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Ph.D. Thesis

Large scale screening of microalgae and cyanobacteria for a presence of bioactive compounds

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I declare, that I developed my dissertation by myself according to my own result and with listed literatures.

Prohlašuji, že jsem disertační práci vypracovala samostatně, na základě vlastních výsledků a za pomoci uvedené literatury.

Bieleszka Petra

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ABSTRAKT

Tato práce je zaměřena na stanovení přítomnosti inhibitorů trypsinu, elastázy a allelopatických látek ve 182 kmenech řas a sinic. Součástí studie je také určení optimálních růstových podmínek a sledování produkce aktivních látek během kultivace třech vybraných kmenů *Nostoc*, jejichž extrakty vykazují inhibici trypsinu. Pro získání těchto látek jsme vyvinuli izolační a separační metody. V závěru jsme se také pokusili objasnit jejich chemickou strukturu vysoce účinnou kapalinovou chromatografií ve spojení s hmotnostní detekcí (HPLC/MS).

ABSTRACT

This thesis is focused on the large scale screening of 182 algal and cyanbacterial strains as a source of trypsin and elastase inhibitors, as well as, allelopathic active compounds. Further, we studied three trypsin inhibitory active strains of cyanobacterial genus *Nostoc*. We studied their optimal growth parameters, maximal production of active compounds during the cultivation, isolation and separation methods as well. Finally, we tried to determine the chemical structures of the active compounds by high performance liquid chromatography with the connection to mass detector (HPLC/MS).

1. INTRODUCTION

Algae and cyanobateria are autotrophic organisms which can be found in almost every conceivable habitat. Their broad occurrence supposes presence of very interesting compounds. They are well known for their potential to produce secondary metabolites with a wide variety of bioactivities (Carmichael, 1992; Patterson, 1994), but they have received much attention as rich sources of novel bioactive compounds applicable to the production of medicines and agricultural chemicals. The systematic screening for presence of antitumour, antiviral, antibiotic, antifungal, imunosuppressive and enzyme inhibitors compounds is still in progress all over the world.

The aim of this thesis is to determine protease inhibitors, especially trypsin and elastase. The main role of these inhibitors is to protect organisms against excessive activity of proteases. Protease inhibitor dysfunction has been implicated in thrombosis, emphysema, cirrhosis, immune hypersensitivity, glomerulonephritis, pancreatitis, mental disorders, encephalomyelitis and in diseases characterised by connective and other tissue self-destruction. The other interesting studied compounds were allelopathic compounds, which are involved in inter- or intra- species competition. For this purpose the large scale screening of 182 algal and cyanobacterial strains were carried out.

Afterward, we focused on three trypsin inhibitory active strains of cyanobacterial genus Nostoc. We studied their optimal growth parameters, maximal production of active compounds during the cultivation, optimal isolation and separation methods. In addition, we tried to determine the chemical structures of active compounds. These targets were achieved by using of various biotechnological methods and analytical techniques, especially high performance liquid chromatography with the connection to mass detector (HPLC/MS), ion chromatography (IC) and visible absorption spectroscopy.

1.1 CYANOBACTERIA

Cyanobacteria (Greek: *cyanos* = blue) are a phylum of bacteria that obtain their energy through photosynthesis. They are often referred to as blue-green algae. They get their name from the bluish pigment phycocyanin, which they use to capture light for photosynthesis.

They also contain chlorophyll a, the same photosynthetic pigment that plants use. Other common forms are red or pink from the pigment phycoerythrin.

Cyanobacteria could be found in diverse habitats in: fresh water, sea, soil dessert, rock, trunk of tree, hot spring, etc. Fossil traces of cyanobacteria are claimed to have been found from around 3.8 billion years ago. The oxygen atmosphere was generated by numerous cyanobacteria during the Archaean and Proterozoic Eras. Many Proterozoic oil deposits are attributed to the activity of cyanobacteria. The other great contribution of the cyanobacteria is the origin of plants.

Relative to nutrition cyanobacteria are divided in three types: facultative chemoheterotrophs, or those organisms capable of growing in the dark on an organic carbon source and of growing phototrophically in the light; obligate phototrophs, or organisms that can grow only in the light on an ionorganic medium; photoheterotrophs, or those cells that are able to use organic compounds as a source of carbon in the light but not in the dark.

The cyanobacteria include unicellular, colonial, and filamentous forms, with the most advanced type of morphological structure attained being the multiseriate branched filament. The Cyanophyceae can conveniently be divided into five orders: Chroococcales, Chamaesiphonales, Pleurocapsales, Nostocales and Stigonematales. In this thesis the most studied.

This thesis is focused on terrestrial and benthic Nostoc species from order Nostocales, which are filamentous without true branching. The occurence of heterocysts and akinetes are very common for genera such as Nostoc, Anabaena and Aulosira.

Additionaly, cyanobacteria are very important organisms for the health and growth of many plants. They are one of very few groups of organisms that can convert inert atmospheric nitrogen into an organic form, such as nitrate or ammonia. Cyanobacteria also form symbiotic relationships with many fungi, forming complex symbiotic "organisms" known as lichens. Some of them, especially Spirulina has long been valued as a food source and in tropical countries. The popularity of Spirulina is primarily as a "health food", being sold in stores as a dried powder or in tablet form. Many other species of cyanobacteria produce populations, which are toxic to humans and animals.

1.2 BIOACTIVE COMPOUNDS PRODUCE BY CYANOBACTERIA

Certain cell metabolites, generally produced during the stacionary growth phase of microbial culture, are called secondary metabolites (Schlegel, 1985). They constitute some of

the most important biotechnological products for mankind. A multitude of substances are involved, including organic acids, carbohydrates, aminoacids and peptides, vitamins, growth substances, antibiotics, enzymes and toxic compound. Bioactive chemicals of this nature lead to carry out research on the relevant molecules, their structures, in vitro synthesis and modes of action.

Cyanobacteria are primary producers and they can cope with high and low temperatures, suboptimal and supraoptimal light intensities, low availability of essential nutrients and other resources. Possessing a multitude of physiological, biochemical and molecular strategies provides cyanobacteria with biosynthetic pathways resulting in a richness of complex organic molecules belonging to the category of bioactive chemicals.

The search for bioactive chemicals from cyanobacteria is becoming steadily more promising and they are recognized as a rich source of compounds with pharmacologically as well as structurally interesting secondary metabolites.

1.2.1 Cyanotoxin

Cyanobacterial toxins are secondary metabolites which do have harmful effects on cells, tissues and organisms (Carmichael, 1992). They can be classified as hepatotoxins (e.g.microcystins, nodularin and cylindrospermopsin) and neurotoxins (e.g. anatoxin-a, anatoxin a(s) and aphantoxin).

1.2.1.1 Hepatotoxic cyanopeptides

Cyanobacterial peptides (cyanopeptides) are among the most ubiquitously found potentially hazardous substances in surface waters used by humans. Though these substances are natural in origin, eutrophication (i.e. excessive loading with fertilising nutrients) has caused massive cyanobacterial proliferation. Thus, cyanopeptides now occur with unnatural frequency and concentration.

Globally the most frequently found cyanobacterial toxins in blooms from fresh and brackish waters are the cyclic hepatotoxic peptides of the microcystin and nodularin family. They pose a major challenge for the production of safe drinking water from surface waters containing cyanobacteria with these toxins. The cyclic peptides contain either five (nodularins) or seven (microcystins) amino acids, with the two terminal amino acids of the linear peptide being condensed (joined) to form a cyclic compound.

The target organ range in mammals is the liver. In aquatic environments, these toxins usually remain contained within the cyanobacterial cells and are only released in substantial amounts on cell lysis. Along with their high chemical stability and their water solubility, this containment has important implications for their environmental persistence and exposure to humans in surface water bodies.

At the present time, it is known that microcystins are produced by bloom forming species of *Microcystis, Anabaena, Oscillatoria (Planktothrix),* and *Nostoc*, by a species of *Anabaenopsis* and by a soil isolate of *Haphalosiphon hibernicus.* Nodularins have been found only in *Nodularia spumigena.* Further species may yet be demonstrated to produce microcystin.

1.2.1.2 Neurotoxic alkaloids

Mass occurrences of neurotoxic cyanobacteria caused animal poisonings. Three families of cyanobacterial neurotoxins are known (1) anatoxin-a and homoanatoxin-a, which mimic the effect of acetyl choline, (2) anatoxin-a(S), which is an anticholinesterase and (3) saxitoxins, also known as paralytic shellfish poisons (PSPs), which block nerve cell sodium channels.

Anatoxin-a has been found in *Anabaena, Oscillatoria* and *Aphanizomenon,* homoanatoxin-a from *Oscillatoria,* anatoxin-a(S) from *Anabaena,* and saxitoxins from *Aphanizomenon, Anabaena, Lyngbya* and *Cylindrospermopsis.*

Fig 1: General features of the cyanotoxins

Toxin group	**Primary target organ in mammals**	**Cyanobacterial genera**
Cyclic peptides		
Microcystins	Liver	*Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis*
Nodularin	Liver	*Nodularia*
Alkaloids		
Anatoxin-a	Nerve synapse	*Anabaena, Planktothrix (Oscillatoria), Aphanizomenon*
Anatoxin-a(S)	Nerve synapse	*Anabaena*
Aplysiatoxins	Skin	*Lyngbya, Schizothrix, Planktothrix (Oscillatoria)*
Cylindrospermopsins	Liver	*Cylindrospermopsis, Aphanizomenon, Umezakia*
Lyngbyatoxin-a	Skin, gastro-intestinal tract	*Lyngbya*
Saxitoxins	Nerve axons	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*
***Lipopolysaccharides* (LPS)**	Potential irritant; affects any exposed tissue	All

1.2.2 Allochemicals and akin substances

The substances, can only be classified as allelochemicals (1) if they are excreted from living cells under natural conditions in biologically active concentrations, (2) if they are stable enough to affect plants and microorganisms, and (3) if their presence/renewal in nature is demonstrated (Inderjit and Dakshini, 1994). The presence of substantial concentrations of extracellular toxins may be related either to continuous liberation from the intracellular pools as a result of cell death and lysis during the stationary phase or to the active excretion as a defence against predatory zooplankton and/or competitors for nutrients. Furthermore a possible residual production during biosynthetic activities has been discussed (modified after Negri et al., 1997).

Cyanobacterial allelochemicals show much structural variety and include cyclic peptides, alkaloids, organic acids, and long-chain fatty acids. Almost majority of allelochemicals are regarded as secondary metabolites (Whittaker & Feeney, 1971; Rice, 1984; Keating, 1997). Extracellular metabolites have been suggested to be one factor contributing to the formation and/or maintenance of cyanobacterial blooms (Keating 1977, 1978), and inhibiting effect on bacteria and phytoplancton (Keating 1977, 1978, 1999; Pushparaj, 1999).

Some of cyanobacterial toxins (saxitoxin, anatoxin-a, microcystin and nodularin) also have allelopathic effects. For instance, anatoxin-a and microcystin-LR produced by *Anabaena flos-aquae Brebisson* cause paralysis of the motile green alga *Chlamydomonas Ehrenberg* (Kearns & Hunter, 2001) and microcystin-LR extracted from Microcystis aeruginosa Kutzing inhibits photosynthesis and induces loss of nitrogenase activity in *Nostoc muscorum C. Agardh* and *Anabaena Bory BT1* (Singh, 2001).

1.2.3 Other bioactive compounds

To date, high number of cyanobacterial metabolites has been isolated and characterized from cultured strains. So far, more than 600 peptides or peptidic metabolites have been described from various taxa (Welker&Döohner, 2005). Most compounds have been isolated from *Oscillatoriales* and *Nostocales*. A major part of cyanobacterial secondary metabolites are peptides or possess peptidic substructures. The majority of these oligopeptides are

assumed to be synhesized by non-ribosomal peptide synthetase or polyketide synthase (Schmidt 2005). Based on the molecular structure can be classified (Welker&Dőohner, 2005):

- Aeruginosins (*Microcystis, Planktothrix, Nodularia*)
- Microginins (*Microcystis, Planktothrix, Nostoc*)
- Anabaenopeptins (*Anabaena, Aphanizomenon,Microcystis, Planktotrix*)
- Cyanopeptolins (*Anabaena, Lyngbya, Microcystis*)
- Microsystins,nodularins (*Microcystis, Planktothrix, Nodularia, Anabaena, Hapalosiphon*)
- Microvoridins (*Microcystis, Planktothrix, Nostoc)*
- Cyclamides (*Microcystis, Nostoc,Oscillatoria, Stigonema, Westelliopsis)*
- Cryptophycins (*Nostoc)*

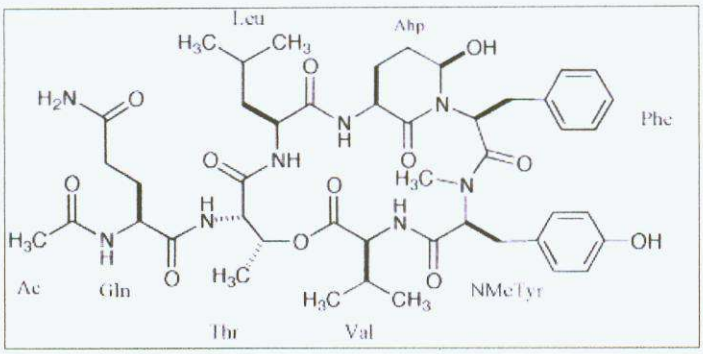

Fig. 2: Example of the cyanobacterial peptide structure Nostopeptin BN 920 (Ploutno, 2002)

These bioactive substances exhibited various antibiotic, antifungal, antiviral, antineoplastic, imuunosuppressive and enzyme inhibitors properties that have potential for biotechnology. Therefore screening programmes for these bioactivities are underway. For example, cryptophycins, which are antitumour agents from cyanobacteria (Golakoti et al. 1995) that inhibit microtubule dynamics during mitosis, were discovered during screening tests for antibacterial activity of *Nostoc spp.* Both the structure and the synthetic pathway of cyanobacterin have been described (Jong et al. 1984), following the discovery that this substance, which is produced by *Scytonema hofmanni C. Agardh*, shows both algicidal and antibacterial properties (Mason, Gleason 1981). *Nostoc spp.*, on the other hand, produces nostocyclamide, a cyclic hexapeptide (Juttner et al. 2001), and muscoride A, an oxazole alkaloid peptide (Nagatsu et al. 1995). Some cyanobacteria, such as *Nostoc*, secrete antibiotics called bacteriocins that kill related strains of the alga. Other cyanobacteria secrete antibiotics that are active against a wide range of cyanobacterial and eukaryotic algae.

1.3. PROTEASE INHIBITORS

1.3.1 Protease

Protease refers to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Proteases differ in their ability to hydrolyze various peptide bonds. Each type of protease has a specific kind of peptide bonds it breaks.

Proteases are essential for the synthesis of all proteins, controlling protein composition, size, shape, turnover and ultimate destruction. In medicine, proteases represent important potential targets for medical intervention because of their important regulatory roles in life.

In the environment, proteases are key regulators of the life of insects and other agricultural pests, key regulators of growth and health of farm animals, and principal regulators of plants and marine food sources. Research into these relatively under-studied proteases has the potential to contribute spectacularly to the economy by improving plant and animal health through enhanced growth and treatment/prevention of parasite infections, crop protection through new herbicides and pesticides, and increased or faster production of food resources.

Some human proteases (kallikreins, metalloproteases, cathepsins) have been identified as important prognostic indicators of diseases. A number of other proteases are experimental vaccines in current development to fight infectious diseases caused by parasite and viral infections (diseases like malaria, schistosomiasis, and Dengue fever). Proteases associated with toxins such as *Clostridium tetani* (tetanus toxin) and *Bacillus anthracis* (anthrax toxin) are also being investigated as possible vaccines (Puente 2003).

1.3.1.1 Classification of proteases

Proteases are divided into two broad categories on the basis of type of attack on the protein: they are exo- and -endo. Proteinases or endopeptidases attack inside the protein to produce large peptides. Peptidases or exopeptidases attack ends or fragments of protein to produce small peptides and amino acids.

These two groups are further sub-divided on basis of the mechanism of action at the active site (Rawlings, 1993). Thus proteinases are divided into 4 groups: serine, cysteine,

ıspartic and metallo proteinases. The peptidases are classified on the action pattern thus ıminopeptidase cleaves amino acids from the amino end: carboxypeptidase cleaves amino ıcids from the carboxyl end dipeptidyl peptidase cleaves two amino acids; dipeptidase splits a lipeptide and tripeptidase cleaves an amino acid from a tripeptide.

The serine proteinases comprise two distinct families. The chymotrypsin family ncludes the mammalian enzymes such as chymotrypsin, trypsin, elastase or kallikrein and the ıubstilisin family which includes the bacterial enzymes such as subtilisin. The general 3D ıtructure is different in the two families but they have the same active site geometry and then :atalysis proceeds via the same mechanism. The serine proteinases exhibit different substrate ıpecificities which are related to amino acid substitutions in the various enzymes interacting with the substrate residues.

The cysteine proteinases includes the plant proteases such as papain, actinidin or bromelain, several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated) as well as several parasitic proteases (e.g Trypanosoma, Schistosoma). Papain is the archetype and the best studied member of the family.

Most of *aspartic proteinases* belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin and chymosin as well as lysosomal cathepsins D and processing enzymes such as renin, and certain fungal proteases (penicillopepsin, rhizopuspepsin, endothiapepsin). A second family comprises viral proteinases such as the protease from the AIDS virus (HIV) also called retropepsin.

The *metallo proteinases* may be one of the older classes of proteinases and are found in bacteria, fungi as well as in higher organisms. They differ widely in their sequences and their structures but the great majority of enzymes contain a zinc atom which is catalytically active.

1.3.2 Protease inhibitors

A molecule that can prevent the function of a protease is known as an inhibitor. Many inhibitors directly interfere with the function of a protease by binding in its active site crevice, denying access by normal peptide substrates. Some naturally occurring inhibitors bind to the outside surface of a protease and either mask the active site or influence the shape of the protease so that peptide substrates cannot enter the active site.

Inhibitors can block the regulatory functions of proteases in conception, birth, developmental biology, digestion, growth, maturation, ageing, diseases and death of all organisms. Genetic or environmental conditions can result in an over- or under- abundance of proteases or of their natural inhibitors/activators, leading to abnormal physiology and disease.

Small proteases inhibitors molecules are very effective medicines, and are currently available in man for treating HIV/AIDS, stroke and coronary infarction; high blood pressure, Alzheimer's disease, arthritis, osteoporosis, inflammatory syndromes, cancers and diabetes. Other protease inhibitors are being developed to treat parasitic, fungal, and viral infections.

Inhibition of proteolytic activity is also employed for the prevention of unwanted degradation of proteins during their isolation and characterization; and to study the regulatory aspects of specific proteolytic events as they relate to cellular processes.

1.3.2.1 Serine proteinase inhibitors

The irreversibility of proteinase inhibition achieved by the serine proteinase inhibitor has made them the principal inhibitors controlling both intra- and extracellular proteolytic pathways. Serine proteinase inhibitors regulate such diverse physiological processes as coagulation, fibrinolysis, complement activation, angiogenesis, apoptosis, inflammation, neoplasia and viral pathogenesis. However, their precise mechanism of action in many of these processes remains controversial. The primary function of most members of the serine proteinase inhibitor family is to neutralise overexpressed serine proteinase activity (Travis, 1983). Serine proteinase inhibitor dysfunction has been implicated in thrombosis, emphysema, cirrhosis, immune hypersensitivity, mental disorders and in diseases characterised by connective and other tissue self-destruction (Stein, 1995).

The occurrence of serine proteinase inhibitors in plants has been known since 1938 and they are extremely widespread in their distribution throughout the plant kingdom (Vogel, 1968; Dechary, 1977; Ryan, 1973). Several macromolecular inhibitors have been isolated from yeast (Bidlingmeyer, 1972; Saheki, 1974), *Neurospora crassa* (Tschesche, 1974), *Aspergillus sojae* (Lakowski, 1971) and various smaller peptide inhibitors from the *Actinomycetes* (Ryan, 1973, Suda, 1972).

Cyanobacteria are becoming famous for the various peptide protease inhibitors, which have been isolated and identified in cultures during the last years (Borowitzka, 1999). Aeruginosins are linear peptides isolated from *Microcystis aeruginosa* with inhibitory activity on thrombin, plastin and trypsin respectively (Murakami et al., 1995). Also the cyclic peptides

of cyanobacteria – microcystins and nodularins – are well known for their proteinase inhibitor effects. Several unusual depsipeptides such as micropeptin (Reshef, 2006), microcystilide, cyanopeptolin, oscilapeptin and nostocyclin, can be mentioned in this connection (Weckesser et al., 1996). Various proteinase inhibitory peptides (nostopeptin, nostoginin, banyascyclamide, banyasin, banyaside) were isolated from the hydrophilic extract of a natural bloom of the cyanobacterium *Nostoc sp.* (Pluoutno, 2002).

In nature, such compounds threaten aquatic animals that feed on cyanobacterial cells. Of particular interest are the effects on the microcrustaceans *Daphnia spp.*, which are among the grazers of planktonic cyanobacteria. The lethal molting disruption of *Daphnia spp.* causing Microviridin J, a newly discovered protease inhibitor produced by *Microcystis* strain UWOCC MRC (Jaspars, 1998; Rohrlack, 1999, 2004). Recent findings showed that cyanobacterial metabolites isolated from *Planktothrix* inhibit the digestive enzyme trypsin and it can result in the death of the *Daphnia* (Rohrlack, 2005).

1.4 ALLELOPATHY

Allelopathy in aquatic environments may provide a competitive advantage to angiosperms, algae, or cyanobacteria in their interaction with other primary producers. Allelopathy can influence the competition between different photoautotrophs for resources and change the succession of species, for example, in phytoplankton communities.

Mass developments of certain species of cyanobacteria are frequently observed in mesoand eutrophic water bodies, owing to morphological, physiological and biochemical mechanisms. The allelopathy is one example which may explain the frequent dominance of cyanobacteria over other algal groups. Other reasons favoring cyanobacteria are their tolerance against extreme physical and chemical conditions and the ability for fixation of molecular nitrogen under simultaneous oxygenic photosynthesis.

Allelochemicals produced by algae can influence other algae in their close vicinity, their own growth potential (autotoxicity), associated microorganisms (e.g. epiphytes), and nearby higher plants and animals (Inderjit 1994). Aquatic allelochemicals often target multiple physiological processes. The inhibition of photosynthesis of competing primary producers seems to be a frequent mode of action. Multiple biotic and abiotic factors determine the strength of allelopathic interactions.

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The chemical nature of relevant microalgal metabolites is reported as mainly organic acids or phenolic compounds (Ahluwalia, 1998). Phenols and brominated phenolics agents produces by some algae are water soluble and acting as potent allelochemics. The growth inhibition caused by *Chlamydomonas reinhardtii* on other microalgae is due to a long chain fatty acid (McCraken *et al.*, 1980). Acrylic acid is identified as the antibacterial and antifungal agent of the chrysophyte flagellate *Phaeocystis poucheti* (Sieburth, 1960). An active principle in extracts from selected strains of *Chlorella*, *Euglena* and *Spirulina* has been found to be the plant hormone jasmonic acid (Ueda *et al.*, 1991). In cultures of several species of blue-green and green algae the presence of cytokinin and gibberllin is demonstrated (Ördög & Pulz, 1996; Stirk *et al.*, 1999). Many other active principles in these extracts have so far not been characterized.

1.5. SCREENING FOR BIOACTIVE COMPOUNDS

A screen is an assay or biological assay that provides a tool that can be used to test for or establish the presence and level of a target activity in a specific sample. Bioassays in a screening program should be rapid, simple to conduct, relevant, capable of being automated, cost effective, and of the potential to deliver high throughput. Appropriate technology should be used to permit low limits of detection. Screens should also be specific for the molecular or cellular therapeutic target of choice. Appropriate additional discriminatory tests outside of the focus of the chosen target activity, such as cytotoxicity measurement for cell-based assays, or isotype specificity tests for molecular assays, are valuable in that they provide additional information relative to the overall value of a potential hit.

