“Investigations on the phylogenetic diversity of urease producing bacteria in soil, the inhibition of urea active transportation and metabolizing in *Bacillus megaterium* DSM 90”

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Declaration of Academic Honesty

Hereby, I declare that I have carried out this PhD research project on my own, having used only the listed resources and tools.

HANY HASSOUNAH Halle (Saale), February 2010.

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1 Index and abbreviations

1.1 Index

1.2 Abbreviations

°C	degree Celsius
µ	micro (10^{-6})
µM	micro molar (µmol/L)
16S rRNA	small subunit ribosomal RNA gene
A	adenine
A436nm	Absorption at 436 nm range
AMMI	Additive Main Effects and Multiplicative Interactions
ARB	from Latin arbor, tree, The ARB program package
ATP	adenosine triphosphate
B.	bacillus
BC	Free cells buffer + CTAB
BDM	Bio dry mass
BMPs	Best Management Practices
BMU	*Bacillus megaterium* urease
BN	Free cells buffer
BPU	*Bacillus pasteurii* urease
BSA	Bovin serum albumin
BU	Free cells buffer + urea
BUC	Free cells buffer + urea + CTAB
BWM	bio wet mass
C_{org}	Organic carbon content
C	cytosine
CCA	canonical correspondence analysis
Cp	Control of the permeabilized cells
CTAB	Cetyl Trimethyl Ammonium Bromide
CV	Column volume
Cw	control of the whole cells
D	Dialysis
Da	Dalton
DNA	Deoxy ribose nucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures
E. coli	Escherichia coli
e.g.	for example
EDTA	Ethylenediaminetetraaceticacid
Fig.	Figure
G	guanine
GC-C-IRM-MS	Gas Chromatography Combustion Isotope Radio Monitoring Mass Spectroscopy
h	Hour(s)
HIC	Hydrophobic interaction column
Hi-Di™ Formamide	Highly deionized formamide
HPH	High pressure homogenizer
IEC	Ion exchange column
Inh.	Inhibitor
IUPAC	International Union of Pure and Applied Chemistry
k	kilo (10^{3})

kb	kilo base
kDa	kilo Dalton
K_I	Inhibitor constant
K_m	Michaelis-Menten´s constant
L	liter
m	milli (10^{-3})
M	molar (mol/L)
ME	B-meracaptoethanol
min.	minute
mL	milliliter
mM	millimolar (mmol/L)
mol	mole
N	Organic nitrogen content
n	nano (10^{-9})
nm	nanometre
nm MDS	non metric multidimensional scaling
OD_{600}	Optical density at 600 nm
OTUs	operational taxonomic units
P	Permeabilized cells
PAGE	polyacrylamide gel electrophoresis
PCA	Principal components analysis
PCR	Polymerase Chain Reaction
PLFA	Phospho lipid Fatty acids Profiles
RDA	redundancy analysis
REPK	Restriction endonuclease Picker
RNA	Ribose nucleic acid
rpm	revolution per minute
rRNA	ribosomal RNA
s	second
SDS	sodium dodecylsulfate
T	thymine
Tab.	table
TAP	T-RFLP Analysis Program
TCA	Trichloric acid
TGGE	Thermal Gradient Gel Electrophoresis
TOC	total organic carbon content
Tot-N	total organic nitrogen content
TRF- ARB	terminal restriction fragment ARB implement tool
TRF- CUT	terminal restriction fragment cutter
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TRFs	Terminal restricted fragments
U	Unit (µmol/min)
U	Uracil
UV	Ultraviolet
v/v	volume to volume
v_{enzyme}	volume of the enzyme solution
V_{max}	maximal velocity
V_{test}	test volume
W	Whole cells

2 Introduction and objectives

2.1 Introduction

Nitrogen is an essential nutrition element for the plants, but soils typically do not supply sufficient quantities for maximum and economic production of non legume crops. Thus the need to apply nitrogen based fertilizers to the agricultural soils arose, from one side to balance the agricultural ecological systems and from the other side to overcome the increasing demand for the agricultural products worldwide. Urea is considered, regarding this issue a desirable choice for fertilization due to its high content of nitrogen, 46 % of the molecular mass is nitrogen, physical and chemical stability, cost efficiency and environment friendly manufacturing procedure and high solubility in water [UNIDO and IFDC, 1998].

Urea has been considered in Europe as slow-release fertilizer [UNIDO and IFDC, 1998], since it must undergo two transformations in soil before it becomes available to most crops [Honti, 1976]. The first transformation is carried out by the means of urease action (urea amidohydrolase E. C. 3.5.1.5) which catalyze the hydrolysis of urea into ammonium and carbonic acid. The second transformation is nitrification in which ammonium is been oxidized in soil into nitrite then to nitrate [UNIDO and IFDC, 1998]. The soil micro flora is playing a remarkable role in this issue, due to its ability to synthesis a special cluster of enzymes which catalyze the degradation and utilization of urea into more suitable nutritional form for the plants [Mobley et al., 1995].

Usually, there are general concerns about the unfavorable cost/benefit ratio of nitrogen fertilizers by producers and consultants. The uncontrolled release or ammonium, due to high urease activity in soil, may lead to reduce the fertilization efficiency up to 20 %, environmental pollution and may damage the germinating seeds due to the high resulting pH and the local ammonia concentration [UNIDO and IFDC, 1998].

There are several strategies to overcome the problems resulted from the uncontrolled urea hydrolysis. These strategies aim to develop Best Management Practices (BMP's), which are cost-efficient operation methods that ensure that fertilizers are used effectively with minimal

impact on the environment [Simihaian, 2002]. One of these approaches is concerned about preventing the excessive urea hydrolysis by applying the urea associated with inhibitor(s) of soil urease activity [Simihaian, 2002]. The actual reaction mechanism of these synthesized inhibitors on urease activity is a topic of a wide span of discussion [Dominguez et al., 2008; Simihaian, 2002].

In this study the phylogenetic biodiversity of the urease-producing bacteria in soil and the effect of preselected phosphoric amide derivatives on the microbial urease activity and the urea active transportation in some bacterial models were investigated.

2.1.1 State of knowledge

2.1.1.1 Soil bacterial diversity dependence on the fertilization management

Investigating the impact of different environmental factors and agricultural managements on the bacterial biodiversity was the scope of several studies [Bossio et al., 1998; Kandeler et al., 1998; Ogilvie et al., 2008]. These studies show that the relative importance of various environmental variables in governing the composition of microbial communities could be ranked in the following order: soil type, time of sampling, specific farming operation, (e.g., cover crop incorporation or side dressing with mineral fertilizer), management system and then the spatial variation in the field [Bossio et al., 1998]. Soil type found to be the primary determinant and the dominant factor on the active bacterial communities [Girvan et al., 2003]. On the other hand comparing samples with the same soil nature shows that the agricultural management practices, particularly inputs of manure and cover crops, can have a significant impacts on the size and activity of soil microbial communities [Powlson et al., 1987].

Many of the long term fertilization-input experiments consider investigating the impact of the fertilization management regime on the bacterial diversity in soil [Clegg et al., 2003; Ogilvie et al., 2008]. The study carried out by Clegg et. al, shows the impact of nitrogen based fertilizers on the microbial communities within the soil [Clegg et al., 2003]. The microbial community structure in this study was assessed using multivariate analysis of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) banding patterns and PLFA (phospholipids fatty acid) profiling. The management regimes assessed were inorganic nitro-

gen (nitrate) fertilizer application and soil drainage. The results of this study clearly demonstrate the impacts of management practice on the bacterial community composition of grassland soils. The application of nitrogen to grassland soils resulted in significant changes to the PLFA community profiles and differences in the PCR-DGGE population structures of the eubacteria and actinomycetes and to a lesser extent the ammonia oxidizers. There was wide variation in the community structure of the pseudomonads and this may have masked any treatment related effects.

Other studies consider investigating the impact of the fertilization management on the microbial community structure [Bossio et al., 1998]. In these studies PLFA profiles were used to investigate the impact of the fertilizer nature: organic, low-input, and conventional on the microbial biomass. The organic system relies on organic sources of nutrients obtained from a vetch winter cover crop, manure, or seaweed and fish powder. The low-input system, which is intermediate between the organic and conventional systems, relies on vetch cover crops as a partial source of nitrogen; it is supplemented with mineral fertilizers and limited amounts of pesticides. The conventional system uses mineral fertilizers, some pesticides, and the only organic matter inputs are in the form of stubble and roots from the previous cash crop of wheat and beans. The main observations of this study conclude that the microbial biomass carbon (MBC), microbial biomass nitrogen (MBN) and substrate-induced respiration (SIR) were consistently lower in conventional than in organic and low input plots. This difference was significant ($P < 0.01$) when all sample dates were considered together, but organic and low input plots did not differ significantly from one another. The PLFA profiles, indicated differences in community composition, were found to be consistent with differences in organic carbon inputs between the organic and conventional farming systems. The third system, low-input, was found to be intermediate between the organic and conventional systems with respect to the mass of organic carbon entering the system and its source of nitrogen fertilizer.

Other studies investigated the impact of the fertilization management on the metabolic activities in the agriculture soils [Enwall et al., 2005]. In this study the effects of different organic and inorganic fertilizers on activity and composition of the denitrifying and total bacterial communities in arable soil was explored. Soils were treated by cattle manure, sewage sludge (organic fertilizers), $Ca(NO_3)_2$, $(NH_4)_2SO_4$ (inorganic fertilizers) beside non-treated unfertilized soil plots. The activity was measured in terms of potential denitrification rate and basal soil respiration. The *nosZ* and *narG* genes were used as functional markers of the denitrifying

community, and the composition was analyzed using denaturing gradient gel electrophoresis of *nosZ* and restriction fragment length polymorphism of *narG*. A fingerprint of the total bacterial community was assessed by ribosomal intergenic spacer region analysis (RISA). It was found that the potential denitrification rates were higher in plots treated with organic fertilizer than in those with only inorganic fertilizer. The basal soil respiration rates were positively correlated to soil carbon content, and the highest rates were found in the plots with the addition of sewage sludge. Fingerprints of the *nosZ* and *narG* genes, as well as the RISA, showed significant differences in the corresponding communities in the plots treated with $(NH_4)_2SO4$ and sewage sludge, which exhibited the lowest pH. In contrast, similar patterns were observed among the other four treatments, unfertilized plots and the plots treated with $Ca(NO_3)_2$ or with manure. The study shows that the addition of different fertilizers affects both the activity and the composition of the denitrifying communities in arable soil on a long-term basis. However, the treatments in which the denitrifying and bacterial community composition was different from each other in a way that the most did not correspond to treatments with the most different activities, showing that potential activity was uncoupled to community composition.

Despite the comprehensive investigation carried out by the previous discussed studies, none has considered to investigating the impact of urea based fertilizers, coupled or uncoupled with urease inhibitors, on the phylogenetic and functional diversity of urease- producing bacteria in soil.

2.1.1.2 Approaches to access the phylogenetic diversity of urease-producing bacteria in soil

Bacteria are an important part of the soil-micro flora due to their abundance in soil, their species diversity and the multiplicity of their metabolic activities [Ranjard et al., 2003]. They play a key role in the biogeochemical cycles of the main and trace elements. Therefore, they are heavily implicated in energy and nutrient exchanges within the soil. They also have the potential to reflect the past history of a given environment [Ranjard et al., 2003]. Regarding the previous facts it is essential to understand the interrelationships between bacteria and their environment and how they respond to various natural or man-made disturbances. Understanding the dynamic response toward the environmental effectors could be achieved by studying the structural and functional diversity of soil bacterial communities.

Soil is a highly heterogeneous environment, which is reflected in the diversity of the physiological and metabolic capabilities of the soil micro flora [Ovreas and Torsvik, 1998]. Previous investigations used re-association kinetics of single stranded DNA to demonstrate that there is also a high genetic diversity among the soil micro flora [Torsvik et al., 1998]. For some investigated forest soil sample it was found that it contained about 4000 – 6000 genome equivalents that could represent between 12000 and 18000 different species [Torsvik et al., 1996].

Generally, there are two approaches which have been adopted to gain an access to the phylogenetic diversity of the bacteria in soil. The first approach is the classical culturable-dependent approach based on isolation and culturing the micro-organisms. The second is the culturable-independent approach based on different molecular biology and nucleic acid surveys.

2.1.1.2.1 Culturable-dependent approach

For at least half a century, it has been known that the major part of the structurally intact bacterial cells in soil appear to lack the capacity to grow on standard laboratory media [Joseph et al., 2003]. Numerous studies examining bacteria from seawater, freshwater, sediments and soil samples have demonstrate that only 0.001 % to 0.3 % of cells from these environments can be grown in laboratories [Amann et al., 1995]. This consensus is based on numerous attempts to compare total microscopic counts with viable counts on laboratory media [Gottschal, 1992]. Nevertheless, culturable-dependent approaches may greatly facilitate other synecological studies obtained from culturable-independent techniques [Janssen et al., 2002]. Early direct observations of soil bacteria was conducted by light fluorescent or electron microscopy ([Bae and Casida, 1973; Zweifel and Hagstrom, 1995]. These observations revealed vast numbers of extremely small and apparently structurally intact bacteria. The total numbers were two to three orders of magnitude higher than the numbers of viable cells as counted by any cultural methods for heterotrophic microorganisms tested [Elsas, 1997].

The adoption of the culturable dependent techniques, parallel to the culturable independent techniques, may have a significant importance. They may provide better understanding for the functional diversity of the bacteria, especially when a complex cascade of genes control the expression of the interested functionality. It may also provide a pure isolated bacterial culture that represent independent functional unit and proving a good model for further analysis

[Daniel, 2004]. Last but not least it may greatly facilitate other synecological studies obtained from culturable independent studies [Janssen et al., 2002].

Heterotrophic bacteria have been assumed to be the dominant types among soil bacteria, since the consumption and mineralization of organic materials represent most of the energy flux through the soil biota [Elsas, 1997]. For this reason, all attempts to culture the majority of soil bacteria have concentrated on the heterotrophic bacteria. For the same reason, the majority of apparently unculturable bacteria in soil are assumed to be heterotrophic.

Few number of studies provide brief comments on the taxonomy of cultured soil bacteria [Elsas, 1997]. One of the studies attempted to summarize a great number of classic taxonomical studies of bacteria prevalent in soil and presents the following ranges (percentage of total viable counts): Arthrobacter: 5 % - 60 %, Bacillus: 7 % - 67 %, Pseudomonas: 3 % - 15 %, Agrobacterium: up to 20 %, Alcaligenes: 2 % - 12 % and Flavobacterium: 2 % - 10 % [Alexander, 1977]. The list suggests variable composition of the culturable bacterial flora of soil. The variability is likely to reflect differences in culture conditions applied as well as true differences between soils. Other studies compared the microflora, in beach forest soil, growing on a number of different nutrient media and found only partial overlap between the populations growing on fairly similar media [Sørheim et al., 1989]. All media indicated a prevalence of gram-negative bacteria, however, the dominance of gram-negatives is commonly assumed to be the rule, particularly in the rhizosphere. On the other hand this view is challenged by those who claim that coryneform bacteria are often more numerous [Elsas et al., 1993].

In experiments with indigenous soil bacteria a relationship between cell dimensions and cultivability have been established [Elsas, 1997]. The outcome of these experiments shows that the small sized bacterial cells, dwarf cells, outnumber other cells and contain a similarly large share of the total bacterial DNA. Nevertheless, it was found that their biovolume is only representing 10 % of the total bacterial biovolume. This suggests that their metabolism accounts for a minor fraction of the total energy flux through the microbial community in soil [Elsas, 1997]. These observations clearly suggest that the major part of the unculturable bacteria in soil is small sized bacterial cells. These cells appear to be structurally intact and metabolically competent at least in having an intact genome. Thus, they carry most of the genetic information (DNA) present in soil. However, their share of the metabolic activity and energy flux

may be moderate because of their small size and apparently low activity [Torrella and Morita, 1981].

Other similar studies adopted the immunofluorescence microscopy to enumerate and characterize cells of *Rhizobium leguminosarum* in soil [Boylen and Mulks, 1978]. Small cells (passing through a 0.4-mm polycarbonate membrane) represented 20 % to 30 % of the total numbers within each of the indigenous populations of four serogroups found in soil. However, attempts to induce growth or nodulation by these small cells were not successful. The fraction of dwarfs within each population increased with soil depth, indicating a relationship between energy availability and the presence of dwarfs within each population. The observations represent a strong case for the hypothesis that dwarf cells are miniature cells of otherwise more normal-sized bacteria.

