasks (Chapter D.2.3) on the podophyllotoxin accumulation, a probably lower DO at the higher initial cell density may have also contributed to the decreased podophyllotoxin accumulation. Relevant experimental data has been provided by TANAKA (2000) and MORENO et al. (1996), who claimed that a limited oxygen concentration, especially during the first days of culture, may lead to the observed differences. Thus, the relatively enhanced oxygen demand of a dense inoculated shake flask, concurrent with a restricted oxygen transfer capacity (lower k_La-value) in a viscous culture broth could have been one reason for the minor podophyllotoxin accumulation. The same might be true for the observed lower PAL and CAD activity at a high inoculation density, which is in agreement with the enzyme activities obtained from an increased filling volume of the shake flasks. Contrary to this assumption, a reduction of the initial cell density (adjustment to the standard shake flask protocol) in the bioreactor, at non-

oxygen-limited conditions, resulted in a distinctively higher podophyllotoxin accumulation, preceded by elevated enzyme activities (Figure D-55). Therefore, an improved oxygen supply at a lower inoculation density can be excluded as the sole reason for an enhanced podophyllotoxin accumulation. For a detailed analysis of the reason behind the observed enzyme induction upon low density inoculation, possible factors such as stress reactions or an improved nutrient supply have to be considered. In this context, it should be referred to the so called "dilution stress" of suspended plant cells after transfer to fresh culture medium at low densities. Although no definite explanation has been given for the noticed growth retardation or even the cell death at low initial cell densities, it can be quote from the literature, that an excessive phosphate intake (SAKANO, et al. 1995), or a drastically lowered pH of the culture medium (HASHIMOTO & AZECHI 1988), may have accounted for this fact. Though, in the present study, the culture pH of the bioreactors with a lower inoculation density (BR_M & BR_N, Table D-4) was found to be equal to the former ones, an influence of excessive phosphate uptake during the first hours after inoculation cannot be excluded. However, the initial biomass concentration utilized for BR_M & BR_N (40 g fw L^{-1}) proved to be sufficient for an almost immediate increase of the dry weight concentration. Moreover, since the final cell mass had been reached without any delay, both bioreactors exhibited a higher specific growth rate and a shorter mass doubling time concomitant with an increased growth index (Table D-5). If this could be attributed to a higher nutrient supply on a per cell basis, then the culture medium inside the bioreactor should be designated as being insufficiently concentrated for a sustained culture growth and podophyllotoxin biosynthesis (Chapter E.5.1).

Besides the stimulating effect on the culture growth both enzymes, the PAL and the CAD, exhibited a higher initial activity, which prevailed, compared to the high-density culture (Figure D-55g & -55h). As stated above, it seems unlikely that this difference can to be attributed to a greater oxygen supply. Since the shape of the presented enzyme kinetics did not change but rather showed a higher induction on the whole, it seems reasonable to expect an intensification of the process behind the enzyme activation. Similar findings have already been described as a light-independent and transient PAL induction by HAHLBROCK & WELLMANN (1973) and HAHLBROCK & SCHRÖDER (1975), even after transfer of the suspended cells into distilled water. In comparison to the present study, the authors found the inducible amount of enzyme activity to be greatly dependent on the degree of dilution and could demonstrate a loss of PAL activity by the application of transcriptional and translational inhibitors. Interestingly enough, the dilution effect was not restricted to a parsley (*Petroselinum hortense*) suspension culture but was also apparent for *Picea abies* L. or *Phaseolus vulgaris* suspensions (BEVAN & NORTHCOTE 1979; MESSNER & BOLL 1993). Apart from the PAL induction, a pronounced CAD activation after subculturing of the *Linum album* suspension at low densities has been described in this study for the first time. In view of the fact that various stress factors, such as wounding, UV-light, a low nutrient level or extreme temperatures have been known to induce enzyme activities, involved in the phenylpropanoid metabolism (DIXON & PAVIA 1995), the transfer of cells into a fresh medium represents a drastic environmental change, equivalent to a stress factor. This, according to HAHLBROCK & SCHRÖDER (1975) and JONES (1984), could be a leaching out of an, so far, unidentified compound from the cells into the medium. Nevertheless, the data presented in Chapter D.5.3, which will be discussed later, provides evidence for a reliance of PAL and CAD induction on distinct culture medium parameters, such as sucrose and phosphate.

Although only a few aspects pertaining to the stimulation of the phenylpropanoid metabolism have been assessed in this chapter, an increase of the enzyme activities and the podophyllotoxin production upon low-density-inoculation of the shake flasks as well as the bioreactor, has been presented. Thereby, the podophyllotoxin accumulation was enhanced and the final biomass retained. Due to the successful improvement of the *Linum album* culture performance by the adjustment of the initial cell density, the described inoculation procedure has been adopted for further optimisation of the culture medium as well as the cell line selection.

E.5 Implications of the culture medium on podophyllotoxin production

E.5.1 Effects of an increased nutrient supply – dynamics of podophyllotoxin accumulation