Direct and indirect methods can be used for the screening of useful biomolecules. Direct assays aim at detection of a specific target product while indirect assays measure the biological activity of the desired product.

1.5.1. Direct assays

Rapid advances in the development of analytical methods and micro-instrumentation allow rapid, selective and highly sensitive analytical procedures for the detection of metabolic products of cyanobacteria. Instruments such as high performance chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS) and nuclear magnetic resonance spectrometry (NMR) are widely employed in the screening.

1.5.2. Indirect assays

Indirect assays measure the biological activity of the desired product. Bio-assays are continuously being developed for the selection of organisms exhibiting antibiotic, antitumor activity and enzyme inhibitory activity. Screening for antitumor activity is carried out with cultures of tumor cells. Screening for antibiotic activities is performed with pathogenic microorganisms. Screening for enzyme inhibitory activity is performed by enzymatic reaction in microtitre plate, when are measured colorimetric changes during this reaction.

1.6. CULTIVATION OF AUTOTROPHIC ORGANISMS

Various bioreactors may be used for cultivation of microorganisms for the production of biological products, study of their physiological characteristics and screening of organisms. They are needed to guarantee a controlled environment with respect to temperature; chemical environment, e.g. pH and nutrients. The stirring of a bioreactor brings the dispersion of air in the nutrient solution; the homogenization to equalize the temperature and the concentration of nutrients throughout the bioreactor; suspension of microorganisms and solid nutrients; and dispersion of immiscible liquids. The cultivation can be carried out in batch, fed-batch or continuous mode.

1.6.1 Batch cultivation

The batch cultivation can be considered to be a closed system. At time zero the nutrient solution in the bioreactor is inoculated with microorganisms and incubation is allowed to proceed. In the course of the entire cultivation, nothing is added, except air with CO_2 (in case of autotrophic microorganisms), an antifoam agent, and acid or base to control the pH. The composition of the culture medium, the biomass concentration, and the metabolite concentration generally change constantly as a result of the metabolism of the cells. The growth of microorganisms can be characterized by four typical phases (Fig.3):

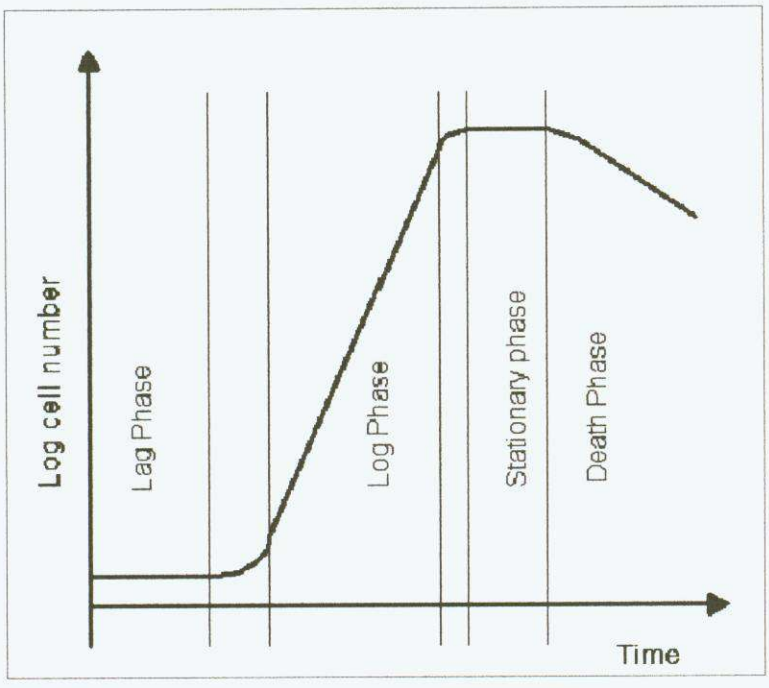


Fig.3: Growth curve of a bacterial culture.

Lag phase, is physicochemical equilibration between microorganism and the environment following inoculation with very little growth.

Log phase, is observed by the end of the lag phase when the cells have adapted to the new conditions of growth. Growth of the cell mass can now be described quantitatively as a doubling of cell number per unit time for bacteria and yeast's, or a doubling of biomass per unit time for filamentous organisms as fungi. By plotting the number of cells or biomass against time on a semilogarithmic graph, a straight line results, hence the term log phase. Although the cells alter the medium through uptake of substrates and excretion of metabolic products, the growth rate remains constant during the log phase. Growth rate is independent of substrate concentration as long as excess substrate is present.

As soon as the substrate is metabolized or toxic substances have been formed, growth slows down or is completely stopped. The biomass increases only gradually or remains constant during this stationary phase, although the composition of the cells may change. Due to lysis, new substrates are released which then may serve as energy sources for the slow growth of survivors. The various metabolites formed in the stationary phase are often of great biotechnological interest.

In death phase the energy reserves of the cells are exhausted. A straight line may be obtained when a semilogarithmic plot is made of survivors versus time, indicating that the cells are dying at an exponential rate. The length of time between the stationary phase and the

death phase is dependent on the microorganism and the process used. The cultivation is usually interrupted at the end of the log phase or before the death phase begins.

1.6.2 Fed batch cultivation

In the conventional batch process just described, all of the substrate is added at the beginning of the fermentation. An enhancement of the closed batch process is the fed-batch cultivation. In the fed-batch process, substrate is added in increments as the cultivation progresses. In the fed-batch method the critical elements of the nutrient solution are added in small concentrations at the beginning of the fermentation and these substances continue to be added in small doses during the production phase.

1.6.3 Continuous cultivation

In continuous cultivation, an open system is set up. Sterile nutrient solution is added to the bioreactor continuously and an equivalent amount of converted nutrient solution with microorganisms is simultaneously taken out of the system. In the case of a homogeneously mixed bioreactor we refer to a *chemostat* or a *turbidistat*. In the chemostat in the steady state, cell growth is controlled by adjusting the concentration of one substrate. In the turbidistat, cell growth is kept constant by using turbidity to monitor the biomass concentration and the rate of feed of nutrient solution is appropriately adjusted.

1.6.4 Nutriens

All microorganisms need for their microbial activity the presence of several nutrients. The autotrophs encompass those organisms that use carbon dioxide as the principal source of cellular carbon. These organisms rely on light and/or the oxidation of inorganic compounds for the generation of metabolic energy; their energy metabolism can thus be classified as photolithotrophic or chemolithotrophic. Since several of the organisms that carry out one of these two types of energy metabolism can also grow at the expense of organic energy sources (facultative autotrophs) or grow better when small amounts of organic carbon compounds are present in otherwise inorganic growth media (mixotrophs).

Mineral nutrients required by microorganisms are species dependent but consists generally of Fe, K, Mg, Mn. Sometimes S, N, Ca, Co, Cu, P, Zn is required.

A wide range of inorganic and organic nitrogen compounds can be utilized to satisfy the requirement for this element. Most phototrophs take up nitrate as the nitrogen source. For biosynthesis, the anion must be reduced. Some cyanobacteria are capable of fixing the nitrogen by heterocysts. Inorganic phosphate is extensively used as a pH buffer and is often added in excess of the growth requirement to fulfil this role. Most microrganisms can utilize sulphate to satisfy the sulphur requirement. Since much sulphur is present in proteins in the form of sulphur-containing amino acids, the sulphate taken up from the environment must be reduced.

1.7 ANALYTICAL TECHNIQUES

In order to discover new bioactive compounds, crude extracts are evaluated by chemical, biological and pharmaceutical screening approaches. Due to the recent increase of the sensitivity of mass spectroscopy (MS), nuclear magnetic resonance (NMR) instruments, and the rapidly growing chemical databases (AntiBase, DNP, CA), allows the dereplication of known compounds and their structure determination. Presently the screening is conducted in combination with high performance liquid chromatography (HPLC), ultraviolet (UV), HPLC-DAAD, HPLC-CD, HPLC-MS, HPLC-NMR-MS or GC-MS systems.

1.7.1. Liquid Chromatography

Chromatography is defined as an analytical technique whereby a mixture of chemicals may be separated by virtue of their differential affinities for two immiscible phases. One of these, the stationary phase, consists of a fixed bed of small particles with a large surface area, while the other, the mobile phase or "eluent", is a fluid that moves constantly through, or over the surface of, the fixed phase. Chromatographic systems achieve their ability to separate mixtures of chemicals by selectively retarding the passage of some compounds through the stationary phase while permitting others to move more freely. Therefore, the chromatogram may be evaluated qualitatively by determining the R_T, or retardation factor, for each of the eluted substances.

Retardation of the solutes by the stationary phase may be achieved by one or a combination of mechanisms. Certain substances, such as alumina or silica gel, interact with the solutes primarily by adsorption, either physical adsoption, in which the binding forces are

weak and easily reversible, or chemisorption, where strong bonding to the surface can occur. Another important mechanism of retardation is partition, which occurs when the solute dissolves in the stationary phase, usually a liquid coated as a thin layer on the surface of an inert particle or chemically bonded to it.

If the liquid phase is a polar substance (e.g. polyethylene glycol) and the mobile phase is nonpolar, the process is termed *normal-phase chromatography*. When the stationary phase is nonpolar (e.g. octadecylsilane) and the mobile phase is polar, the process is *reverse-phase chromatography*.

For the separation of mixtures of ionic species, insoluble polymers called ion exchangers are used as the stationary phase. Ions of the solutes contained in the mobile phase are adsorbed onto the surface of the ion exchanger while at the same time displacing an electrically equivalent amount of less strongly bound ions in order to maintain the electroneutrality of both phases.

The chromatographic separation of mixtures of large molecules such as proteins may be accomplished by a mechanism called exclusion, chromatography or gel chromatography. The stationary phases used are highly cross-linked polymers that have imbibed a sufficient amount of solvent to form a gel. The separation is based on the physical size of the solutes; those that are too large to fit within the interstices of the gel are eluted rapidly, while the smaller molecules follow an irregular path through the pores of the gel and are eluted later. In any chromatographic separation, more than one of the above mechanisms may be occurring simultaneously.

Chromatographic separations may also be characterized according to the type of instrumentation or apparatus used. The types of chromatography that may be used are column, paper, thin-layer, gas and high-performance liquid chromatography. The most often use technique for analysis of bioactive compounds is high-performance liquid chromatography.

In this thesis high-performance liquid chromatography with connection to mass spectrometer was used for the identification, as well as preparative high-performance liquid chromatography for the isolation of active compounds. In addition the ion chromatography was used for study of the biomass growth.

7.1.1 High performance liquid chromatography

In this type of analysis the mobile phase is a liquid that is pumped at moderately high essures through a narrow-bore column. Interaction of the solutes with the stationary phases :curs by adsorption, partition, ion exchange, and exclusion. The reverse-phase partition ıromatography is used extensively because its selectivity for solutes can be adjusted over a ide range by varying the polarity of the mobile phase.

The columns usually used for analytical separations have internal diameters ranging 'om 2 to 4 mm and lengths from 25 to 100 cm. The bonded material is a long-chain nonpolar ubstance (e.g. octadecylsilyl).

The types of detectors most frequently used in HPLC are spectrophotometric, luorometric, and refractometric detectors. The spectrophotometric detectors are fixed- or 'ariable-wave-length photometers that operate in the ultraviolet and visible portions of the ;pectrum. The most commonly employed detector of this type is diode array detector, which .s a sensitive for organic molecules that absorb light within the UV or visible wavelength ;pektrum.

1.7.1.2 Ion chromatography

Ion chromatography is a form of liquid chromatography that uses ion-exchange resins to separate atomic or molecular ions based on their interaction with the resin. Its greatest utility is for analysis of anions for which there are no other rapid analytical methods. It is also commonly used for cations and biochemical species such as amino acids and proteins.

The column packings for ion chromatography consist of ion-exchange resins bonded to inert polymeric particles. For cation separation the cation-exchange resin is usually a sulfonic or carboxylic acid, and for anion separation the anion-exchange resin is usually a quaternary ammonium group ions in solution can be detected by measuring the conductivity of the solution.

In ion chromatography, the mobile phase contains ions that create background conductivity, making it difficult to measure the conductivity due only to the analyte ions as they exit the column. This problem can be greatly reduced by selectively removing the mobile phase ions after the analytical column and before the detector. This is done by converting the mobile phase ions to a neutral form or removing them with an eluent suppressor, which consists of an ion-exchange column or membrane. For cation analysis, the mobile phase is

often HCl or HNO_3, which can be neutralized by an eluent suppressor that supplies OH^-. The Cl^- or NO_3^- is either retained or removed by the suppressor column or membrane. The same principle holds for anion analysis. The mobile phase is often NaOH or $NaHCO_3$, and the eluent suppressor supplies H^+ to neutralize the anion and retain or remove the Na^+. Molecular ions, such as proteins, that have absorption bands in the ultraviolet or visible spectral region can be detected by absorption spectroscopy.

1.7.1.3 Preparative chromatography

Preparative elution chromatography is the most widely employed mode for preparative separations. Elution chromatography can be carried out under isocratic (constant mobile phase composition), gradient (continuous change in mobile phase composition) or step elution conditions. Under these conditions, a feed mixture is injected into the column inlet as a finite volume pulse. The feed components then migrate through the column at different speeds, which are a function of the mobile phase velocity and the distribution of the compounds between the mobile and stationary phases.

Preparative elution chromatography is generally carried out under mass and/or volume overloaded conditions in order to increase the product throughput. In volume overloading, the sample concentration is maintained in the linear region of the isotherm and the volume is increased until the throughput is optimized. A combination of volume and mass overloading is commonly used to maximize throughput in preparative elution chromatography.

1.7.2 Spectroscopy

The most frequently use spectroscopic methods for analysis of bioactive compound are UV/Vis spectroscopy, IR, NMR and mass spectroscopy. In this thesis the most frequently used method was mass spectrometry with connection to HPLC and UV/Vis spectroscopy.

1.7.2.1 Ultraviolet and visible absorption spectroscopy (UV-Vis)

Ultraviolet and visible (UV-Vis) absorption spectroscopy is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. Absorption measurements can be at a single wavelength or over an extended spectral range. Ultraviolet and visible light are energetic enough to promote outer electrons to

higher energy levels, and UV-Vis spectroscopy is usually applied to molecules or inorganic complexes in solution. The UV-Vis spectra have broad features that are of limited use for sample identification but are very useful for quantitative measurements. The concentration of an analyte in solution can be determined by measuring the absorbance at some wavelength and applying the Beer-Lambert Law.

Since the UV-Vis range spans the range of human visual acuity of approximately 400 - 750 nm, UV-Vis spectroscopy is useful to characterize the absorption, transmission, and reflectivity of a variety of technologically important materials, such as pigments, coatings, windows, and filters. This more qualitative application usually requires recording at least a portion of the UV-Vis spectrum for characterization of the optical or electronic properties of materials.

1.7.2.2 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy uses radiofrequency radiation to induce transitions between different nuclear spin states of samples in a magnetic field. NMR spectroscopy can be used for quantitative measurements, but it is most useful for determining the structure of molecules (along with IR spectroscopy and mass spectrometry). The utility of NMR spectroscopy for structural characterization arises because different atoms in a molecule experience slightly different magnetic fields and therefore transitions at slightly different resonance frequencies in an NMR spectrum. Furthermore, splittings of the spectra lines arise due to interactions between different nuclei, which provides information about the proximity of different atoms in a molecule.

1.7.2.3 Mass spectrometry

Mass spectrometry is a powerful analytical technique that is used to identify unknown compounds, to quantify known compounds, and to elucidate the structure and chemical properties of molecules. Detection of compounds can be accomplished with very minute quantities (as little as 10^{-12}g, 10^{-15} moles for a compound of mass 1000 Daltons). This means that compounds can be identified at very low concentrations (one part in 10^{12}) in chemically complex mixtures.

Mass spectrometers use the difference in mass-to-charge ratio (m/z) of ionized atoms or molecules to separate them from each other. Molecules have distinctive fragmentation patterns that provide structural information to identify structural components.

The general operations of a mass spectrometer are (1) creation of gas-phase ions, (2) separation of the ions based on their mass-to-charge ratio and (3) measurement of the ions quantity. The mass spectrometer is consisted of ion source, in our case electrospray (ESI); mass-selective analyzer (quadrupol ion trap) and ion detector.

ESI is very often use ionization technique for sensitive analysis of small, large and labile molecules such as peptides, proteins, organometallics, oligosaccharides, and polymers.

Electrospray ionization generates ions directly from solution by creating a fine spray of highly charged droplets in the presence of a strong electric field (typically 3.5 kV). As the droplet decreases in size, the electric charge density on its surface increases. The mutual repulsion between like charges on this surface becomes so great that it exceeds the forces of surface tension, and ions begin to leave the droplet through what is known as a "Taylor cone". The ions are then electrostatically directed into the mass analyzer. Vaporization of these charged droplets results in the production of singly or multiply-charged gaseous ions.

The advantage of ESI-MS is that ions are formed directly from solution, a feature that has established the technique as a convenient mass detector for high performance liquid chromatography (HPLC).

Immediately following ionization, gas phase ions enter a region of the mass spectrometer known as the mass analyzer. The mass analyzer is used to separate ions within a selected range of mass-to-charge (m/z) ratios. Ions are typically separated by magnetic fields, electric fields, or by measuring the time it takes an ion to travel a fixed distance.

Qudrupol ion trap use ions externally generate by electrospray. The ions are then injected into the trapping volume and then they are ejected and detected as the radio frequency field is scanned. Further, it is also possible to isolate one ion species by ejecting all others from the trap. The isolated ions can subsequently be fragmented by collisional activation and the fragments detected to generate a fragmentation spectrum. The ions passes through the mass analyzer are then detected by the ion detector.

2. AIMS OF THE THESIS

Development of the screening tests for trypsininhibitors, elastase inhibitors and allelopathic compounds.

Determination of the most effective physiological parameters for trypsin inhibitors syntheses.

Isolation and the chemical structure determination of compounds from selected active strains of cyanobacteria.

3. METHODS

3.1 SCREENING OF TRYPSIN INHIBITORS, ELASTASE INHIBITORS AND ALLELOPATHIC COMPOUNDS

3.1.1 Cultivation of algae and cyanobacteria

A large scale screening of 124 freshwater algal and 58 freshwater cyanobacterial cultures has been carried out to identify the presence of trypsin inhibitors, elastase inhibitrors and allelopathic compounds. Algal cultures were obtained from Culture Collection of Autotrophic Organisms at Institute of Botany, Třeboň, Czech Republic (CCALA). Cyanobacterials cultures were gained from Biological Institute, St. Petersburg University, Russian (CALU), Culture Collection of the Institute of Soil Biology, South Bohemia University, České Budějovice, Czech Republic (ISB, Dr. Lukešová) and Culture Collection of Autotrophic Organisms at Institute of Botany, Třeboň, Czech Republic (CCALA). All strains tested were isolated from soil and freshwater samples collected in different habitat.

The cultures were cultivated in 300 ml glass cylinders, bubbled with air and 2% of carbon dioxide. The cylinders were dept in tempered water bath (25°C) with continuous illumination of 70 W/m^2 (Philips, Osram Dulux L, 55W/12-950, made in Italy). The algal cultures were grown in ½ SŠ medium (Šetlík, 1970); and cyanobacteria were grown in Allen&Arnon medium (Allen&Arnon, 1955). The composition of growths media is shown in table Fig. 4.

After two week cultivation, the cultures were harvested by centrifugation (Janetzki T23) at 5 000 r.p.m. The obtained supernatant was directly tested and resultant biomass was lyophilized (Lyovac GT3, Leybold-Heralens) for the following extraction.

A

Solution A	**25.0 ml**
4% $MgSO_4 \cdot 7H_2O$	500.0 ml
1.2% $CaCl_2 \cdot 2H_2O$	500.0 ml
3.8% NaCl	500.0 ml
Microelements stock solution	500.0 ml
(A & A FeEDTA solution 160.0 ml, $MnCl_2 \cdot 4H_2O$ 360 mg, MoO_3 36.0 mg, $ZnSO_4 \cdot 7H_2O$ 44.0 mg, $CuSO_4 \cdot 5H_2O$ 15.8 mg, H_3BO_3 572.0 mg, NH_4VO_3 4.6 mg, $CoCl_2 \cdot 6H_2O$ 8.0 mg, Distilled water 1090.0 ml)	
Solution B	**6.25 ml**
K_2HPO_4	28.0 g
Distilled water	500.0 ml
KNO_3	**0.253 g**
Distilled water	**969.0 ml**

B

20.2% KNO_3	10.0 ml
17.0% K_2HPO_2	2.0 ml
32.9% $MgSO_4 \cdot 7H_2O$	3.0 ml
1.8% Chelaton Fe^{3+}	1.0 ml
7.7% $CaCl_2$	0.2 ml
Microelements stock solution	0.5 ml
H_3BO_3 3.09 g, $MnSO_4.4\ H_2O$ 1.18 g, $CuSO_4.5\ H_2O$ 1.24 g, $ZnSO_4.7\ H_2O$ 1.43 g, $(NH_4)Mo_7O_{24}.4\ H_2O$ 0.88 g, $CoSO_4.7\ H_2O$ 1.4 g, Distilled water 991.7 ml	
Distilled water	983.3 ml
4% KOH (pH 7.2 to 7.6)	

Fig. 4: A: Composition of Allen&Arnon medium. B: Composition of ½ SŠ medium.

3.1.2 Extraction of algal biomass

Freeze dried biomass was disintegrated by sea sand with 100 % methanol (6 ml/0.2 g dry weight), 70% methanol and mixture of methanol/tetrahydrofurane in the ratio 1:1. The extracts were centrifugated at 5 000 r.p.m. The resulting supernatants were tested for enzymatic and allelopathic assay.

3.1.3 Extraction of cyanobacterial biomass

Freeze-dried biomass for screening was disintegrated by sea sand with 70% methanol (0.2 g/ 6 ml dry weight). The obtained extract was centrifugated at 5 000 r.p.m, and the supernatants tested for enzymatic and allelopathic assay.

Freeze-dried biomass for isolation of active compounds were disintegrated (2 times) by sea sand and 100% or 70 % methanol (see chapter 4.2.2; 10 g/ 300 ml dry weight). The

extracts were centrifugated at 5 000 r.p.m, and the suspernatans evaporated afterwards. These evaporators were washed out with 50 ml of hexan or acetone for the pigments removal (see chapter 4.3.3). The obtained remains were diluted in 100 % methanol or water and solutions were liquid-liquid extracted by hexan. Finally, aceton was carefully added to improve the pigments partitionig. The pigments pass into hexan layer and polar compounds remained in the second methanol/acetone layers, which were further evaporated. We used it for the separation on C_8 column of preparative chromatograph.

3.2 PROTEASE INHIBITORY ASSAY

The standard methods for the assay of proteinase inhibitors generally involve spectrophotometry (Grach-Pogrebinsky, 2003) or pH-titration (Kassel, 1970) to determine the decrease (inhibition) of the enzymatic hydrolysis of natural (e.g. casein, haemoglobin) or synthetic (e.g. N-α-benzoyl-D,L-arginine-p-nitroanilide, N-succinyl-ala-ala-ala-p-nitroanilide) substrates caused by the inhibitor, as well as a wide variety of other methods such as vasodilation assays (for kallikrein inhibitors) and milk clotting have also been used.

In this work we used spectrophotometrical method based on cleavage of p-nitroanilide from substrate N*α*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) by trypsin and from N-succinyl-ala-ala-ala-p-nitroanilide by elastase.

Firstly, we had to determine enzyme saturation by substrate, because in this area the speed of enzymatic reaction depends only on the enzyme concentration and achieves the value $2*K_M$. The Michaelis-Menten´s (K_M) constant and maximal speed was determined by time course measurement of enzymatic reaction. The concentration of enzyme was constant while the substrate concentration varied. In the enzymatic reaction we used the concentration of substrate that is close to saturation. Due to the results of these measurements we developed following enzymatic assay.