Regarding the urease-producing bacteria, some studies conduct a cultivation-based method for enumerating ureolytic cells in groundwater [Tyler, 2004]. A most-probable-number (MPN) technique was performed using a broth growth medium containing urea and the pH indicator phenol red. The pH indicator changed color in response to the pH increase which resulted from urea hydrolysis. This method was initially designed to detect urea hydrolysis in clinical bacterial species that are easily cultivated in the laboratory and also are known to hydrolyze urea at a high rate [Christensen, 1946]. Numerous ureolytic groundwater isolates were tested using this medium, but many isolates were unable to grow and those that could grow did not hydrolyze detectable amounts of urea after several weeks of incubation. Due to the incompatibility of this growth medium with the slow growth and relatively low urea hydrolysis rates of environmental ureolytic bacteria, molecular methods to target urease genes were suggested to be an alternative method to detect, characterize, and enumerate groundwater ureolytic bacteria.

2.1.1.2.2 Culturable-independent approach

Culturable -dependent techniques are known for their selectivity and are not considered representative of the extent of the bacterial community [Cowan et al., 2005]. The proportion of cells which can be cultured is estimated to be 0.001 % or at most 0.3 % of the total population [Amann et al., 1995] and few data are available concerning how closely they reflect the actual composition of these communities. Therefore, there is a general agreement that investigating

the environmental bacterial diversity via the non culturable surveys, specially the nucleic acid based surveys, is the suitable approach to have a relative good overview for the bacterial biodiversity in soil [Schutte et al., 2008]. These surveys employ the molecular genetics approaches to access the bacterial biodiversity in an environmental sample, without the need to cultivate all the bacterial species involved in the sample. On the other hand, the traditional microbiological methods have its limits in providing access to the true extend of bacterial biodiversity in soil [Cowan et al., 2005]. Although the nucleic based approaches show that the bacterial diversity is extremely high in soil ecosystems [Borneman et al., 1996; Felske et al., 1998; Zhou et al., 1996] the state of knowledge regarding the factors influencing and shaping this biodiversity is not well developed [Enwall et al., 2005].

Several cultivation independent methods have been used to study and monitor the structure and compositions of microbial communities, Phospholipid Fatty Acid profile (PLFA) [Bossio et al., 1998], Temperature Gradient Gel Electrophoresis (TGGE) [Ogilvie et al., 2008] and Terminal Restriction Fragment Polymorphism (T-RFLP) [Enwall et al., 2005]. The most wide used technique is the T-RFLP, mainly due to the acceptable ratio between labor intensity and the gained information and due to its relative simplicity [Schutte et al., 2008]. T-RFLP techniques have been used to study and analyze the bacterial biodiversity by the means of 16S rRNA gene analysis [Hullar et al., 2006; Katsivela et al., 2005], additional to that, T-RFLP has been used to analyze several functional genes biodiversity, such as those encoding for nitrogen fixation [Rosch et al., 2006; Tan et al., 2003] and methane oxidation [Horz et al., 2001; Mohanty et al., 2006].

The main concept of T-RFLP is based on amplifying the gene encoding the small ribosomal subunit 16S rRNA, which is a prokaryotic biomarker [Weisburg et al., 1991]. Alternatively a functional gene from total community genomic material could be amplified, using polymerase chain reaction (PCR). In T-RFLP, one or both of the primers used are labeled with a fluorescent dye. The resulting PCR product is then digested by one or more restriction enzymes. Typically four base paired recognition site restriction enzymes are used. The sizes and the relative abundances of the fluorescently labeled Terminal Restricted Fragments (TRFs) are determined using an automated DNA sequencer. While the difference in the (TRFs) sizes reflect the sequences differences between them, the phylogenetically distinct populations of organisms can be resolved [Schutte et al., 2008].

T-RFLP shares the problems inherent to any PCR based method [Schutte et al., 2008]. The basic types of PCR artifacts have been shown in controlled laboratory studies and can be divided into two categories: those resulting in sequence artifacts (PCR errors), and those skewing the distribution of PCR products due to unequal amplification (PCR bias) [Acinas et al., 2005]. Sequence artifacts (PCR errors) may arise due to the formation of chimerical molecules [Brakenhoff et al., 1991], the formation of heteroduplex molecules [Crosby and Criddle, 2003] or *Taq* DNA polymerase error [Qiu et al., 2001]. On the other hand PCR bias is thought to be due to intrinsic differences in the amplification efficiency of templates [Polz and Cavanaugh, 1998] or to the inhibition of amplification by the self-annealing of the most abundant templates in the late stages of amplification [Suzuki and Giovannoni, 1996]. However, it remains difficult to translate these results to environmental samples in which target genes are orders of magnitude more highly concentrated than in the simple mixtures of templates generally used in controlled laboratory studies. Nevertheless although these limitations, T-RFLP is still considered a suitable method to asses changes in microbial community structure by monitoring the presence or intensity of specific fragments from the obtained profiles [Franklin and Mills, 2003; Mummey and Stahl, 2003].

Regarding the PCR protocol, approached for T-RFLP analysis, the designing of the primers plays a critical role. Ideally the chosen primers should have high affinity toward the targeted taxonomic group and simultaneously sufficiently universal so that they can amplify all bacterial populations that are of interest. Although the problematic of designing primers which satisfy both of these criteria [Marsh et al., 2000], some bacterial universal primers which cover 76 to 98 percent of the bacterial 16S rRNA gene sequences have been proposed and evaluated [Lane, 1991]. Different PCR primer, have been used to investigate the overall bacterial biodiversity in soil and marine sediments. It was found that the primer pair, UniBac 27f (5-A G A G T T T G A T C (A C) T G G C T C A G -3) and UniBac 1492r (5-T A C G G Y T A C C T T G T T A C G A C T T -3) is providing relatively the most informatics 16S rRNA amplicons [Mahmood et al., 2006]. Simultaneously some other studies were investigating the best primer combination for the urease functional gene (*ureC*), which is considered the biggest urease subunit and the active site holding subunit. These studies conclude the absent of universal primer for this gene that can cover the whole functional taxa. However the best proposed pair of primers found to be L2F (5- A T H G G Y A A R G C N G G N A A Y C C -3) and urecR (5- G G T G G T G G C A C A C C A T N A N C A T R T C-3) [Tyler, 2004]. Different bacterial populations may share the same TRFs length for particular primer–enzyme combination

[Marsh et al., 2000]. Thus the use of only one fluorescently labeled primer may result in underestimating the microbial diversity in a sample [Liu et al., 1997; Marsh et al., 2000].

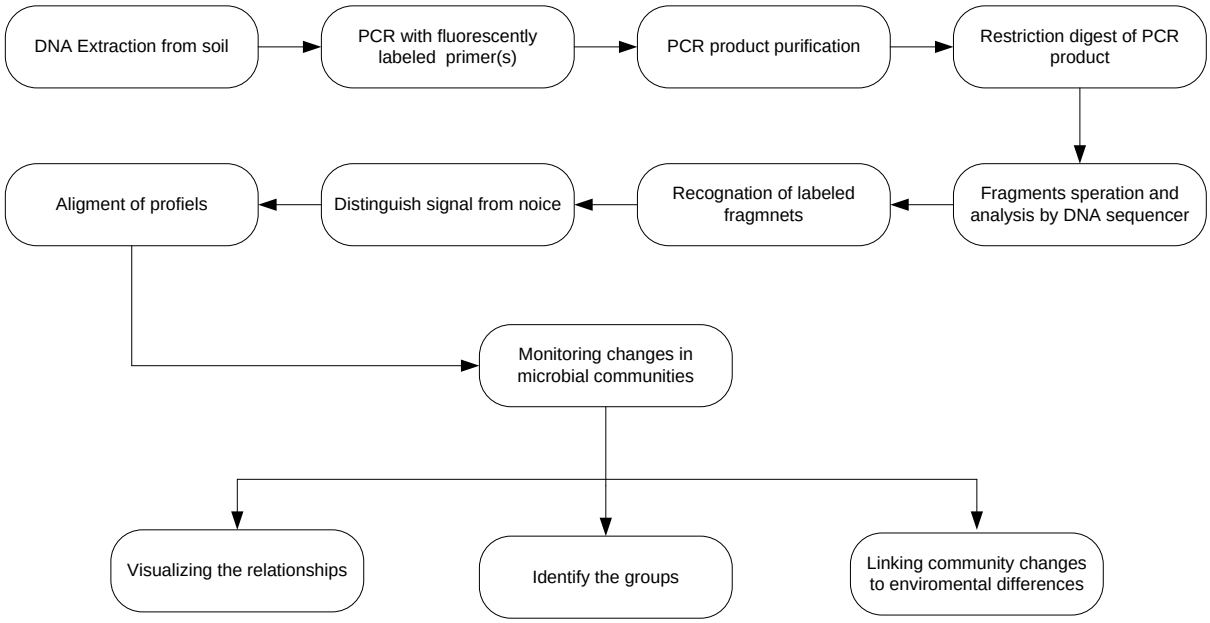


Figure 1 Schematic overview about the steps of microbial community analyses based on Terminal Restriction Length Polymorphism (T-RFLP). Beginning with the extraction of DNA from the environmental sample (soil), then PCR is carried out employing fluorescent labeled primers, the PCR product after that is the subject of restriction enzymes action, followed by the separation of the TRFs and the recognition of labeled fragments. The analysis of the data obtained includes distinguishing signals from noise, alignments of the profiles and monitoring the changes in microbial communities. The end results could be interpolated by visualizing of the relationships, recognition the identity of the groups and linking the community changes to the environmental differences.

Phylogenetic biodiversity discrimination of bacterial populations by T-RFLP analysis relies on detecting the 16S rRNA gene sequence polymorphisms using restriction enzymes [Liu et al., 1997]. Typically, enzymes that have four base-pair recognition sites are used due to the higher frequency of these recognition sites. It has been found that for relatively high richness microbial communities, above 50 operational taxonomic units (OTUs) the terminal node in phylogenetic analysis, there is some lowering in the discrimination resolution and thus it was recommended that the T-RFLP should be used in middle to low richness microbial communities [Engebretson and Moyer, 2003].

Choosing of suitable restriction enzyme for the proposed experimental conditions could be aided by the silico digestion analysis to evaluate the discrimination resolution and to define the best primer-enzyme combination. Using tools such as the T-RFLP analysis program (TAP), T-RFLP ARB implemented tool TRF-CUT [Ricke et al., 2005] and Restriction En-

donuclease Picker (REPK) [Collins and Rocap, 2007] may aid achieving this goal. Although the great aid that these tools may provide, the output results of these programs should be used in caution, as only a small fraction of the total bacterial diversity has been described and sequenced. Therefore the fitness of the enzymeprimer combination should be empirically verified.

Terminal restricted fragments (TRFs) are usually separated by the means of capillary or polyacrylamide gel electrophoresis. Wherein the electrophoresis mobility of the T-RFs are compared to those of known size in an internal standard, such as ROX 500. Each T-RF is expressed as a peak which has a defined size, representing the amplicon size, and defined height and area, which represent the amount of the amplicon in the sample. T-RFLP analysis using capillary gel electrophoresis was found to be more precise and reproducible rather than other methods such as DGGE [Behr et al., 1999].

The first step to analyze the T-RFLP profiles obtained from the capillary gel electrophoreses is distinguishing signal from noise. There are many approaches dealing with this issue, such as the fixed threshold method [Lueders and Friedrich, 2003], which is considered the simplest one. In this method the noise is eliminated by an arbitrary chosen value for the detection threshold, for example 50 fluorescent units. Anything below this threshold will be ignored and only the peaks that overcome this threshold will be processed. Although its simplicity this method is not taking in account the variation of the experimental conditions. These conditions may vary slightly from one run to another and thus the resulting profiles may eliminate a lot of important information or introduce non necessary noise.

Another approach which has evolved from the previous method is the constant percentage threshold method [Sait et al., 2003]. In this method a matrix of all TRFs size and peak area in a defined T-RFLP profile is created and standardized by computing the proportional of the total area for each peak in the T-RLFP profile. The variable percentage threshold then will be chosen in such a way that the ratio between the total peak area and number of peaks is minimized. The drawback of this method is in the case of high number of peaks above the variable percentage threshold. This may represent the amount of the injected DNA rather than the real richness of the microbial community. Thus the final obtained results may affect the statistical value of the variable percentage threshold [Abdo et al., 2006].

To overcome most of the problems introduced by the previous methods, a statistical method has been developed to distinguish between noise and signal based on a statistical theory [Abdo et al., 2006]. The idea of this method is to standardize the data by dividing the area of each peak by the total peak area of that particular T-RFLP profile, to overcome the variation of DNA injected amount. The standard deviation of the dataset is then computed assuming that the true mean is zero. Peaks with a relative area larger than users defined standard deviations, usually between 2 to 3 times, from the mean are identified as true signal and may furthered processed. This method can be more sensitive to identifying smaller peaks than the other two previously described methods.

Alignment of the T-RFLP profiles is the next step. The fact that slight differences in size estimation due to run to run variability arise, thus there is a need for profile alignment before any further statistical processing of the T-RFLP data. This point could be applied by grouping the fragments size to length categories, even by applying nearest integer rounding approach, manual binding [Blackwood et al., 2003] and cluster based statistical approaches [Abdo et al., 2006]. The last approach is overcoming the other ones as it depends on its parameters on an empirical data and applied consistently and the automated procedures allow an objective analysis of large data sets with statistical justification [Schutte et al., 2008].

The concept of the statistical approach for peak clustering based on pooling and sorting all the interesting peaks from different profiles together. Then removing any resulting doubling in peaks intensity, performing peak clustering by binding peaks with sizes close enough to be grouped in one bin (e.g., within a radius of ±1 bp). This procedure continued to group the fragments until no more peaks are fulfilled the previous conditions. The intensities of the binned peaks are summed into single peaks [Abdo et al., 2006].

However changes in microbial communities goes along with the problem of subjective analysis of the complex, noisy and intensive data sets obtained from the analysis of T-RFLP profiles [Schutte et al., 2008]. Thus visualizing the relationships between different microbial communities, identifying the key groups and clarifying the effect of environmental conditions on these groups should be simplified and carried out in a simple and representative manner.

There is several methods deal with the visualization of the relationship between microbial groups, the principal component analysis (PCA) [Clement et al., 1998], non metric multi di-

mensional scaling (nm MDS) [Denaro et al., 2005] and additive main effects and multiplicative interactions (AMMI) [Culman et al., 2006]. All these methods reduce the dimensionality of the data, which are then plotted into 2D or 3D representative plots. In the case of PCA as much as possible set of variables are described with few variables. The nm MDS arrange the T-RFLP profiles in a plot in a way that the distance between two different profiles is representing the real similarity to dissimilarity between them as accurate as possible, considering at the same time minimizing the resulted Kruskal constrain as possible [Rees et al., 2004]. AMMI could be more useful than the other two methods as it reflects the effect of the environmental factors on the microbial communities beside providing a statistical support for the differences between the communities and the actual relation between them [Culman et al., 2006].

The groups of microbial communities are identified by clustering the T-RFLP profiles depending on computing the pair wise distance based on different similarities indexes, such as Jaccard, Euclidean or Bray–Curtis indexes. The output of this clustering may aid the in visualizing the relations between the different groups and subgroups besides spotting the key playing groups under different defined experimental or environmental conditions.

The structure of the microbial communities is depending on variations of the environmental conditions. Combining data gathered about the related environmental variables with T-RFLP profiles may enhance and expand the value of the final analysis. Methods such as canonical correspondence analysis (CCA) [Cao et al., 2006] and redundancy analysis (RDA) [Blackwood et al., 2003] have been used to link the environmental factors with the changing of the microbial communities structure.

2.1.1.3 Active urea transportation

Bacterial urease is an intracellular cytoplasmic enzyme [Mobley and Hausinger, 1989]. Thus, the urea has to pass through the cell membrane to be in contact with the urease and further to be processed into ammonia and carbon dioxide. Urea is a small polar molecule which is believed to has the ability to diffuse through the bacterial cell membrane at high concentrations [Jahns and Kaltwasser, 1989]. However it was found that in some bacterial species under certain environmental stresses, such as low pH or nitrogen deficiency, the urea can be actively

transported through the cell membrane. This phenomena could be achieved by the means of energy dependent or putative active urea transporters [Greenwood et al., 1998; Jahns and Kaltwasser, 1989; Scott et al., 2000].