Considering the data presented in the previous chapters, one strategy for an enhanced productivity of the *Linum album* suspension culture was to raise the nutrient availability for the cells. Since the knowledge associated with the culture requirements has been limited, the following studies, related to the culture optimisation, were carried out on an empirical basis. In addition, it was assumed that a simple increase of the medium concentration would be beneficial for three reasons: First of all, the MS-medium, at standard concentration of the presumably oxygen-limited shake flask studies, yielded an equivalent podophyllotoxin content when compared to the non-oxygen-limited bioreactor studies (diluted MS-medium). Secondly, a higher podophyllotoxin production was achieved at a lower inoculation density, which inevitably ought about a 10% increase of the final medium concentration inside the bioreactor, which was thirdly concurrent with an elevated nutrient availability on a per cell basis (due to a lower cell concentration). Although an influence of the postulated dilution stress cannot be excluded (compare Chapter D.4), one may argue that the biosynthetic provision by L-phenylalanine as the ultimate substrate for the phenylpropanoid metabolism, is dependent on an adequate carbon (shikimate pathway) and nitrogen (transamination by glutamate) supply (see Figure A-5). Thus, with respect to the approximate 20% of fixed carbon, which is channelled through the shikimate pathway under normal conditions (HERRMANN 1995), an appropriate substrate supply may represent a crucial factor for a sustained podophyllotoxin biosynthesis. Furthermore, OOSTDAM et al. (1996) stated that for the carbon skeleton of one mole of coniferin (coniferyl alcohol-β-D-glucoside) about two moles of glucose are required, which favour a situation where carbohydrates do not represent a limiting factor. Finally, a sufficient nitrogen supply, though not being directly incorporated into phenylpropanoids and lignans, can be characterised as essential for the enzyme and L-phenylalanine formation. For these reasons, the work reported in the present chapter was undertaken to clarify (1) whether the early cessation of podophyllotoxin accumulation can be imputed to lack of nutrient availability (e.g. carbohydrates), (2) if a higher oxygen saturation would lead to any further culture improvement and (3) to elucidate which kind of correlation exists between the primary metabolism (culture growth) and podophyllotoxin production.

As depicted by the data presented in Chapter D.5.1 the accumulation of podophyllotoxin in BR_O & BR_P continued for two additional days if compared to the 14% less concentrated MS culture medium, which has been utilized in BR_M & BR_N (Figure D-56a). Moreover, the higher nutrient supply in the case of BR_O & BR_P resulted in a shorter mass-doubling time and an increase of the growth index (Table D-5). If the unaffected growth yield (dw-increase per consumed carbohydrates) is also taken into account, it appears that the cells have used an equivalent amount of carbohydrates when related to the ultimate biomass increase. Thus, it seems unlikely that the greater podophyllotoxin production could be attributed to either an elevated carbohydrate provision or a pronounced utilisation for secondary metabolites. However, the efficient uptake of relevant ions from the culture medium (decrease of conductivity), concomitant with a similar protein content of the cells, hints to an alternative consumption or storage of the surplus of nitrogen found in the culture medium. If these results are compared to the literature (CHISHAKI & HORIGUCHI 1997; GERSHENZON 1984; YEOMAN & YEOMAN 1996), it becomes apparent that the general view of an induction of the secondary metabolism at a low nutrient supply or culture medium exhaustion cannot be directly applied to the podophyllotoxin accumulation characteristics by *Linum album* suspension cultures. Unlike MORARD & HENRY (1998) who succeeded in enhancing the solamargine production in a half-concentrated MS culture medium, the podophyllotoxin accumulation showed a more inverse relationship. In this context it has to be mentioned that neither the carbohydrate feeding (fed-batch culture, BR_Q & BR_R) nor the application of a high oxygen saturation (DO 100%, BR_S & BR_T) aided to the podophyllotoxin accumulation of the cells.

Though an enhanced production of phenolics or of ginseng saponin, at an increased carbohydrate supply, has been described elsewhere (LUX-ENDRICH, et al. 2000; ZHANG & ZHONG 1997), in the case of the *Linum album* culture the podophyllotoxin accumulation was restrained on the expense of a prolonged and high yielding biomass formation. Nevertheless, since the approach of an intermittent carbohydrate feeding strategy may interfere with other metabolic processes during culture growth one has also to think about an earlier supplementation of the sucrose-solution or the lack of any further medium compounds in a non-carbohydrate limited culture (ZHANG & ZHONG 1997).

Apart from the absolute values of secondary metabolites accumulated by the cells, biotechnologists have been especially concerned about the kinetics of product formation during the culture period. Since, with respect to the timing, the production of a chosen compound is generally related to the biomass formation, any approach for a culture improvement should consider both, the primary as well as the secondary metabolism. In this context, a possible interrelation of the podophyllotoxin production with an active primary metabolism, represented by the culture growth and protein formation, will be discussed in the next section.

At first sight the pattern of podophyllotoxin accumulation by *Linum album* suspensions corresponds to a time of pronounced biomass increase (Figure D-56a & -56b). Moreover, with respect to BR_A – BR_O, it seems to be positively correlated with a high value of the specific growth rate and short mass doubling time (Table D-5). Thus, in accordance with KADKADE (1982) and CHATTOPADHYAY (2002), who characterised the podophyllotoxin production of *Podophyllum peltatum* and *Podophyllum hexandrum* cultures respectively, a growth-associated production by the *Linum album* suspensions could also be assumed. However, a direct relationship between secondary metabolite production and culture growth has been rarely described and could be depicted only for a few examples, e.g. anthocyanins, negatively correlated to cell division (SAKUTA, et al. 1994), and rosmarinic acid, positively correlated to cell division (RAZZAQUE & ELLIS 1977). Furthermore, pertaining to the described storage of starch granules by the *Linum album* suspensions, one should be careful about relating the formation of any compound to the increase of biomass (SU 1995).