3.2.1 Trypsin inhibitory assay

Trypsin was dissolved in 50 mM Tris-HCl (pH 7.5) to prepare a 150 units/ml solution. After, 55 µl enzyme solution, 55 µl 0.4 M Tris-HCl, 15 µl of water and 10 µl of test solution were added to each microtiter plate well and preincubated at 37°C during 30 minutes. Then 115 µl of substrate solution (4.6 mg of BAPNA dissolved in 100 µl of dimethylsulphoxide

and made up to 10 ml with 50 mM Tris-HCl pH 7.5) was added to start the reaction. After the following 30-minutes incubation at 37°C, developed p-nitroanilide was measured on Tecan Sunrise reader at 410 nm.

$$\text{Trypsin} + \text{BAPNA} + H_2O \rightarrow \text{N-Benzoyl-L-arginin} + \text{p-nitroanilide}$$

In each samples set the "blank" was always prepared. There was used methanol instead of test solution. The absorbance of the blank represented a maximal absorbance of the enzymatic reaction and we attributed it to 100% trypsin activity. Values of absorbance obtained from the sample preparations were compared with this value.

This approach was applied only for medium samples. The absorption spectrum of p-nitroanilide at 410 nm in biomass extract was overlaped by pigments spectrum.

Three methods for elimination of this effect were developed. The first is based on solid phase extraction of enzymatic reaction product on C18 cartridge by 60% methanol. In this way the pigments remained on the cartridge and p-nitroanilide was measured on UV/VIS spectrophotometer at 410 nm.

The second applied method was black gelatine method based on the gelatine plates prepared from gelatine and water soluble nigrosin (Šafařík, 1988). To detect the trypsin inhibitors the buffered trypsin solution was mixed with an extract in the ratio 1:1, and after 5-30 minutes of incubation 20 µl of the mixture were placed on a gelatine-coated plate. The hydrolysis was allowed to proceed at room temperature about 30 minutes. The plates were thoroughly washed with hot running water (50-60°C). Discovery of colourless spots on a blue background indicated the absence of proteinase inhibitor in the sample. The gelatine layer in the place of applied drops containing the inhibitors remained unhydrolyzed.

The last one was based on measuring of absorption differences at the beginning and at the end of enzymatic reaction. The absorbance was immediately measured after the substrate addition and at the end of enzymatic reaction at 410 nm. The last mentioned method was used for all trypsin inhibitory assays.

3.2.2 Elastase inhibitory assay

Elastase was dissolved in 50 mM Tris-HCl (pH 8.6) to prepare a stock solution 0.04 units/ml. A 30 µl enzyme solution, 30 µl 0.4 M Tris-HCl (pH 8.6), and 65 µl of test solution were added to each microtiter plate well and preincubated at 37°C during 30 minutes. Then 65 µl of substrate solution (1 mg of N-succinyl-ala-ala-ala-p-nitroanilide dissolved in 7 µl of dimethylsulphoxide and made up to 1 ml with 50 mM Tris-HCl pH 8.6) was added to start the reaction. The absorbance in microtitre plate was immediately measured on Tecan Sunrise reader at 410 nm. The developed p-nitroanilide was measured after next 30-minutes incubation at 37°C.

3.3 HPLC/DAD/MS analysis

3.3.1 Analytical scale HPLC

Analytical scale chromatography was performed on the high performance liquid chromatograph (HPLC) Agilent 1100 with MSD SL-Ion Trap mass spectrometer. Trap mass spectrometer was composed of electrospray ion source (ESI). The spray needle was at a potential of 5 kV and 4800 kPa coaxial flow of nitrogen was used to stabilize the spray. The counter electrode was a heated (199 °C) stainless-steel capillary held at a potential of 10 V. The tube-lens offset was 20 V, and the electron multiplier voltage was -800 V. Helium gas was introduced into the ion trap at a pressure of 1 mtorr to improve the trapping efficiency of the sample ions introduced into the ion trap. The background helium gas also served as the collision gas during the collisionally activated dissociation (CAD) experiments.

HPLC system was consisted of a vacuum degasser, binary pump, column thermostat and diode-array detector (DAD). The extracts were separated on the reverse phase column Zorbax, XDB C8 (4.6 x 150 mm, 5 µm) at 35°C using the linear gradient of methanol and water from 30% to 100% of methanol within 30 min (Fig. 5). The mobile phase contained 0.1% formic acid for improvement of ionisation. The flow rate was 0.6 ml/min; the injection volume was 20 µl and the detection wavelengths were 220, 233, 280 and 440 nm. The ion trap was optimized for ions with rate m/z 900 and electrospray analyses were carried out in positive mode.

The data analyses were performed using CHEMSTATION Software and Windows^{NT} (Microsoft, Eugene, OR, USA) operating system.

Fig. 5: Table of gradient (analytical HPLC):

Time (min)	Flow (ml/min)	% A (methanol)	% B (water)
0	0.6	30	70
1	0.6	30	70
25	0.6	100	0
30	0.6	100	0
32	0.6	30	70

3.3.1 Preparative scale HPLC

Preparative scale experiments (the purification of active fraction) were performed on the LabAlliance^{TM} preparative HPLC system connected through the splitter directly to mass spectrometer. The preparative HPLC system was consisted of two dual piston preparative pumps Prep 100, DeltaChromTM column thermostat and gradient controller LabAlliance TM (Watrex, Prague).

The separation of extracts was performed on the reverse phase column Reprosil 100 C8 (25 x 250 mm, 5 μm) at 35°C. Methanol and water containing 0.1% formic acid were used as the mobile phase at a flow rate 15 ml/min with linear gradient from 30% to 100% of methanol during 30 min (Fig. 6). The effluent from column was split up to two parts. The first part (1 ml/min) flowed into ESI/MS/MS; the rest was collected in the fraction collector. This allowed us to collect the fractions accoarding their ration m/z (mass/charge).

The detection wavelengths were 220, 233, 280 and 440 nm and the injection volume was 1ml. The ion trap was optimized for ions with rate m/z 900 and electrospray analyses were carried out in positive mode. This separation was used to determine individual fraction activity and following active fraction isolation.

Fig. 6: Table of gradient (preparative HPLC):

Time (min)	Flow (ml/min)	% A (methanol)	% B (water)
0	1	30	70
1	15	30	70
25	15	100	0
30	15	100	0
32	15	30	70

3.4 OPTIMIZATION OF GROWTH PARAMETERS

After optimization of trypsin inhibitor assay, alagal and cyanobacterial extracts were tested for enzymatic assay. According to the inhibitory activity, three active strains were chosen for more detailed studies.

We studied the influence of temperature (25°C, 30°C and 35°C) in comparision with illumination (30, 70, 100 and 120 $W.m^{-2}$). From the results, the growth speed for each strain was determined. Dependence of these values on temperatures and light intensities allow to assed optimal growth parameters.

3.4.1 Cultivation

The cultivation was carried out in 100 ml glass cylinders bubbled with air and 2% of carbon dioxide. The cylinders were dept in tempered water bath (25°, 30°, 35°C) with continuous illumination of 30, 70, 120, 150 $W.m^{-2}$ (Philips, Osram Dulux L, 55W/12-950, made in Italy). Nostoc cultures were grown in Allen&Arnon medium. The cultivation was allowed to proceed to deplet of nitrate or stopping the culture growth.

3.4.2 Determination of growth speed

Determination of growth speed is usually realised by the optical density measurement of homogenous biomass suspensions. In the case of non-homogenous suspension, due to quick filaments sedimentation on the bottom of cuvette, the measurements are not realizable.

Nostoc strains form the clumps therefore the optical density measurement was supplied by nitrate consumption measurement in cultivation medium. It could be supposed that decreasing of nitrate concentration in cultivation medium is caused by biomass growth.

Measurement was carried out on ICS-90 Ion chromatography system Dionex which contained detection stabilizer DS5, precolumn Ion Pac® AG9-HC (4×50 mm) and analytical column Ion Pac® AS9-HC (4×250 nm). The mobile phase was 9 mM sodium carbonate (Na_2CO_3), and as the regeneration solution we used 50 mN H_2SO_4. The samples were separated by isocratic gradient with flow rate 1 ml/ min, afterwards.

3.4.3 Determination active compound production

During the cultivation, we also studied the production of bioactive compounds. Cultivation of biomass was carried out in column bioreactor (8 liters) with continuous illumination 70 $W.m^{-2}$ (Philips, Osram Dulux L, 55W/12-950, made in Italy). Bioreactor was tempered at 25°C and bubbled with 2 % CO_2. The Nostoc strains were grown in Allen&Arnon medium and detection of active compounds production was realized by 24 hour sampling of 200 ml suspension. The collected samples were centrifuged at 5 000 r.p.m and lyophilized. After extraction we analysed them on the HPLC/MS.

3.5 ALLELOPATHIC ASSAY

Allelopathic activity was tested using the suspension of Anabaena variabilis (Pushparaj, 1999). One millilitre of this suspension was pored into Petri dishes of 6 cm in diameter. Then, agar was added and the dishes were mixed by carefully wheeling. The Petri dishes were left to pre-incubate in the incubator for 24 hours at 30°C, afterwards.

After the incubation, two sterile targets were put on the surface of agar. On the first target 15 µl of algal or cyanobacterial extract were added, and on the second one was added 25 µl of extract. The Petri dishes with these targets were placed into the incubator again. Every day apperance of inhibition zone was controled as well. The first signs of allelopathic activity were detected after one week incubation.

3.6 CHEMICALS

Nα-benzoyl-D,L-arginine-4-nitroanilde hydrochloride (lyophilized powder), N-succinyl-ala-ala-ala-p-nitroanilide (lyophilized powder), methanol, acetone, hexane, formic acid, Tris and all common chemicals were purchased from Sigma-Aldrich, dimethyl solfoxide and hydrochloric acid from Fluka.

4. RESULTS

4.1 SCREENING OF ALGAE AND CYANOBACTERIA FOR THE PRESENCE OF TRYPSIN INHIBITORS, ELASTASE INHIBITORS AND ALLELOPATHIC COMPOUNDS

The first step of the screening was the cultivation of 124 algal and 58 cyanobacterial strains in inorganic medium. Afterwards, the resultant biomass was centrifugated, lyophilized and extracted. Each of these extracts was tested to detect trypsin inhibitors, elastase inhibitors and allelopathic compounds presence.

4.1.1 Trypsin inhibitory assay

The trypsin inhibitory assay was derived from Cannell (1987) experiments and was carried out in microtitre plates. On purpose of this thesis, we lowered the substrate concentration at 36 $U.ml^{-1}$ and next enzyme saturation curve was measured with the substrate concentration ranging between 0.317 $mM.l^{-1}$ and 1.276 $mM.l^{-1}$. Finally, the obtained results of p-nitroanilide absorbance were plotted as shows the figure 7. It illustrates that substrate concentration 1.057 $mM.l^{-1}$ is sufficient for enzyme saturation. We used this concentration in our next experiments as well.

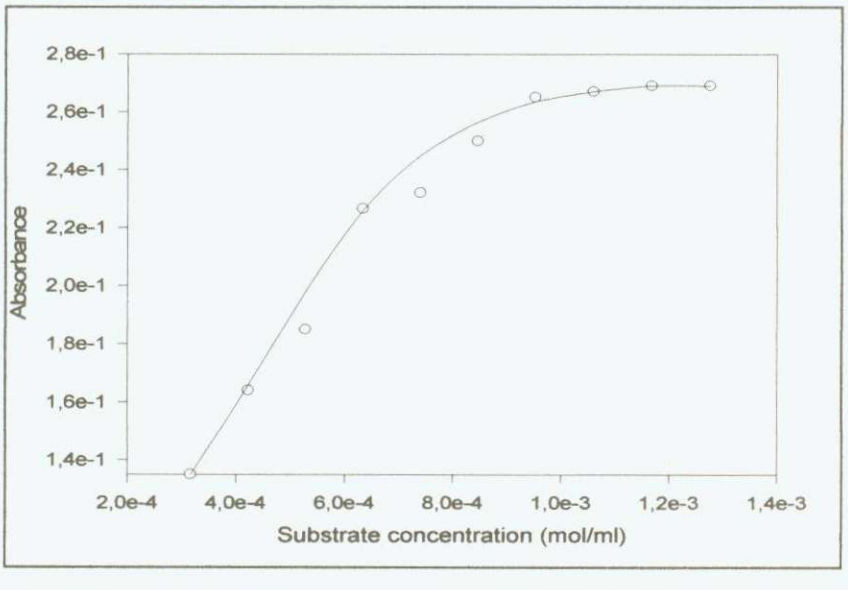


Fig. 7: Determination of trypsin saturation by substrate BAPNA.

1.1.1 Methods for elimination of disturbance effect of pigments

In view of the fact that algal and cyanobacterial extracts contains pigments there are me difficulties with over lapping of their absorption spectra with absorption spectrum of nitroanilide (product of enzymatic reaction) at wavelenght 410 nm. Hence it is necessary to minate this effect.

The first method to eliminate the effect is solid phase extraction of enzymatic reaction oduct on C_{18} cartridge by 60% methanol. In this way the pigments remained on the rtridge and p-nitroanilide was measured on UV/VIS spectrophotometer at 410 nm. ditionally, the elution was checked by HPLC analysis, whose results are showed in g. 8. Figure 8 A shows chromatogram with two peaks belonging to p-nitroanilide (1) and bstrate (2), which were determined by recording of HPLC spectra (Fig.8 B). On the romatogram no peaks of pigments were observed.

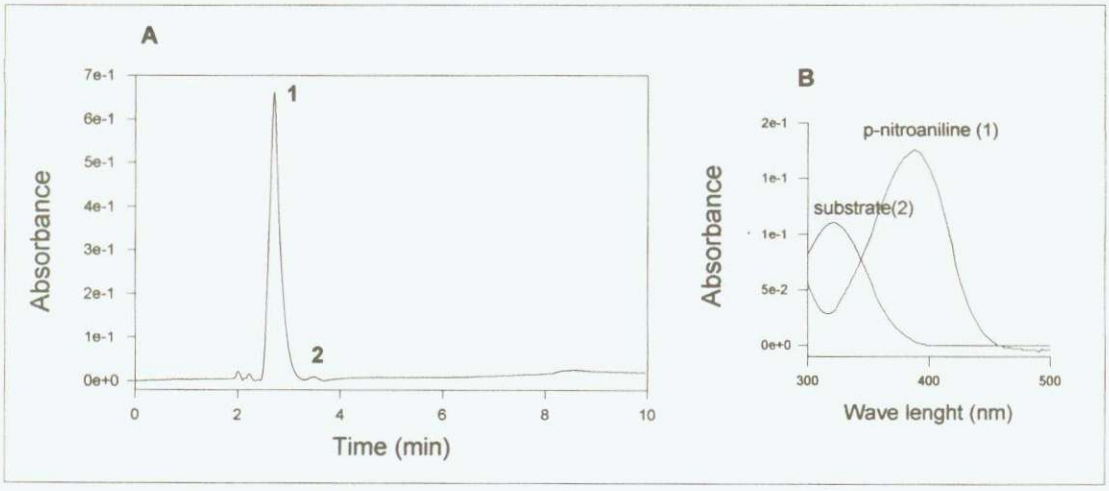


Fig. 8: A: Chromatogram of p-nitroanilide (1) and substrate (2)
B: Spectra of p-nitroanilide (1) and substrate (2)

Sufficient amount of a solvent for the p-nitroanilide elution was tested by the ogressive elution using 0.5 ml, 1.0 ml, 1.5 ml and 2.0 ml of 60% methanol. The Fig. 9 ows that one millilitre of 60% methanol is adequate to elute the whole amount of nitroanilide. Due to this elution all the pigments were removed. Hence this method is too ne-consuming and for the large scale screening is not suitable.

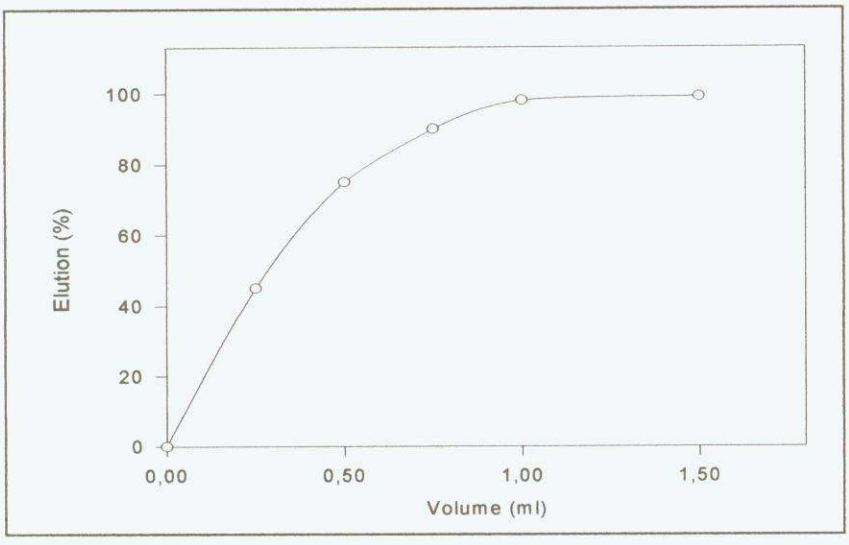


Fig. 9: Elution of p-nitroanilide from C_{18} cartridge by 60% methanol.

The second studied method was black gelatine method based on the gelatine plates (Fig. 10) prepared from gelatine and water soluble nigrosin. To detect the trypsin inhibitors the buffered trypsin solution was mixed with an extract in the ratio 1:1, and after 15-30 minutes of incubation 20 µl of the mixture were placed on a gelatine-coated plate. The hydrolysis was allowed to proceed at room temperature about 30 minutes. The plates were thoroughly washed with hot running water (50-60°C). Discovery of colourless spots on a blue background indicated the absence of proteinase inhibitor in the sample. The gelatine layer in the place of applied drops containing the inhibitors remained unhydrolyzed (Šafařík, 1988).

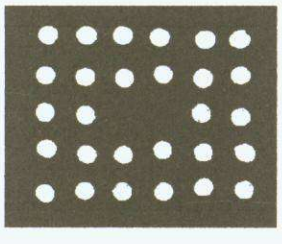


Fig. 10: Black gelatine plate with hydrolyzed spot (white) and unhydrolyzed (dark).

This methodology was not influenced by pigments, as well as it was very rapid, but the preparation of gelatine plates was complicated.

The last tested method was based on colour background subtraction. The absorbance was immediately measured (410 nm) after substrate addition and at the end of enzymatic reaction again. The first absorbance value was subtracted from the last one, and then the real

absorbance value was obtained. Furthermore, the inhibitor activity was determined after comparison of this value with absorbance of a blank (reaction mixture without inhibitor). This relation is showed by the following equation in Fig.11. The mentioned method was very rapid and simple. Finally, it was used for next trypsin inhibitory assays.

$$I(\%) = 100 - \left[100 * \frac{(A_{60} - A_{30})_S}{(A_{60} - A_{30})_B} \right]$$

Fig. 11: Equation for calculation of sample inhibitory activity.
I(%)-inhibitory activity of samples, A_{60}-absorbance at the end of the reaction, A_{30}-absorbance after addition of substrate, S-samples, B-blank (reaction mixture without inhibitor).

4.1.2 Elastase inhibitory assay

In the same way as trypsin inhibitory assay, the saturation of elastase (0.04 $U.ml^{-1}$) by substrate was determined as well. The methodology was derived from Feinsteine (1973) using soluble substrate N-succinyl-ala-ala-ala-p-nitroanilide, which was tested in five concentrations (from 0.44 $mM.l^{-1}$ to 2.66 $mM.l^{-1}$). Obtained results of absorbance were plotted on the figure 12. The figure illustrates that substrate concentration 2.22 $mM.l^{-1}$ is sufficient for enzyme saturation. We used this concentration in our next experiments as well.

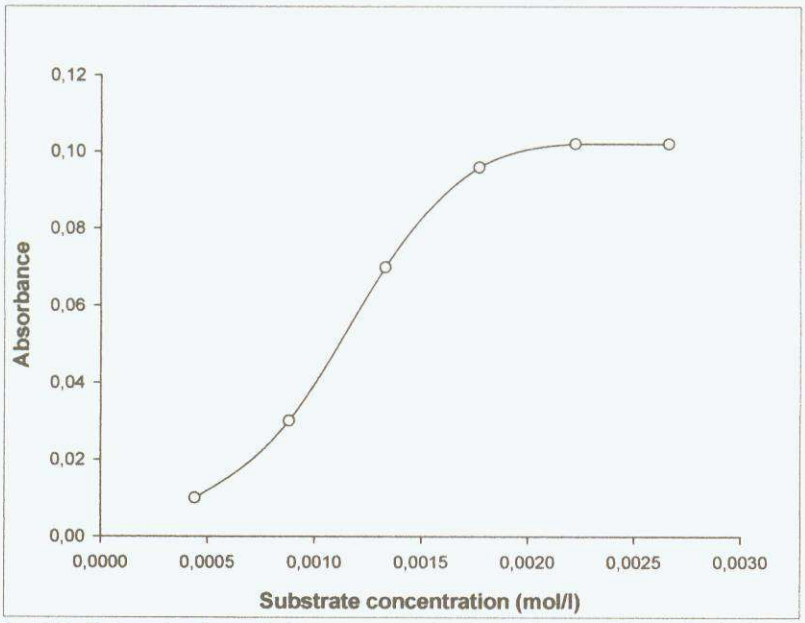


Fig. 12: Determination of elastase saturation by substrate N-succinyl-ala-ala-ala-p-nitro-anilide.

4.1.3 Results of the screening

The screening of algal strains was carried out by the extraction with sea sand and methanol/tetrahydrofurane (1:1). In this way both lipophilic and hydrophilic components passed to the extract. Before testing of enzyme inhibitory (trypsin, elastase) assay and allelopathic assay the extraction solvent had to be evaporated, because tetrahydrofurane inhibits enzymes. In addition, tetrahydrofurane is aggressive against the plastic materials (microtitrate well plate) as well as toxic for microorganisms.

The following Fig. 13 illustrates the inhibitory activity of 124 algal strains. If the inhibition of enzyme had been ≥ 50 %, the extract would have been considered as inhibitory active. In the case of allelopathic activity, the active extract exhibited inhibition zone ≥ 10 mm. According to these conditions about 22 % of algal strains showed trypsin inhibitory activity, 24 % of algal strains showed elastase inhibitory activity and 15% of algal strains exhibited allelopathic activity.

Fig. 13: The results of trypsin inhibitory, elastase inhibitory and allelopathic activities in the algal strains.