It was shown that urea up-taking in bacteria is achieved only by the passive diffusion process [Sachs et al., 2007]. Measurement of urea permeability through bacterial membranes was found to have a permeability coefficient (P) of 10^7 to $4X10^6$ cm/s, such as in other bilayers [Tien, 1974]. Later, some studies showed some physiological evidences for high affinity, energy-dependent urea uptake in some bacteria such as *Alcaligenes eutrophus, Klebsiella pneumoniae* and *Bacillus megaterium* [Jahns and Kaltwasser, 1989; Jahns et al., 1988]. Besides that, the putative active urea transport systems for short chain amids or urea in *Helicobacter pylori, Pseudomonas aeruginosa* and *Rhodococcus* sp. have been reported [Chebrou et al., 1995; Cussac et al., 1992; Greenwood et al., 1998; Parish et al., 1997; Wilson et al., 1995]. At least three urea uptake systems have been identified in bacteria: the Yut, UreI and ABC urea transporter.

Yut urea transporters are well known in *Yersinia* sp. that infects the mammalian gastric canals. This system enables the bacteria to survive the low pH conditions by introducing the urea to the cell and employing the resulted ammonia in buffering the cytoplasm [de Koning-Ward and Robins-Browne, 1997]. In *Helicobacter pylori* another urea transport system, UreI, has an analogue function to Yut. This system, which is pH regulated also introduces the urea into the cell, so the cells have the ability to maintain the pH in their pre-cytoplasm. Thus helping the cells to survive in the stressed environmental conditions and colonize the mammalian gastric canal [Marcus et al., 2005].

ATP-binding cassette (ABC) transporters is a super family of evolutionarily conserved proteins spanning from bacteria to humans [Dassa and Bouige, 2001]. One of characteristic features that the movement of molecules through ABC transporters, coupled to ATP hydrolysis, can be outward or inward [Holland and Blight, 1999]. In bacteria, ABC transporters may export substrates, including drugs and antibiotics, or facilitate the uptake of essential nutrients [Higgins, 1992]. Furthermore, a common characteristic of ABC transporters is their location either at the plasma membrane as well as their extremely conserved role in the translocation of molecules through different cell barriers [Ponte-Sucre, 2007]. The primary structure of these transporters usually contain the “Walker A and B consensus motifs” twice in the same

polypeptide chain, denoting the presence of two nucleotide binding sites [Pedersen, 2005]. Despite the structural variations found among ABC transporters, most of these proteins recognize similar dyes, toxic ions [Van Veen, 2001]. In prokaryotes ABC transporters are involved in the up-taking of small solutes such as histidine [Holland and Blight, 1999], maltose [Ehrmann et al., 1998], peptides, or urea [Jahns and Kaltwasser, 1989].

The active urea transportation in *B. megaterium* DSM90 was shown to be achieved by the means of an energy dependent high affinity ABC transporting system [Jahns and Kaltwasser, 1989]. This energy-dependent system is encoded by the urtABCDE gene cluster [Beckers et al., 2004]. Significant differences in the uptake rates were observed during growth with different nitrogen sources, suggesting that the formation of the system is under nitrogen control.

Table 1 Urea uptake and urease activity in *Bacillus megaterium* grown with different nitrogen sources or incubated without nitrogen [Jahns and Kaltwasser, 1989].

Nitrogen source	Urease activity (units/g protein)	Urea uptake activity (units/g protein)
20 mM nitrate	240	5.0
10 mM urea	193	4.9
20 mM ammonium	33	1.1
1 mM ammonium	234	2.7
10 mM glutamine	28	0.1
8 g nutrient broth/l	6	0
without nitrogen	64	0.6

It was observed that the rate-limiting step in the overall reaction of uptake is the translocation of urea through the cell membrane and not its subsequent intracellular hydrolysis by urease. This is furthermore suggested by the observation that under different growth conditions, urease activities were much higher than the urea uptake activities as shown in Table 1. Cells grown with nitrate as the nitrogen source took up urea rapidly, when glucose was present as an energy source. Uptake was markedly reduced in the absence of glucose [Jahns and Kaltwasser, 1989]. The *in vitro* urease activity, however, was not affected by the glucose presence. Various metabolic poisons, such as inhibitors of the electron transport (azide and cyanide) caused a strong decrease in urea uptake. Urease activity, however, was not affected to a significant extent by these tested metabolic inhibitors [Jahns and Kaltwasser, 1989].

Radioactive ^{14}C-urea was used to investigate the ABC transportation system [Jahns and Kaltwasser, 1989]. In these experiments, urea at low concentration conditions, i.e. 25µM, has been actively transported in a rate which is overcoming the diffusion rate of urea in such concentrations. Permeabilization of the bacterial cells by CTAB reduced the uptake of urea up to 90 % due to the destruction effect of the process on the cell membrane proteins, including the ABC transporters. Using azides, which suppress the ATP regeneration, leads to total inhibition of the active transportation process, which prove the energy dependence nature of this transportation system.

The analysis of large variety of compounds that can be substrates or blockers of ABC transporters revealed that the design of these chemosensitizers should, in general, include one protonable nitrogen as well as a planar aromatic region [Ponte-Sucre, 2007]. In addition, the compounds should be highly lipophilic and able to form numerous and strong H-bond interactions with the ABC transporter [Seelig, 1998]. Natural inhibitors include flavonoids extracted from plants. They have a very low toxicity and increase the cellular accumulation of substances like the fluorescent substrate rhodamine due to a selective inhibition of ABC transporters [Kitagawa, 2006]. Further details about these blockers and their potential role in urease and urea uptake inhibition will be discussed comprehensively in the next chapter.

2.1.1.4 Microbial urease activity inhibition by phosphoric amide derivatives

Bacterial urease (urea amidohydrolase; EC 3.5.1.5) is a nickel-dependent enzyme that catalyzes the hydrolysis of urea to ammonia and carbamic acid. The latter, spontaneously hydrolyzes to form carbonic acid and an additional molecule of ammonia [Mobley et al., 1995].

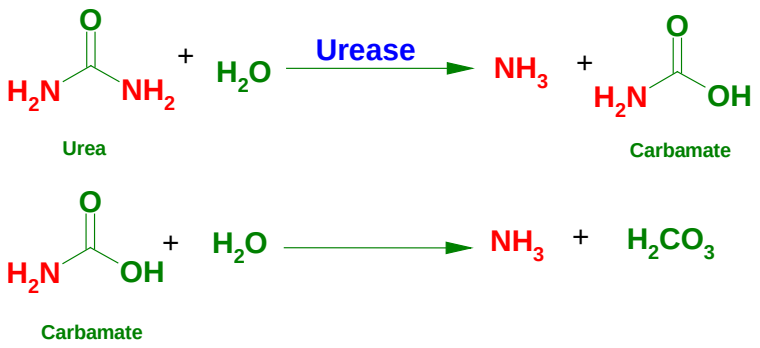


Figure 2. Reaction mechanism of urea hydrolysis by urease into ammonia and carbonic acid which further decompose in to carbon dioxide

Ureases have been isolated from a wide variety of organisms, including plants [Bacanamwo et al., 2002], fungi, and bacteria [Mobley et al., 1995]. Urea hydrolysis in most bacteria is used as a nitrogen or carbon source for anabolic processes [Sachs et al., 2007]. On the other hand, some mammalian gastric pathogenic bacteria species, such as *Helicobacter pylori* employ the resulted ammonia from the hydrolysis process in buffering the pre-cytoplasm and maintain the pH into appropriate range for surviving and colonizing the host gastric canal [Marcus et al., 2005].

Jack bean *(Canavalia ensiformis)* urease (JBU) consists of six subunits, each made of 840 amino acids. Microbial ureases, such as *Klebsiella aerogenes* urease (KAU), have three subunits (α, β and γ) with 101, 106, and 506 amino acids residues, respectively. These subunits form the biological active unit by forming a trimer complex [Benini et al., 2001]. The (αβγ) fragment of KAU is highly homologous to the single subunit of JBU. The structure of *Bacillus pasteurii* urease (BPU) reveals an analogous (αβγ) quaternary structure and a very similar active site geometry to (KAU) and (JBU) [Ciurli et al., 1999], Figure 3. Crystallographic structures of urease are available for only limited number of bacterial ureases [Benini et al., 2001; Benini et al., 1999; Dominguez et al., 2008], however, the highly conserved amino acid sequences of all known bacterial ureases and the constant presence of two Ni ions have been observed. The two Ni ions are bridged by the carboxylate group of the carbamylated lysine (Lys_{R220}) in native BPU and coordinated by some surrounding histidine and aspartic residues [His_{R249} and His_{R275} for (Ni_I) and His_{R137}, His_{R139}, and Asp_{R363} for (Ni_{II}) [Dominguez et al., 2008; Mobley et al., 1995].

Dealing with *Bacillus megaterium* urease attracts a special interest. *Bacillus megaterium* is a common soil bacteria [Vary et al., 2007], acquire an ABC urea active transportation system and have been used as a soil inoculants in agriculture and horticulture [Huang, 2008]. Unfortunately, there is a shortage of literature that deals with *B. megaterium* urease. The lack of any crystal structure or detailed systematic information about BMU hindrances any speculations about the behavior of BMU toward different inhibitors.

Several classes of compounds are known to show considerable inhibitory activity against urease. Up to 14000 individual inhibitors have been tested and verified [Simihaian, 2002]. Table 2 summarizes the major chemical classes of the urease inhibitors that have been comprehensively studied.

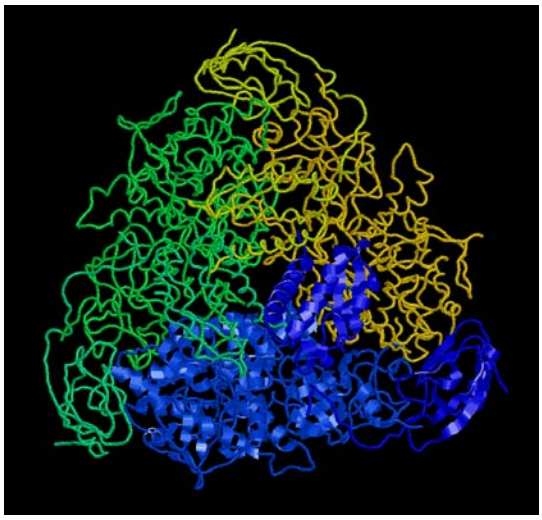


Figure 3. Crystal structure of the biological unit of urease from *Bacillus pasteurii* inhibited with beta mercapoethanol. PDB entry number (1UBP), each (αβγ) fragment is represented with different colour.

Furthermore, the effect of heavy metals, such as complexes of bismuth, mercury and vanadium beside the halogens compounds on urease activity have been investigated [Mobley and Hausinger, 1989].

Among all the known inhibitors of urease, the most efficient are phosphoric di- and triamide derivatives and its derivatives [Dominguez et al., 2008]. This group includes the *N*-*n*-butylthiophosphorictriamide (NBTPT) [Carmona et al., 1990; Gill et al., 1999], phenylphosphorodiamidate (PPDA) [Hendrickson et al., 1987; Niclas, 2007; O'Connor and Hendrickson, 1987] beside other novel phosphoric triamides [Hucke et al., 2001; Hucke et al., 2002; Hucke

et al., 2004; Hucke et al., 2005; Hucke et al., 2006; Michel et al., 2000a]. The PPDA is considered the standard reference for new novel urease inhibitors [Hanisch, 2008]. A very high activity of phosphorodiamidic acid, the simplest structure in this group, results from its close similarity to the transition state of the enzymatic reaction. It was observed that the main disadvantage to the widespread application of this class of inhibitors is their susceptibility to hydrolysis, particularly at low pH [Vassiliou et al., 2008].

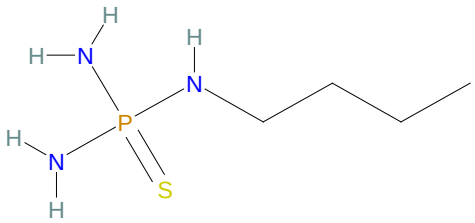


Figure 4. The chemical structure of NBTPT

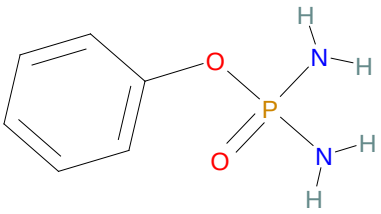


Figure 5. The chemical structure of PPDA

The proposed urease inhibition mechanism by PPDA and NBTPT consist of three main steps: (I) Transporting of the inhibitor toward the active site of the enzyme. The transportation domain of the inhibitor (see Figure 6) is partially responsible for the solubility and mobility of the inhibitor in solutions [Simihaian, 2002]. (II) The formation of the active inhibitor form of PPDA and NBTPT. Both PPDA and NBTPT are in the strict sense only precursors of the actual inhibitors. After the binding to the active centre of the enzyme both of them should hydrolyze to form DAP [Benini et al., 1999]. (III) The last step could be considered the actual inhibition step. In this step it was suggested that the oxygen atom bonded to the phosphorus can bridge the Ni ions, whereas the surrounding nitrogen and/or oxygen atoms bind to (Ni_I) or (Ni_{II}). Thus, in studies carried out on crystals of BPU obtained from PPDA solutions (PDB code 3UBP) it has been established that a diamidophosphoric acid (DAP) molecule, the PPD enzymatic hydrolysis product, is present in the active site. DAP is coordinated to (Ni_I) and (Ni_{II}) by the P-O moiety, whereas one oxygen and one nitrogen bind to (Ni_I) and (Ni_{II}), respectively. The second nitrogen atom of DAP points away toward the cavity opening [Benini et al., 2001; Musiani et al., 2001]. In the absence of crystallographic data for any other phosphorotriamide derivatives-urease complex, the marked ability of these inhibitors can be explained on the basis of it's tridentate nature. In this system the lateral alkyl chain can point toward the cavity opening. As a consequence, the probability of urea reaching the (Ni_I) atom is greatly reduced when the active site is locked by these molecular domains. The designing of new urease inhibitors has been carried out by altering the

Table 2. Overview about the main classes of urease inhibitors.

Class of compound	Example	Effect	Mechanism	K_I *	Application	Refrence
Substrate analog	Methylurea	Formation of a stable enzyme-inhibitor complex	Mixed	>25 mM	Mineral fertilizer	[Amtul et al., 2002; Hasan, 2000; Mobley et al., 1995; Musiani et al., 2001]
	Thiourea	Formation of a stable enzyme-inhibitor complex	ND**	>25 mM	Mineral fertilizer	[Amtul et al., 2002; Hasan, 2000; Mobley et al., 1995; Musiani et al., 2001]
Hydroxamic acid derivatives	Acetohydroxamic acid	Formation of chelate complex with the Ni-ion in the active center	Competitive	2 µM	Enzyme Structure analysis and Mechanism of Urease action Medicine	[Amtul et al., 2002; Mobley et al., 1995; Musiani et al., 2001]
Phosphoroamide	PPDA	Formation of a stable enzyme-inhibitor complex	Competitive	<0.1 nM	Mineral fertilizer, Medicine	[Amtul et al., 2002; Mobley et al., 1995; Musiani et al., 2001]
Phosphate***	H_3P0_4	Formation of a stable enzyme-inhibitor complex	Competitive	0.12 µM	Storage of manure	[Amtul et al., 2002]
Thiols	2-Mercaptoethanol	Formation of chelate complex with the Ni-ion in the active center	Competitive	0.55 mM	Enzyme Structure analysis and	[Mobley and Hausinger, 1989; Musiani et al., 2001]
Boron containing	Boric acid	Formation of chelate complex with the Ni-ion in the active center	Competitive	0.33 mM	Medicine	[Mobley et al., 1995]

* All the K_I values were based on the purified *K. aerogenes* urease inhibition experiments. These experiments were carried out in pH 7.75 buffer at 37C by Todd and Hausinger (unpublished data). Methylurea inhibition studies were performed by Breitenbaich and HaLlsinger (unpublished data)[Mobley and Hausinger, 1989].
** ND= not determined.
*** The deprotonated compound inhibits poorly; a total phosphate concentration of approximately 250 mM is required to provide 0.12 µM H_3PO_4 at this pH.

Chemical structure of the active domain(s) based on the data of BPU-inhibitors structures complexes. [Dominguez et al., 2008].