In conclusion, the following passage implies an accumulation characteristic by *Linum album* suspensions different from that found in *Podophyllum* cultures. It became evident that the kinetics of podophyllotoxin formation were strongly dependent on the culture conditions as well as on the cell line (compare Chapter D-6). In view of the fact that the biomass increase can be uncoupled from podophyllotoxin accumulation at low oxygen saturations or by carbohydrate feeding (BR_Q & BR_R) and on account of a distinct increase of product formation at a retarded culture growth (see Table D-5, BR_U & BR_V and BR_W & BR_X), it is tempting to expect another regulatory factor to be involved. Although the vast majority of results published in the literature, proposed an elevated metabolite accumulation at slow culture growth or during the stationary phase (KNOBLOCH & BERLIN 1980; MEI, et al. 1996; TATICEK, et al. 1991), no conclusive inference can be drawn for the *Linum album* culture. Due to the described induction of podophyllotoxin accumulation by different culture conditions concomitant with a lower or higher specific growth rate, the podophyllotoxin biosynthesis seems not to be dependent, either directly or inversely, on the growth rate. This is in agreement with studies which confirmed a growth independent diosgenin biosynthesis (DRAPEAU, et al. 1986), a variable pattern of lignan formation in different culture media (PÁSKA, et al. 1999) and a pronounced effect after transfer from the shake flask to the bioreactor system on the characteristics of ajmalicine accumulation (KNOBLOCH & BERLIN 1980).

E.5.2 Effects of an increased nutrient supply – phenylpropanoid metabolism activity

Due to the fact that protein biosynthesis may interfere with the availability of L-phenylalanine as the ultimate substrate for PAL reaction, it is reasonable to consider a relationship between the phenylpropanoid metabolism and protein formation. A survey of the literature reveals that merely in a few cases a lower concentration of phenylpropanoid derivatives could be related to a reduced PAL activity (JANAS, et al. 2000; WHETTEN & SEDEROFF 1995). Thus, as reviewed by ANTEROLA et al. (2002), JONES (1984) and MARGNA (1977), the concept of PAL as the rate limiting control point for phenylpropanoid biosynthesis (DIXON, et al. 1996; HISAMINATO, et al. 2001), conflicts with the lack of quantitative correspondence. It seems as if PAL represents an enzyme whose catalytic potential, though influenced by various factors, remains sufficient under physiological conditions. Moreover, L-phenylalanine usually accumulates only in low intracellular concentrations as it is readily metabolised for (1) protein synthesis, (2) conversion to cinnamate, or (3) in some cases, acts as a precursor for aromatic alkaloid synthesis. Therefore, it can be argued, that L-phenylalanine might represent a limiting factor for the carbon channelling into the phenylpropanoid metabolism (KNOBLOCH & BERLIN 1980; MARGNA 1977; RÜHMANN, et al. 2001). Since for cell maintenance protein biosynthesis is of paramount importance, L-phenylalanine will be consumed preferentially by the primary metabolism, which dictates the portion of substrate available for PAL activity. If this argumentation is applied to the data presented in the previous chapters, it becomes apparent that, after a lag-phase of several days, the induction of podophyllotoxin accumulation coincided with the time when the maximum protein concentration had already been reached.

The observation described above was not restricted to the shake flask culture but could also be proven by bioreactor studies. Moreover, the second peak of PAL activity, though irregular resolved in the case of the *Linum album* suspensions (approx. third day of culture, Figure D-12 & -14 & -32), was also reported for parsley (HAHLBROCK 1976), fungal infected potato (FRITZEMEIER, et al. 1987) and bean suspension cultures (BEVAN & NORTHCOTE 1979). It has been argued if naphthylacetic acid (MS-medium constituent) or depletion by culture medium nutrients might be involved in a two-phased PAL induction. However, the results obtained from *Linum album* are indicative for a relationship between termination of protein synthesis and an alternative L-phenylalanine utilization. Thus, dynamics of protein accumulation seemed to influence the second induction of PAL as well as the onset of podophyllotoxin biosynthesis. Thereby L-phenylalanine becomes available for PAL reaction, not only because of the lower sink strength, but also as a result of protein degradation (MATSUKI 1996). For that reason it has to be tested whether, as indicated by KAKEGAWA et al. (1995) and HATTORI et al. (1999) for other plant species, podophyllotoxin accumulation by *Linum album* cultures can be enhanced by the addition of L-phenylalanine into the culture medium.

According to the questions raised at the beginning of this chapter, it can be concluded that: (a) though an adequate energy provision can be viewed as being indispensable for cell survival, it turned out that feeding of sucrose or elevated oxygen concentrations did not improve the yield of podophyllotoxin. Furthermore, kinetics of product formation were (b) highly influenced by the culture conditions, appeared to be (c) independent of the growth rate and seem to be (c) correlated with the L-phenylalanine supply. Provided that the phenylpropanoid metabolism is not crucial for culture maintenance, its activity will be low under conditions where the substrate supply confines metabolite formation. Further indications have been suggested for a competition between two biochemical pathways for one common precursor. Since a comparable maximum protein concentration was formed by the cultures even at different conditions, it can be speculated whether a surplus of L-phenylalanine would yield a higher amount of podophyllotoxin. These results are in agreement with ANTEROLA et al. (2002), who found the carbon supply for phenylpropanoid metabolism in *Pinus taeda* suspensions to be controlled in terms of L-phenylalanine availability. In addition, neither the enzymatic steps catalysed by PAL, nor by 4CL and CAD proved to be truly rate-limiting but were rather regulated according to the carbon flux through the phenylpropanoid and monolignol pathway.