Algal strains	**Allelopathic activity (volume of extract)**		**Trypsin inhibition in medium**	**Trypsin inhibition in biomass**	**Elastase inhibition in biomass**
	15 µl	**25 µl**	**[%]**	**[%]**	**[%]**
Chlorococcum sp.	-	-	-	-	-
Dictyosphaerium chlorelloides	-	-	-	-	-
Chlorococcum sp.	-	-	-	-	71.0
Chlorosarcinopsis sp.	-	-	-	-	-
Chlamydomonas sp.	-	-	-	-	86.2
Pseudococcomyxa simplex	-	-	-	-	-
Planophila terrestris	-	-	-	-	-
Chlorosarcinopsis minuta	-	-	-	-	-
Monodus subter.	-	-	-	-	-
Gald. sulp. (Mt Lawu)	-	-	-	-	-
Gald. sulp. (Azowen)	-	-	-	92.1	-
Gald. sulp. (Iceland)	-	-	-	50.7	-
Gald. sulp. (Salvador)	-	-	-	92.1	-
Gald. sulp. (Rio Tinto)	-	-	-	-	-
Gald. sulp. (Devils)	-	-	-	64.3	-
Coelastrum reticulatum	-	-	-	-	-
Pleurastrum sarcinoideum	-	-	-	-	-
Chorosarcinopsis gelatinosa	-	-	-	-	-
Scenedesmus dimarmis	-	-	-	-	-
Monodus subter.	-	-	-	-	-
Gald. sp. (5)	-	-	-	-	-
Gald. sp. (6)	-	-	-	-	-
Gald. sp. (5) /S	-	-	-	67.1	-
Gald. sp. (3) /S	-	-	-	-	-
Gald. sp. (1)	+	+	-	99.3	-
Gald. sp. (2)	+	+	-	-	-

Algal strains	Allelopathic activity (volume of extract) 15 µl	25 µl	Trypsin inhibition in medium [%]	Trypsin inhibition in biomass [%]	Elastase inhibition in biomass [%]
Gald. sp. (4)	+	+	-	-	-
Gald. sp. (4) /S	-	-	-	77.9	-
Gald. sp. (1) /S	-	-	-	50.7	-
Gald. sp. (3) /S	+	+	-	-	-
Gald. sp. (6) /S	-	-	-	-	67.0
Planophila terrestris /S	+	+	-	-	-
Dictyosphaerium chlorelloide s/S	-	-	-	-	-
Chlorococcum sp. /S	-	-	-	-	-
Chlorosarcinopsis sp. /S	-	-	-	-	-
Chlamydomonas sp. /S	-	-	-	-	-
Chlorococcum sp. /S	+	+	-	-	74.5
Pseudococcomyxa simplex /S	+	+	-	-	44.1
Spongiochloris spongiosa /S	+	+	-	-	-
Scenedesmus cf opoliensisvar/S	-	-	-	-	63.5
Monoraphidium saxatile	-	-	-	59.8	-
Scenedesmus sp. /S	-	-	-	-	-
Enallax sp. /S	-	-	-	97.7	-
Coelastrella multistriata /S	-	-	-	-	-
Coelastrum sphaericum /S	+	+	-	53.9	-
Scotiellopsis terrestris /S	-	-	-	-	-
Ankistrodesmus spiralis /S	+	+	-	84.7	93.7
Scenedesmus pecsensis /S	-	-	-	-	-
Coelastrella multistriata /S	-	-	-	-	-
Scotiellopsis oocystiformis /S	-	-	-	-	-
Scotiella chlorelloides /S	-	-	-	-	-
Dictyococccus varians /S	-	-	-	85.3	77.9
Synechocc. termophilus	-	-	-	-	-
Synechoccoc. termophilus	-	-	-	-	-
Phormidium sp. (gray)	-	-	-	-	-
Phormidium sp. (blue)	-	-	-	-	-
Phormidium sp. Zehnder 64	-	-	-	-	-
Phormidium sp. Marvan	-	-	-	-	-
Trentepohlia sp.	-	-	-	-	-
Trentepohlia sp./S	-	-	-	45.6	-
Scotiellopsis terrestris HINDÁK1968/14 /S	-	-	-	-	-
Monodus subteratus /S	-	-	-	-	-
Chlorosarcinopsis aggregata ARCE/UTEX 779/ S	+	+	-	59.3	52.4
Scotiellopsis terrestris HINDÁK 1963/59 /S	-	-	-	96.7	-
Scotiellopsis terrestris /REISIGL/ B, HINDÁK 1963/58 /S	-	-	-	52.0	-
Scotiellopsis rubescens VINATZ.VINATZER/Insbruck V 195 /S	-	-	-	-	-
Pleurastrum sarcinoideum LUKEŠOVÁ 1986/11 /S	+	+	-	-	-
Spirulina/ S	-	-	-	-	-
Scenedesmus sp. Nečas 1965/N-508 /S	-	-	-	-	-
Scenedesmus cf. Gutwinskii KOVAČIK 1983/9 /S	+	+	-	53.1	90.3
Scenedesmus dimorphus /TURP./KUTZ., KOMÁREK 1962/36 /S	-	-	-	-	45.6
Coelastrum reticulatum HINDÁK, 1965/85 /S	-	-	-	-	-

Algal strains	Allelopathic activity (volume of extract) 15 µl	25 µl	Trypsin inhibition in medium [%]	Trypsin inhibition in biomass [%]	Elastase inhibition in biomass [%]
Spongiochloris spongiosa VISCHER 1942/318 /S	+	+	-	-	-
Scotiellopsis terrestris /REISIGL/B, HINDÁK 1963/58	-	-	-	99.2	-
Spirulina	-	-	-	-	86.2
Scotiellopsis rubescens VINATZ. VINATZER, Insbruck V 195	-	-	-	-	-
Monodus subteratus	-	-	-	-	-
Coelastrum reticulatum HINDÁK, 1965/85	-	-	-	-	-
Chlorosarcinopsis gelatinosa VINATZER 1975/Innsbruck V 211	-	-	-	-	-
Spongiochloris spongiosa VISCHER 1942/318	-	-	-	67.2	93.8
Scenedesmus sp. NEČAS 1965/N-508	-	-	-	-	-
Pleurastrum sarcinoideum LUKEŠOVÁ 1986/11	-	-	-	-	-
Chlorosarcinopsis aggregata ARCE/VITEX 779	-	-	-	-	-
Scotiellopsis terrestris HINDÁK 1963/59	-	-	-	-	73.1
Scotiellopsis terrestris HINDÁK 1968/14	-	-	-	-	-
Scenedesmus dimorphus /TURP./ KUTZ., KOMÁREK 1962/36	-	-	-	-	
Scenedesmus cf. Gutwinskii KOVAČIK 1983/9	-	-	-	85.4	90.3
Chlorosarcinopsis gelatinosa VINATZER 1975/Innsbruck V 211/S	-	-	-	78.0	73.1
Chlorella ellipsoidea D. Ves, La, VII/97	-	-		-	88.3
Chlamydomonas sp. D. Ves, Ua, IV/97, ORAN	+	+		-	-
Monodopsis subterranea D. Ves, Ub, IV/99	-	-	-	58.8	86.9
Chlorella vulgaris 100/89, Smrz /S	+	+	-	-	-
Geminella terricola Malacky, N7b, X/95, RK/S	+	+	-	63.3	44.1
Spongiochloris cf. irregularis Hlincova hora,H3, V/2000 /S	-	-	-	-	86.9
Spongiochloris excentrica Malacky, A 3a, 1993	-	-	-	-	-
Dictyochloris sp. Kuba, VI/91/S	-	-	-	-	-
Borodinellopsis Sibir, 7, 1991	-	-	-	-	-
Geminella terricola Malacky, N7b, X/95, RK	-	-	-	-	-
Borodinellopsis Sibir, 7, 1991	-	-	-	55.3	-
Spongiochloris cf. irregularis Hlincova hora,H3, V/2000	-	-	-	60.2	-
Phormidium sp. green-blue	-	-	-	-	-
Pleurastum sarcinoideum Lukeš	-	-	-	-	93.1
Phormidium sp.	-	-	-	-	91.7
Chlamydomonas sp. D. Ves, Ua, IV/97, ORAN	-	-	-	-	-
Chlorella ellipsoidea D. Ves, La, VII/97	-	-	-	-	-
Chlorella vulgaris 100/89, Smrz	-	-	-	-	-
Spongiochloris excentrica Malacky, A 3a, jaro 1993	+	+	-	-	-
Dictyochloris sp. Kuba, VI/91	+	+	-	-	55.9
Anabaena doliolum	-	-	-	86.5	93.8
Chlamydomonas debaryana	-	-	-	-	-
Heterococcus pleurococcoides Elster 98/18/30	-	-	-	-	98.1
Stichococcus bacilaris Elster 1998/28/51	-	-	-	-	-
Muriella cf. Kubečková 1999/32	-	-	-	-	76.8
Sphaerocystis oleifera 98/26/16	-	-	-	-	-
Coccomyxa curvata Elster 1998/10/48	-	-	-	-	-
Chlorella minutissima	-	-	-	-	50.0
2 *Chlorella sp.* Kubečková 1992/2	-	-	-	-	69.3

Algal strains	Allelopathic activity (volume of extract) 15 µl	25 µl	Trypsin inhibition in medium [%]	Trypsin inhibition in biomass [%]	Elastase inhibition in biomass [%]
Bracteococcus minor	-	-	-	-	-
Chlorella homosphaera cf. 1998/5	-	-	-	-	-
Chlorella homosphaera	-	-	-	-	-
Chlorella sp. Elster	-	-	-	-	-
Elliptochloris	-	-	-	-	61.8
Pseudococcomyxa simplex Elster 98/25	-	-	-	-	-
Chlorella sp. Elster 6/2	-	-	-	-	80.1

S = *Strains were exposured to the stress conditions (Kopecký, 2000).*

* *The complete taxonomical classification has not been done yet.*

The screening of cyanobacterial strains was carried out with 70% methanol extracts. In this way a smaller amount of pigments was extracted, without need to evaporate the extraction solvent as it was required in the case of algal extracts.

In the following Fig. 14 there are 58 strains of genus Nostoc. About 41% of them exhibited the trypsin inhibitory activity in biomass, but only 3 % of them showed this activity in the cultivation medium.

In addition, 32% of Nostoc strains exhibited the elastase inhibitory activity, whereas allelopathic activity was not observed here.

Fig. 14: The results of trypsin inhibitory, elastase inhibitory and allelopathic activities of Nostoc strains.

Nostoc strains	Allelopathic activity (volume of extract) 15 µl	25 µl	Trypsin inhibition in medium [%]	Trypsin inhibition in biomass [%]	Elastase inhibition in biomass [%]
Nostoc deaph	-	-	-	-	-
Nostoc ofcale	-	-	-	-	-
Nostoc clipsespor	-	-	-	83.6	98.6
N.muscorum 85	-	-	-	-	87.0
N. muscorum 86	-	-	-	-	50.6
Nostoc sp. Lukešová 5/96	-	-	-	-	-
Nostoc sp. Lukešová 20/97	-	-	-	72.9	-
Nostoc sp. 3/97	-	-	-	-	-
Nostoc sp. Lukešová 27/97	-	-	63.4	96.3	-
Nostoc muscorum Lukešová 14/86	-	-	-	-	84.2
Nostoc sp. Lukešová 116/96	-	-	68.4	94.0	-
Nostoc sp. Lukešová 51/1991	-	-	-	88.4	76.9
Nostoc sp. Lukešová 19/96a	-	-	-	-	-
Nostoc muscorum Lukešová 27/97	-	-	-	-	91.8
Nostoc sp. Lukešová 18/89	-	-	-	-	-
Nostoc sp. Lukešová 2/89	-	-	-	81.0	-
Nostoc muscorum	-	-	-	-	97.9
Nostoc edeaphicum Lukešová 1/88	-	-	-	-	-
Nostoc sp. Lukešová 6/96	-	-	-	88.0	-
Nostoc sp. Lukešová 30/93	-	-	-	69.0	62.0
Nostoc sp. Lukešová 2/91	-	-	-	-	93.9

Nostoc strains	Allelopathic activity (volume of extract) 15 µl	25 µl	Trypsin inhibition in medium [%]	Trypsin inhibition in biomass [%]	Elastase inhibition in biomass [%]
Nostoc linkia Roth.str. Gromov, 1962/10, CALU – 129	-	-	-	-	-
Nostoc sp. str. S. Lhotsky, CALU – 268	-	-	-	96.7	-
Nostoc muscorum Ag. str. Lefevre, CALU 304	-	-	-	-	-
Nostoc sp. S. Lhotsky, CALU – 327	-	-	-	-	-
Nostoc sp. str. S. Lhotsky, CALU – 379	-	-	-	-	-
Nostoc muscorum (Ag) Elenk., CALU – 526	-	-	-	-	-
Nostoc muscorum (Ag) Elenk. str. Tretijakova, CALU - 542	-	-	-	98.3	-
Nostoc muscorum (Ag) Elenk. str. Tretijakova, CALU – 543	-	-	-	91.2	96.1
Nostoc muscorum (Ag) Elenk. str. Tretijakova, CALU – 545	-	-	-	-	-
Nostoc muscorum (Ag) Elenk. str. Tretijakova, CALU – 546	-	-	-	91.2	-
Nostoc edaphicum Kondr. str. Gromov, 1977/760, CALU - 760	-	-	-	-	-
Nostoc sp. str. Avilov, 1980/T.0,6 II, CALU – 803	-	-	-		-
Nostoc sp. str. Miklos Szekeres, PR – 17, CALU – 870	-	-	-	-	-
Nostoc sp. str. Gromov, 1967/15(alfa13), CALU – 907	-	-	-	-	-
Nostoc sp. str. Gromov, 1967/30(alfa30), CALU – 913	-	-	-	-	-
Nostoc sp. str. Gromov, 1967/33(alfa33), CALU – 914	-	-	-		-
Nostoc sp. str. Gromov, 1985/35, CALU – 915	-	-	-	72.3	-
Nostoc linckia f. ellipsosporum (Desur.) Elenk. str.Gromov, 1988, CALU – 979	-	-	-	-	-
Nostoc linckia f. muscorum (Ag.) Elenk. str. Gromov, 1988, CALU – 980	-	-	-	93.2	-
Nostoc linckia f. muscorum (Ag.) Elenk. str. Gromov, 1988, CALU – 981	-	-	-	61.0	-
Nostoc linckia f. piscinale (Kutz.) Elenk. str. Gromov, 1988, CALU – 982	-	-	-	93.7	97.8
Nostoc linckia f. str. Gromov, 1988, CALU – 983	-	-	-	87.3	81.1
Nostoc commune (Vauch.) Elenk. str. Gromov, 1988, CALU – 984	-	-	-	89.8	53.9
Nostoc sp. str. Gromov, CALU – 991	-	-	-	-	-
Nostoc sp. str. Gromov, CALU – 992	-	-	-	97.6	63.9
Nostoc linkia F. muscorum (A) Elenk., CALU – 993	-	-	-	-	-

Nostoc strains	Allelopathic activity (volume of extract) 15 µl	25 µl	Trypsin inhibition in medium [%]	Trypsin inhibition in biomass [%]	Elastase inhibition in biomass [%]
Nostoc sp. str. Gromov, CALU – 994	-	-	-	86.1	93.9
Nostoc sp. str. Gromov, CALU – 995	-	-	-	98.2	85.6
Nostoc sp. str. Gromov, CALU – 996	-	-	-	88.9	-
Nostoc sp. str. Gromov, CALU – 997	-	-	-	91.4	-
Nostoc sp. str. Gromov, CALU – 998	-	-	-	90.9	83.3
Nostoc sp. CALU – 1191	-	-	-	-	-
Nostoc sp. CALU-979	-	-	-	-	-
Amorphonostoc sp. CALU-521	-	-	-	-	-
Stratonostoc linkia sp. CALU-453	-	-	-	-	53.9
Nostoc sp. strain LN-6	-	-	-	-	91.4
Amorphonostoc sp. CALU-374	-	-	-	-	53.9

In accordance with the results listed in the previous Fig. 14, three active strains were chosen for more detail investigation: ***Nostoc sp.* Lukešová 116/96**, ***Nostoc sp.* Lukešová 6/96** and ***Nostoc sp.* Lukešová 30/93.** We carried out the physiological experiments to determine the optimal growth parameters (temperature, light intensity), and during the cultivation we also studied a production of active compounds. Simultaneously we analysed the influence of solvents on active compounds extractions and tried to determine the structure of active compounds.

4.2 OPTIMIZATION OF GROWTH PARAMETERS

4.2.1 Determination of growth speed

The determination of growth parameters was based on growth speeds measurement at different temperatures and different light intensities, which was carried out by the measuring of nitrate consumption by ion chromatography. The samples (2 ml) were collected in 24 hour interval until the depleting of nitrate. The first sampling was realised immediately after the inoculation. The content of nitrate from the following the cultivation days was subtracted from the content of the first sampling point. The difference was assigned to the amount of

nitrate used for growth of biomass. On the basis of this correlation the growth curve was created afterward (Fig. 16).

In figure 15 A, there is showed ion chromatogram of medium after the inoculation. There are peaks of chloride, nitrate, phosphate and sulphate. The most important peak is the peak of nitrate with retention time 13.5 minutes and its area corresponds to concentration of 350 mg/l. This is the initial content of nitrate.

The part B of the figure 15 shows ion chromatogram of medium after the following cultivation. It illustrates that nitrate peak in retention time 13.5 minutes was depleted. Therefore, it could be supposed that nitrate lost in medium was consumed by growing biomass.

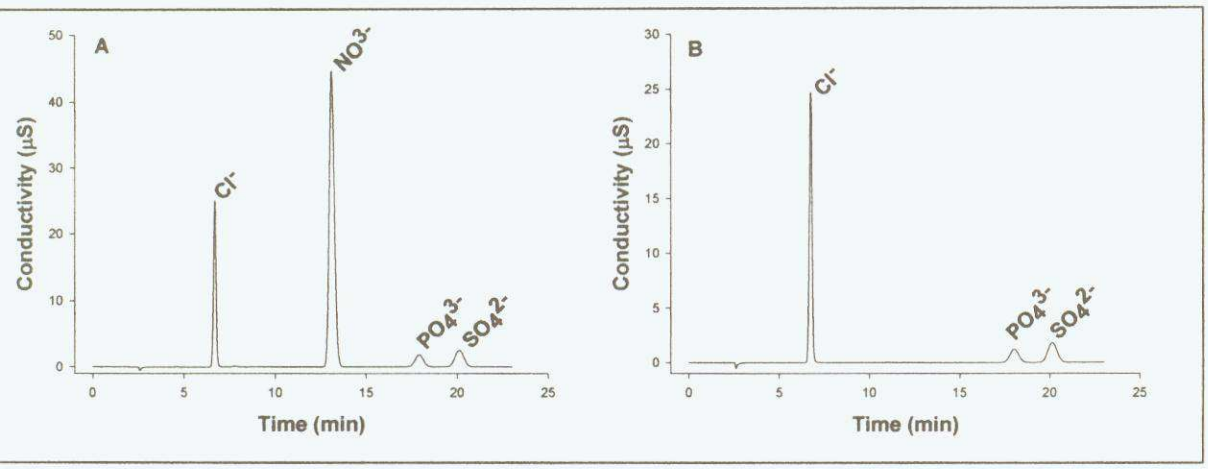


Fig. 15: Ion chromatogram of Allen&Arnon medium. A: Medium at the beginning of the cultivation (350 mg/lNO_3^-), B: Medium at the end of the cultivation (0 mg/ml NO_3^-).

Using the analysis of nitrate consumption the growth curve was created as dependence of consumed nitrate on time. The fig. 16 shows the growth curves of three chosen active Nostoc strains at temperature 30°C.

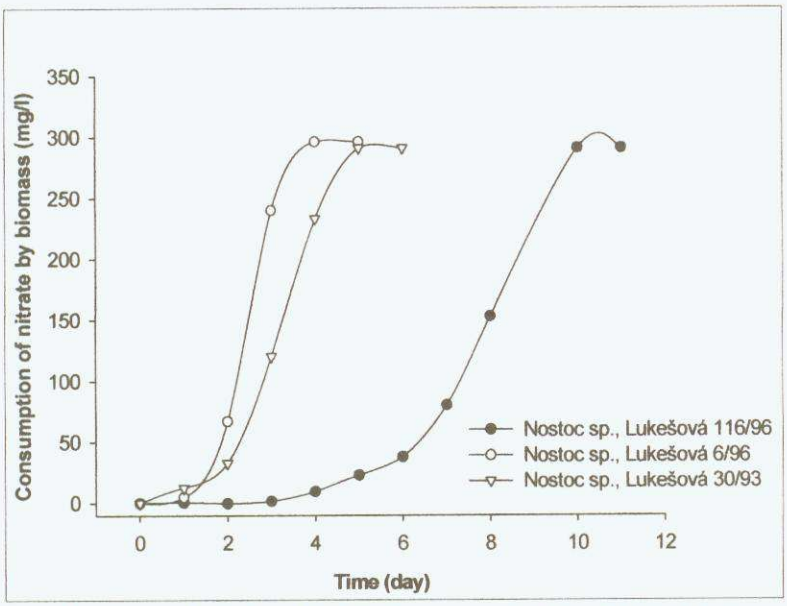


Fig. 16: Growth curve of *Nostoc sp.* Lukešová 116/96, *Nostoc sp.* Lukešová 6/96, *Nostoc sp.* Lukešová 30/93 in bioreactor at 30°C.

By plotting the content of nitrate against time on a semilogarithmic graph, a straight line results. The growth speed was determined after the following application of the linear regression (y = ax + b; a ~ growth speed). Finally, the growth speeds was used for comparison of growth at three different temperatures (25, 30 and 35°C) and four different light intensities (30, 70, 120 and 150 $W.m^{-2}$). Dependence of these values on light intensities illustrates next three figures 17, 18 and 19.

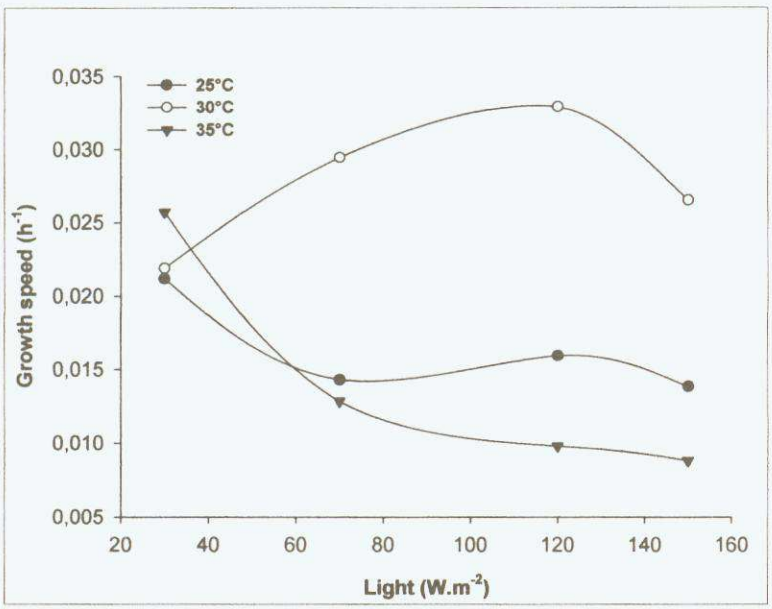


Fig. 17: Growth speed of *Nostoc sp.* Lukešová 116/96 at 25°C, 30°C and 35°C under illumination 30, 70, 120 and 150 $W.m^{-2}$.

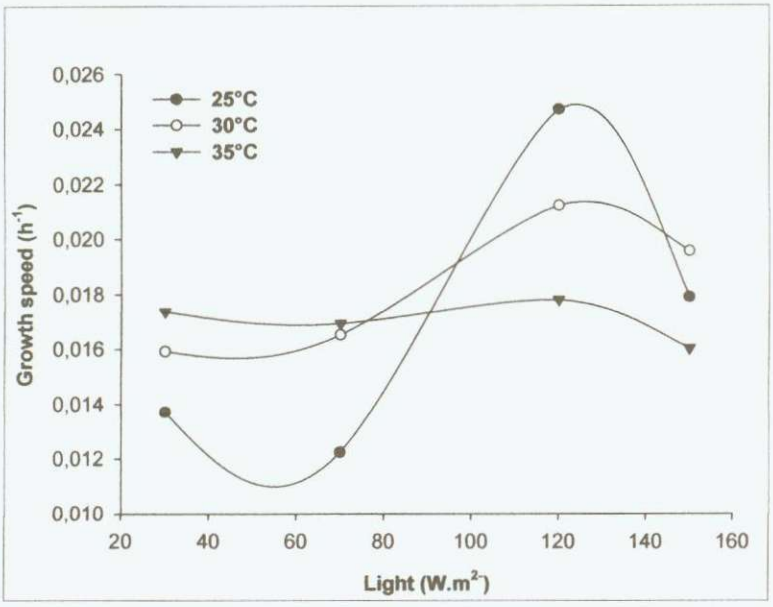
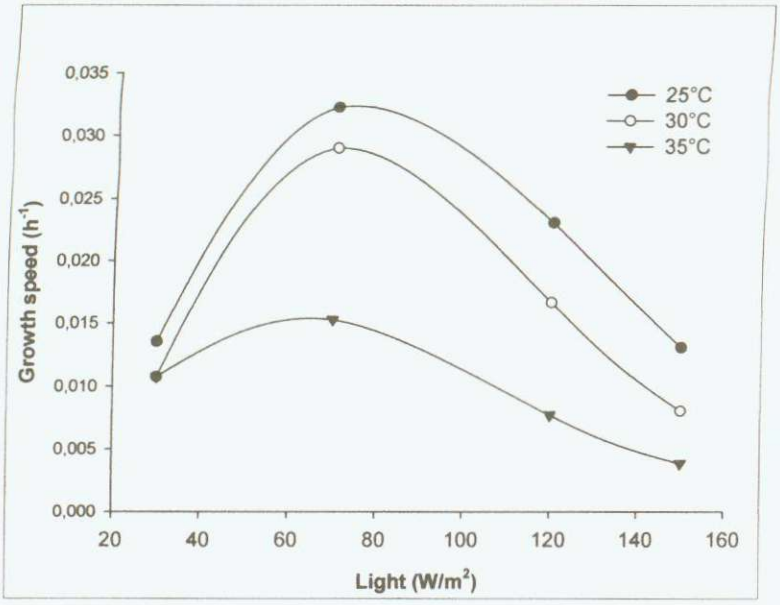


Fig.18: Growth speed of *Nostoc sp.* Lukešová 6/96 at 25°C, 30°C and 35°C under illumination 30, 70, 120 and 150 $W.m^{-2}$.



ig.19: Growth speed of *Nostoc sp. Lukešová 30/93* at 25°C, 30°C and 35°C under illumination 30, 70, 120 and 50 $W.m^{-2}$.