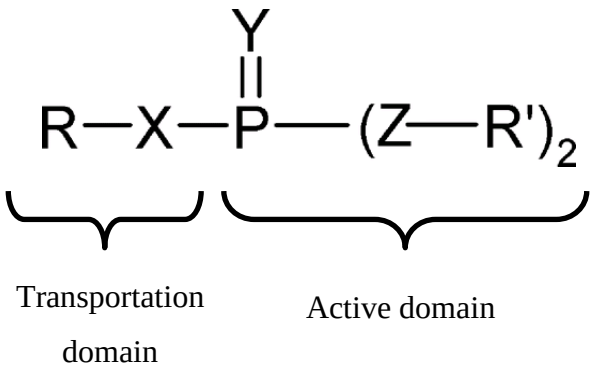


Figure 6. The backbone structure of phosphoric amide derivatives compound

Speculations about novel urease inhibitors could be based on the backbone structure of the PPDA, as shown in Figure 6. In the proposed structures the P=Y moiety (Y could be O or S) adopts the function of DAP's P=O moiety, whereas the surrounding nitrogen and/or oxygen atoms (X=O, NH; Z = O, N) can bind to (Ni_I) and/or (Ni_{II}) in a way similar to that observed for DAP. The lateral chain (R = alkyl, haloalkyl, aryl, alkyl-aryl, heteroalkyl, heteroaryl, alkyl-heteroalkyl, alkylheteroaryl) can be placed in the cavity opening. The introduction of alkylic chains on the terminal nitrogens and/or oxygens (R´= ethyl, *n*-butyl) was proposed to modify the connection pattern of these atoms with the (N_I) atoms as well as with the residues involved in the active site [Dominguez et al., 2008].

2.2 The objectives of the study

In this study three working scopes have been considered. The first one is related to the total and urease-producing bacterial communities in agricultural soil environments. The nature and dynamic changing in these communities as a response for different fertilization managements should be investigated. The gained information will potentially evolve better fertilization management strategies by altering the soil associated micro-flora in a way minimizing the negative impact of non controlled urease activity.

Secondly the active urea transportation by the urea ABC active transporters in *Bacillus megaterium* DSM90 and the effect of some pre-selected phosphoric amide derivatives urea-

hydrolysis inhibitors on this active transportation process is to be investigated. The scope of this point was to clarify the ABC transporter/inhibitors interaction nature and the possible mechanisms of the inhibition processes. The knowledge which may be gained from this investigation should be employed to evaluate the potential alternatives targets for the urease hydrolysis inhibitors.

The last point concerns about the effect of the preselected phosphoric amide derivatives on the *Bacillus megaterium* DSM90 urease activity. This point of investigation is dedicated to spot the potential role of these inhibitors on the urease activity and inhibition mechanism of the proposed inhibitors.

3 Materials and methods

3.1 Chemicals

All the chemicals which have been used in this work, otherwise mentioned, are listed in the following table.

Table 3 Materials and chemicals which have been used in this work

Chemical compound	Supplier	Ordering N°.	Lot.N°.	Purity
Acetic acid, 100%	Carl Roth GmbH & Co (Karlsruhe)	3738.2	31898758	100 %
Acetone	Carl Roth GmbH & Co (Karlsruhe)	9372.6	40573541	≥ 99 %
Acryl amide	Carl Roth GmbH & Co (Karlsruhe)	7906.2	21679242	≥ 99,9 %
Agar-Agar, class I	Carl Roth GmbH & Co (Karlsruhe)	5210.2	5892600	N. S.
Aluminum chloride hexahydrate	Sigma-Aldrich Chemie GmbH (Steinheim)	23,707-8	S32649-166	99 %
Ammonium heptamolybdate tetra hydrate	Carl Roth GmbH & Co (Karlsruhe)	3666.1	20458675	≥99 %
Ammonium peroxo disulfat (APS)	Carl Roth GmbH & Co (Karlsruhe)	9592.3	16675673	≥ 98 %
Ammonium sulfate	Carl Roth GmbH & Co (Karlsruhe)	9212.1	5463297	≥ 99 %
Barium chloride	Sigma-Aldrich Chemie GmbH (Steinheim)	B0750-100G	056K3708	≥ 99 %
Benzonase®	Merck KGaA (Darmstadt)	1.016.950.001	K37522595 728	250 U/µl
Boric acid	Carl Roth GmbH & Co (Karlsruhe)	P010.1	10249875	≥ 99 %
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH	5479	382994/1 52860	≥ 96 %
Bromophenol blue Na-salt	Carl Roth GmbH & Co (Karlsruhe)	A512.1	9253253	N. S.
Calcium chloride	Carl Roth GmbH & Co (Karlsruhe)	A119.1	29678497	≥ 94 %
Cobalt (II) chloride	Sigma-Aldrich Chemie GmbH	60818	408231	≥ 98 %
Cobalt (II) chloride hexahydrate	Carl Roth GmbH & Co (Karlsruhe)	T889.1	26897580	≥ 99 %
Coomassie Brilliant blue G 250	Carl Roth GmbH & Co (Karlsruhe)	9598.1	19890322	N. S.
Copper (II) chloride dihydrate	Merck KGaA (Darmstadt)	1.027.330.250	K35637333 604	≥ 99 %
Copper (II) sulfate	Merck KGaA (Darmstadt)	102.790	A354690	≥99 %
CTAB	Sigma-Aldrich Chemie GmbH	52365	1292654	≥99 %
D(+)-Glucose-Monohydrate	Merck KGaA (Darmstadt)	1.083.421.000	K32578642 402	N. S.
Di-Potassium hydrogen phosphate	Merck KGaA (Darmstadt)	1.051.015.000	A368601 220	98-100 %

Chemical compound	Supplier	Ordering N°.	Lot.N°.	Purity
Disodium hydrogen phosphate dihydrate	Carl Roth GmbH & Co (Karlsruhe)	4984.3	46683303	≥ 99 %
Ethanol	KMF Laborchemie Handels GmbH (Lohmar)	KMF.12-052.2500	2308061510U	≥ 99 %
Ethylendiamintetraaceticacid (EDTA)	Carl Roth GmbH & Co (Karlsruhe)	8043.1	37465498	≥ 99 %
Glycerin	Carl Roth GmbH & Co (Karlsruhe)	3783.2	29897686	≥ 99 %
Glycine	Carl Roth GmbH & Co (Karlsruhe)	3908.2	37789648	≥ 99 %
Guanidine hydrochloride	Carl Roth GmbH & Co (Karlsruhe)	37.1	25253354	≥ 99 %
Hydrochloric acid 37% fumigated	Carl Roth GmbH & Co (Karlsruhe)	4625.2	25465386	N. S.
Iron (III)-chloride	Sigma-Aldrich Chemie GmbH (Steinheim)	F7134-100G	017K3463	N. S.
Isopropyl alcohol	Carl Roth GmbH & Co (Karlsruhe)	6752.5	28466114	≥ 99 %
L-glutamine	Sigma-Aldrich Chemie GmbH	G3126-100G	056K0149	≥99 %
Lithium chloride	Sigma-Aldrich Chemie GmbH	213233	1255268	N. S.
Lithium chloride	Sigma-Aldrich Chemie GmbH (Steinheim)	213233	1255268	N. S.
Magnesium chloride Hexahydrate	Carl Roth GmbH & Co (Karlsruhe)	2189.2	2356965	≥ 99 %
Magnesium sulfate-Heptahydrate	Carl Roth GmbH & Co (Karlsruhe)	P027.1	22896847	≥ 99 %
Meat extract	Carl Roth GmbH & Co (Karlsruhe)	5770.1	15784631	N. S.
Mercaptoethanol	Carl Roth GmbH & Co (Karlsruhe)	4227.2	28625	N. S.
Methanol	Carl Roth GmbH & Co (Karlsruhe)	4627.6	37467008	≥ 99 %
N,N´-Methylene-bis-acrylamide	Carl Roth GmbH & Co (Karlsruhe)	7867.1	27679371	2 x Crys.
Nessler´s Reagent	Sigma-Aldrich Chemie GmbH (Steinheim)	72190	71240	N. S.
Nickel (II) chloride hexahydrate	Sigma-Aldrich Chemie GmbH (Steinheim)	22,338-7	61106 U20066	N. S.
Peptone	Merck KGaA (Darmstadt)	1.072.131.000	VM884113 731	N. S.
Phenol	Carl Roth GmbH & Co (Karlsruhe)	40.2	19679080	≥ 99 %
Phenol red	FEINCHEMIE K.-H. Kallies KG (Sebnitz)	-	-	N. S.
Phosphoric acid, 85 %	Carl Roth GmbH & Co (Karlsruhe)	6366.1	35573842	N. S.
Potassium bromide	Sigma-Aldrich Chemie GmbH (Steinheim)	P9881-500G	066K0754	≥ 99 %
Potassium chloride	KMF Laborchemie Handels GmbH (Lohmar)	KMF.08-248.1000	5J001570	≥ 99 %
Potassium dihydrogen phosphate	Riedel-de Haën Laborchemi-kalien GmbH & Co. KG (Seelze)	30407	91760	≥ 99 %
Potassium iodide	Sigma-Aldrich Chemie GmbH (Steinheim)	207969-100G	1279738	99 %

Chemical compound	Supplier	Ordering N°.	Lot.N°.	Purity
Potassium nitrate	Carl Roth GmbH & Co (Karlsruhe)	P021.2	50468953	≥ 99 %
Protein Molecular Weight Marker	Fermentas GmbH (St. Leon-Rot)	#SM0431	1811	N. S.
Sodium azide	Carl Roth GmbH & Co (Karlsruhe)	K305.1	11253405	≥ 99 %
Sodium chloride	Carl Roth GmbH & Co (Karlsruhe)	3957.2	16895929	≥ 99 %
Sodium citrate tribasic Dehydrate	Sigma-Aldrich Chemie GmbH (Steinheim)	C7254-1KG	115K0039	≥ 98 %
Sodium dodecyl sulfate	Carl Roth GmbH & Co (Karlsruhe)	1057.1	14895724	N. S.
Sodium hydroxide	Carl Roth GmbH & Co (Karlsruhe)	6771.1	3892596	≥ 99 %
Sodium hypochlorite	Sigma-Aldrich Chemie GmbH (Steinheim)	23,930-5	18981	N. S.
Sodium metavanadate	Sigma-Aldrich Chemie GmbH (Steinheim)	590088-5G	02913EB	99,9 %
Sodium Molybdate	Sigma-Aldrich Chemie GmbH	71756	418956/1	≥99 %
Sodium molybdate dehydrate	Merck KGaA (Darmstadt)	1.065.210.100	A523521 404	≥ 99 %
Sodium nitroprusside dihydrate	Carl Roth GmbH & Co (Karlsruhe)	HN34.1	25892697	≥ 99 %
Sodium selenite	Sigma-Aldrich Chemie GmbH (Steinheim)	S5261-10G	125K2611	98 %
Sodium sulfate	Riedel-de Haën Laborchemi-kalien GmbH & Co. KG (Seelze)	13464	01930-121	N. S.
Sodium sulfite	Carl Roth GmbH & Co (Karlsruhe)	P033.1	16348477	≥ 99 %
Tetramethylethylenediamine	Carl Roth GmbH & Co (Karlsruhe)	-	11250517	N. S.
Trichloroacetic acid (TCA)	Carl Roth GmbH & Co (Karlsruhe)	8789.1	16253891	≥ 99 %
Tris hydroxymethyl aminomethane	Carl Roth GmbH & Co (Karlsruhe)	4855.3	24678188	≥ 99 %
Urease	Carl Roth GmbH & Co (Karlsruhe)	X999.3	33463142	≥ 99 %
Urease From Jack bean	Sigma Aldrich Chemie GmbH (Steinheim)	U1875-25ML	086K7030	N. S.
Zinc chloride	Sigma Aldrich Chemie GmbH (Steinheim)	Z0152-100G	105K0091	N. S.
Zinn (II) chloride dihydrate	Sigma-Aldrich Chemie GmbH (Steinheim)	20,803-5	S35432-356	98 %

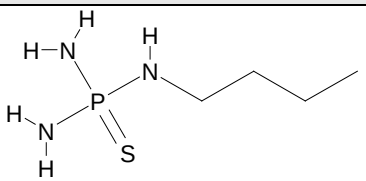
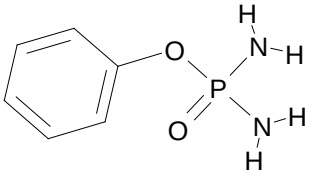
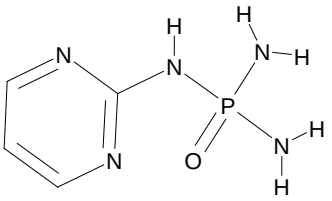
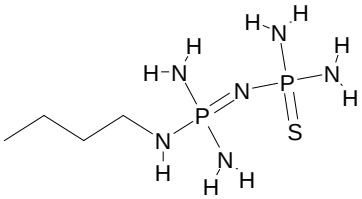
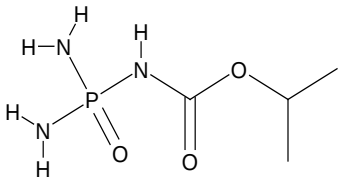
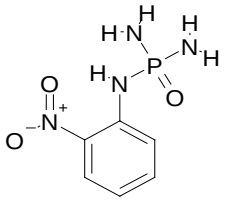
3.2 Bacterial strains

Bacillus megaterium (DSMZ 90), *Corynebacterium glutamicum* (DSMZ 1412), *Sporosarcina ureae* (DSMZ 317), *Sporosarcina pasteurii* (DSMZ 33), *Bacillus subtilis* (DSMZ 10) and *Escherichia coli* (DSMZ 8871) were purchased from the German Collection of Micro-organisms and Cell Cultures (DSMZ), Braunschweig, Germany.

3.3 Urea nitrification and hydrolysis inhibitors

All urease inhibitors were provided by SKW Piesteritz GmbH, Wittenberg, Germany, an overview about the provided inhibitors could be found in the following table:

Table 4. List of urea nitrification and hydrolysis inhibitors used in this work

Inhibitor code	Supplied Synonyms and IUPAC n	Molecular weight [g/mol]	Chemical structure	References
Inh I.	NBTPT N-butylphosphorothioic triamide	167,16		[Kolc et al., 1984]
Inh II.	PPDA phenyl diamidophosphate	172,12		[Held et al., 1976]
Inh III.	SKWP204 N-2-pyrimidinylphosphoric triamide	173,11		[Hucke et al., 2005]
Inh VI.	P96/04 N-[diamino(butylamino) phosphoranylidene] phosphorothioic triamide	244,24		[Hucke et al., 2005]
Inh. V	P204/98 isopropyl (diaminophosphoryl) carbamate	181,13		[Michel et al., 2000b]
Inh. VI	P101/04 N-(2-nitrophenyl) phosphoric triamide	216,13		[Hucke et al., 2006]

3.4 Bacterial growth media

3.4.1 DSMZ1 complex nutrient agar media

The medium was prepared according to the DSMZ recommendations. 5.0 g of peptone, 3.0 g of meat extract and, if solid nutrient media is desired, 15.0 g of agar were dissolved in 800 mL of distilled water. The pH was adjusted to pH 7.00 by 1 M HCl and the volume completed up to 1000 mL. Sterilization took place by autoclaving at 121 °C for 20 min.

3.4.2 Schlegel liquid mineral media

3.4.2.1 Stock solutions used in Schlegel mineral media preparation

50 % v/w Glucose, 1.22 mg/mL $FeCl_3$ hexahydrate, 0.2 mg/mL $MgSO_4$ heprtahydrate and 2 mg/mL $CaCl_2$ were prepared as separate stock solutions and, otherwise mentioned, autoclaved at 121 °C for 20 min independently.

3.4.2.2 Hoagland stock solution

Modified Hoagland trace element solution was prepared according to [Atlas, 1995]. 1 g $AlCl_3$, 1 g KI, 0.5 g KBr, 0.50 LiCl, 7 g $MnCl_2x4\ H_2O$, 11.00 g H_3BO_3, 1 g $ZnCl_2$, 1 g $CuCl_2$, 1 g $NiCl_2$, 1 g $CoCl_2$, 0.5 g $SnCl_2x2\ H_2O$, 0.5 g $BaCl_2$, 0.50 g Na_2MoO_4, 0.1 g $NaVO_3xH_2O$, and 0.5 g Na_2SeO_3 were dissolved each separately in 10 mL distilled water before mixing, and the pH of each solution was adjusted to pH 7.0. After pooling the different preparation the final pH was adjusted to pH 3.5, and the total volume was adjusted to 3.6 L by distilled water. The flaky yellow precipitate which is formed after mixing transforms after a few days into a very fine white precipitate. The solution was shacked thoroughly before administrating into the mineral media. Sterilization took place by autoclaving at 120 °C for 20 min.