E.5.3 Modification of the inorganic nitrogen composition

In terrestrial plants the phenylpropanoid metabolism represents a major sink for assimilated carbon, which is mainly converted to lignin. Thus, it has been estimated that 30-45% of plant organic matter is primarily derived from L-phenylalanine, whose putative importance for podophyllotoxin accumulation in *Linum album* suspensions has been clearly stated in the previous chapter. Apart from the competition for L-phenylalanine in protein biosynthesis, accumulation of secondary metabolites and lignin formation, one has also to consider equimolar amounts of ammonium as being liberated by PAL reaction. Pertaining to the low nitrogen availability and its importance for plant growth, RAZAL et al. (1996) had provided evidence for a nitrogen recycling associated with the phenylpropanoid metabolism. Thereby, the ammonium, which is being released at the entry point of the phenylpropanoid metabolism, is incorporated into the amide nitrogen of glutamine via glutamine synthetase and becomes available for further conversions. However, since no lignin could have been detected in the *Linum album* cultures at all, the fraction of L-phenylalanine consumed by the phenylpropanoid metabolism can be supposed to be much lower here than for protein biosynthesis. Moreover, unlike field-grown plants, suspension cultures do not have to compete for nitrogen in the soil with abiotic and biotic processes such as erosion, leaching and microbial consumption.

In culture media nitrogen is mostly provided in form of inorganic ions, such as ammonium and nitrate, which are consumed by different mechanisms and proved to be key factors for the activity of the secondary metabolism (ERTOLA, et al. 1995; HIRASUNA, et al. 1991). Interestingly enough, the nitrogen source of culture media, utilized for the experiments with *Linum album*, did not seem to be insufficient, since substantial amounts of nitrate were released, presumably by cell lysis at the end of the culture period (see Figure D-2, Figure D-11, Figure D-34). It seems likely that some of the nitrate had been stored inside the vacuole and remained in its oxidised form, not being reduced by the nitrate reductase. A similar biphasic uptake and storage of nitrate has been suggested by FERRARI et al. (1973), JACKSON et al. (1973) and ROSE et al. (1972), who draw the conclusion of a reducible metabolic pool (cytoplasm) and a storage pool (vacuole), in which the latter was not readily accessible for enzymatic reduction to ammonium. Nevertheless, although the *Linum album* cells obviously preferred the ammonium-ion to nitrogen provision, the utilization of nitrate for protein biosynthesis did not seem to be restricted, since considerable amounts of protein were accumulated during cultivation in a modified MS-medium with nitrate as the sole nitrogen source (med._b, Figure D-40). These results are in accordance with the general view of the nitrate- (NR) and nitrite reductase (NiR) induction by carbohydrates, NAD(P)H or nitrate itself in the absence of an adequate irradiation for light mediated control of enzymatic activity, which represents a typical feature of nitrate utilization by roots (BECKER, et al. 1992; CRAWFORD 1995; LILLO 1994).

Except of the importance of ammonium and nitrate for the primary metabolism, an induction or repression of secondary metabolite formation in plant cell suspensions has often been attributed to the quantitative as well as qualitative inorganic nitrogen composition of culture media. Since both ions do not only interfere in cell growth but have also been proven to exhibit diverse effects on secondary metabolite formation (HIRASUNA, et al. 1991), usually the modification of the nutrient composition has to take an adequate nitrogen supply into consideration. It is typical of such approaches, that the influence of a revised culture medium can vary drastically, regarding to the metabolite production, depending on the plant species and inherent cell line characteristics.
For instance, ZHONG & WANG (1998) were able to show that a high nitrate to ammonium ratio or even nitrate as the sole inorganic nitrogen source favoured metabolite formation by suspension cultures of *Panax quinquefolium* L. In turn, a culture medium in which nitrogen was entirely supplied by nitrate did not yield positive results in the case of triterpenoid saponin production by *Saponaria officinalis* L. suspensions (FULCHERI, et al. 1998). It rather appeared that only by a simultaneous supply of nitrate and ammonium the culture performance could be improved, a fact which has frequently been described in plant cell *in vitro* culture.

Furthermore, apart from the nutritional components provided by the culture medium, another factor, with respect to the release of ammonium by the PAL reaction, has been discussed by HATTORI et al. (1999). The authors argued that an induction of PAL, concomitant with the unexpected repression of veratryl alcohol biosynthesis by the ligninolytic fungus *Phanerochaete chrysosporium*, after the addition of L-phenylalanine could be attributed to a liberation of ammonium by PAL-catalysed deamination. As a consequence, in the case of the *Linum album* suspension, the optimisation of the culture medium in terms of the inorganic nitrogen supply was rather empirical. Only the reappearance of excess nitrate in the culture medium during the late growth phase and prolonged enzyme activities in the case of ammonium as the sole inorganic nitrogen source indicated a positive effect on podophyllotoxin accumulation by the improved MS-medium.

Obviously, as shown in Figure D-46, the higher ammonium to nitrate ratio of the improved culture medium caused a prevailing low pH during the first growth period. This acidification has generally been attributed to the mechanism of ammonium uptake, which is accompanied by about equimolar proton production, due to an increased H^+-ATPase activity (Von WIRÉN, et al. 2000). However, the reason behind toxic effects of ammonium at low nitrate concentrations or the lack of any counter-ion (e.g. carboxylate anions of organic acids) for pH-control is still not fully understood. Apart from a possible growth retarding effect of a low pH (MATSUMOTO, et al. 1971; MORARD & HENRY 1998), which was also apparent in the case of the *Linum album* suspension (Figure D-56b), additional implications of the ammonium and nitrate-ion in physiological processes should to be taken into consideration. Therefore, it has been discussed whether a high ammonium concentration might lead to a repression of cation uptake or whether the lack of nitrate, which represents an important osmoticum and signal for gene expression, can give rise to an imbalance of the intracellular hormone level (BLIGNY, et al. 1997; Von WIRÉN, et al. 2000). In consequence, no definite explanation can be given for the positive effect of the improved culture medium on podophyllotoxin accumulation. This is due to uncertainty about the cellular implications of inorganic nitrogen supply, putative additional ammonium sources from PAL reaction, amino acid catabolism, photorespiration and a fade of ammonia (NH_3) by free diffusion (LANG & KAISER 1994; RAVEN, et al. 1993; SCHNEIDER, et al. 1996). Typical of suspended cells, the *Linum album* suspensions preferentially took up the reduced and energetically more efficient ammonium-ion when both inorganic nitrogen forms were present. Furthermore, a distinctively higher mass doubling time and lower specific growth rate (Table D-5) might have supported an enhanced podophyllotoxin production, probably because of a greater L-phenylalanine availability (compare Chapter E.5.1). A similar inverse relationship between retarded growth and accelerated biosynthesis of phenolic compounds in young leaves of apple trees has been demonstrated by RÜHMANN et al. (2001). The authors argued that a slow leave growth, caused by a reduced nitrogen supply, might have led to a pronounced hydroxycinnamic acid formation and thereby, could have added to a well established defence system against pathogens.