According to the curves maxima (see Fig. 17 to Fig. 19), the optimal temperature and ght intensity for the individual strains were determined. In the Fig. 20, the similarity etween growth speeds of *Nostoc sp.* Lukešová 116/96 and *30/93* is possible to notice. In omparison with *Nostoc sp.* Lukešová 6/96 their growth speeds were higher. So, the growth f *Nostoc sp.* Lukešová 6/96 was the lowest of these two strains.

Fig. 20: Optimal physiological parameters of Nostoc sp., Lukešová 116/96, 6/96 and 30/3.

Strain	Temperature (°C)	Light intesity ($W.m^{-2}$)	Growth speed (h^{-1})
Nostoc sp. Lukešová 116/96	30	120	0.0329
Nostoc sp. Lukešová 6/96	25	120	0.0247
Nostoc sp. Lukešová 30/93	25	70	0.0323

4.2.2 Production of trypsin inhibitors during the cultivation

Production of active compounds during the cultivation was studied by 24 hour sampling of 200 ml Nostoc suspension. After the centrifugation (5,000 r.p.m) and the lyophilization, the extracts were prepared. The following HPLC/MS analysis evaluated the relevant active peaks, using ChemStation® software. Final dependence of relevant active peaks areas on the cultivation time corresponds to the production of the active compound during the cultivation.

The Figure 21 shows a comparison of the growth curve of *Nostoc sp.* Lukešová 116/96 with the active compound production during the cultivation. In this strain, two active compounds with molecular ion peaks m/z 775.5 and 997.5 were proved (see to 4.4.1). Their maximal production was detected in biomass during stacionary phase of growth. The content in the cultivation medium was low, however at the stationary phase of growth minor accumulation of ion m/z 775.5 was observed. This could be explained by release of the active compound from necrotic cells.

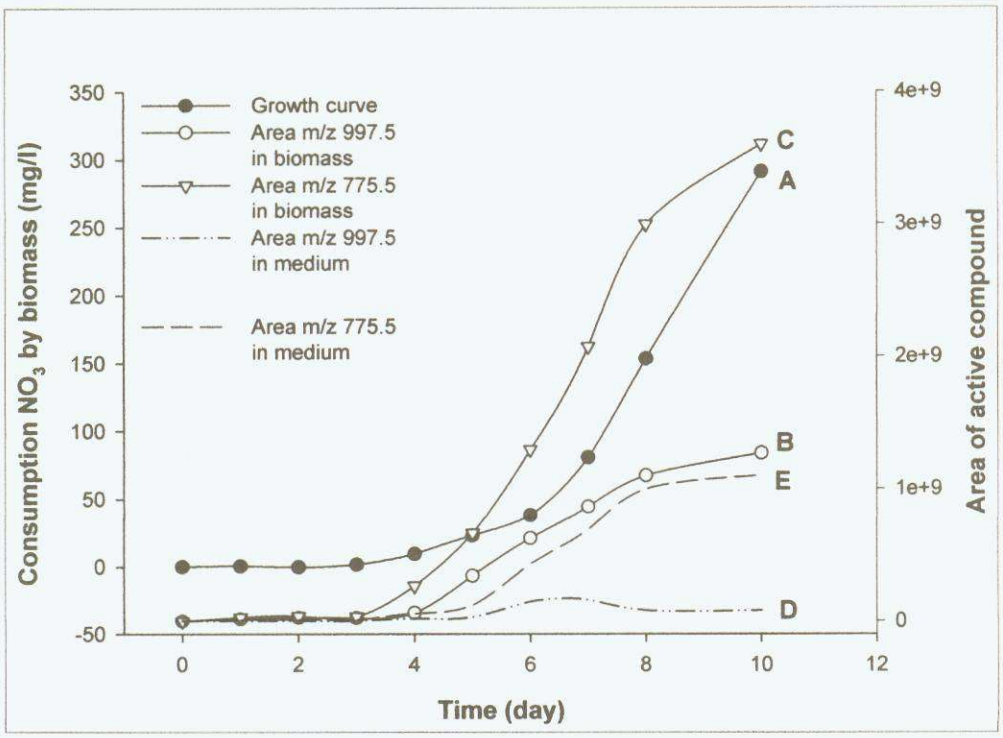


Fig. 21: Comparison of the growth curve of *Nostoc sp.* Lukešová 116/96 with the production of the active compounds m/z 997.5 and 775.5. **A**: Growth curve. **B**: Area of m/z 997.5 in biomass. **C**: Area of m/z 775.5 in biomass. **D**: Area of m/z 997.5 in medium. **E**: Area of m/z 775.5 in medium.

The Figure 22 shows a comparison of the growth curve of *Nostoc sp.* Lukešová 6/96 with the active compound production during the cultivation. In this strain the occurrence of an active compound with molecular ion m/z 705.5 was observed (see to 4.4.1). The maximal compound production was detected during logarithmic phase of growth and the medium curve shows that this compound was not released into the medium.

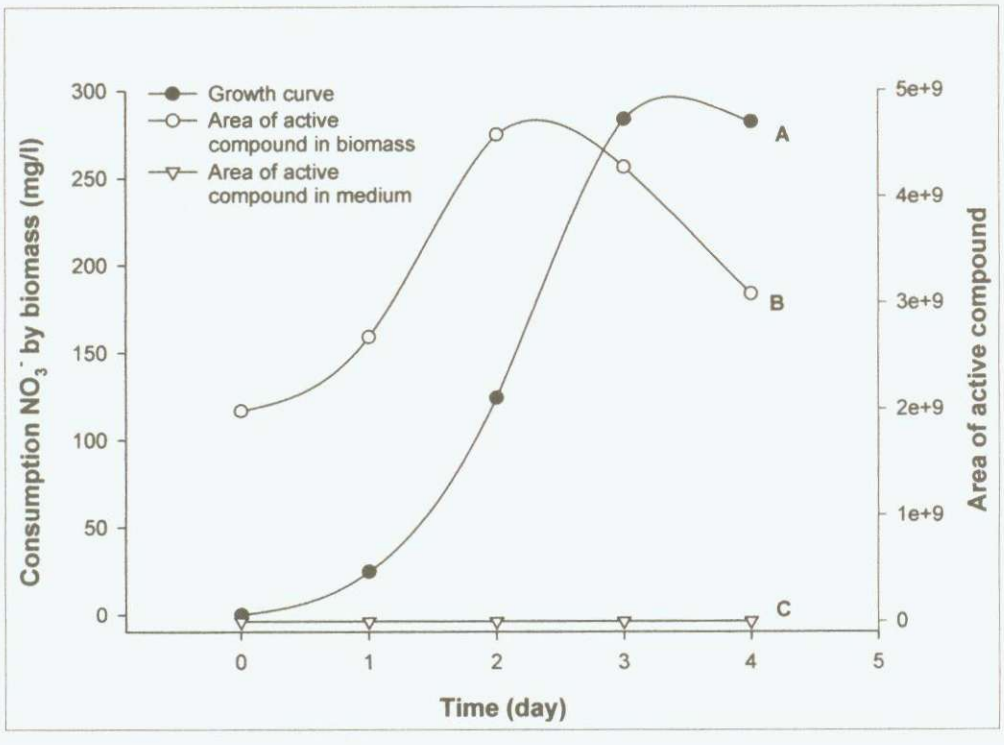


Fig. 22: Comparison of the growth curve of *Nostoc sp.* Lukešová 6/96 with production of the active compound m/z 707.5. **A**: Growth curve. **B**: Area of m/z 707.5 in biomass. **C**: Area of m/z 707.5 in medium.

Figure 23 shows a comparison of the growth curve of *Nostoc sp.* Lukešová 30/93 with the active compound production during the cultivation. In the strain the occurrence of an active compound with molecular ion m/z 865.5 (see to 4.4.1) was observed. The maximal compound production was detected during logarithmic phase. The content in the cultivation medium was infinitesimal, however it could be explained by release of the active compound from necrotic cells.

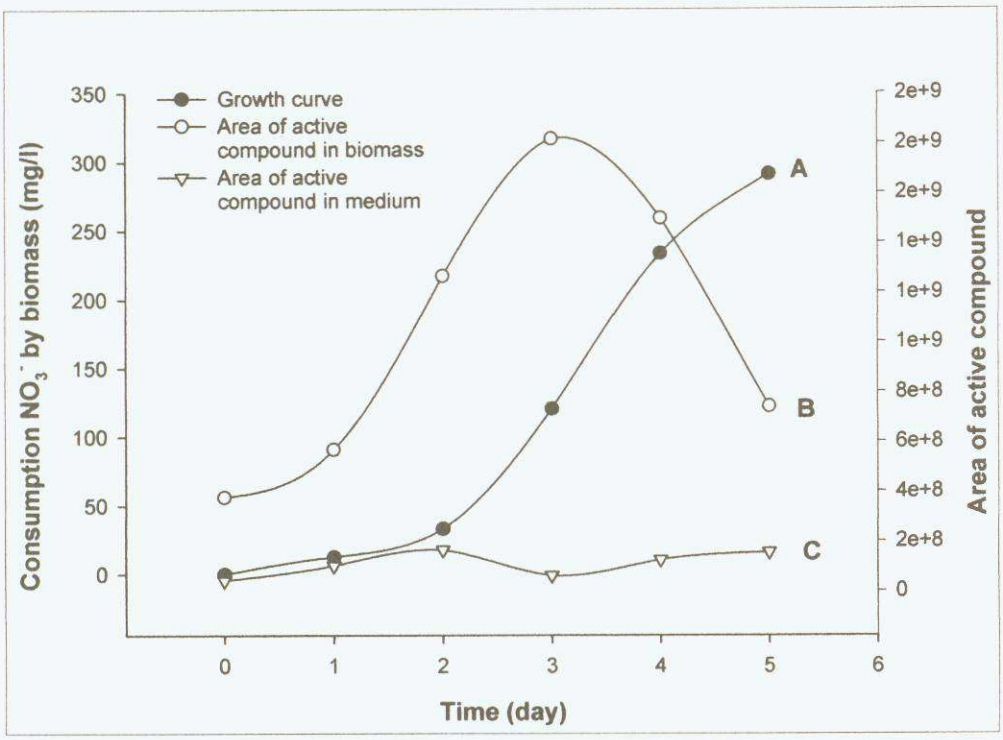


Fig. 23: Comparison of the growth curve of *Nostoc sp.* Lukešová 30/93 with production of the active compound m/z 865.5. **A**: Growth curve. **B**. Area of m/z 865.5 in biomass. **C**: Area of m/z 865.5 in medium.

4.3 ISOLATION AND CHARACTERISATION OF THE SUBSTANCES WITH TRYPSIN INHIBITORY ACTIVITY

4.3.1 Determination of active compounds

The biomass of three active *Nostoc* strains was obtained by the cultivation in bubbled column bioreactor at the optimal physiological parameters. After centrifugation, lyophylization and extraction with 70% methanol the extract was separated on C8 column of preparative HPLC/MS. Continually the individual fractions were collected according to the ratio *m/z*. By next trypsin inhibitory assay the active fraction was determined.

The following figure 24 shows total ion chromatogram of extract obtained from *Nostoc sp.* Lukešová 116/96 with the active molecular ions m/z 775.5 and 997.5.

The same procedure was carried out with two last strains. The active molecular ion m/z 707.5 was detected in the fractions from *Nostoc sp.* Lukešová 6/96 (Fig. 25) whereas the active molecular ion m/z 865.5 from *Nostoc sp.* Lukešová 30/93 (Fig. 26).

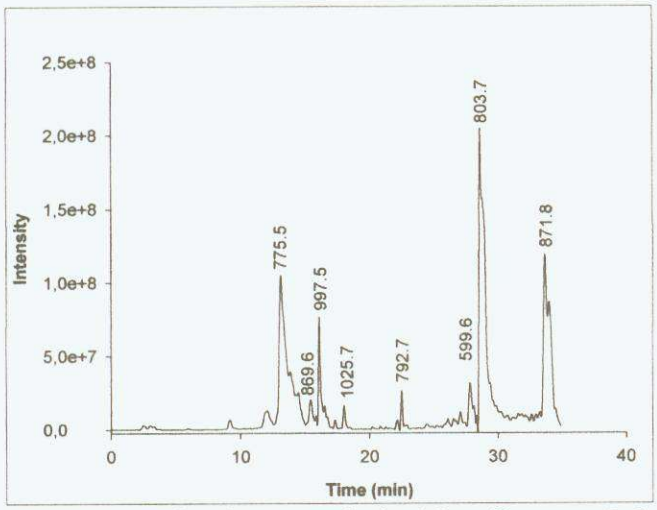


Fig. 24: Total ion current chromatogram of crude extract obtained from *Nostoc sp.* Lukešová 116/96 recorded on analytical HPLC/MS.

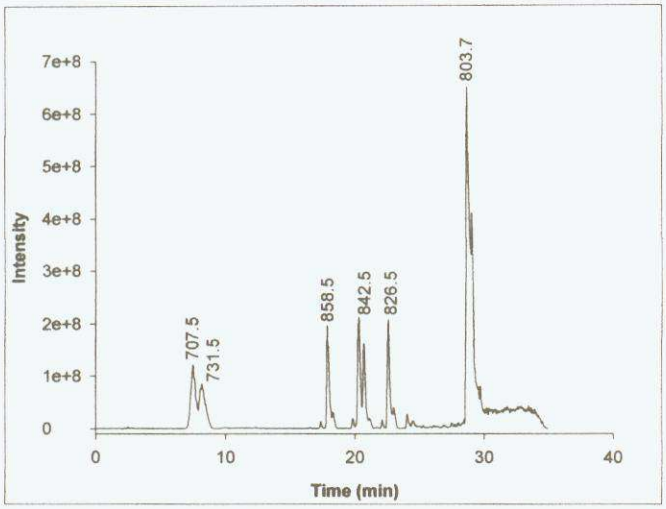


Fig. 25: Total ion current chromatogram of crude extract obtained from *Nostoc sp.* Lukešová 6/96 recorded on analytical HPLC/MS.

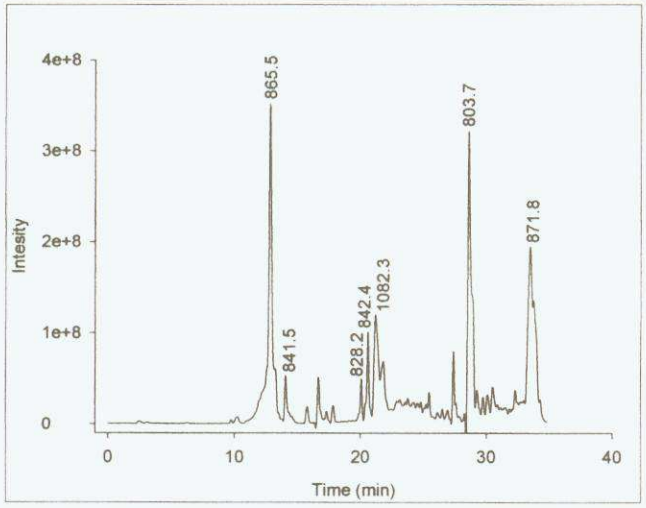


Fig. 26: Total ion current chromatogram of crude extract produced by *Nostoc sp.* Lukešová 30/93 recorded on analytical HPLC/MS.

4.3.2 Influence of solvent on extraction efficiency

The influence of various solvents on the quantitative extraction of active compound was tested. So, the same amount of lyophilized biomass (0.2 g) was extracted with the same volume (6 ml) of 100% methanol, 70% methanol, methanol:tetrahydrofurane (1:1) and acetone. Moreover, the supercritical fluid extraction (SFE) was used. The resulting extracts were tested by HPLC/MS and the areas of the active peaks are showed in the Fig. 27 to 29.

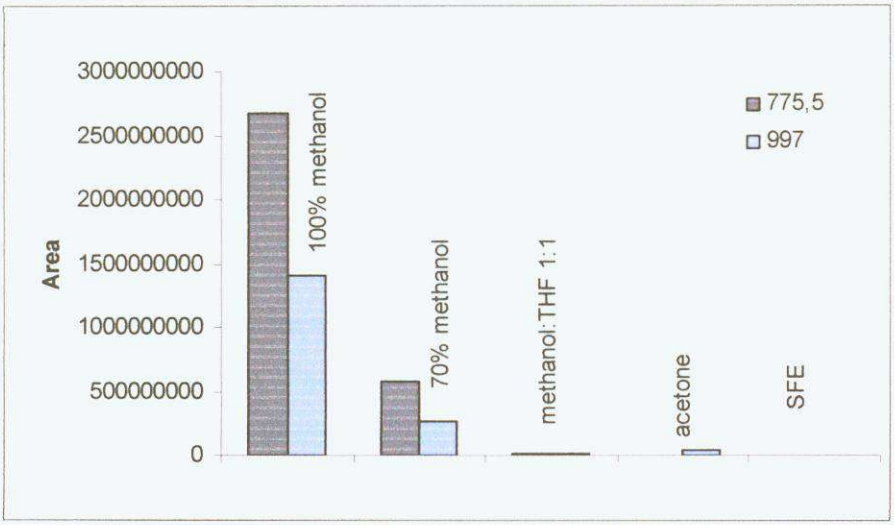


Fig. 27: Comparison of the solvent efficiency with the extraction of the active compounds m/z 775.5 and 997 produced by *Nostoc sp. Lukešová* 116/96.

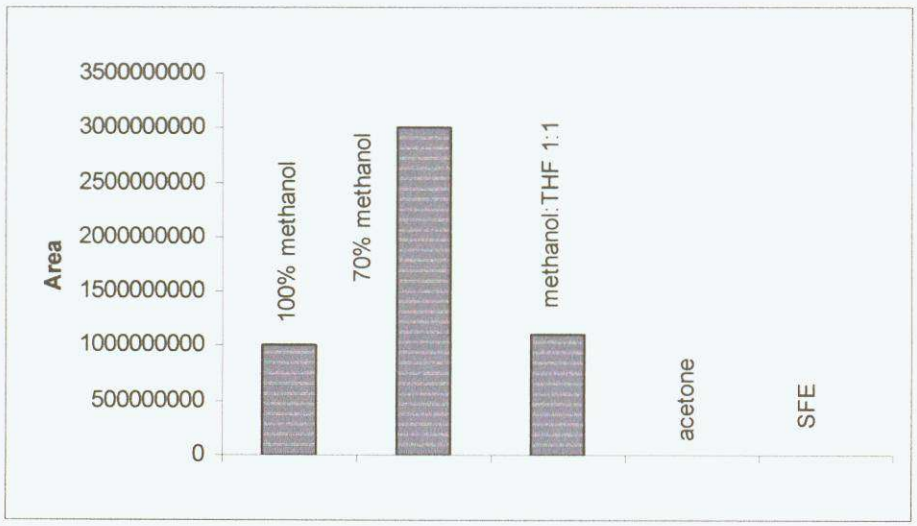


Fig. 28: Comparison of the solvent efficiency with the extraction of the active compound m/z 707.5 produced by *Nostoc sp.* Lukešová 6/96.

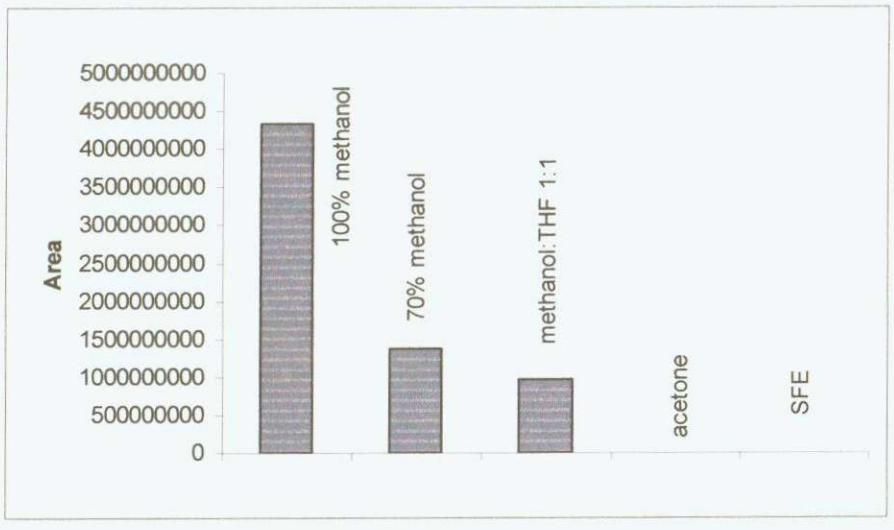


Fig. 29: Comparison of the solvent efficiency with the extraction of the active compound m/z 865.5 produced by *Nostoc sp.* Lukešová 30/93.

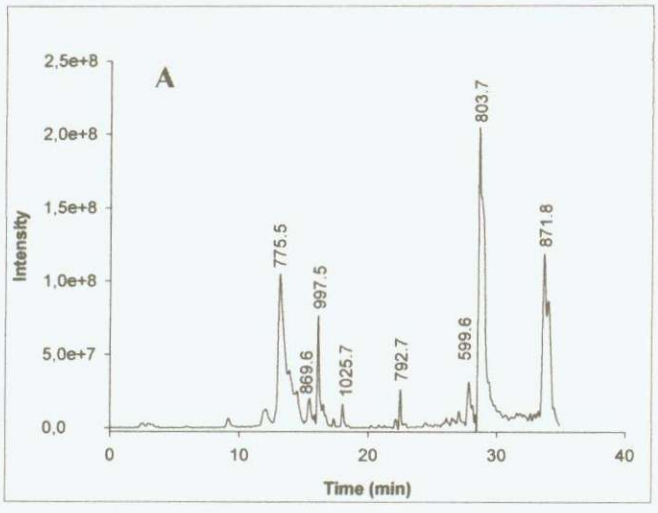
Corresponding to above mentioned results, 100 % methanol is the most efficient extraction solvent for *Nostoc sp., Lukešová* 116/96 and *Nostoc sp. Lukešová 30/93.* The most efficient extraction solvent for *Nostoc sp. Lukešová 6/96* is 70% methanol. Therefore, these solvents were consequently used for quantitative isolation of the relevant active compounds.