3.4.2.3 Schlegel mineral medium with nitrate

The medium was prepared according to [Jahns and Kaltwasser, 1989; Schlegel et al., 1961]. 4.474 g of $Na_2HPO_4x2H_2O$, 1.5 g of KH_2PO_4, and 2.022 g of KNO_3 were dissolved into 850

mL of distilled water, the pH was adjusted to pH 6.8 ± 0.2 by 1 M HCl or NaOH and the volume was adjusted to 985 mL. Sterilization took place by autoclaving at 121 °C, 20 min. After cooling 10 mL sterile 50 % glucose stock solution, 1 mL sterile 1.22 mg/mL $FeCl_3$ x $6H_2O$ stock solution, 1 mL sterile 0.2 g/mL $MgSO_4x7H_2O$ stock solution, 1 mL sterile 20 mg/mL $CaCl_2$ stock solution and 2 mL Hoagland stock solution were added.

3.4.2.4 Schlegel mineral medium with glutamine

The medium was prepared according to [Jahns and Kaltwasser, 1989; Schlegel et al., 1961]. 4.474 g of $Na_2HPO_4x2H_2O$, 1.5 g of KH_2PO_4, and 1.46 g of glutamine were dissolved into 850 mL of distilled water, the pH was adjusted to pH 6.8 ± 0.2 by 1 M HCl or NaOH and the volume was adjusted to 985 mL. Sterilization took place by autoclaving at 121 °C, 20 min. After cooling 10 mL sterile 50 % glucose stock solution, 1 mL sterile 1.22 mg/mL $FeCl_3$ x $6H_2O$ stock solution, 1 mL sterile 0.2 g/mL $MgSO_4x7H_2O$ stock solution, 1 mL sterile 20 mg/mL $CaCl_2$ stock solution and 2 mL Hoagland stock solution were added.

3.4.3 Christensen agar media

This media was been used as a selective media to differentiate and isolate the urease-producing bacteria [Christensen, 1946]. The pH indicator in this media turns to red as a response to high pH value. The increasing of the pH is a direct result of the urea hydrolysis and ammonium liberation by the action of urease. The medium was prepared according to [Christensen, 1946]. 20 g urea, 15 g agar, 5 g NaCl, 1 g peptone 1 g glucose and 0.012 g of phenol red were dissolved in 800 mL of distilled water. The pH was adjusted to 6.8 ± 0.2 by 1 M HCl and the volume was adjusted to 1000 mL. Sterilization took place by autoclaving at 121 °C for 20 min.

3.5 Buffers and reagents

The main buffer which has been used in this study is Na-K phosphate buffer, the pH and the co-additives may slightly vary depending on the application. Otherwise mentioned 50 mM Na-K-phosphate buffer war prepared by mixing equal volumes of 50 mM KH_2PO_4 and

50 mM Na_2HPO_4 solutions, then the pH was adjusted to the desired pH by altering the ratio between the two components.

3.5.1 Biuret reagent

This reagent was prepared to determine the protein concentrations in aqueous solutions according to [Gornall et al., 1948]. To prepare 50 mL of Biuret reagent, 0.839 g of K-Na-tartarat 4 H_2O, 0.5 g NaOH, 0.125 g $CuSO_4x5H_2O$ and 3.125 g of KI were dissolved in 50 mL of distilled water.

3.5.2 Bradford reagent

Bradford reagent was used to determine the protein concentration in aqueous solutions, the reagent was prepared according to [Bradford, 1976], 100-mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL 95 % Ethanol, 100 mL 85 % phosphoric acid and the mixture was diluted with distilled water up to 600 ml, the solution was filtered, 100 mL glycerol was added and the total volume was completed upto1 L by distilled water. The reagent was used after 24 hours of preparation.

3.6 Radioactive ^{14}C urea

^{14}C radioactive urea was purchased from American Radiolabel Chemicals Inc., Saint Louis, USA. The specific activity of the preparation was 56 mCi/mmol, the concentration was 1 mCi/ml and it was dissolved in absolute ethanol.

3.7 Soils samples

3.7.1 Soil samples for the culturable–independent soil bacteria analysis

Total number of 32 soil plots was analyzed in this study. The soil sampling was carried out in June 2008 from individual plots in the long term experimental site of SKW Piesteritz, Cun-

nersdorf, 12 ° 13 'east longitude 51 ° 24' north altitude 130 - 140 m above sea level, the area of each plot was 10 m^2. The soil samples were collected from the top 5 cm to 25 cm depth with a stainless steel piston sampler. All the soil samples were transferred to the lab on dry ice and stored at -80 °C within half an hour prior to analysis [de Lipthay et al., 2004].

Generally the experimental plots could be separated into two major groups the first group, fertilization treatment, related to the effect of the fertilization management on the bacterial communities. This group was consisting of four different treatment groups: group (A) is without any treatment, group (B) was treated with KAS, group (C) was treated with PIAGRAN® 46, granulate urea with 46 % nitrogen content, and group (D) was treated with ALZON®, granulate urea with 46 % nitrogen content and Dicyandiamid as nitrification inhibitor. 180 kg ha^{-1} $year^{-1}$ total amount of nitrogen which was administrated for groups (B), (C) and (D). Each treatment group was analysed in the form of four treatment-identical plot-different replicates. Crops cultivation history for this experimental site could be summarized as following: in 2002 green maize was cultivated, in 2003 and 2004 winter wheat was cultivated, , in 2005 winter rape, in 2006 winter wheat, in 2007 winter barley and in 2008 the experimental sites were cultivated by winter rape.

Figure 7 Eagle-eyed view of the soil sampling location, the long term experimental site of SKW Piesteritz, Cunnersdorf. The exact location of the plots is indicated by the white doted box.

Table 5 Overview of the first experimental group, related to the fertilization management effect on the bacterial community structure in soil.

Experimental site	Cunnersdorf			
Experimental group	Fertilization treatment			
Treatment group	A	B	C	D
Treatment name	No treatment	KAS	PIAGRAN	ALZON
Fertilizer	-	KAS	urea	urea
Urea nitrification inhibitor	-	-	-	Dicyandiamid
Number of Plots	4	4	4	4

Table 6 Overview about the relative position of the different treatment plots in the fertilization treatment experiment. The number which is associated with the letters is representing the plot replicate number.

A_4	B_4	-	C_4	-	D_4	-
D_3	C_3	-	A_3	-	B_3	-
-	-	D_2	B_2	C_2	-	A_2
B_1	-	-	-	D_1	A_1	C_1

On the other hand the second major experimental group, the inhibitor treatment, was construct to investigate the effect of the different urea hydrolyze inhibition treatments on the soil bacterial communities. This group was consisting also of four different treatment groups: group (E) is without any treatment, group (F) was treated with PIAGRAN® 46, granulate urea with 46 % nitrogen content, finally groups G and H was treated with PIAGRAN®, granulate urea with 46 % nitrogen content with urease inhibitor (VI), N-(2-nitrophenyl) phosphoric triamide, with 0.05 % and 0.15 % administrating concentration respectively. 150 kg ha^{-1} $year^{-1}$ total amount of nitrogen which has been applied for the groups (E), (G) and (H), each treatment group was analysed in the form of four treatment-identical plot-different replicates. Crop cultivation history for this experimental site could be summarized as following: in 2007 winter wheat was cultivated and in 2008 the experimental site was cultivated by winter barely.

Table 7 Overview of the second experimental group, related to the inhibitor management effect on the bacterial community structure in soil.

Experimental site	Cunnersdorf			
Experimental group	Inhibitor treatment			
Treatment group	E	F	G	H
Treatment name	Blank	PIAGRAN	P 33/06/F-3	P 03/06/F-3
Fertilizer	-	urea	urea	urea
Urea hydrolysis inhibitor	-	-	0.05 % N-weight Inh VI	0.15% N-weight Inh VI
Number of Plots	4	4	4	4

Table 8 The relative position of the different treatment plots in the inhibitor treatment experiment. The number which is associated with the letters is representing the plot replicate numbers.

G_4	F_4	E_4
E_3	H_3	G_3
H_2	E_2	F_2
F_1	G_1	H_1

3.7.2 Soil samples for culturable-dependent soil bacteria analysis

For the culturable-dependent analysis of soil bacteria two soil plots have been analyzed in this study. The first soil sample was obtained in 2002 from the long term experimental site of SKW Piesteritz, Cunnersdorf, Germany. 12 ° 13 'east longitude 51 ° 24' north altitude 130 - 140 m above sea level. This soil plot has not been treated with any kind of fertilization or nutrient enrichment treatment for at least 5 years prior of sampling.

The second soil sample was obtained from Thyrow, Trebbin, Germany, 13 ° 15 'east longitude 52 ° 14' north altitude 37 m above sea level. This soil plot has been treated with potassium ammonium sulphate (KAS) for the last 5 years prior of sampling.

Both soil samples were collected from the top 5 cm to 25 cm depth with a stainless steel piston sampler. After that they were air dried, sieved and stored at room temperature in air tight polyethylene prior to any further analysis.

3.8 Analytical techniques

3.8.1 Protein concentration assay

3.8.1.1 Bradford test

Calibration of the reagent was done by BSA in Tris HCl pH 8.0 buffer and the assay was carried out by mixing of 50 µL of the unknown protein concentration solution with 2 mL of the reagent and the absorption was measured at 595 nm against a blank, the linear range of the reagent lays between 5 µg and 500 µg protein / mL.

3.8.1.2 Biuret Test

Calibration of the reagent was carried out using BSA in 50 mM phosphate buffer pH 7.5 and the assay was carried out by mixing 0.9 mL of the unknown protein concentration solution with 95 µL of 16 % NaOH and incubation at 99 °C for 5 min, then 0.36 mL of the reagent was added and incubation for 30 min at 37 °C took place, the extension was measured at 546 nm against a blank. The linear range of the assay lies between 5 mg and 60 mg / L.

3.8.2 Urease activity assay

3.8.2.1 Nessler assay

Nessler assay was used as a quick robust assay to characterize the urease activity after IEC or HIC purification treatment. The assay was calibrated by using free ammonia in 50 mM K-Na phosphate buffer pH 7.5. The linear range of the assay was found to stand between 0.05 and 0.7 µmol NH_4. The assay was carried out according to [Worthington, 1993]: 850 µL urea substrate was incubated with 100 µL assay buffer and 50 µL of urease at 30 °C after 10 min of incubation 50 µL of 1.5 M TCA was added to stop the reaction and centrifugation at 16000 g, 4 °C for 3 min. took place, 100 L of the supernatant was mixed with 100 µL distilled water and 700 µL Nessler reagent and the extension at 436 nm against a blank was measured. The urease activity was determined based on the following equation:

$$UreaseActivity[U/mL] = \frac{E_{436nm} \cdot V_{Test}\,[\mu L] \cdot ED}{K_{436nm} \cdot V_{Probe}\,[\mu L] \cdot V_{Enzymesolution}\,[mL] \cdot t\,[\mathrm{min}]}$$

An international unit of urease activity (IU) corresponds to 1 µmol formed ammonia per minute at pH 7 and 25 °C.

3.8.2.2 Indophenol assay

Indophenol assay was used to characterize the urease activity and inhibition kinetics, the assay was calibrated by using free ammonia in 50 mM K-Na phosphate buffer pH 7.5 and the linear range of the assay was found to stand between 0.0625 and 2 mM NH_4. the assay was

carried according to [Weatherburn, 1967]: the unknown enzyme concentration solution was mixed with the assay buffer in the ratio of 1:2, 60 µL of the mixture was incubated with 340 µL of substrate solution at 30 °C for 10min., 60 µL of the reaction mixture were mixed with 600 µL of reagent A (Phenol + Sodium Nitroprusside Dihydrate) and 600 µL of reagent B(Sodium hydroxide + 5 % sodium hypochlorite), after 30 min. of incubation at room temperature the extension was measured at 625 nm against a blank. To determine the urea hydrolysis velocity the following equation was used:

$$Velocity[mM/\min]\frac{E_{625nm}}{K_{625nm}\left[\frac{1}{mM}\right]\cdot t\,[\min]}$$

3.8.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS Page was used to investigate the protein content in aqueous solution quantitavely and qualitatively, mainly to investigate the efficiency of the purification procedure. The unknown protein solution was denaturized in a sample buffer containing mercaptoethanol at 99 °C for 5 min, then was applied into PAGE, which consist of 4.5 %T stacking gel and 14.5 %T resolving gel, the electrophoresis protocol was carried out in 2 phases: first one 300 V, 80 mA for 6 min and secondly 300 V 60 mA for 40 min, the gel was stained afterward by coomassin blue dye overnight.

3.8.4 Analysis of soil (TOC) and (Tot-N)

The soil analysis was performed by the department of isotope biogeochemistry (UFZ, Leipzig, Germany). Gas Chromatography Combustion Isotope Ratio Monitoring Mass Spectrometry system (GC–C-IRM–MS) was applied to determine total organic carbon content (TOC) and total organic nitrogen content (Tot-N) for the 32 soil samples [Cichocka et al., 2008; Rosell et al., 2007]. All samples were measured in at least double replicates. The Dumas pyrolytical conversion was carried out on a HTP reactor (HT-O, HEKAtech, Wegberg, Germany) coupled with a reduction oven. The pyrolysis products and reference gases were transferred into an IRMS (MAT 523, Thermo Fisher, Germany) by a ConFlow III open split unit. The reaction temperature in the HTP reactor was held at 1020 °C, the oxygen carrier gas flow was adjusted to 10 mL/min and the reduction reaction temperature was held at 650 °C. Sam-

ples were calibrated using ammonium sulphate as nitrogen reference and cellulose as carbon reference. The error associated to the system, accuracy and reproducibility, was commonly about ± 0.5‰.

3.9 Methods and experiments

3.9.1 Analysis of urease-producing bacteria bio-diversity in soil

3.9.1.1 Culturable -dependent analysis for the soil bacteria

3.9.1.1.1 Screening of urease-producing bacteria in soil samples

1 g soil was suspended in 10 mL 0.9 % saline solution and stirred with Teflon coated magnetic stirrer for 30 min at approximately 200 rpm [Janssen et al., 2002]. A serial dilution up to 10^{-5} was carried out using 0.9 % saline solution. 200 µL aliquots from 10^{-1}, 10^{-3} and 10^{-5} dilutions were stricken on Petri dishes containing Christensen agar medium [Christensen, 1946]. Each set of plates were incubated even in room temperature 25 ± 2 °C or 30 ± 2 °C. The number of positive urease colonies appeared on the plates was counted at regular intervals, after 24, 48 and 72 hours. Each count represents the mean value from a series of three plates at each dilution and was calculated based on the dry weight of the soil and dilution factors. Each colony show positive result, red colour around the colony, was picked by sterile toothpick and further stricken on other fresh Petri dishes for the verification proposes.

3.9.1.1.2 Identification of the isolated urease producing bacteria

The identification of the positive isolated urease-producing bacteria was carried out by the means of 16S rRNA and *ureC* sequencing and blasting the sequence against the gene databank provided by NCBI. To isolate the genetic material from the targeted bacteria, a single colony was suspended in 30 µL distilled water and incubated at 99 °C for 10 min. Then the lysate was centrifuged for 2 min at 13200 rpm and the supernatant was used for the PCR assay [Süßmuth, 1999]. The PCR protocol for 16S rRNA and *ureC* was carried out as mentioned in section 3.9.1.2.3.1 and 3.9.1.2.3.2. The purified PCR products were sequenced using the service of MWG Biotech AG, Ebersberg, Germany. A minimum of 20 ng/100 base pairs concentration of DNA was provided for sequencing and respective primers (10 pmol/µL)

were sent along with the DNA samples. The obtained sequences were compared against the gene data bank provided by NCBI using the BLAST service [Altschul et al., 1990].

3.9.1.1.3 Phylogenetic analysis of urease-producing bacteria

Approximately 104 partially sequenced *ureC* and 355 partially sequenced 16S rRNA genes originated from uncultured soil bacterium were retrieved from the GenBank database at NCBI. After importing the sequences obtained from this study the nucleotide sequences were converted to their deduced amino acid sequence prior to alignment using Bioedit© computer software [Hall, 1999]. Distance analyses were performed by clustalX© computer software using the neighbour-joining algorithm [Thompson et al., 1997]. The phylogeny between the bacterial communities was represented by a non-rooted phylogenetic tree using phyolegenetic tree drawer Phylodraw computer software [Choi et al., 2000].

3.9.1.2 Culturable -independent analysis for the soil bacteria

3.9.1.2.1 DNA extraction from soil

DNA extraction from soil was carried out using a specific kit for DNA extraction from soil, FastDNA® spin kit for soil from MP-Biomedicals, Germany. DNA isolation protocol was carried out according to the manufacture recommendation.