E.5.4 Comparison of the *Linum album* system with *Podophyllum* cultures

Considering the available knowledge, the data obtained in these studies should now be contrasted with similar approaches, published elsewhere. Since, as presented in previous chapters, a reliable bioreactor system for the *Linum album* X4-cell line was set up and has been further improved on the basis of nutritional aspects, the system may serve as a point of reference for comparable *in vitro* cultures. To this day, only a few reports are available, dealing with the establishment of an alternative source for sustained podophyllotoxin production, independent of the natural plants habitat. Due to the limited number of plant species and *in vitro* cultures which accumulate the desired compound in relevant amounts, scientists have mainly focused on *Podophyllum hexandrum* and *Podophyllum peltatum* cultures for biotechnological purposes. Not only did the *in vitro* cultures of both species reveal poor growth characteristics but they unfortunately also exhibited a drastically reduced biosynthetic capacity (GIRI & NARASU 2000; Van UDEN 1993). In view of the fact that, generally, a high-producing cell suspension can only be obtained from a well established callus, KADKADE (1982) has provided evidence for the impact of the parent plant material and for a positive effect of L-phenylalanine provision by the culture medium. Although the author found a maximum podophyllotoxin content of 0.7% (on dw basis), which surpassed the values obtained from the X4-cell line (0.4% on dw, if applied to the improved culture medium), one also has to take into account the ultimate production values. However, no quantitative data on biomass formation has been provided. Due to the fact that an eight-week-old callus was used for lignan extraction, inferior growth characteristics can be assumed. Besides, a more recent study published by GIRI et al. (2001) aimed for a higher podophyllotoxin accumulation by *Agrobacterium*-mediated transformation of *Podophyllum hexandrum* embryos, which were further used for initiation of callus and suspension cultures. Nevertheless, the highest accumulation rates also did not exceed a value of 0.7% – no quantitative data on biomass formation was given.

More elaborated studies on the production of podophyllotoxin have been provided by CHATTOPADHYAY, who reported the establishment and optimisation of *Podophyllum hexandrum* cultures in a 3-L-bioreactor (CHATTOPADHYAY, et al. 2001; CHATTOPADHYAY, et al. 2002). The data revealed a pronounced lag-phase of at least one week for both the biomass as well as the podophyllotoxin accumulation. In addition, contrary to the *Linum album* system (compare Chapter E.5.1), the authors postulated a rather growth-associated process with respect to the metabolite formation. Further parameters descriptive of the culture performance, such as the growth yield (0.15 by *P. hexandrum*, 0.44 by *L. album*), the growth index (2.25 by *P. hexandrum*, 7.03 by *L. album*) and the specific growth rate (0.14 d^{-1} by *P. hexandrum*, 0.24 d^{-1} by *L. album*) confirmed the superior characteristics of the *Linum album* bioreactor culture. However, a comparison of the specific growth rate with other well-established suspension cultures, such as *Catharanthus roseus* (0.25 d^{-1}), *Hyoscyamus muticus* (0.23 d^{-1}), *Berberis wilsonae* (0.15 d^{-1}) or *Nicotiana tabacum* (0.64 d^{-1}) characterizes the *Linum album* X4 cell line as a typical cell suspension (BREULING, et al. 1985; GAO & LEE 1992; SINGH & CURTIS 1994). In conclusion, one ought to refer to an improved productivity of the *Podophyllum hexandrum* culture in an optimised medium by CHATTOPADHYAY et al. (2003). It was shown that the adjustment of the phosphate level, concomitant with a reduced inorganic nitrogen concentration, led to a maximum podophyllotoxin production of 4.9 mg L^{-1}. Nevertheless, this was still ten-times lower than the value of 53.0 mg L^{-1}, which was attained in BR_W & BR_X by using the improved culture medium (line X4). Finally, it should be noted that even higher values were reached by another potent cell suspension (line-A, Chapter E.6) and that closely related podophyllotoxin derivatives were found in concentrations as high as 4% on dw in hairy root cultures of *Linum album* at our institute.

E.6 Selection of a high-producing cell line

Although not mentioned before, line selection represents one of the traditional and most effective approaches for an optimisation of secondary metabolite production in both cell *in vitro* culture and agricultural plantation. Due to the heterogeneity of a plant species even under the same growth conditions, with respect to molecular or biochemical aspects, it is reasonable to pre-select potent individuals for the establishment of a high-producing plant line (COLLIVER, et al. 2002; VERPOORTE, et al. 2002). However, the screening of a heterogeneous population for variant cell clones, containing the highest levels of the required and not visibly observable compound can be an intricate challenge. In the case of the various *Linum album* cultures, which have been established at our institute (PETERSEN & ALFERMANN 2001), cell line selection was mainly based on HPLC-techniques, rather than enzymatically characterization or visual assessment. Only the intensive red histochemical phloroglucinol/HCl staining (Figure D-48), which was predominantly intracellularly located and has neither been perceived for line-X4, nor for line-X4F, led us to the assumption of an active phenylpropanoid metabolism. Even though the phloroglucinol/HCl reagent has been known for its wide range of responses to various aromatic or aliphatic aldehydes (CLIFFORD 1974), it has been widely used, due to its characteristic red colour on cinnamaldehydes and lignins (DAVIDSON, et al. 1995; EBERHARDT, et al. 1993).