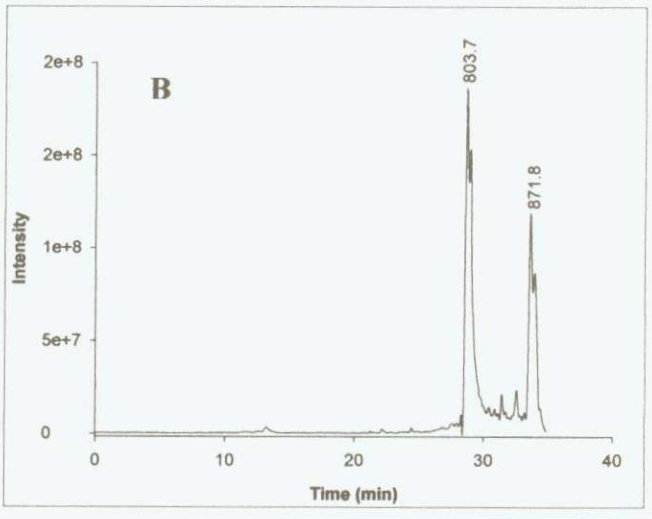
4.3.3 Pre-separation techniques

Pre-separation techniques for the active compounds were developed for *Nostoc sp.* Lukešová 116/96, 6/96 and 30/93. On the basis of the previous results the harvest of biomass was carried out during the stationary phase of growth in case of strain *Nostoc sp. Lukešová 116/96,* and in the log phase for *Nostoc sp.* Lukešová 6/96 and 30/93. Afterwards, the obtained lyophilized biomass was extracted with relevant extraction solvent. In this way significant amount o pigments pass to the extract. Therefore, we developed the methodology for pre-separation.

For *Nostoc sp.* Lukešová 116/96 was used the methodology which is shown in the Fig. 30. Firstly, the crude extract was evaporated under vacuum, and the obtained evaporator was washed out with hexane. As a result, considerable amount of pigments was removed. The remaining solid evaporator was dissolved in methanol and the rest of pigments were removed by liquid-liquid extraction using hexane. For better partition, acetone was added to the mixture. Finally, two layers were obtained. One of them - green hexane/acetone layer didn´t contain the active compound (Figure 31 B) and other one - yellow methanol/acetone layer

The figure 31 A shows the crude extract from *Nostoc sp.* Lukešová 116/96 with the ıctive compound at retention time 13.3 min with m/z 775.5. Additionally, the active :ompound with m/z 997.5 at retention time16.1 min was observed. Next figure 31 B shows ıexane layer with non-polar pigments (m/z 803.7 and m/z 871.8). Other figure 31 C shows nethanol layer, which contains the active compounds. In comparison with figure A the peak vith m/z 871.8 in the figure C was highly reduced. After the previous procedure, the pre-;eparated extract was obtained.





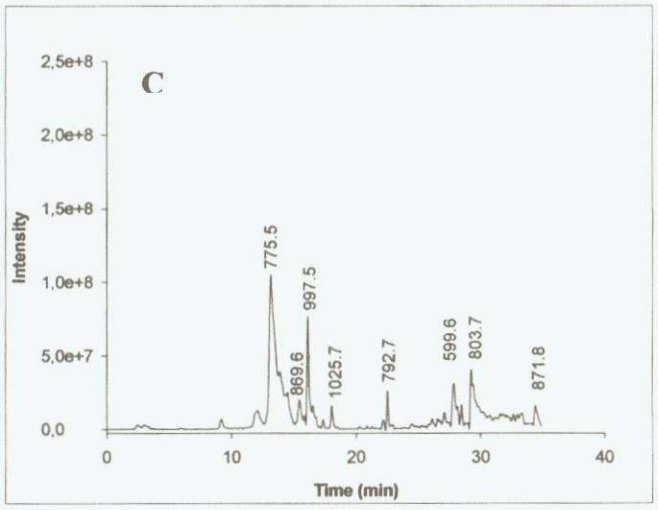
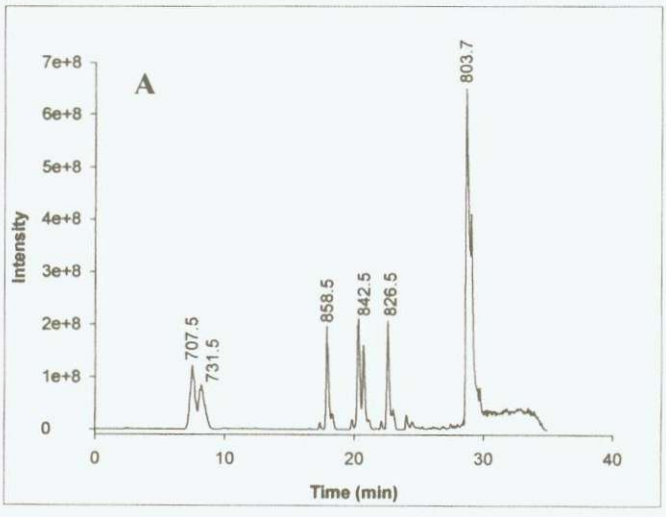


Fig. 31: Total ion current chromatograms of extract from *Nostoc sp. Lukešová* 116/96 with active compound m/z 777.5 and m/z 997.5. A: Crude extract. B: Hexane layer. C: Methanol layer.

Figure 32 A shows the crude extract from *Nostoc sp., Lukešová 6/96* with the active compound m/z 707.5 at retention time 7.5 min. Figure 32 B shows hexane layer with non-polar pigments (m/z 803.7 and m/z 871.8). Figure 32 C shows methanol layer which contains the active compound. In comparison with figure A the peak with m/z 871.8 in the figure C was reduced to 50%. After the previous procedure, the pre-separated extract was obtained.



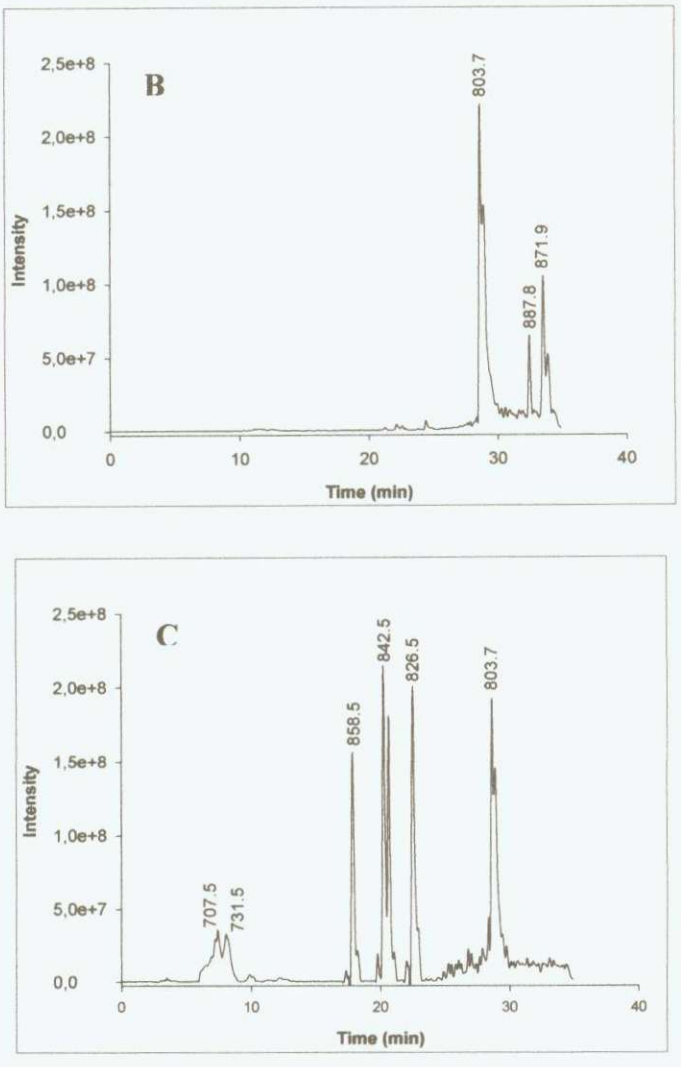


Fig. 32: Total ion current chromatograms of extract from *Nostoc sp.* Lukešová 6/96 with active compound m/z 707.5. A: Crude extract. B: Hexane layer. C: Methanol layer.

Figure 33 A shows the crude extract obtained from *Nostoc sp. Lukešová 30/93* with the active compound m/z 865.5 at retention time 12.5 min. Figure 33 B shows hexane layer with non-polar pigments (m/z 803.7 and m/z 871.8). Figure 33 C shows methanol layer which contains the active compound. In comparison with figure A the peak with m/z 871.8 in the figure C was highly reduced. After the previous procedure, the pre-separated extract was obtained.

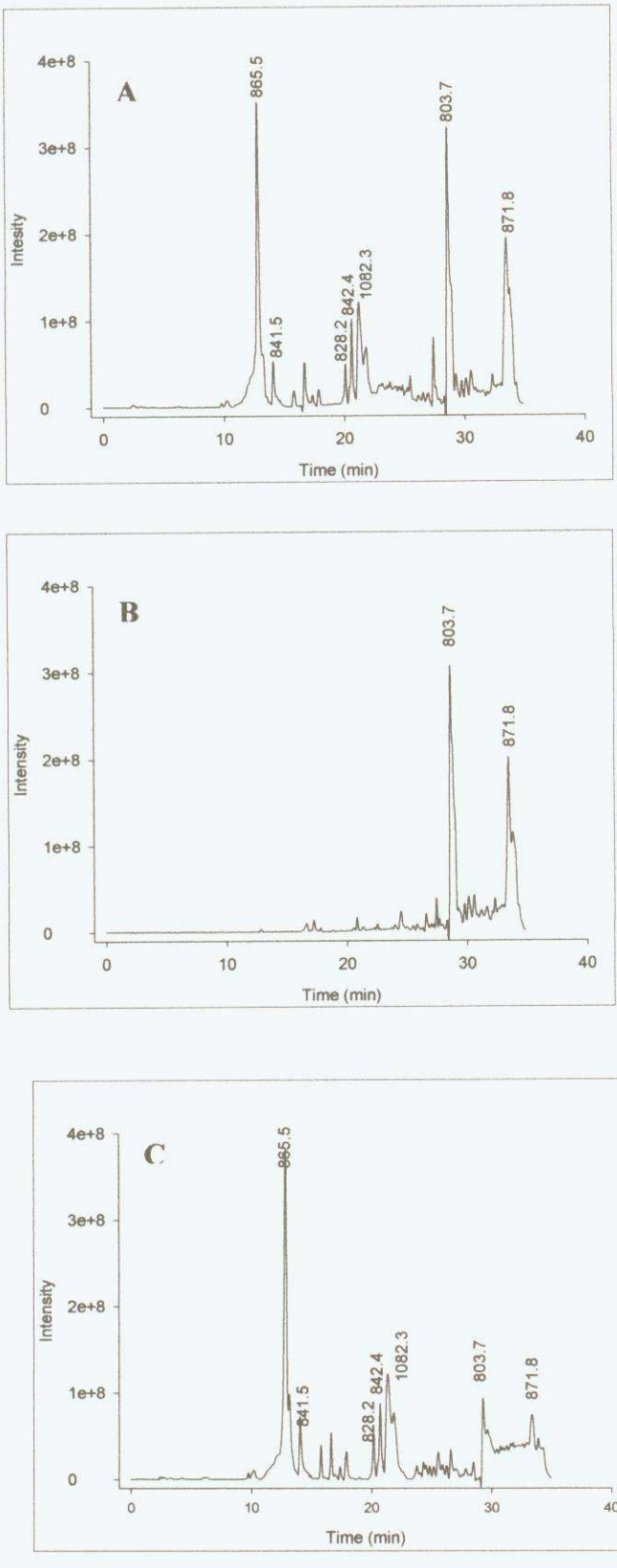


Fig. 33: Total ion current chromatograms of extract from *Nostoc sp.* Lukešová 30/93 with active compound m/z 865.5. A: Crude extract. B: Hexane layer. C: Methanol layer

4.3.4 Preparative HPLC/MS

The pre-separated extract obtained from *Nostoc sp,* Lukešová 30/93 was injected repeatedly on C_8 column of preparative HPLC/MS. The active peaks were collected according to the ratio m/z. Afterwards, the collected peaks were mixed together, evaporated and resuspended in 1 ml of methanol. This pure fraction was analyzed by NMR analysis.

In addition, HPLC-ESI-MS^n of the pre-separated extract in the positive ion mode was realized. Fig. 34 shows mass spectrum of *Nostoc* sp. Lukešová 30/93 with the protonated molecular ion $[M+H]^+$ at m/z 865. The product-ion spectrum of the protonated molecular ion shows that the $[M+H]^+$ at *m/z* 865 loses small, neutral fragments (NH_3, H_2O, CO or $H_2NCOCH_2NH_2$) accompanying by loss of 176 corresponding to arginine side chain. Resulting most intensive ion at *m/z* 689 contains information on the cyclic peptide moiety and shows that the small neutral fragments (NH_3, H_2O, CO and $CONH_2$) are again lost in its fragmentation. The gas-phase chemistry leading to the formation of the ions is highly dependent on the site of initial protonation to form the molecular ion. The protonation occurs initially on the most basic nitrogen atom of arginine residue and this side chain amino acid is ejected in the first stage of CID as a neutral species. The high intensity of the molecular ion also supports the presence of Arg in the side chain because of his high proton affinity. The residual peptide moiety is formed as the mixture of linear isobaric ions present in the product-ion spectrum at *m/z* 689. This linear peptide moiety fit to the following amino acid sequence [N-MetAla–Val–Leu/Ileu–Phe-Leu/Ileu-Met]. The final structure confirmation will be done by detailed NMR analysis of the active fraction.

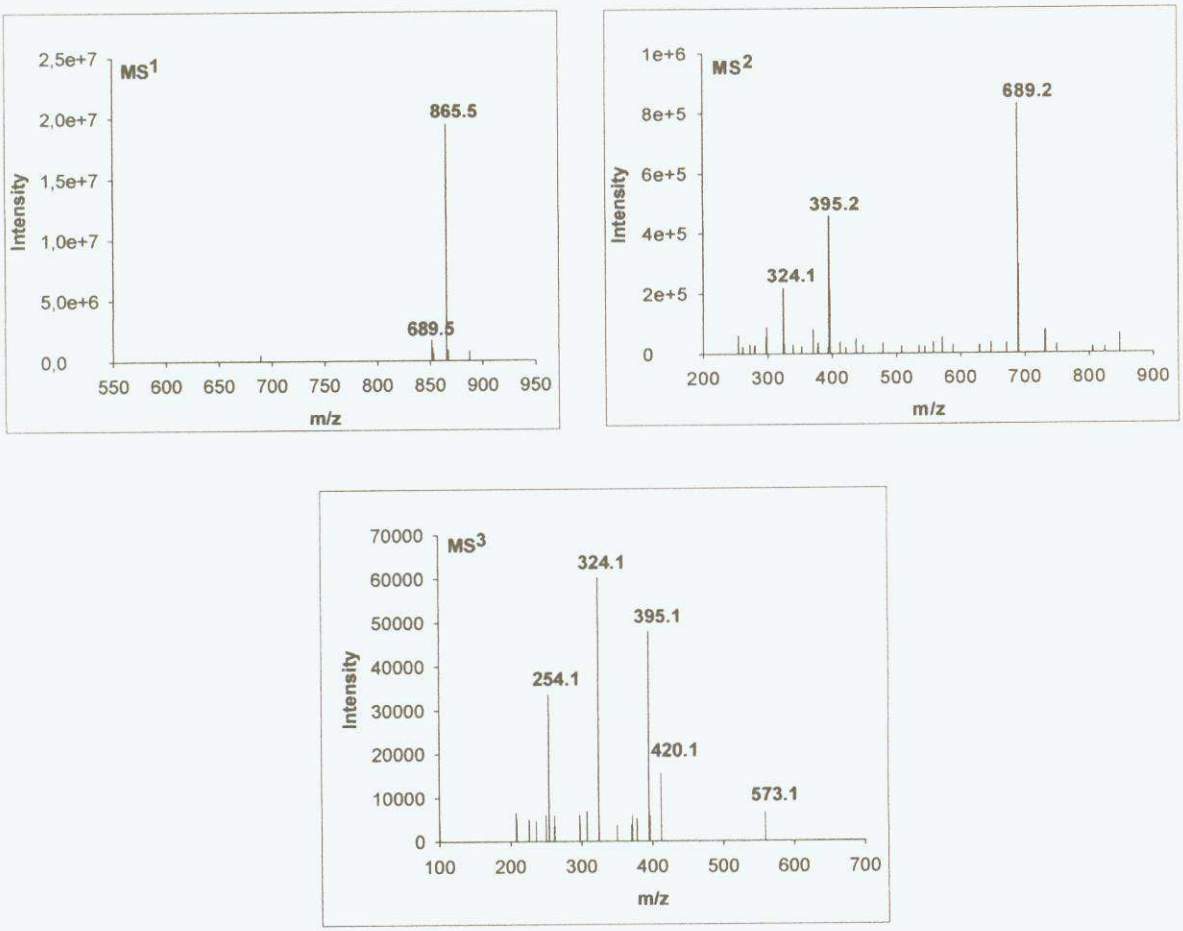


Fig. 34: MS^1 – MS^3 spectra of active compound m/z 865.5 produced by *Nostoc sp.* Lukešová 30/93.

5. SUMMARY AND DISCUSSION

Proteolytic enzymes are essential for most physiological processes, but their overexpression or unregulated actions can lead to many debilitating diseases associated with the CNS and cardiovascular systems, inflammatory and neurodegenerative conditions, and viral and parasitic infections (Puente, 2003). It also seems likely that the regulation of protease precursors, protease folding, endogenous protease inhibitors, cofactors, receptors and transporters can all indirectly influence protease activity. So, extensive research and resources targeted to the development of potent and selective protease inhibitors has been carried out all over the world.

Steadily over the years, the focus of interest has been shifted from plants towards the microorganisms as a new source of natural products. The production of bioactive metabolites by bacteria is preferred over plants and animals. This is attributed to: 1) their ability of the cultivation in any quantity and any condition, since, they are being found in extreme environments i.e. polar ice, geothermal vents, dark cave-sand and deep-sea sites; 2) Bacteria are symbiotically important to animals and plants; 3) most of natural antibiotic are attributed to bacteria and fungi.

This thesis was focused on microalgae and cyanobacteria as a source of metabolites with serine inhibitory activity and allelopathic activity. The primary function of most members of the serine proteinase inhibitor family is to neutralise overexpressed serine proteinase activity (Travis, 1983). In addition, it seems that the production of protease inhibitors is a common protection strategy in terrestrial plants against herbivores (Bowles, 1998). The trypsin inhibitors produced by cyanobacteria threaten aquatic animals that feed on cyanobacterial cells. Of particular interest are the effects on the microcrustaceans *Daphnia spp.*, which are among the grazers of planktonic cyanobacteria. The lethal molting disruption of *Daphnia spp.* causing Microviridin J, a newly discovered protease inhibitor produced by *Microcystis strain* UWOCC MRC was reported (Jaspars, 1998; Rohrlack, 2004).

On this purpose, we carried out the large scale screening with more than 124 algal strains and 58 cyanobacterial strains. The following trypsin inhibition was exhibited by 22 % of algal and 41% of *Nostoc* strains. In addition, 24 % of algal and 32% of *Nostoc* strains showed elastase inhibitory activity. The presence of trypsin inhibitors in the cultivation medium was observed just in 3 % of algal strains.

The large scale screening was also focused on allelopathic activity, which may play role in the interspecific competition. The release of organic compounds by algae and cyanobacteria affect other species - their potential competitors for nutrient resources. Rice (1984) suggested that algal toxins play a role in phytoplankton succession by affecting some species more than others. Until now, some cyanobacterial allelochemicals have been isolated and characterised (Landsberg, 2002). In this thesis the screening of previously mentioned strains for the allelopathic activity was carried out. We observed that 15 % of the algal strains exhibited the allelopathic activity. Furthermore, the *Nostoc* strains did not exhibit the allelopathic activity.

On the basis of trypsin inhibitory assay, three of the most active Nostoc strains were chosen for the thesis studies. At the beginning of the research, the optimal physiological parameters were investigated. *Nostoc sp.* Lukešová 116/06, *Nostoc sp.* Lukešová 6/96 and *Nostoc sp.* Lukešová 30/93 were cultivated at different temperatures and at different light intensities. Finally, the individual growth curves were monitored and after their evaluation, the optimal growth parameters were determined (see chapter 4.2.1).

Further fractionation of crude extracts by preparative HPLC with connection to MS allowed us to determine the fraction with trypsin inhibitory activity (see chapter 4.4.1). On the basis of these results, we studied the production of the active compounds during the cultivation (see chapter 4.2.2). The maximal production during log phase of growth was observed in *Nostoc sp.* Lukešová 6/96 and *Nostoc sp.* Lukešová 30/93. This could be proof of a fact that these compounds are synthesized as primary metabolites, whereas in case of *Nostoc sp.* Lukešová 116/96 the production of the active compound was observed during stationary phase of growth curve. The content of the active compound in the cultivation medium was infinitesimal, however it could be explained by release of the active compound from necrotic cells.

Additionally, we developed the pre-separation technique for three mentioned active *Nostoc* strains (see chapter 4.3.3). The following separation of active compound for MS and NMR analysis was carried out only for *Nostoc sp.* Lukešová 30/93. The structure determination was realised on the basis of the fragmentation mechanism in the ion trap as well as supported by previous comparison with information from the literature (see chapter 4.4.4). We proposed the active compound fit to the following amino acid sequence [N-MetAla–Val–Leu/Ileu–Phe-Leu/Ileu-Met], however the accurate confirmation of the structure should be realized by NMR analyses.

Due to the fact that trypsin inhibitors from *Nostoc sp.* Lukešová 30/93 was produced in logarithmic phase of growth when the density of culture is lower than in stationary phase, the obtained amount of lyophilized biomass was too low. Hence the cultivation of biomass for isolation of bioactive compound in bubbled column bioreactor was repeated several times. We obtained the pure active fraction with m/z 865.5, but this amount for NMR analysis was insufficient, so the signal obtained from NMR was not interpretable. Therefore, it could be concluded that continuation of the submitted studies allow the next research in the field of bioactive compounds production by various algal and cyanobacterial strains.

6. APPENDIX

Algological Studies (in print):

Genus *Nostoc* a source of novel trypsin inhibitors

By PETRA DRÁPALOVÁ [1], DALIBOR ŠTYS[3], ALENA LUKEŠOVÁ[4] and JIŘÍ KOPECKÝ[2]

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[2] Institute of Microbiology, Academy of Sciences, Třeboň, Czech Republic
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With 7 figures and 3 tables in the text

Abstract: In the extract of fifty newly isolated strains of the genus *Nostoc* were identified numerous new molecules showing the capacity of protease inhibition. The growth conditions of the most active molecule, found in the strain *Nostoc* sp., Lukešová 30/93, were studied in detail. We have examined the optimal physiological parameters (growth phase, light intensity and culture temperature). The highest active compound concentration was usually found 3 days after inoculation in culture grown at optimum conditions (light intensity 70 $W.m^{-2}$ and temperature 25°C). Similar observation seemed to be found other strains examined in the study. Thus, we may conclude that what concerns the trypsin inhibiting secondary metabolites we have not found any other dependency on the cell growth phase, light intensity or temperature than that leading to optimum of growth conditions. We propose that the production of the trypsin inhibitor is coincident with conditions under which the colony of the microorganism prospers mostly. The possibility that these compounds serve the anti-grazing function, prominent in time of expansion, is logical and consistent with previous observation. The trypsin inhibitor examined in detail is a cyclic oligopeptide containing non-coded amino acids. As such this compound is highly interesting from the point of view of both pharmacology – probably not degradable by ordinary proteinases – and from the point of view of mechanism of inhibition.

Key words: Cyanobacteria, *Nostoc*, trypsin inhibitors, LC/MS, peptides

Introduction

Nostoc species are terrestrial and benthic cosmopolitan microorganisms often forming mucilaginous layers on soil and in the aquatic environment on stones and mud (Doodds et al. 1995). Many secondary metabolites with new chemical structures have been isolated from these organisms (Patterson et al. 1994, Moore 1996, Harada 2004). Significant part of these metabolites possess an interesting biological activities including antibacterial (Pergament & Carmeli 1994), antifungal (Biondi et al. 2004), antitumor (Gerwick 2001), anti-HIV (Schaeffer & Krylov 1999), immunosuppressive (Zhang et al. 1997), various enzyme

inhibitors (Radau et al. 2003, Yamaki et al. 2005), as well as angiotensin-converting enzyme inhibitory activity (Okino et al. 1993). Apart from well known genera of freshwater and brackish cyanobacteria (*Microcystis*, *Anabaena*, *Oscillatoria)* some strains of *Nostoc* also produce potent hepatotoxic cyclic peptides microcystins which have been known for more than 20 years (Carmichael 1988, Carmichael 1992, Rinehart et al. 1988, Kuiper-Goodman et al. 1999).