3.9.1.2.2 Nucleic acid material quality evaluation and extraction verification

The quantity and quality of the extracted nucleic acid material were evaluated photometrical by Thermo Scientific NanoDrop^{TM} 1000 Spectrophotometer. Beside that the extracted nucleic material were applied on 0.8 % agarose gel electrophoresis, stained with ethidium bromide and the resulted gel were analyzed.

3.9.1.2.3 Polymerase chain reaction

3.9.1.2.3.1 PCR for 16S rRNA

To identify the biodiversity of urease-producing bacteria in soil and to obtain a suitable amount of the genetic material for the T-RFLP analysis, PCR was carried out for each of the soil-extracted genetic material. For a total volume of 25 µl for each PCR probe, the PCR mixture consisted of 12.5 µl Mastermix from quiagen®, 1 µl 6-carboxyfluorescein (FAM)-labelled primer, UniBac27f primer (5-AGAGTTTGATC(AC)TGGCTCAG-3) 5 pmol/µl from Biomers.net [Lane, 1991], 1 µl UniBac1492r primer (5-TACGGYTACCTTGTTACGACTT-3) 5 pmol/µl from Biomers.net [Lane, 1991], 8.5 µl distilled DNAse free water and 2 µl of DNA template.

The PCR protocol regarding the DNA template concentration was optimized. Up to 1000 times dilution of the original concentrations of the template DNA were prepared by double distilled DNAse free water and has been employed in the PCR protocol.

The thermal profile of the 16S rRNA PCR was as follows: initial denaturation (94 °C for 10 min) followed by 35 cycles of denaturation (94 °C for 60 s), annealing (55 °C for 90 s), and extension (72 °C for 120 s) and a final elongation step for 6 min at 72 °C.

3.9.1.2.3.2 PCR for ureC

To investigate the functional diversity of urease-producing bacteria in soil and to obtain a suitable amount of the genetic material for the T-RFLP analysis, PCR was carried out for each of the soil-extracted genetic material. For a total volume of 25 µl for each PCR probe, the PCR mixture consisted of 12.5 µl Mastermix from quiagen®, 1 µl 6-carboxyfluorescein (FAM)-labelled primers FAM-L2Fprimer (5-ATHGGYAARGCNGGNAAYCC-3) 50 pmo l/ µl from Biomers [Tyler, 2004], 1 µl urecR (5-GGTGGTGGCACACCATNANCATRTC-3) primer 5 pmol/µl from Biomers.net [Tyler, 2004], 8.5 µl water and 2 µl of DNA template.

Also in this case the PCR protocol regarding the DNA template concentration was optimized; up to 1000 dilution of the original concentrations of the template DNA was prepared by double distilled DNAse free water and been used in the PCR.

The *ureC* PCR thermal profile was as follows: initial denaturation (94 °C for 10 min) followed by 35 cycles of denaturation (94 °C for 60 s), annealing (59 °C for 90 s), and extension (72 °C for 120 s) and a final elongation step for 6 min at 72 °C.

Purification of the PCR products was carried out using peqGOLD cycle-Pure Kit from PeQLab, Germany. In the case of non specific PCR products or primer dimmer formation, agarose gel extraction was carried out using Qiagen DNA gel extraction kit, and the purification protocols was carried out according to the manufacture recommendations.

The quality and quantity of the PCR product before and after purification was analysed after applying the PCR products on 1.5 % agarose gel with TAE stained with ethidium bromide.

3.9.1.2.4 Restriction enzyme treatment

Restriction treatment for the PCR products was carried out using different restriction enzymes. The restriction enzymes which have been used were MspI for the 16S rRNA amplicons and KpnI for the *ureC* amplicons both enzymes were purchased from NEW ENGLAND BOILABS. The employing of the restriction enzymes was based on the gained information after analysing the restriction patterns in silico using the REPK web service [Collins and Rocap, 2007]. In vitro screening of 8 different restriction enzymes, elected from the previous step, was carried out and the ones which show the best T-RFLP profiles were employed in the afterward restriction treatment.

The concentration of each PCR product was measured on 1.5 % agarose gel by the aid of GenSnap and GeneTools software (SYNGENE). The amount of DNA suspension equivalent to 10 ng/µl was introduced in the restriction treatment.

For each probe the total reaction volume was 10 µl. The reaction mixture consisted of 10 ng of purified PCR product, 10 units of the restriction enzyme, equivalent amount of suitable manufacture-recommended buffer and the volume was completed up to 10 µl by sterile DNAse free distilled water. Samples were incubated at 37 °C for 2.5 hours for MspI and overnight for KpnI. Termination of the restriction treatment was carried out by thermal inactivation of MspI at 65 °C and at 80 °C for KpnI.

3.9.1.2.5 Restricted DNA purification

After restriction treatment the reaction mixture were buffered by of 3 M Na acetate, pH 5.5 in the ration of 1:10 and the fragmented DNA amplicons were precipitated by absolute ethanol in the ration of 2.5:1. The probes were centrifuged at 15000 g for 20 min and the supernatant was discarded carefully. Additional 300 µl of 70 % ethanol was added and after 50 min of incubation at 4 °C the probes were centrifuged again at 15000 g for 15 min. after removing the supernatant the probes was placed in a centrifugal vacuum concentrator, Eppendorf model 5301 Concentrator, at 40 °C for 30 min until dryness.

3.9.1.2.6 T-RFLP analysis

The final preparation for the T-RFLP analysis was carried out after the total dryness of the probes by adding 0.4 µl of internal marker GeneScan-500 ROX Standard (APPLIED BIOSYSTEMS) and 20 µl HiDi for each sample, the probes were incubated at 95 °C for 5 min and then were cooled down on ice for 5 min. The fluorescently labelled T-RFs were size separated on capillary electrophoresis (ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and T-RFLP electropherograms were analyzed with Genemapper software version 3.7 (APPLIED BIOSYSTEMS).

3.9.1.2.7 Analysis of the T-RFLP profiles

3.9.1.2.7.1 T-RFLP profiles processing

T-RFLP electropherograms were analyzed with Genemapper software version 3.7 (APPLIED BIOSYSTEMS). The software was configured to export all the peaks which are higher than 1 fluorescence unit in intensity and more than 2 points in width. The minimal cut off peak, was chosen to be 100 and the maximal cut off peak to be 500 bp. Distinguishing the real signal from the electronic noise was carried out using statistical determination of threshold approach [Abdo et al., 2006]. Peaks which were higher than three times the standard deviation value of the standardized peaks dataset were considered as true peaks.

3.9.1.2.7.2 Statistical analysis for the processed T-RFLP profiles

Similarities between the different T-RFLP profiles were determined by a non metric multidimensional scaling (nm-MDS). This method is a multivariate ordination technique based on square root transformed data using a Bray–Curtis similarity matrix [Rees et al., 2004]. Nm-MDS is an ordination technique that attempts to preserve the ranked order of the similarity of communities as an inverse function of the distance between the points representing such as T-RFLP profiles, on a graphical plot [Rees et al., 2004]. The greater the similarity of T-RFLP profiles from each soil sample the closer they are plotted together, profiles with the lowest similarity are plotted furthest apart. Kruskal stress values which should be gained ideally lay between 0.1 and 0.29. In order to obtain more supporting integral picture of how the bacterial communities are responding to the different fertilization managements, statistical analyses were performed on the T-RFLP processed data. The significance between different treatment groups was calculated using a one way analysis of similarities (ANOSIM) test. ANOSIM R values of 1 indicate that replicates within the same plot are more similar to each other than to any samples from the other plots, whereas an R value of 0 indicates that there is as much variation within-plot as between plots being compared. In deciding whether plots were different, both the R value and the significance value were considered. Nm-MDS and ANOSIM analysis have a generality of application due to lack of assumptions and flexibility to use unbalanced data sets [Clarke and Warwick, 1994].

Vector fitting representing the environmental variables to analyse their effect on the microbial communities was carried out. The interpretation of the environmental variables was carried out in a way that the length of the vectors is reflecting the magnitude of the environmental variable and the relative direction of these vectors is representing the dependency of these vectors on each others, the goodness of fitting value (r^2) and the significant value (P) were based on 1000 permutations.

All statistical analyses and interpretation were performed with the statistical software R program version 2.7.1 [R Development Core, 2005] and the R statistical package Vegan [Jari Oksanen, 2008].

3.9.2 Active urea uptake by *Bacillus megaterium*

3.9.2.1 Cultivation of *Bacillus megaterium* DSM90

3.9.2.1.1 Activation, rehydration and agar plates stock cultures

Activation of *Bacillus megaterium* DSM90, obtained in the form of lyophilized pellet, was carried out according to the recommendations of DSMZ. 10 mL of liquid nutrient media were added to the lyophilized bacterial pellet and the rehydration took place for 30 min. The suspension was streaked out on a nutrient agar Petri dishes and was incubated at 30 °C for 24 hours. After growth the Petri dishes were preserved at 4 °C and fresh cultures were prepared each two weeks. All the procedures were carried out under restrict sterile conditions.

3.9.2.1.2 Stock cryo-cultures 50 % glycerol

A single colony from the agar plate stock cultures, prepared from the previous step, was used to inoculate 100 mL of liquid nutrient growing medium in 500 mL Erlenmeyer shaking flasks with 4 baffles. The flasks were incubated at 30 °C at 120 rpm for 24 hours until the OD_{600} is ranged between 2 and 3. 1 mL of 99 % glycerol was added to 1 mL of stock culture, final concentration 50 %, in a cryo-tube and stored at - 80 °C.

3.9.2.1.3 The pre-cultures for the mineral media

490 mL of DSMZ1 complex liquid nutrient media in 2 L Erlenmeyer shaking flasks with 4 baffles, were inoculated with 10 mL overnight grown pre-culture, in DSMZ1 liquid nutrient media, of OD_{600} approx. 2. The main culture was grown at 30 °C, at 100 rpm (in thermostat Multitron II, Infors, Bootmingen, Switzerland), to an OD_{600} ranged between 2.5 and 3.00. After growth, the whole culture was centrifuged at 5000 g for 10 min. The supernatant was discarded and the pellet was re-suspended in appropriate volume of Schlegel mineral media to wash the cells and remove and traces of the DSMZ1 complex liquid nutrient media. The washing step was carried out for 2 successive rounds. Then the supernatant was discarded and the pellet was re-suspended in appropriate volume of mineral media to gain an OD_{600} of 25. All the previous steps were carried out under restricted sterile conditions.

3.9.2.1.4 Growth behavior of B. megaterium DSM90 in DSMZ1 liquid nutrient media

The growth behavior of *B. megaterium* DSM90 in DSMZ1 liquid nutrient media, was monitored after inoculation of 490 mL of liquid media in 2 L Erlenmeyer shaking flasks with 4 baffles, with 10 mL overnight grown pre-culture of OD_{600} approx. 2. The main culture was grown at 30 °C, at 100 rpm (in thermostat Multitron II, Infors, Bootmingen, Switzerland), and the OD_{600} was measured over time in fixed time intervals.

3.9.2.1.5 Growth behavior of Bacillus megaterium DSM90 in Schlegel liquid mineral media

The growth behavior of *B. megaterium* DSM90 in Schlegel liquid mineral media, with KNO_3 or glutamine as nitrogen source, was investigated after inoculation of 490 mL of liquid media in 2 L Erlenmeyer shaking flasks with 4 baffles, with 10 mL inoculums, cultivated previously in liquid nutrient media as described in 3.9.2.1.3, of OD_{600} approx. 25. The main culture was grown at 26 °C, at 100 rpm in thermostat Multitron II, Infors, Bootmingen, Switzerland) and the OD_{600} was measured over time in fixed time intervals.

3.9.2.2 Active urea uptake experiments

3.9.2.2.1 Urea uptake assay suspension

To investigate the urea active transportation in bacteria, *Bacillus megaterium* DSM90 biomass was produced as described in section 3.9.2.1.5. The cultivation took place at 26 °C, 100 rpm for 16 hours until the OD_{600} reached approx. 3. The preparation of the assay reaction mixture was carried out according to [Jahns and Kaltwasser, 1989]. After cultivation, the bacterial biomass was obtained by centrifugation at 5000 g for 30 min, two successful washing steps with 50 mM Na-K-phosphate buffer pH 7 was carried out in order to remove any residual of the cultivation media. To obtain an equivalent protein concentration of 1 mg/ml, the final OD_{600} for the radioactive assay was adjusted to 30 with 50 mM K-Na phosphate buffer containing 0.5 % glucose [Jahns and Kaltwasser, 1989].

3.9.2.2.2 Experimental procedure

The prepared bacterial suspension, OD_{600} 30, were transferred to small caped polypropylene bottles and incubated at 30 °C, 100 rpm for 30 min. CTAB was administrated, 15 mg/mL final concentration, when the Permeabilization effect is desired. The urease hydrolysis inhibitor was added to the bacterial suspension, 0.25 µM final concentrations, to investigate the effect of these inhibitors on the overall uptake procedure. After 5 min. of incubation 70.27 µL of radioactive ^{14}C urea solution was added with final urea concentration of 1 mM or 25µM of urea and 0.5 mcpm/mL radioactivity, the final volume of the reaction mixture was 2970.27 µL.
Directly after adding the ^{14}C urea, 500 µL samples were obtained from each probe at fixed time intervals, 30 sec, 2 min, 3 min and 5 min. These probes were centrifuged directly after sampling at 16000 g for 30 sec. at 10 °C. 200 µL of supernatant transferred to scintillation disposable tube, 3 mL of scintillation solution were added and the radioactivity of was measured in scintillation counter. The radioactive measurements are representing the free radioactive urea which has not been up taken by the bacteria.

In the previous experiments, sodium azide has been used as positive control for the active urea transportation inhibition [Jahns and Kaltwasser, 1989]. Sodium azide is a biocide which has been used frequently as a preservative, especially in bulk reagents and stock solutions, in many laboratories. Sodium azide acts as a bacteriostatic by inhibiting cytochrome oxidase in gram-negative bacteria [Lichstein and Soule, 1944]; binding irreversibly to the heme cofactor in a process similar to the action of carbon monoxide [Li and Palmer, 1993].

3.9.2.2.3 The stability of the urea active transportation system over time

This experiment is designed to investigate the stability of the active transportation system over time. The idea of this experiment is to determine the optimum window time at which the urea uptake experiment yield its best results. In this experiment the bacterial cells were incubated in the reaction suspension without adding the ^{14}C urea for prolonged time intervals 0, 60, 120, 180 and 240 min. The activity of the transportation system was then been determined by adding the radioactive urea and measuring the urea uptake over 2 min as mentioned in 3.9.2.2.2.

3.9.3 Isolation and purification of *B. megaterium* DSM90 urease

To investigate the inhibitor kinetics of the pre-selected phosphoric amide derivatives on the microbial urease, pure form of microbial urease is to be obtained. For this purpose *B. megaterium* urease was chosen as a model of the microbial urease, based on the reasons discussed previously in section 2.1.1.4. The purification procedure was initially performed according to [Benini et al., 1996].

3.9.3.1 Cell disruption and enzyme liberation

After cultivation of *B. megaterium* DSM90 as mentioned in section 3.9.2.1.5, the cells were harvested by centrifugation at 5000 g for 20 min and washed by 50 Na-K phosphate buffer pH 7, for 2 successful rounds. The obtained wet biomass was re-suspended in double amount of Na-K phosphate buffer with 100 U/mL of Benzonase® and 2 mM $MgCl_2$. the cells were undergo a high press homogenizer treatment for 4 rounds at 750 bar, 4 °C to disruption the cells and liberate the enzyme into the suspension media. Cell debris and membrane fragments were removed by differential centrifugation at 10000 g and 100000 g, respectively for 20 min at 4 °C.

3.9.3.2 Purification of the enzyme by chromatography

The chromatographic purification of the enzyme was carried out using the chromatographic Äkta Explorer device, Amersham Bioscience and the data registration and evaluation was done by Unicorn 4.1, software, Amersham Bioscience.

The evaluation of the purification efficiency qualitatively was carried out by pooling the fractions which show urease activity, measured by Nessler reagent, and evaluate the purity of the pooled samples by SDS-PAGE after acetone precipitation beside the protein concentration, measured by Bradford reagent.