With respect to earlier results pertaining to a possible interrelation of precursor availability and podophyllotoxin accumulation, the high and long-lasting PAL, 4CL and CAD activity (Figure D-50 & -57) was in good alignment with a regulation of these enzymes according to the metabolic demand (ANTEROLA, et al. 2002). Moreover, similar to the X4-line, the accumulation of podophyllotoxin was induced at a time when the protein concentration was receding (Figure D-51 & -52). However, line-A proved to be sensitive to a high ammonium/nitrate ratio of the improved medium, which might have been imputed to a lower ability to utilize nitrate under a low pH of the culture medium after ammonium consumption (OGAWA, et al. 1996). As already implied in Chapter D.6.2, it can be speculated whether the reduced fresh weight of line-A correlated with a minor uptake of osmotically active nitrate which led to a lesser intracellular water content. This view is supported by a high residual conductivity and low culture medium pH in the case of BR_U & BR_V, but needs to be proven by further quantification of the definite inorganic nitrogen content. All facts considered, it should be noted that even if the standard MS-medium had been used for cultivation of line-A suspensions without any further modifications, the culture yielded a podophyllotoxin production as high as 130 mg L^{-1}, which encourages an elaborate improvement of culture growth and production characteristics.

F. SUMMARY

The research presented in this study was carried out to establish and improve an airlift bioreactor system for the supply of podophyllotoxin (ptox) by *Linum album* Ky. (Linaceae) suspension cultures. Since the foremost interest was not restricted to maintaining but rather to increasing the rate of production, the experiments traced for an elucidation of decisive factors involved in the control of ptox accumulation. Therefore, data was provided for inherent characteristics of the shake flask as well as the bioreactor system, which had a distinctive influence on culture dynamics by means of the inoculation procedure, shear effects and the oxygen supply. Moreover, a sufficiently high dissolved oxygen saturation (DO) of the culture broth proved to be a prerequisite for ptox accumulation, which has been further enhanced by various nutritional and cell-line-interrelated aspects. In summary, the following statements reveal how the adaptation of growth conditions and the selection of high-producing cell lines have contributed to a definite enhancement of the ptox content and production values (data on dry weight basis). Additionally, the airlift bioreactor system has been portrayed in terms of its potentiality for a further system improvement:

■ **system characteristics**

Essential variables, such as the culture growth, ptox formation and the oxygen transfer rates were examined for the shake flask and the bioreactor system. The results feature the shake flasks as valuable tools for strain development, evaluation of basic culture parameter and screening for rate-limiting factors, which can be performed by extended parallel experiments. However, the data revealed an inferior oxygen delivery especially under the constraints of a high density culture. Thereby, any other approach for culture improvement might get masked by a lack of oxygen.

As far as the airlift bioreactor is concerned, it became possible to adjust the oxygen saturation to the culture's requirements. Due to excessive foam formation the aeration rate was restricted to moderate values and a separate ceramic-sparger was incorporated into the system for pure oxygen supplementation. Thus, the constraints of a low mass transfer were avoided and both, the culture mixing and oxygen provision could have been regulated independently. Nevertheless, the circulation capacity proved to be insufficient at a higher cell load, which will probably be circumvented by the application of a stirred-tank bioreactor.

■ **cell stress**

When the cells were transferred to the bioreactor system, problems were encountered regarding a discolouration of the medium followed by a culture decay. It has been proven that a cell line with an active phenylpropanoid metabolism, incubated in a shear environment at low oxygen concentrations, gave rise to an extracellular red compound upon a rapid increase of the oxygen saturation. Similarly adverse reactions have never been observed in standard shake flasks before and were avoided by ensuring a sufficiently high and stable oxygen saturation during inoculation and culture growth.

■ **shake flask culture**

1.6 mg ptox g^{-1}
25.0 mg ptox L^{-1}

Preliminary experiments on the production dynamics in shake flasks revealed a transient accumulation of ptox with a minimum DO of 40%, measured directly under the liquid surface. Maximum ptox yields were found around the 10^{th} day of culture and the main inorganic nutrients (NH_4^+, NO_3^-, PO_4^{3-}) were taken up by the cells at

different rates. NO_3^- proved to be incompletely metabolised, as indicated by its reappearance in the medium at the onset of cell lysis. Additionally, evidence for a positive relationship between the oxygen saturation and ptox yield was provided. Thereby, it was shown that in oxygen deprived cultures ptox formation can be completely blocked, whereas only a minor reduction of PAL, 4CL and CAD activity (enzymes involved in the phenylpropanoid pathway) was recognized.

■ **aeration rate**

0.6 mg ptox g^{-1}
7.0 mg ptox L^{-1}

In alignment with the shake flask culture, the induction of ptox accumulation upon improved oxygen delivery could be verified by elevated aeration rates of the bioreactor system. However, increased shear rates necessitated the exchange of polypropylene glycol with a silicone-emulsion for foam control. As indicated by low ptox-values, a DO of 22% still appeared to be insufficient.

■ **oxygen supplemention**

1.0 mg ptox g^{-1}
10.0 mg ptox L^{-1}

As stated above, the implementation of a ceramic-sparger for pure oxygen injection guaranteed a high and stable DO at 60%. Even though an improved ptox content of the cells and better production rates were achieved, it became apparent that the values found in shake flasks could not be restored and that additional factors needed to be optimised.