Among broad range of bioactive secondary metabolites synthesized by cyanobacteria the members of the serine protease inhibitors represents one of the most important ones (Radau 2000). Proteases play crucial functions in organisms all over the phylogenetic tree, starting from viruses, bacteria, protozoa, algae, fungi, or higher plants and ending with animals and humans, both at the physiological level and in infection mechanisms. Regulation of proteolytic activity in biological processes is essential, and many pathways involving protease enzymes use also specific inhibitors to control the balance between desired and undesired products (Neurath 1989). Failures of one or more of these enzymes may cause a state of imbalance between protease and anti-protease (endogenous protease inhibitors - serpins) and may lead to an excess of proteolytic activity and to the development of various diseases (thrombosis, asthma, multiple sclerosis and pancreatitis). The serine proteases of the trypsin superfamily are by far the most versatile and widely studied proteolytic enzymes. Some protease inhibitors catalyze tumour progression have shown good anti-tumor activity without the side effects of known cytotoxic drugs (Rohrlack *et al,* 2003). The proteases in general are key factors in determining the infectiveness of various pathogens, ranging from viruses, bacteria, fungi to protozoa. Their inhibition thus represents an attractive target in therapy and pharmaceutical research (Leung 2000, Tossi 2000, Zlatoidsky 1996, 1999).

Cyanobacteria represent rich and unexplored source of active compounds of new bioactive compounds of new chemical structures. We now report the results of natural compound library based on the secondary metabolites produced by fifty isolates of the genus *Nostoc* active against trypsin. We found that the trypsin inhibition activity is common if not generic in *Nostoc* and that numerous different compounds exhibit this activity. We describe the most active strain *Nostoc* sp., Lukešová 30/93 in more detail. The optimal physiological parameters (light intensity, growth temperature) and the changes in production of the active compound during cultivation were estimated under controlled culture condition. We also found that, at least in this strain and for this compound, there is no active efflux of it in the medium.

Materials and methods

Organisms and culture conditions

Cyanobacterial strains were obtained from the Collection of Algal Strains at the Biological Institute, St. Petersburg University, Russian (CALU); Culture Collection of the Center of Phycology, Institute of Botany, Třeboň, Czech Republic (CCALA), Culture Collection of the Institute of Soil Biology, South Bohemia University, České Budějovice, Czech Republic (ISB, Dr. Lukešová). All strains tested were isolated from soil and freshwater samples collected in different habitat. Cyanobacteria were purified essentially by methodology described earlier (de Chazal, 1996). Unialgal, but not necessarily axenic cyanophyte cultures were determined by microscopic observation and repurified by further plating if necessary. Since almost all strains have been collected from natural environment, no information on optimal culture conditions were available, therefore we apply following conditions as a standard culture conditions for screening work. Cyanobacterial isolates were placed in the medium of Allen and Arnon (Allen & Arnon, 1955) and grown in 350 ml glass tubes bubbled

by mixture of air and 2 % carbon dioxide at constant temperature (28°C) under low continuous illumination with banks of cool-white fluorescent lights of 50 W/m^2 (Philips, Osram Dulux L, 55W/12-950). After two weeks of cultivation, the strains were harvested by centrifugation (Hettich 320 centrifuge) at 5 000 r.p.m. The supernatant was directly tested and biomass was lyophilized (Lyovac GT3, Leybold-Heralens).

The experiments for optimization of growth parameters were carried out at temperatures 20°C, 25°C, 30°C and 35°C, light intensities 30, 70, 120 and 150 $W.m^{-2}$. The growth rate of the selected active *Nostoc* strain was measured indirectly from nitrate consumption measured by ion chromatography (Dionex, IC90, USA).

Table 1. List of *Nostoc* strains tested for presence of trypsin inhibitors.

No	Cyanobacterial species/strain	Source	No.	Cyanobacterial species/strain	Source
1	*Nostoc sp., Lukešová 5/96*	ISB	26	*Nostoc muscorum, 543*	CALU
2	*Nostoc sp., Lukešová 20/97*	ISB	27	*Nostoc muscorum, 545*	CALU
3	*Nostoc sp., Lukešová 3/97*	CCALA	28	*Nostoc muscorum, 546*	CALU
4	*Nostoc sp., Lukešová 27/97*	ISB	29	*Nostoc edaphicum, 760*	CALU
5	*Nostoc muscorum, Lukešová 14/86*	ISB	30	*Nostoc sp., 803*	CALU
6	*Nostoc sp., Lukešová 116/96*	ISB	31	*Nostoc sp., 870*	CALU
7	*Nostoc sp., Lukešová 51/91*	ISB	32	*Nostoc sp., 907*	CALU
8	*Nostoc sp., Lukešová 19/96a*	ISB	33	*Nostoc sp., 913*	CALU
9	*Nostoc muscorum, Lukešová 27/97*	ISB	34	*Nostoc sp., 914*	CALU
10	*Nostoc sp., Lukešová 18/89*	ISB	35	*Nostoc sp., 915*	CALU
11	*Nostoc sp., Lukešová 2/89*	ISB	36	*Nostoc linckia, 979*	CALU
12	*Nostoc muscorum*	ISB	37	*Nostoc linckia, 980*	CALU
13	*Nostoc sp., Lukešová 1/88*	ISB	38	*Nostoc linckia, 981*	CALU
14	*Nostoc sp., Lukešová 6/96*	ISB	39	*Nostoc linckia, 982*	CALU
15	*Nostoc sp., Lukešová 30/93*	ISB	40	*Nostoc linckia, 983*	CALU
16	*Nostoc sp., Lukešová 2/91*	ISB	41	*Nostoc commune, 984*	CALU
17	*Nostoc linkia, 129*	CALU	42	*Nostoc sp., 991*	CALU
18	*Nostoc sp., 268*	CALU	43	*Nostoc sp., 992*	CALU
19	*Nostoc muscorum, 304*	CALU	44	*Nostoc linka, 993*	CALU
20	*Nostoc sp., 327*	CALU	45	*Nostoc sp., 994*	CALU
21	*Nostoc punctiforme, 374*	CALU	46	*Nostoc sp., 995*	CALU
22	*Nostoc sp., 379*	CALU	47	*Nostoc sp., 996*	CALU
23	*Nnostoc linkia, 453*	CALU	48	*Nostoc sp., 997*	CALU
24	*Nostoc muscorum, 526*	CALU	49	*Nostoc sp., 998*	CALU
25	*Nostoc muscorum, 542*	CALU	50	*Nostoc sp., 1191*	CALU

Extraction and partial purification of the active compounds

Freeze-dried biomass was disintegrated by sea sand and extracted with 70% methanol (6 ml per 200 mg of dry biomass). The extract was centrifuged at 5 000 r.p.m. and crude supernatant was used directly for enzymatic assay.

Freeze dried biomass for isolation of active compound was disintegrated (2 times) by sea sand and extracted with 100% methanol (300 ml/10 g dry weight). The extract was centrifuged at 5 000 r.p.m. and supernatant evaporated under vacuum. The residue was dissolved in water/acetone and partitioned between water and hexane for removal of pigments. Water/acetone fraction containing the active compounds was applied on C_8 preparative HPLC.

Analysis

Analytical scale chromatography (screening analysis) was performed on the high performance liquid chromatograph (HPLC) Agilent 1100 with MSD SL-Ion Trap mass spectrometer and electrospray ion source (ESI). HPLC system consists of a vacuum degasser, binary pump, column thermostat and diode-array detector (DAD). Column used was Zorbax, XDB C8, 4.6 x 150 mm, 5 μm. The purification of active fraction was performed on the preparative HPLC connected through the splitter directly to mass spectrometer (1100 MSD SL-Ion Trap). The preparative system consists of two dual piston preparative pumps Prep 100, DeltaChrom^{TM} column thermostat and gradient controller LabAlliance ^{TM} (Watrex, Prague). Preparative column used was Reprosil 100, C8, 25 x 250 mm, 5 μm.

The mobile phase composition was the same for both the analytical and preparative scale separation: column temperature 35°C, linear gradient methanol/water from 30% to 100% of methanol within 30 min. The mobile phase contained 0.1% formic acid for improvement of ionisation. The flow rate was 0.6 ml/min for analytical column and 15 ml/min for preparative column. The detection wavelengths were 220, 233, 280 and 440 nm.

The ion trap mass spectrometer was optimized for ions with *m/z* ratio 900 in positive mode. The data acquisition and analysis was performed using CHEMSTATION Software under Windows^{NT} (Microsoft, USA) operating system. The spray needle was at a potential of 4.5 kV, and nitrogen sheet gas flow of 20 (arbitrary units) was used to stabilize the spray. The counter electrode was a heated (200 °C) stainless-steel capillary held at a potential of 10 V. The tube-lens offset was 20 V, and the electron multiplier voltage was -800 V. Helium gas was introduced into the ion trap at a pressure of 1 mTorr to improve the trapping efficiency of the sample ions introduced into the ion trap. The background helium gas also served as the collision gas during the collisionally activated dissociation (CAD) experiments. A typical experimental protocol for direct infusion MS/MS experiments consists of infusing a solution of a purified fraction from preparative HPLC in 30% methanol with 0.1% formic acid into the ion-trap mass spectrometer via a 250-μL syringe at a flow rate of 50 μL/min.

Trypsin Inhibition Assay

The assay was based on colorimetric techniques (Cannell, 1988) and was carried out in microtitre plates. Trypsin was dissolved in 50 mM Tris-HCl (pH 7.5) to prepare a 150 units/ml solution. A 15 μl enzyme solution, 55 μl 0.4 M Tris-HCl, 55 μl of 0.05 M Tris-HCl and 10 μl of test solution were added to each microtiter plate well and were preincubated at 37°C for 30 minutes. Then 115 μl of substrate solution (4.6 mg of BAPNA was dissolved in 100 μl of dimethylsulphoxide and made up to 10 ml with 50 mM Tris-HCl pH 7.5) was added to begin the reaction. The absorbance was immediately measured at 410 nm. The developed colour was measured after incubation at 37°C for 30 minutes. Photometric microplate reader Sunrise (TECAN) and related equipment (Magellam 2.01 software) was used for the *in vitro* enzyme assays.

Chemicals

Nα-benzoyl-D,L-arginine-4-nitroanilde hydrochloride (BAPNA), trypsin from porcine pancreas (lyophilized powder, 2,000 BAEE units/mg), methanol, formic acid, Tris and all

common chemicals were purchased from Sigma-Aldrich, dimethyl solfoxide (DMSO) and hydrochloric acid from Fluka.

Results

Extraction and screening for trypsin inhibitors

Depending on the solvent used in the extraction method, different quantities and types of compound can be extracted from analyzed material. In the literature, many kinds of extraction schemes are described: some highly selective for a certain group of compounds, others very general, using an array of consecutive steps going from nonpolar (e.g. hexane) to highly polar (e.g. water) solvents. In this study to assess the chances of finding bioactive compounds in cyanobacteria, we have compared several extraction solvents by testing the extracts for trypsin inhibition activity. From the solvents tested (methanol, water-methanol, methanol/THF, acetone and supercritical fluid extraction) the maximum yield of compounds exhibiting the trypsin inhibition activity was reached for 70 % aq. methanol (Fig.1.). In addition to the reasonable extraction efficiency, this solvent effectively remove pigments which are a serious obstacle to the both bioassay and chromatographic separation and also provides stabile response to trypsin inhibition assay. Concerning to the extraction efficiency we found two successive extraction steps sufficient for 95 % extraction.

To determine the most suitable *Nostoc* species for the further physiological experiments, all 50 strains were compared in the ability to produce a compounds with inhibitory activity to trypsin. Vast majority of strains inhibiting trypsin over 50% were the filamentous heterocystous representatives originated from soil. Most of these bioactive isolates were sessile, isolated from soil crusts, stones in water channels or walls of buildings.

Among the 50 strains reported here, we found 24 strains showing inhibition activity above 50 % (Table 2). These results demonstrated that production of trypsin inhibitors is widespread feature in the genus *Nostoc*. The results revealed to be encouraging with few selected strains that showed 'high' (arbitrarily defined as higher than 65%) inhibitory activity of the extract against the trypsin (*Nostoc* sp. Lukešová *30/93* showed 100% inhibition, *Nostoc* sp. Lukešová 6/96 revealed 80% inhibition, and *Nostoc* sp. 998 around 65% inhibition). Among all 24 active *Nostoc* strains only two strains released in moderate amount the compounds with trypsin inhibitory activity to the culture medium (*Nostoc sp.* Lukešová 20/97, *Nostoc linckia* 983). Among 26 strains found non-active in Table 2, only 7 strains showed no detectable activity (0%) in this assay and remaining 19 strains showing inhibition activity below 50 % (usually between 20% to 30%). The hypothesis that the low activity in the later strains may be, at least in part, associated with lower efficiency of the extraction or differences in inhibition specificity rather than that these strains lack the activity is thus quite probable. The collection of *Nostoc* strains yielded a much higher proportion of bioactive extracts than other cyanobacterial or green algae collections or even higher plants (Grabley, S. & Thiericke, R. 1999).

The most active strain (100% of trypsin inhibitory activity) was *Nostoc* sp., *Lukešová 30/93*. This strain was selected for additional physiological studies in the laboratory growth experiments to evaluate the productivity and chemical structure evaluation of the active substance.

Table 2. Occurrence of the trypsin inhibition activity within the fifty *Nostoc* strains tested.

No	Cyanobacterial species/strain	Activity (%)	No.	Cyanobacterial species/strain	Activity
1	*Nostoc sp., Lukešová 5/96*	-	26	*Nostoc muscorum, 543*	+
2	*Nostoc sp., Lukešová 20/97*	+	27	*Nostoc muscorum, 545*	-
3	*Nostoc sp., Lukešová 3/97*	-	28	*Nostoc muscorum, 546*	+
4	*Nostoc sp., Lukešová 27/97*	+	29	*Nostoc edaphicum, 760*	-
5	*Nostoc muscorum, Lukešová 14/86*	-	30	*Nostoc sp., 803*	-
6	*Nostoc sp., Lukešová 116/96*	+	31	*Nostoc sp., 870*	-
7	*Nostoc sp., Lukešová 51/91*	+	32	*Nostoc sp., 907*	-
8	*Nostoc sp., Lukešová 19/96a*	-	33	*Nostoc sp., 913*	-
9	*Nostoc muscorum, Lukešová 27/97*	-	34	*Nostoc sp., 914*	-
10	*Nostoc sp., Lukešová 18/89*	-	35	*Nostoc sp., 915*	+
11	*Nostoc sp., Lukešová 2/89*	+	36	*Nostoc linckia, 979*	-
12	*Nostoc muscorum*	-	37	*Nostoc linckia, 980*	+
13	*Nostoc sp., Lukešová 1/88*	-	38	*Nostoc linckia, 981*	+
14	*Nostoc sp., Lukešová 6/96*	+	39	*Nostoc linckia, 982*	+
15	*Nostoc sp., Lukešová 30/93*	+	40	*Nostoc linckia, 983*	+
16	*Nostoc sp., Lukešová 2/91*	-	41	*Nostoc commune, 984*	+
17	*Nostoc linkia, 129*	-	42	*Nostoc sp., 991*	-
18	*Nostoc sp., 268*	+	43	*Nostoc sp., 992*	+
19	*Nostoc muscorum, 304*	-	44	*Nostoc linka, 993*	-
20	*Nostoc sp., 327*	-	45	*Nostoc sp., 994*	+
21	*Nostoc punctiforme, 374*	-	46	*Nostoc sp., 995*	+
22	*Nostoc sp., 379*	-	47	*Nostoc sp., 996*	+
23	*Nostoc linkia, 453*	+	48	*Nostoc sp., 997*	+
24	*Nostoc muscorum, 526*	-	49	*Nostoc sp., 998*	+
25	*Nostoc muscorum, 542*	+	50	*Nostoc sp., 1191*	-

Identification of active compounds Nostoc *sp., Lukešová 30/93*

The compound exhibiting trypsin inhibitory activity was further evaluated by chemical characterization of the crude extracts by RP-HPLC with photodiode array detector and ion trap mass spectrometer. Figure 2 shows the reconstructed total ion current (TIC) chromatogram (m/z 100–2000), UV trace monitored at 280 nm and bioactivity to trypsin of the 70 % aq. methanol extract of this strain. The TIC of the crude extract illustrates the complex composition of secondary metabolites. There are 13 peaks detected eluting according to their decreasing polarities on the reverse phase HPLC column.

We were able to detect the presence of several known and also unknown peaks by this HPLC method. Majority of the peaks could not be identified with any entry in the accessible databases. In 0.1% formic acid in the mobile phase, the protonated molecular ion was usually dominating in the mass spectrum of the eluted peaks (Fig. 3A). However, even in mass spectra of pure compounds the peak with the highest intensity often was not necessarily the protonated molecular ion $[M+H]^+$ and $[M+Na]^+$, $[M+K]^+$ and in some cases also lose of water $[M+H-H_2O]^+$ or amino group $[M-NH_2]^+$ can have higher ion intensities. In peptide mixtures like cyanobacterial extracts are, this can lead to complex mass spectra with a number of apparent molecular ion peaks exceeding the number of actually present congeners. Therefore, series of peaks corresponding to adducts or neutral loses were evaluated to confirm the molecular ion $[M+H]^+$.

Table 3. Molecular ions $[M+H]^+$ and characteristic fragment ions of the individual peaks of the crude extract from the strain *Nostoc* sp. Lukešová 30/93.

peak No.	*t_R (min)*	*$[M+H]^+$*	*informative fragments*	*compound*
1	12,8	865	689, 576, 391, 260	not identified
2	13,4	807	775, 757	not identified
3	14,0	841	809, 791	not identified
4	17,5	844	663	anabaenopeptin A
5	18,0	858	677	anabaenopeptin A homologue
6	20.2	828	663	anabaenopeptin D
7	20.8	842	677	anabaenopeptin D homologue
8	21,4	1081	898	nostopeptolide
9	22,8	866	733	anabaenopeptin T
10	24,6	579	149, 301, 579	phthalate
11	28.0	803[1]	413	phthalate adduct
12	29,0	933[2]	655	chlorophyll *a* adduct
13	33,0	871	593	phaeophytin *a*

[1] $[M+Na]^+$ adduct
[2] $[M+H_2O+Na]^+$ adduct

Resulting full-scan mass spectra and corresponding fragment spectra (MS^2) were analyzed for indicative fragments or series of fragments by comparison to mass spectra of known peptides based on literature data. Fragment patterns of likely known peptides were compared to respective theoretical fragment spectra based on empirical data (Harrison & Young 2005, Qin et all 1999, Paizs, B. & Šuhaj S. 2005) by identifying fragments with matching masses. For putative new structural variants the hypothetical molecules were created by exchanging amino acids in variable positions by amino acids that have been reported for particular positions (Wolker & von Dohren 2006). Further, modifications like chlorination or methylation were also considered.

The first peak eluting at 12,8 min. with dominant molecular ion at *m/z* 865 did not fit with any known cyanobacterial peptide. Two subsequent peaks eluting at 13,4 min. (*m/z* 807) and 14,0 min. (*m/z* 841) were also not identified yet. Additional two peaks eluting at 17,5 min. and 18,0 min. were identified as known variant of anabaenopeptin A and his homologue (Fig. 2.). These two peptides designated as anabaenopeptin A (*m/z* 844) and his homologue (*m/z* 858) were identified by presence of both protonated molecular ions $[M+H]^+$, *m/z* 844 and 858 together with sodium adducts $[M+Na]^+$, *m/z* 866 and *m/z* 880 and potassium adducts $[M+K]^+$, *m/z* 882 and *m/z* 896, respectively, generated during gas phase ionization as shown in full scan spectrum (Tab 3). The fragment spectrum of the protonated molecule $[M+H]^+$ (*m/z* 844) provides the main fragment at *m/z* 663 corresponding neutral loss of side chain amino acid (Tyr). Analogically the same fragmentation pattern was revealed for homologues peak *m/z* 858 where the dominant ion was at *m/z* 677. The later have the same molecular ion (*m/z* 858) as known peptide Oscillamide. We distinguish among these two peptides on the basis of fragmentation pattern. In the fragment spectrum a number of other shared fragment masses indicated indeed the anabaenopeptin A homologue and not Oscillamide.

Another pair of peaks were identify as anabaenopeptin D (*m/z* 828 retention time 20,2 min.) and his homologue (*m/z* 842 retention time 20,8 min.), respectively. These peptides were identified by their characteristic fragmentation pattern (Tab 3). The fragment spectrum of the protonated molecule $[M+H]^+$, *m/z* 828 provides the main fragment at *m/z* 663 which correspond neutral loss of 165 (Phe). Analogically the same fragmentation pattern was revealed for homologues peak *m/z* 842 where the dominant ion was at *m/z* 677 which also correspond neutral loss of 165 (Phe). Both anabaenopeptins A and D were originally isolated

from the hepatotoxic and neurotoxic *Anabaena flos-aquae* strain NRC525-17 (Harada et al. 1995).

Likewise, the last two peaks were designated as nostopeptolide (*m/z* 1081, retention time 21.4 min.) and anabaenopeptin T (*m/z* 866, retention time 22.8 min.). The mass spectrum of nostopeptolide shows the intensive protonated molecular ion $[M+H]^+$=1081 together with double charged molecule $[M+H]^{2+}$=541. The characteristic fragmentation pattern of the protonated molecule $[M+H]^+$=866 of the anabaenopeptin T is consistent with cyclic backbone of all anabaenopeptins, confirming the chemical nature of the identified peak. The anabaenopeptins A, D and their homologues, (*m/z* 844, 858 and *m/z* 828, 842 Da) together with anabaenopeptin T were detected with relatively low intensities. This may lead to an underestimation of their presence as evidenced by HPLC/MS (TIC) analyzes (Fig. 2) although their absolute concentration can be in the same range as others compounds. This could be explained by relatively low proton affinity of side chain amino acid residue in all these peptides (Phe for anabaenopeptins A, D and their homologues and Ile for anabaenopeptin T). Similar effects have been observed also with microcystin variants (Yuan et all 1999).

The other observed peaks were common contaminants found in all extracts analyzed like two peaks of phthalates (PHT) and two peaks of chlorophylls. The first peak of phthalates eluting at 24,6 min. have characteristic series 149, 301, 579 and second eluting at 28 min. with very intensive pseudomolecular ion at *m/z* 803 corresponding to the $[M+Na]^+$ adduct. The fragment spectrum of the sodium adduct clearly proved the characteristic loose of 390 corresponding to monomer of bis-(2-ethylhexyl)phthalate. The last two peaks eluting at 29 min. (*m/z* 933) and 33 min. (*m/z* 871) corresponds to chlorophyll *a* adduct $[M+H_2O+Na]^+$ and their degradation product phaeophytin a, respectively. Both these peaks were identified on the basis of characteristic loss of 278 corresponding of phytol side chain in the MS^2 spectrum.

Since the strain *Nostoc* sp. Lukešová 30/93 showed consistent trypsin inhibition activity we applied the bioactivity-directed fractionation of the crude extract to determine the active substance. This work led to the compound with *m/z* 865 as the only one active component of the extracted mixture (Fig. 2C). The direct infusion of purified active fraction to ion trap mass spectrometer was used to sequencing the peptide structure according to the following procedure. In the first stage, a protonated molecule was subjected to collisionally activated dissociation (CAD) to produce the first generation, product-ion mass spectrum. In the next step, a given *b* ion (*b* is an acylium ion or equivalent) was selected from the product-ion spectrum and then subjected to another stage of CAD.