3.9.3.2.1 Ion exchange chromatography (IEC)

The protein supernatant obtained from the cell eruption procedure, mentioned in section 3.9.3.1, was loaded into a Q Sepharose™ Fast Flow (Anion-exchange resin), XK 26/20 chromatography column. The ion exchange chromatographic procedure was carried out by applying a linear gradient from 0 M to 1 M NaCl in K-Na phosphate buffer pH 7.5. The fractions which show urease activity way pooled together prior to any furthered treatment.

3.9.3.2.2 Hydrophobic interaction chromatography

The ionic strength of the obtained active fractions, from the IEC step, were raised to 1 M KCl and. The resulting solution was loaded onto a Phenyl Sepharose ® 6 Fast Flow XK 16/20 column and developed using 50 mM K-Na phosphate buffer pH7.5 a linear gradient from 1 M to 0 M of KCl and the factions which show urease activity were pooled together.

3.9.3.3 Stability of the purified urease

To investigate the purified urease stability over time, 200 µL samples from each purification step were obtained, stored at room temperature and at -20 °C for maximum 5 weeks period and the activity of the urease were measured over time.

3.9.4 Kinetic studies for the purified enzyme

3.9.4.1 K_M and V_{max} determination

To characterize the enzyme kinetics of the chromatographically purified urease from *B. megaterium* DSM90, Indophenol analytical assay was used. The quantity of the librated ammonia during the hydrolysis reaction of urease was representing the reaction velocity V [mM / min]. To determine the V_{max} and K_M values, the hydrolysis reaction urease was carried out for different substrate concentrations, in room temperature for fixed time intervals using 50 mM K-Na phosphate buffer pH 7.5 as a reaction buffer. The Interpretation of the obtained data was carried out in MATLAB program, version 7.0.1, The MathWorks Inc., using a non linear fitness for the Michaels-Menten equation on the obtained data and the differences in devia-

tions between the experimental data and Michaels-Menten equation was minimized, a random fitting for the equation was carried out for 1000 trials and the best fit was considered.

3.9.4.2 K_I determination

To characterize the inhibition effect of the preselected inhibitors on the purified urease, measurements of the enzymatic reaction under the specified experimental conditions mentioned in section 3.9.4.1 was carried out varying the substrate and the inhibitor concentrations. Besides the non-linear fitting of the data, as mentioned in the previous section, double-reciprocal representation of the data based on Line-Weaver Burk equation was carried out to evaluate the obtained data and to determine the inhibition constant (K_I).

3.10 Instruments and devices

Table 9 instruments and devices which have been used in this work

Instruments and devices	Model	Manufacture
Bio safety cabinet, Class II	Nu-440-400E	ZAPF Instruments, Sarstedt, Germany
Conductivity Meter	Qcond 2200	VWR International GmbH, Darmstadt, Germany
Desalting column	PD-10	Amersham Biosciences, Uppsala, Sweeden
Autoclave	Varioklav	Thermo Fisher Scientific Inc., MA, USA
Balance	BP221S	Sartorius AG, Göttingen, Germany
	BL310	
	APX-153	Denver Instrument GmbH, Göttingen, Germany
Centrifuge	Centrifuge 5415 R	Eppendorf AG, Hamburg, Germany
	Hermle ZK 630	
	Avanti™ J-30I	Beckmann Coulter GmbH, Krefeld, Germany
Centrifuge rotors	16 A4-44	Eppendorf AG, Hamburg, Germany
	JA-30.50	Beckmann Coulter GmbH, Krefeld, Germany
	JA-10	Beckmann Coulter GmbH, Krefeld, Germany
Chromatography device	Äkta Explorer (P-900, UV 900, pH/C-900, Box 900, Frac-950)	Amersham Biosciences, Uppsala, Sweeden
	XK 26/20	
	XK 16/20	
	XK 5/5	

Chromatography-resins	Q Sepharose™ Fast Flow	
	Phenyl Sepharose® 6 Fast Flow	
Dialyses membrane	Spectra/Por® 7 Dialysis Membrane MWCO 15,000	Spectrum Laboratories Inc., Rancho Dominguez, USA
Element analyzer	ConFLO III	Thermo Fisher Scientific Inc., MA, USA
Filter	Steritop-GP 0.22 µm	Millipore Corporation, Billerica, USA
Gel capillary electrophoresis	ABI prism 3100 Genetic analyser	Applied Biosystems
Gel documentation system	Gene Genius bio imaging system	Syngene,Cambridge, United Kingdom.
High pressure homogenizer	Emulsiflex C5	Avestin Inc., Ottawa, Canada
Incubator	Innova 4230	New Brunswick Scientific Co., Inc., New Jersey, USA
	Multitron II	Infos Bottmingen, Switzerland
PCR thermo cycler	T personal Thermocycler	Biometra biomedizinische Analytik GmbH, Göttingen, Germany
pH-Meter	inoLab pH Level 2	Wissenschaftlich-Technische Werkstätten, Weilheim, Germany
Photometer	Genesys 6	ThermoSpectronic Rochester, New York, USA
	UVIKON 930	Kontron Instruments GmbH, München, Germany
Pipette	0,5 – 10 µL	Biohit Deutschland GmbH, Rosbach v. d. Höhe, Germany
	2 – 20 µL	Eppendorf AG, Hamburg, Germany
	10 – 100 µL	
	20 – 200 µL	
	100 – 1000 µL	
	500 – 5000 µL	
Shaking table	Rocky 1000	Fröbel Labortechnik, Lindau, Germany
SDS-PAGE-Device		Amersham Biosciences, Uppsala, Schweden
Speedvac concentrator	5301	Eppendorf AG, Hamburg,Germany
Super loops	Superloop™ 50 mL	Amersham Biosciences, Uppsala, Sweden
	Superloop™ 150 mL	
Thermo mixer	Thermostar comfort	Eppendorf AG, Hamburg,Germany
Thermoblock 1,5 mL / 2,0 mL	Thermostat plus	Eppendorf AG, Hamburg, Germany
Thermostat	DC 10	Thermo Haake, Karlsruhe, Germany
Ultra low temperature freezer	C 340	New Brunswick Scientific Co., Inc., New Jersey, USA
Ultrasonic bath	SONOREX SUPER 10 P Digital DK 255 P	BANDELIN electronic, Berlin, Germany
Ultrasonic Disintegrator	Sonifier® W-450 D	BRANSON Digital Sonifier, Danbury, USA
Vacuum System	MZ 2C	VACUUBRAND GmbH + COKG, Wertheim, Germany
Vortexing	labDancer S40	VWR International GmbH, Darmstadt, Germany

4 Results

4.1 Analysis of urease-producing bacteria bio-diversity in soil

4.1.1 Culturable-dependent analysis of urease-producing soil bacteria

4.1.1.1 Screening of urease-producing bacteria in soil samples

In order to enumerate the number of urease-producing bacteria in the investigated soil samples, screening of the two provided soil samples was performed as mentioned in the section 3.9.1.1.1. Christensen media, which contains urea and phenol red as pH indicator, is routinely used for determining urease activity in rapidly growing bacterial species that are constitutively expressing urease or that are capable of hydrolyzing urea at a high rate. Numbers of colonies grown on the plates are counted after 24 and 48 hours of inoculation at each dilution from the plates incubated at room temperature and 30 °C. Under the mentioned growing conditions the urease-producing bacteria growth was recognized by forming a diffused pink color in the media around the grown colony. Figure 8 is an illustration for a typical obtained result from this experiment. The results obtained from this experiment are represented in Table 10.

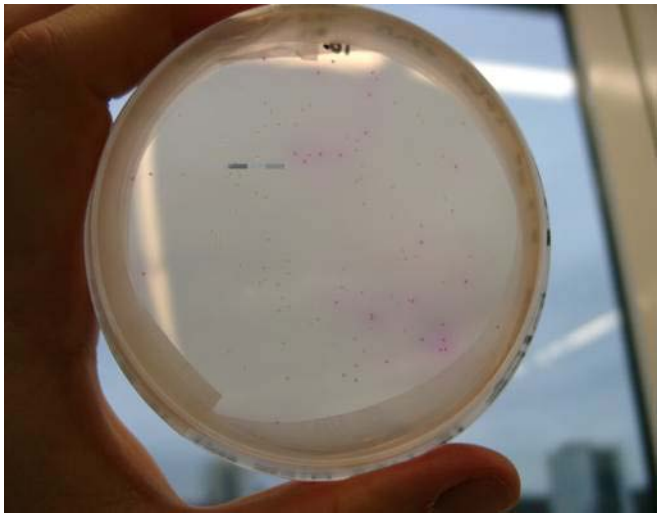


Figure 8 Colonies around which the medium turned red-violet in color are identified as urease producing microorganisms

The results which have been obtained from this experiment show that the total numbers of culturable soil bacteria in both soil samples are roughly the same. The number and the percentage of urease-producing bacteria was found to be remarkably more in the soil samples originated from Thyrow location, KAS treated location. The number of colonies was found to be increasing as a response for the temperature increasing from 20 °C to 30 °C. On the other hand it leads to a decreasing in the urease-producing bacteria counts. Higher dilution factors were associated with increasing in the number of the identified urease-producing bacteria. The following table is over viewing the results obtained in this experiment.

Table 10: Enumeration of urease positive microbial population on Christensen agar plates

Soil	Temp. [°C]	Dilution factor	N° of colonies (average)				
			Total		positive urease		Percentage
			24 h	48 h	24 h	48 h	[%]
1 (Cunnersdorf)	30	10^{-1}	595	ND*	34	ND*	5.60
		10^{-3}	666	ND*	63	ND*	9.40
		10^{-5}	7	9	2	2	22
	20	10^{-1}	8	11	1	2	18.18
		10^{-3}	0	0	0	0	0
		10^{-5}	0	0	0	0	0
2 (Thyrow)	30	10^{-1}	383	ND*	61	ND*	15.90
		10^{-3}	5	ND*	1	ND*	20
		10^{-5}	0	0	0	0	0
	20	10^{-1}	251	ND*	60	ND*	24
		10^{-3}	8	ND*	2	ND*	25
		10^{-5}	0	0	0	0	0

ND* = not determined.

4.1.1.1.1 Identification of the isolated urease producing bacteria

Genomic DNA of 8 urease-producing isolates was obtained and used as template in the 16s rRNA PCR reaction as mentioned in section 3.9.1.1.2. The amplified products were analyzed on 1 % agarose gel along with a MassRuler™ DNA ladder mix. Degenerate primers were used to amplify a fragment length of about 394 bp of the *ure*C gene. Construction of degenerate primers and PCR conditions are as described in section 3.9.1.2.3. Figure 9 shows the amplified products which have been analyzed on 1 % agarose gel along with a MassRuler™ DNA ladder mix marker.

For most of the samples along with the expected 394 bp PCR product, lower and higher length fragments were amplified. Hence PCR products were purified by gel purification method. Latter concentrations of the products are estimated from 1 µL electrophoresis of purified product on 1 % agarose gel.

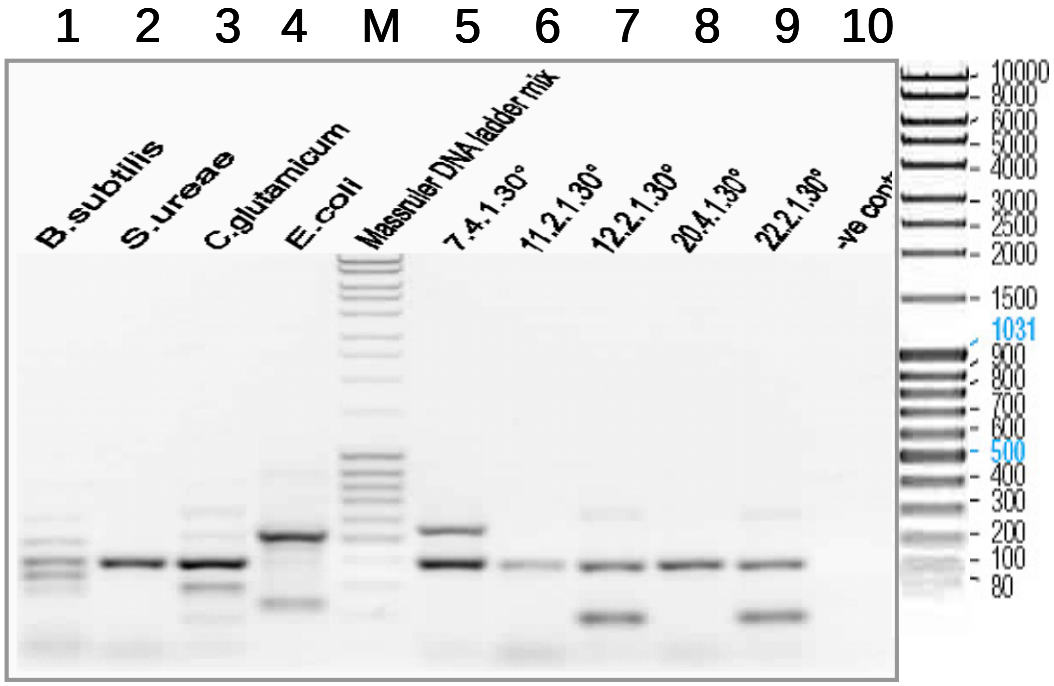


Figure 9 1 % agarose gel analysis of *ureC* gene PCR amplification products of urease-producing control strains and urease producing isolates: 1 is *Bacillus subtilis*, 2 is *Sporosarcina ureae*, 3 is *Corynebacterium glutamicum*, 4 is *E. coli*, M is MassRuler™ DNA ladder mix marker 5 to 9 are isolate A to E and 10 is a negative control.

The purified *16s rRNA* and *ureC* PCR products were sequenced by MWG Biotech AG, Ebersberg, Germany. The good quality clipped lengths were processed and a multiple sequence alignment was done using ClustalW program. After considering the similar sequences the unique sequences were undergone identification processes employing the BLAST search. BLAST search results which show high percent similarity (98-100 % for 16s rRNA and > 75 % for *ureC*) to the database sequences were considered for the identification processes. Table 11 show some of the identified bacteria based on 16S rRNA and *ureC* sequence analyses.

Analysis of the identified bacteria shows that all of them are spore formation strains [Holt et al., 1975; Onyenwoke et al., 2004; Vary et al., 2007]. According to this the predominance of these identified strains in the isolation and identification procedures could be explained, as

these strains, due to spore formation, are stable over the prolonged storage times, up to 2 years. Some of the isolated bacteria could not be identified based on the 16S rRNA amplicons due to the lack in similarities within the compared databanks, such as isolates D, E and F.

Table 11. 16s rRNA and ureC sequences analysis and culturable bacteria identification.

Isolate N°	16s rRNA			ureC		
	Proposed result*	Ass no	Similarity [%]	Proposed result	Ass no	Similarity [%]
A	*Lysinibacillus sphaericus*	FJ844477.1	99	*Lysinibacillus sphaericus*	CP000817.1	93
B	*Bacillus megaterium*	EU221330.1	99	No entry	No entry	NE
C	*Bacillus megaterium*	GQ203108.1	99	No entry	D14439.1	NE
D	ND	ND	ND	*Bacillus sp. T03-2A*	AY618590.1	78
E	ND	ND	ND	No entry	No entry	NE
F	ND	ND	ND	No entry	No entry	NE
G	*Firmicutes bacterium*	EU810844.1	99	ND	ND	ND
H	*Bacillus muralis*	FM875868.1	99	ND	ND	ND

*ND= not has been determined, NE= no entry in the database.

4.1.1.1.2 Phylogenetic analysis of urease-producing bacteria

4.1.1.1.2.1 16S rRNA gene phylogenetic analysis

As mentioned in section 3.9.1.1.3, 355 at least partially sequenced 16s rRNA genes originated from uncultured soil bacterium were retrieved from the GenBank database at NCBI. After the importing the sequences obtained from this study, distance analyses were performed by clustalX© computer software using the neighbour-joining algorithm [Thompson et al., 1997]. The phylogency of the bacteria was represented by a radial non rooted tree using phy-olegenetic tree drawer Phylodraw computer software, Figure 10 [Choi et al., 2000].