■ **inoculation density**

1.6 mg ptox g^{-1}
20.0 mg ptox L^{-1}

Distinct differences between both systems, in relation to the initial cell density and overall nutrient supply, led to the assumption of an imperfect inoculation procedure. A reduction of the initial cell density (60 g fw L^{-1}) to approximate levels which were also used for the shake flask studies (40 - 50 g fw L^{-1}) led to a further increase of ptox at a DO of 60%. Such results could be imputed to a higher inoculation stress (enzyme induction) or to an advanced nutrient availability. Vice versa, shake flask studies at an elevated inoculation density gave proof of lower enzyme activities and of a minor ptox content.

■ **medium concentration**

2.5 mg ptox g^{-1}
30.0 mg ptox L^{-1}

Since not only the cell mass of 7-days old culture flasks but also some spent medium was used for the inoculation of the bioreactor, the readjustment of the overall nutrient concentration to the standard MS-medium additionally aided the ptox production (low inoculation density, DO 60%). In this case, however, no altered enzyme activities were observed.

■ **improved medium**

4.0 mg ptox g^{-1}
50.0 mg ptox L^{-1}

By modifications of the culture medium (reduced inorganic nitrogen content, higher ammonium to nitrate ratio) the cells indeed showed retarded growth and a marked increase of ptox accumulation (low inoculation density, adjusted culture medium concentration, DO 60%). Moreover, a drastic reduction of the medium nitrate content, after high-temperature sterilization in the presence of sucrose, has to be taken into account for nutrient formulation.

■ **cell line selection**

10.0 mg ptox g^{-1}
130.0 mg ptox L^{-1}

Screening of various *Linum album* cell suspensions yielded line-A, which offered a superior production capacity if compared to the previously utilized line-X4. A high ptox accumulation was paralleled by a prolonged PAL, 4CL and CAD activity. Unfortunately, due to a drastic culture medium acidification, the cells could not be applied to the improved medium, but offer a potential system for further production enhancement.

As illustrated in the preceding chapters, a suitable bioreactor system for the supply of ptox by *Linum album* suspension cultures has been established successfully and has yielded the highest *in vitro* production of ptox reported so far. Data descriptive of the accumulation dynamics and phenylpropanoid pathway activity, characterized the ptox formation as not being interrelated with the culture growth, but presumably as being controlled by precursor availability. Besides, an enhanced ptox production could have been positively correlated with an increased or prolonged PAL, 4CL and CAD activity in several cases. Thus, the phenylpropanoid metabolism appeared to be strongly influenced by the culture conditions and supposed carbon throughput. However, pertaining to the distinctive induction of ptox accumulation at a sufficiently high oxygen saturation, other decisive control sites should be expected.

In conclusion, the proven feasibility of the *Linum album* system might enable further research by biochemical as well as molecular approaches, since the product yield needs to be enhanced for commercial application. It should be noted that the endangered natural resources and the drawbacks of *Podophyllum*-plantation provide compelling arguments in favour of expanded research and scale-up of the ptox production by *Linum album* cell suspensions, when coupled with a high interest in semisynthetic ptox-derivatives for the treatment of cancer.

G. LIST OF INSTRUMENTS & COMPONENTS

INSTRUMENTS

Autoclaves	
Webeco, Type No.5	Webeco, Lüdenscheid, Germany
Varioclav®, Type 500E	H+P, Labortech., Oberschleißheim, Germany
Bioreactor system	
Airlift bioreactor	University in-house manufactured
Mass flow meter, 5850TR	Westphal, Ottobrunn, Germany
Polarographic electrode	Mettler Toledo, Gießen, Germany
Micro-optode, MICROX system	PreSens GmbH, Regensburg, Germany
Water bath, RMT 6-DS Lauda	Dr. Wobser, Lauda-Königshofen, Germany
Pump, Baun FE211	B. Braun, Melsungen, Germany
Control device, Infors HT®	Infors GmbH, Einsbach, Germany
Centrifuges	
Eppendorf, 5415	Eppendorf, Wesseling-Berzdorf, Germany
Eppendorf, 5804 R	Eppendorf, Wesseling-Berzdorf, Germany
Sorvall Superspeed, RC-5B / Rotor SS34	Kendro Laborating, Langenselbold, Germany
HPLC-system	
Waters 996 photodiode array detector	Waters GmbH, Eschborn, Germany
Waters 510 gradient pump	Waters GmbH, Eschborn, Germany
HPLC-column, GROM-SIL 120 C_{18}, 5 µm	GROM, Herrenberg, Germany
HPLC-pre-column, GROM-SIL 120 C_{18}, 5 µm	GROM, Herrenberg, Germany
Freeze-dryer	
Freeze-dryer, Alpha 1-4	Martin Christ GmbH, Osterode, Germany
Vaccum pump, Pfeiffer	Pfeiffer, Asslar, Germany
AAS, Type 2280 / Analyst 100	Perkin-Elmer, Überlingen, Germany
Ceramic sparger, CeraMICS	BIM GmbH, Gießen, Germany
Conductivity electrode, LF91 (KLE 1/T)	WTW, Weilheim, Germany
Deionised water system, E-Pure D4632	Barnstead, Leverkusen, Germany
pH electrode	Mettler Toledo, Gießen, Germany
Refractometer	Krüss, Hamburg, Germany
Rotary shaker, G53	New Brunswick Scientific, New Jersy, USA
Spectrophotometer, Uvicon 930	Kontron Instruments, Tegimenta, Suisse
Sterile filter, KLEENPACK 2PV2	Pall, Dreieich, Germany
Ultra sonic bath, Sonorex super RK 103H	Bandelin electronics, Berlin, Germany
Ultra Turrax, T25B	IKA Labortechnik, Staufen, Germany
Vaccum pump, CVC2	Vaccumbrand GmbH, Wertheim, Germany