Representative product-ion mass spectrum of protonated molecular ion $[M+H]^+$ at m/z 865 of purified active fraction proved the cyclic peptide character (Fig. 3A) however, molecular mass differs from all known peptides described in the genus *Nostoc* yet. Additionally the UV absorption in methanol indicated the presence of a conjugated diene (λ_{max} 220 nm), and also clear presence of aromatic ring (λ_{max} 280 nm) as is shown on Fig. 3B.

We proposed that the active compound consists of seven amino-acid residues, six in the backbone and one in the linear side chain. The product-ion spectrum of the protonated molecular ion (Fig. 3A) shows that the $[M+H]^+$ at *m/z* 865 loses small, neutral fragments (NH_3, H_2O, CO or $H_2NCOCH_2NH_2$) accompanying by loss of 176 corresponding to arginine side chain. Resulting most intensive ion at *m/z* 689 contains information on the cyclic peptide moiety and shows that the small neutral fragments (NH_3, H_2O, CO and $CONH_2$) are again lost in its fragmentation. The gas-phase chemistry leading to the formation of the ions is highly dependent on the site of initial protonation to form the molecular ion. The protonation occurs initially on the most basic nitrogen atom of arginine residue and this side chain amino acid is ejected in the first stage of CID as a neutral species. The high intensity of the molecular ion also supports the presence of Arg in the side chain because of his high proton affinity. The residual peptide moiety is formed as the mixture of linear isobaric ions present in the product-

ion spectrum at *m/z* 689. This linear peptide moiety fit to the following amino acid sequence [N-MetAla–Val–Leu/Ileu–Phe-Leu/Ileu-Met]. The final structure confirmation will be done by detailed NMR analysis of the active fraction.

The effect of physiological parameters on growth of *Nostoc* sp., *Lukešová 30/93*

It is well known that in batch culture the optical conditions change considerably along the whole growth curve. When only a restricted part of the growth curve (i.e. linear phase) is considered, it is possible to compare growth rates of individual strains with relatively high accuracy. To characterize the growth of *Nostoc* sp. Lukešová 30/93 we have started with a detailed examination of the specific growth rate dependence on culture temperature and irradiance (Fig. 4.). Cultures were adapted to the indicated temperatures for at least two generation and then diluted to the original biomass concentration. At the lowest light intensity (30 $W.m^{-2}$) the growth was poor and practically the same for all culture temperatures. The growth rate optimum of the *Nostoc* sp. Lukešová 30/93 includes 25°C and 30°C and the growth rate at 20°C and 35°C was distinctly lower. The maximum specific growth rate (μ_{MAX}=0,033 h^{-1}) is attained at temperature 25°C and light intensity about 70 $W.m^{-2}$. The potential biomass yield depends to a large extent on the light saturation characteristics of the particular strain and on their resistance to photoinhibition at high light intensities. The maximum growth rate is reached at relatively low light intensity (70 $W.m^{-2}$). The light intensities higher than those value remarkably inhibits the photosynthetic activity and consecutively also the growth rate of the culture. During the exponential phase of growth an approximate doubling time of 20 hours was reached. The maximum daily output of biomass at that stage was 0.19 mg/ml/day. The culture entered stationary phase of growth 6 days after inoculation, when dry mass reached the value 1.6 g/l.

The effect of physiological parameters on production of active compound

In order to discover differences in the concentration of bioactive compound we investigated the roles of growth phase, light and temperature on the endogenous content of the active compound. Light was chosen as a major growth factor because it was shown previously to influence significantly cyanobacterial peptides synthesis (Tonk et al. 2005). Influence of growth phase was studied because it is another important factor in the respect of nutrient limitation during the growth for nitrogen-fixing cyanobacteria. The temperature as the last physiological parameter tested play crucial role in the membrane permeability and, therefore, affects also whole metabolism.

The strain *Nostoc* sp. Lukešová 30/93 was found as relatively rich source of the various low molecular weight peptides. The active metabolite was the peak eluting in the retention time of 12.4 min. (*m/z* 865). We followed the production of the inhibitor during a two-week growth cycle. Samples (200 ml) of the culture were collected everyday, centrifuged at 5 000 r.p.m. and lyophilized during whole experiment. The 70% methanol extracts were analyzed by the $HPLC/DAD/MS^n$ as described in the Material and methods section. The relative content of the active compound was quantified on the basis of peak area in selected ion monitoring mode (SIM) for *m/z* 865. The results clearly demonstrate that the highest amount of active compound was produced at the beginning of the linear phase of growth about 3 days after inoculation (Fig. 5.).

The strain *Nostoc* sp. Lukešová 30/93 continuously accumulated the active compound endogenously and did not release it during the growth. Active compound was not detected in

the growth medium within whole two-week growth cycle with except at the end of the growth phase. Perhaps some peptides leaked out of the death cells in the old culture. This hypothesis is supported by the fact that we found very low extracellular concentration of active compound in old cultures and particularly under high light levels variant only. Our results thus support the proposal that there are probably no active efflux mechanisms for these peptides in the genus *Nostoc*.

The influence of light and temperature on production of active compound was also studied separately. We tested four light intensities (30, 70, 120 and 150 $W.m^{-2}$) and four temperatures (20°C, 25°C, 30°C and 35°C). The Figure 6 shows that the highest amount of active compound was produced at low light intensity 30 W/m^2. Total content of the active compound per dry weight decreased steeply with increasing light intensity (Fig. 6.). So this compound doesn't appear light protective function and its production is inhibited by light. In general it may be concluded that the intracellular content of the active compound showed a significant positive correlation with maximal specific growth rate reached at the end of exponential phase at optimal growth conditions (Fig. 4.).

The effect of temperature on the production of active compound can be seen on the Fig. 7. The increasing of culture temperature led to a decline in active compound intracellular content. The maximum cellular content was again observed at the maximum growth rate was reached on the end of exponential phase of growth.

Several trends could be distinguished:
(i) the production of active compound is not in direct proportion to cell density (maximal production is on the beginning of the linear phase of growth)
(ii) increasing irradiance had a negative effect on production of active compound with trypsin inhibition activity. Thus, saturating irradiances suppressed active compound production and caused a decrease in cellular content by the same manner as the effect on the growth rate.
(iii) the effect of temperature led to a decline in both active compound productivity and intracellular content. The maximum cellular content was observed at the maximum growth rate was reached. Therefore, the active compound production by *Nostoc* sp. Lukešová 30/93 was shown to be tightly both temperature- and light-dependent.

Discussion

From general point of view the secondary metabolites are substances of low molecular weight (up to 3.000 Daltons) with high structural variability and appearing to have no explicit role in the internal metabolism of the producing organism. The basic question, however, arise why are secondary metabolites synthesized. It is generally accepted that they form certain kind of chemical defence mechanism (Haney et al.1995). While the natural function of cyanobacterial intracellular metabolites so far is unknown some special cases of extracellular metabolites were identified with allelopathic activity in aquatic phototrophic populations in the interaction between competing microalgal organisms of the same habitat (Suikkanen et al. 2005, 2006, Legrand et al. 2003, Gross 2003). In previous screening studies (Suikkanen et al. 2004, Smith & Doan, 1999), dealing with this subject, algicidal properties have been observed generally in a restricted number of cyanobacterial genera: *Anabaena*, *Calothrix*, *Fischerella*, *Nostoc*, *Oscillatoria* and *Scytonema*. It was suggested by Flores, E. & Wolk, C.P. (1986) that some cyanobacterial strains, such as *Nostoc* sp. ATCC 29132, protect themselves and other species from antibiotic activity by producing a masking protein that inhibits the activity. In the course of this work it was observed that this same *Nostoc* cleared the *Calothrix* WA 96/8 in the presence of proteinase K, but not in its absence. Dittmann et. al. (2001) suggested that some

of the cyanobacterial peptides (i.e. microcystins) may act as intercellular signal molecules in the cells.

The current knowledge about trypsin inhibitory chemicals and their regulatory mechanisms in aquatic microorganisms is low. The function of trypsin inhibitor was suggested to be antifeedant activity (Rohrlack et al. 2003, 2004).

Although some papers about application of cyanobacteria in biotechnology was published elsewhere (Marwick et al. 1999, Armstrong et al.1991, Rossi et al. 1997), little mention is made of application of cyanobacteria in pharmaceutical or medicinal chemistry. In contrast to many other classes of prokaryotes, there is a direct correlation between growth phase and conditions and secondary metabolite production in cyanobacteria. In separate studies carried out by Rossi et al. (1997) or Armstrong et al. (1991), bioactive metabolites were either produced throughout the period of exponential growth or synthesized during the stationary growth phase in batch culture. This is the reason that cyanobacteria require tighter control of culture parameters for potential biotechnology secondary metabolite production. For this reason detailed studies were undertaken to determine the growth characteristics of a laboratory maintained strains subjected to variations in both physical (light or temperature response) and chemical conditions (growth media composition).

Our data revealed a trypsin inhibitory is widespread in the *Nostoc* genus and quite probably upon use of broader trypsin proteinase panel we will be able to detect this activity in all assays. In the detailed study using selected strain exhibiting the highest anti-trypsin activity, the peak of the production of the active compound may be identified with the early growth phase at optimal growth conditions, i.e. when cell divide most frequently. This leads us to the hypothesis that anti-trypsin activity is connected with expansion of microorganism colonies under optimal growth conditions. Perhaps it may be really the mechanism of protection against the grazers (as suggested by Rohrlack et al. 2003, 2004) applied in conditions when the organism expands on the expense of other organism in the same niche and has the capacity to produce energetically demanding "luxurious" compounds. From the biotechnological point of view that is again quite promising case since the optimal growth conditions are combined with maximum production, both allowing the intensification of the production process.

The identity of the active compound, not generally as a surprise, is a cyclic peptide containing non-coded amino acids. Thus the inhibition mechanism represents a research topic of general interest since it may lead to design of new inhibitors of various types. This opens many new projects in structural biochemistry and, possibly, also chemical synthesis.

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Fig. 1. Comparison of solvent efficiency on extraction of compounds with trypsin inhibition activity from lyophilized *Nostoc* biomass.

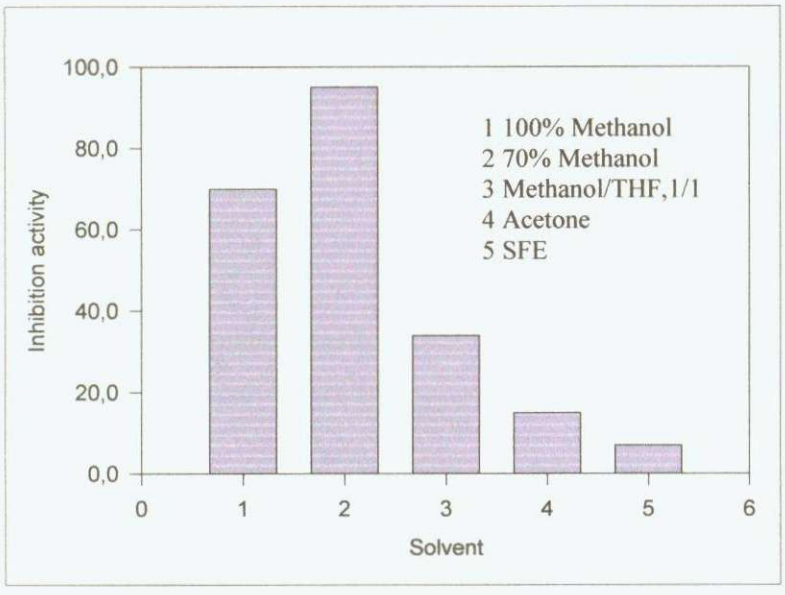


Fig. 2. Analysis of the crude extract. A. total ion currents (TIC). B. UV 280 nm. C inhibition activity.

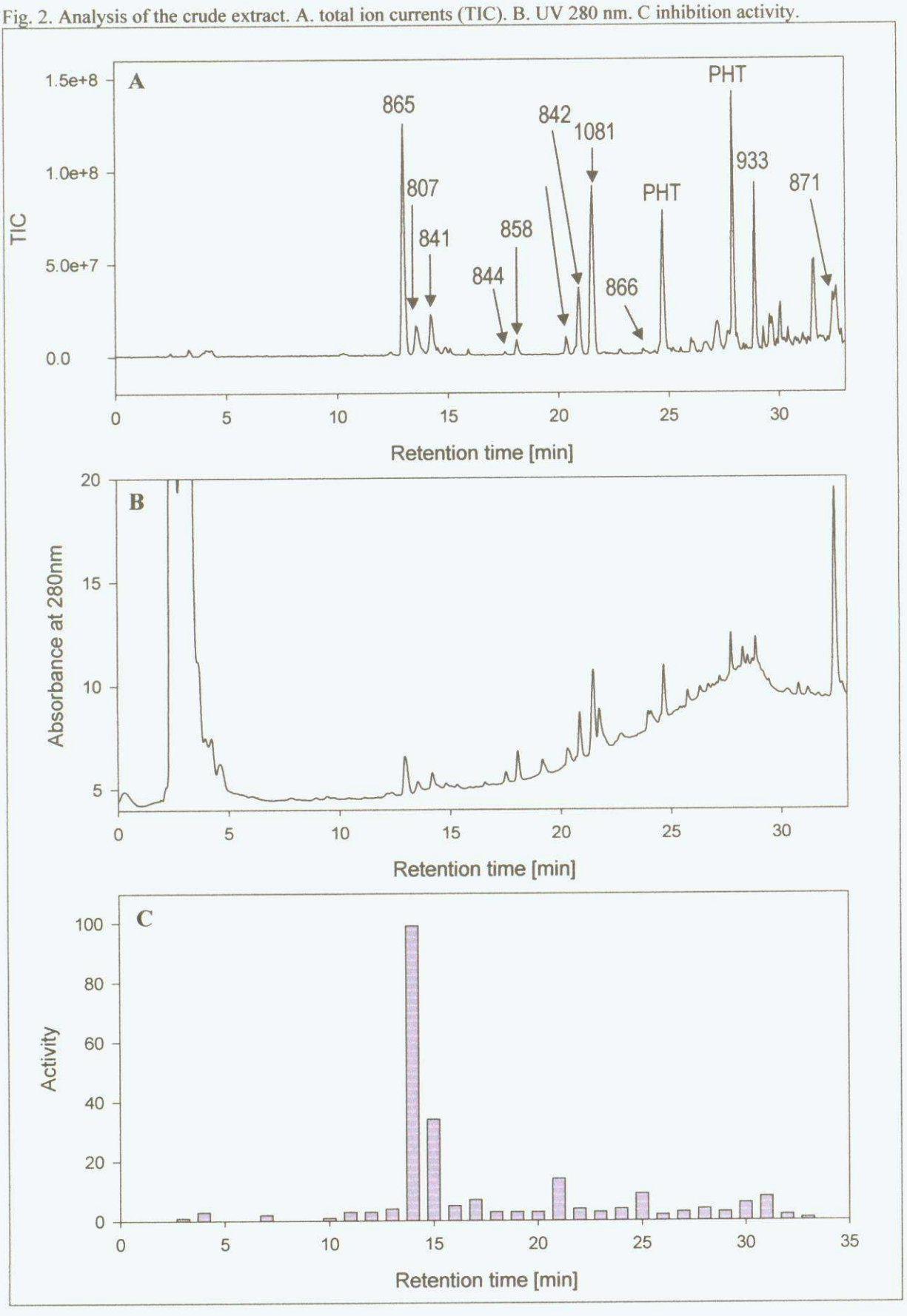
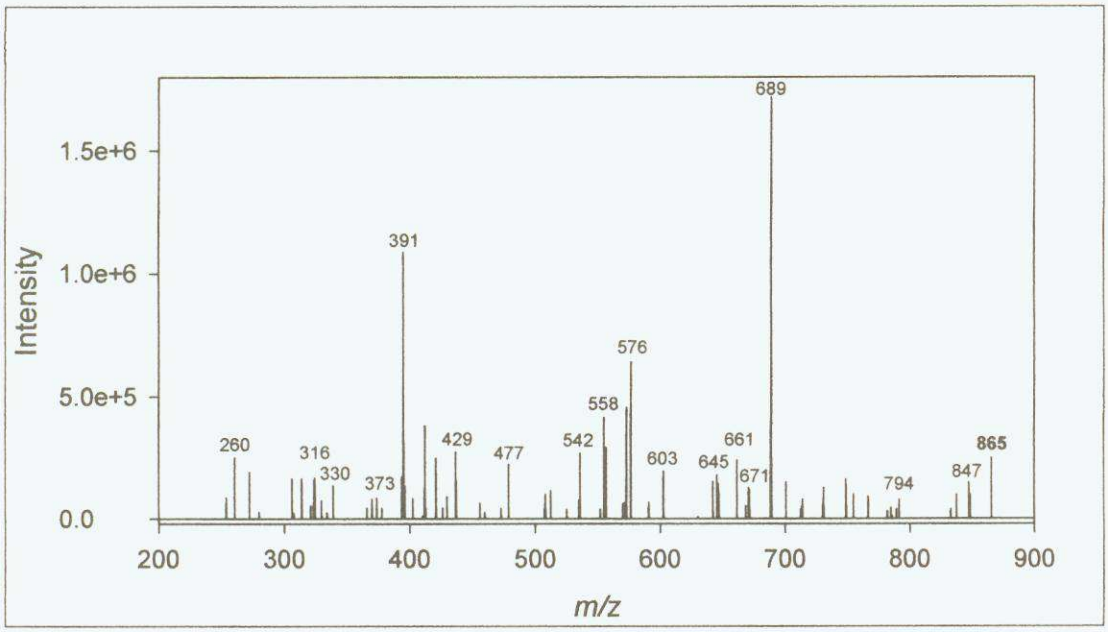


Fig. 3. Chemical structure evaluation of the active compound.

A. The product-ion spectrum of the molecular ion $[M+H]^+$ at *m/z* 865.



B. UV spectrum of the active compound.

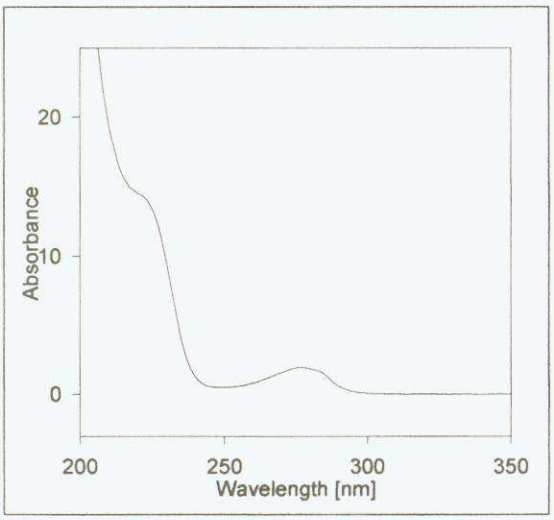


Fig. 4. Specific growth rate.

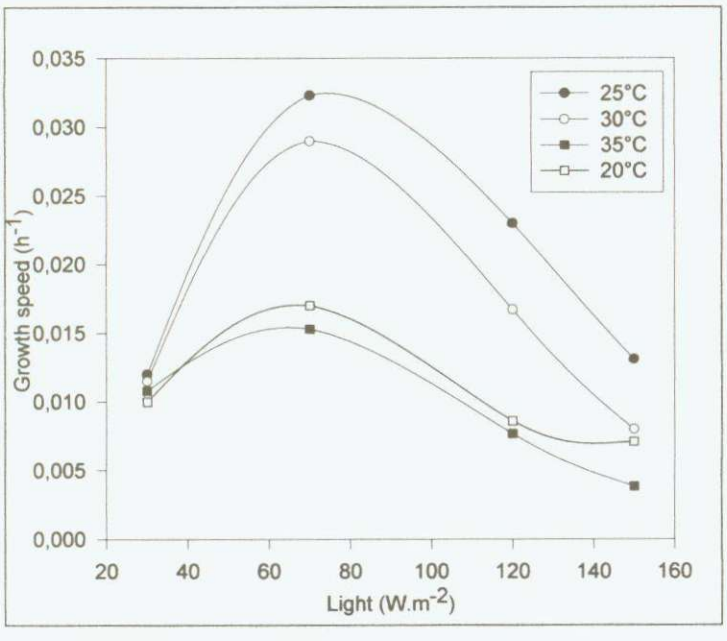


Fig. 5. Production of the active compound during the growth.

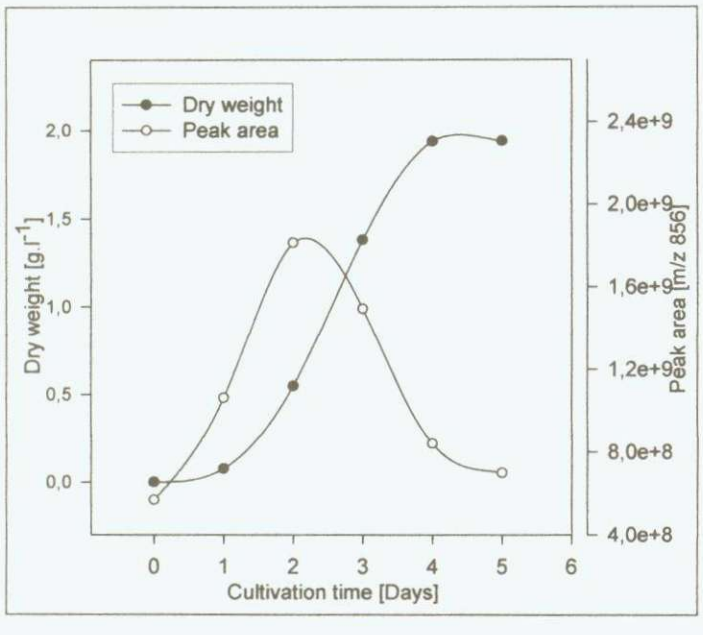


Fig. 6. Influence of light on the active compound biosynthesis.

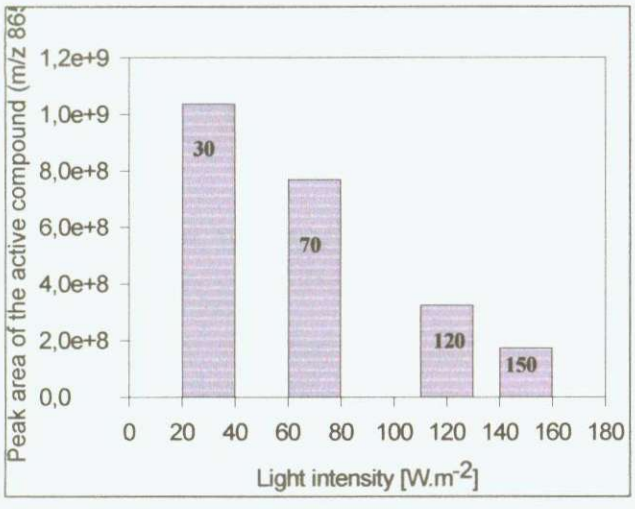
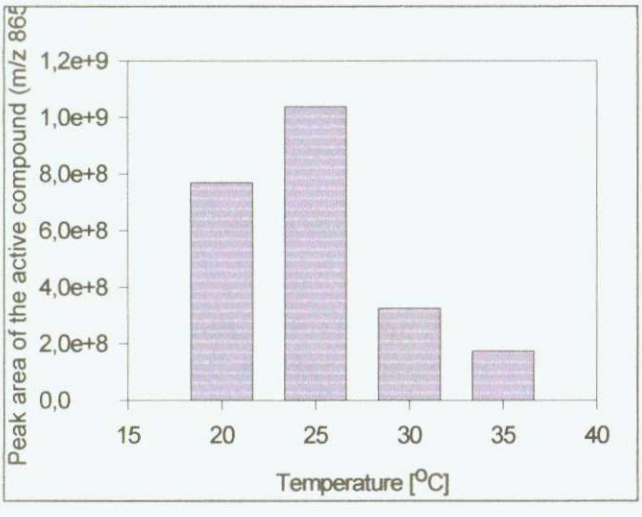


Fig. 7. Influence of temperature on the active compound biosynthesis.



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