COMPONENTS

Acetone, GR for analysis	Merck KgaA, Darmstadt, Germany
Acetonitrile, HPLC gradient grade	J.T. Baker, Deventer, Netherlands
Adenosine triphosphate	Biomol Feinchem., Hamburg, Germany
Ammonium molybdate (IV), p.a.	Carl Roth GmbH, Karlsruhe, Germany
Ammonium nitrate, GR for analysis	Merck KgaA, Darmstadt, Germany
Boric acid, p.a.	Carl Roth GmbH, Karlsruhe, Germany
Calcium, Titrisol® standard	Merck KgaA, Darmstadt, Germany
Calcium chloride dihydrate, Ph Eur	Merck KgaA, Darmstadt, Germany
Cellulose acetates filter, 0.2 µm	Satoriums AG, Göttingen, Germany
Cobalt(II) chloride hexahydrate, p.a.	Ferak, Berlin, Germany
Coenzyme A, Grade II, free acid	Boehringer, Mannheim, GmbH, Germany
Coniferyl alcohol, purum	Fluka Chemie GmbH, Buchs, Suisse
Coomassie® Brilliant-blue, for electrophoresis	Merck KgaA, Darmstadt, Germany
Copper(II) sulfate pentahydrate, p.a.	Fluka Chemie GmbH, Buchs, Suisse
1,4-Dithiothreitol (DTT), p.a.	Carl Roth GmbH, Karlsruhe, Germany
Ethanol ablsolute, GR for analysis	Merck KgaA, Darmstadt, Germany
Iron(II) sulfate heptahydrate, GR for analysis	Merck KgaA, Darmstadt, Germany
β-Glucosidase from almonds, >1000 U/mg	Serva, Hamburg, Germany
Glycine, p.a.	Carl Roth GmbH, Karlsruhe, Germany
Hydrazine sulfate, GR for analysis	Merck KgaA, Darmstadt, Germany
Hydrochloric acid 36-38%, baker analysed	J.T. Baker, Deventer, Netherlands
myo-Inositol, for biochemistry	Merck KgaA, Darmstadt, Germany
Iron, Titrisol® standard	Merck KgaA, Darmstadt, Germany
Magnesium sulfate heptahydrate, GR for analysis	Merck KgaA, Darmstadt, Germany
Manganese, Titrisol® standard	Merck KgaA, Darmstadt, Germany
Manganese(II) sulfate, monohydr., GR for analysis	Merck KgaA, Darmstadt, Germany
D(-)-Mannitol, for determination of boric acid	Merck KgaA, Darmstadt, Germany
$NADP^+$, for biochemistry 98-99%	Biomol Feinchem., Hamburg, Germany
Naphthylacetic acid, for biochemistry	Merck KgaA, Darmstadt, Germany
Naphthyldiamine, p.a.	Merck KgaA, Darmstadt, Germany
Methanol, GR for analysis	Merck KgaA, Darmstadt, Germany
Nicotinic acid, for biochemistry	Merck KgaA, Darmstadt, Germany
Nitrogen, 5.0	Messer Griesheim, Oberhausen, Germany
Oxygen, technical grade	Messer Griesheim, Oberhausen, Germany
Phenol, baker analysed	J.T. Baker, Deventer, Netherlands
o-Phosphoric acid, baker analysed	J.T. Baker, Deventer, Netherlands
Photo-Rex®	Carl Roth GmbH, Karlsruhe, Germany
(-)-Podophyllotoxin, HPLC	Carl Roth GmbH, Karlsruhe, Germany
Polypropylene glycol, for GC	Merck KgaA, Darmstadt, Germany
Potassium, Titrisol® standard	Merck KgaA, Darmstadt, Germany

Potassium iodide, GR for analysis	Merck KgaA, Darmstadt, Germany
Potassium nitrate, GR for analysis	Merck KgaA, Darmstadt, Germany
Potassium dihydrogen phosphate, GR for analysis	Merck KgaA, Darmstadt, Germany
di-Potassium hydrogen phosphate, GR for analysis	Merck KgaA, Darmstadt, Germany
Pyridoxine hydrochloride, for biochemistry	Merck KgaA, Darmstadt, Germany
Silicone antifoam, Antifoam A	Sigma-Aldrich, Steinheim, Germany
Sodium chloride, crystal, baker analysed	J.T. Baker, Deventer, Netherlands
Sodium hypochloride (12%), techn. grade	in-house chemical supply
Sodium hydroxide, pellets, GR for analysis	Merck KgaA, Darmstadt, Germany
Sodium molybdate dihydrate, GR for analysis	Merck KgaA, Darmstadt, Germany
Sodium nitroprusside	Analar, Essex, England
Sodium sulfite, GR for analysis	Merck KgaA, Darmstadt, Germany
Sucrose (“Kölner Raffinade Zucker”)	Pfeifer & Langen, Köln, Germany
Sulfanilamide, GR for analysis	Merck KgaA, Darmstadt, Germany
Sulfuric acid, baker analysed	J.T. Baker, Deventer, Netherlands
Thiamine chloride hydrochloride, for biochemistry	Merck KgaA, Darmstadt, Germany
Titriplex III (Na_2-EDTA), GR for analysis	Merck KgaA, Darmstadt, Germany
Tris (TRIZMA BASE), reagent grade	Sigma-Aldrich Chemie GmbH, Steinheim
Zinc sulfate heptahydrate, GR for analysis	Merck KgaA, Darmstadt, Germany

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