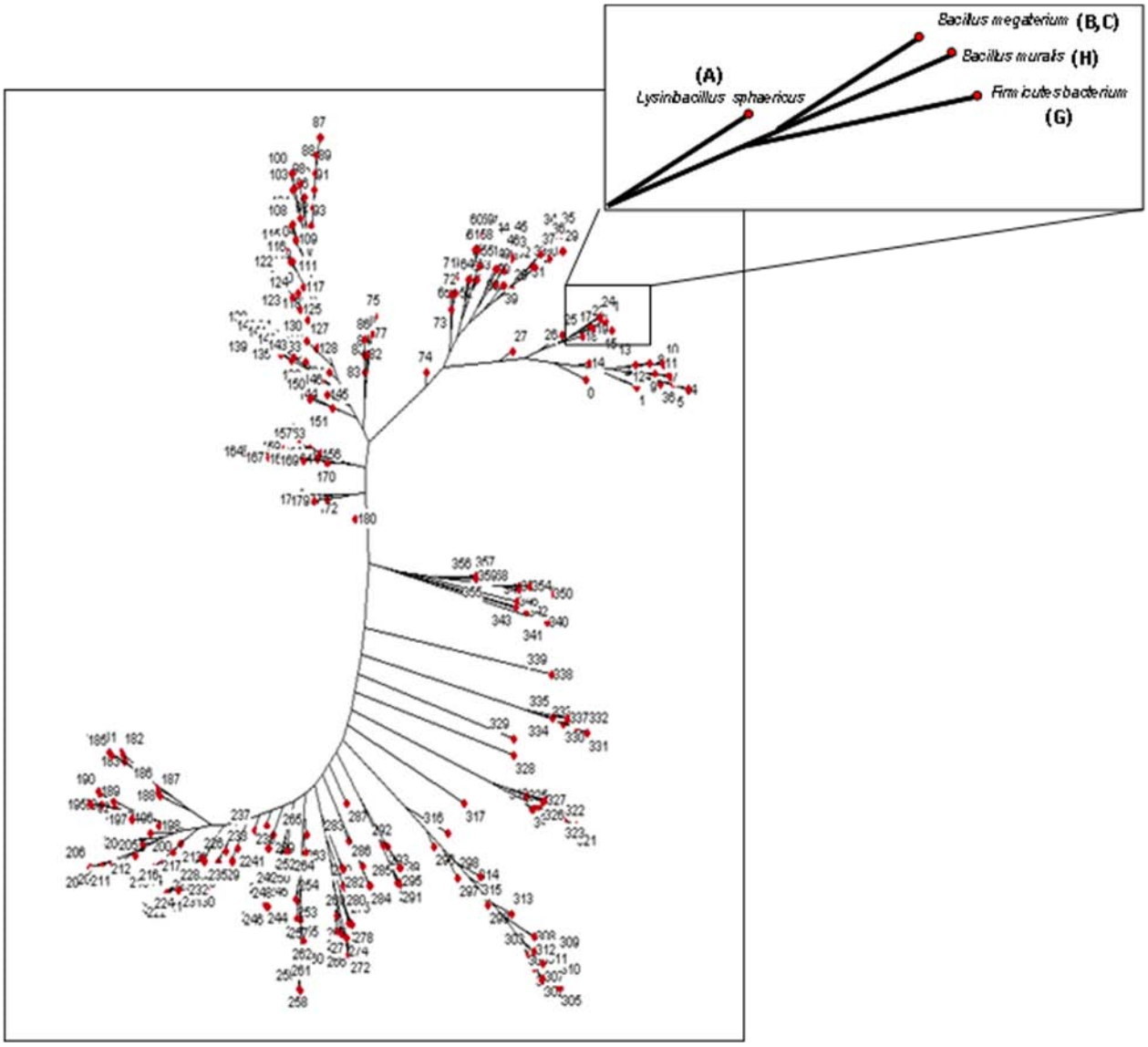


Figure 10 Phylogram of *ureC* gene for urease producing soil microorganisms, determined by distance analysis using the neighbour joining algorithm in ClustalX. Each number in representing a unique clone obtained from the Gene databank at NCBI, for the key of the accession numbers please refer to the attachments. Taxa in boxes indicate sequences obtained from this study, A, B, C, G and H.

The resulted phlylogram consistent with the accepted 16S rRNA based phylogeny for prokaryotes. The phylogenetic analysis shows that the isolates obtained from this study are sharing the same hypothetical taxonomic unit node, relatively closely related to each other. Long storage period in room temperature for the soil samples, up to 2 years, may provoke unfavourable conditions for the bacterial growth. Thus only bacteria which have the ability to form spores, such as *Bacillus sp*. could survive and grow after while in more favourable conditions.

4.1.1.1.2.2 ureC gene phylogenetic analysis

A phylogeny for *ureC* gene sequences was also constructed and compared to the 16S rRNA phylogeny for the same group of species to determine if *ureC* was a suitable phylogenetic marker for prokaryotes. 105 partially sequenced *ureC* genes originated from uncultured soil bacterium were retrieved from the GenBank database at NCBI. For the NCBI association numbers and the corresponded code please refer to the attachments (Table 18 and Table 19). After the importing the sequences obtained from this study, distance analyses were performed by clustalX© computer software using the neighbour-joining algorithm [Thompson et al., 1997]. The phylogency of the bacteria was represented by a non rooted tree using phyolegenetic tree drawer Phylodraw computer software [Choi et al., 2000], refer to Figure 11.

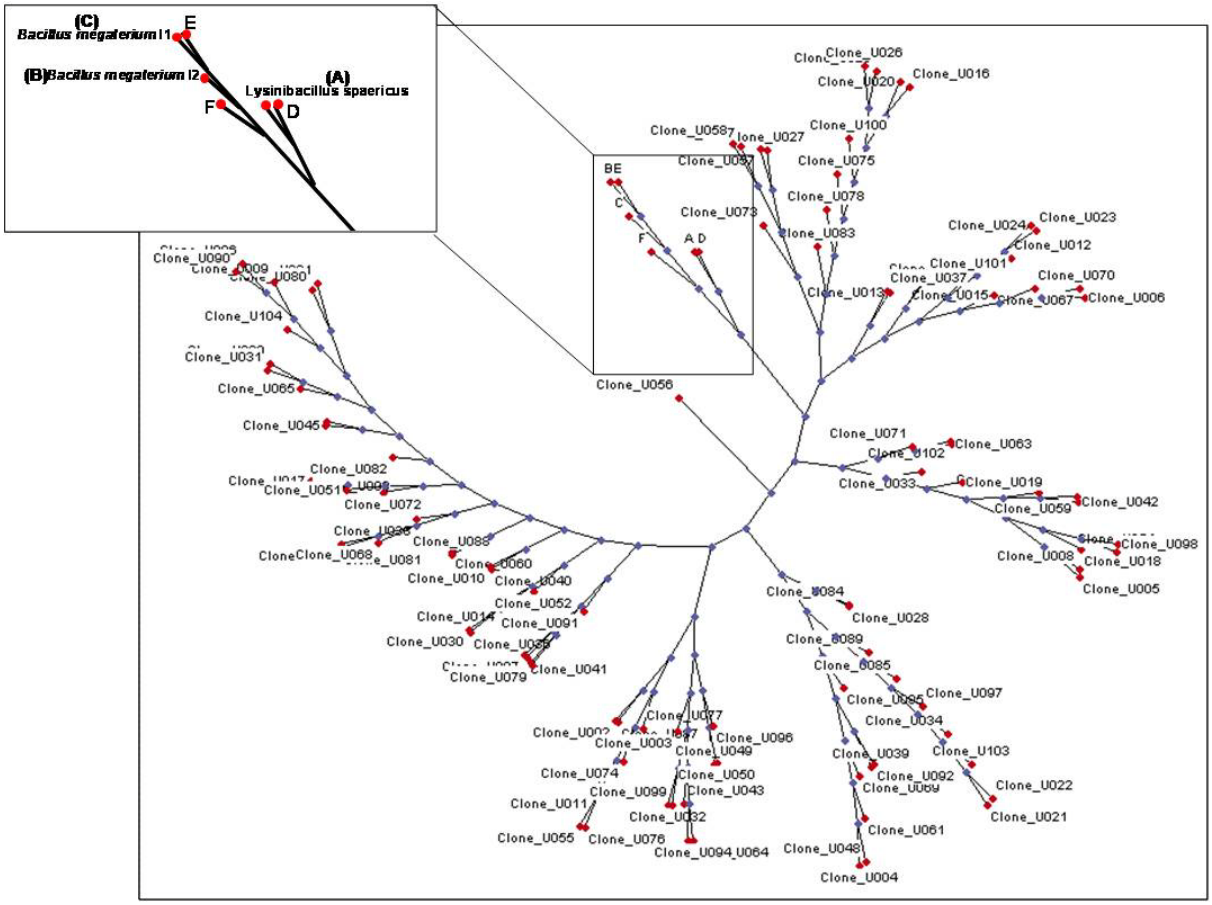


Figure 11 Phylogram of *ureC* gene for urease producing soil microorganisms, determined by distance analysis using the neighbour joining algorithm in ClustalX. Each number in representing a unique clone obtained from the Gene databank at NCBI, for the key of the accession numbers please refer to the attachments. Taxa in boxes indicate sequences obtained from the resent.

Generally, the analysis demonstrated that *ureC* genes exhibit a partial congruence with the 16S rRNA based phylogeny. The previous phylogram shows that the isolated bacteria are

sharing the same internal hypothetical taxonomic unit, clearly related to each other. The obtained results show that under the specified experimental conditions the *ureC* could be consider as good polygenetic marker as 16S rRNA is.

4.1.2 Analysis of culturable- independent urease-producing soil bacteria

4.1.2.1 DNA extraction from soil

In order to obtain a sufficient and representing amount of soil bacteria genomic material, DNA extraction from soil was carried out using as mentioned3.9.1.2.1. The spectroscopic evaluation of the extracted DNA is shown in the following figures.

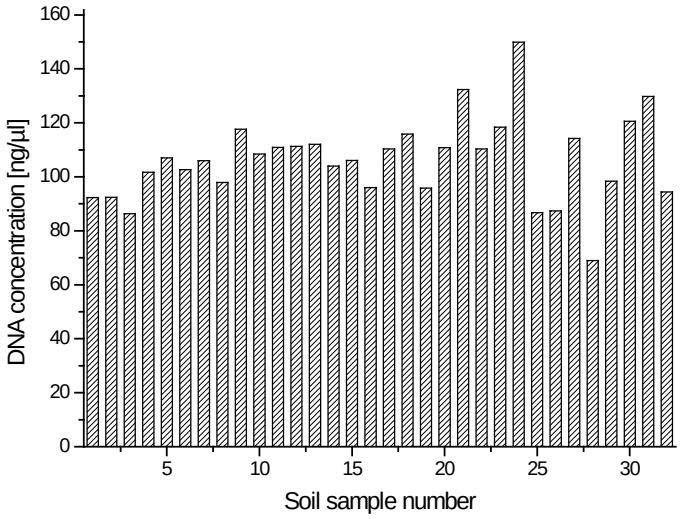


Figure 12 the observed concentration of the extracted DNA from different soil plots by the FastDNA® spin kit.

The mean value of the extracted DNA concentration was found to be 106 ng/µl with a standard deviation of 15.4. Further spectroscopic analysis, 260:280 and 260:230 ratios which reflect the efficiency and purity of DNA extraction regarding the co-extracted proteins and humic acids impurities respectively, was carried out to stand on the actual quality and quantity of the extracted DNA.

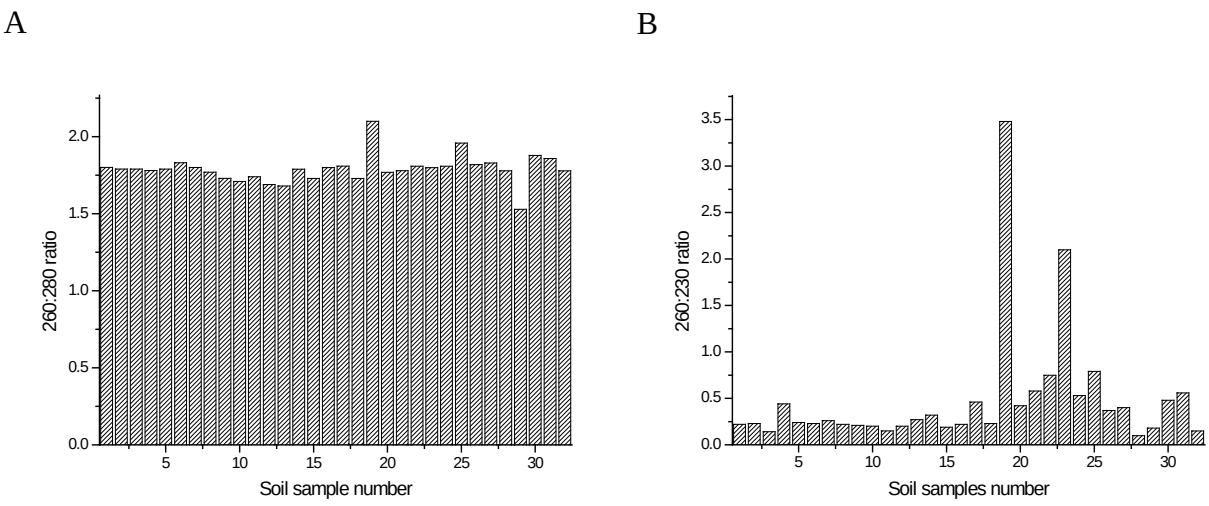


Figure 13. The spectroscopic analysis of the extracted DNA from soil. A the 260:280 ratio, which is an indicator for the purity of DNA extraction regarding the co-extracted proteins impurities for different soil plots B the 260:230 ratio, which is an indicator of the purity of DNA extraction regarding the co-extracted humic acid compounds.

The mean value of the 260:280 ratios, which is an indicator for the DNA extraction purity regarding the protein contamination, was found to be 1.7 with a standard deviation of 0.09. Values below 1.8 indicate a high purity DNA extraction [Sambrook et al., 2000]. On the other hand the mean value of 260:230 ratios, which is an indicator for the DNA extraction purity regarding the humic acid compounds contamination, was found to be 0.47 with a standard deviation of 0.65. Values greater than 2 indicate a pure DNA extraction regarding the humic acid like compounds contamination. The obtained mean values indicate a high contamination with humic acid compounds. These contaminants may influence negatively on the performance of the post extraction molecular manipulation processes such as PCR.

Further analysis for the DNA extracts on 0.8 % gel electrophoreses to validate it qualitatively was carried out and the results is shown in Figure 14. The stained gel shows a significant yield of the extracted DNA. Some degradation in the DNA was observed which may be referred to the mechanical shearing forces used in the extraction procedure. Nevertheless the amount of the extracted DNA quantitatively and qualitatively was suitable for further PCR procedures.

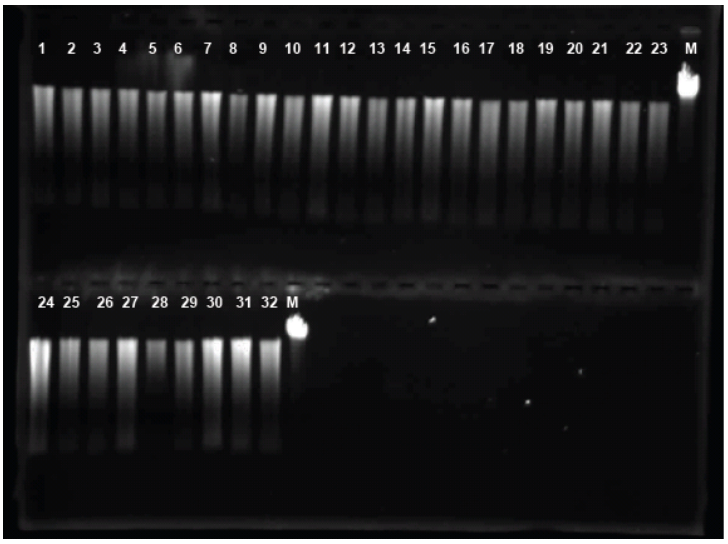


Figure 14 (0.8 %) agarose gel electrophoresis shows the DNA extraction quality, each lane represents different soil sample, the marker which have been used, the M lane, lambda genome marker for comparison porpoises.

Bearing in mind the relatively high amount of humic acid compounds and probably phenol impurities, a dilution of the DNA extracts has been approached to minimize the negative effect of these contaminants on PCR post-extraction procedures.

4.1.2.2 Polymerase chain reaction

4.1.2.2.1 PCR for 16S rRNA amplification

PCR for 16S rRNA amplification was carried out to provide a suitable biodiversity-representative amount of the genomic material for T-RFLP analysis.

To minimize the negative influence of the co-extraction contaminants, an optimization for the PCR regarding the template concentration was carried out. Serial dilution of the extracted DNA templates was carried out and the PCR was done under the specified conditions mentioned in section 3.9.1.2.3.1. The resulted PCR products were applied on 1.5 % agarose gel electrophoresis for the analysis purposes and the resulted stained gel is shown in Figure 15.

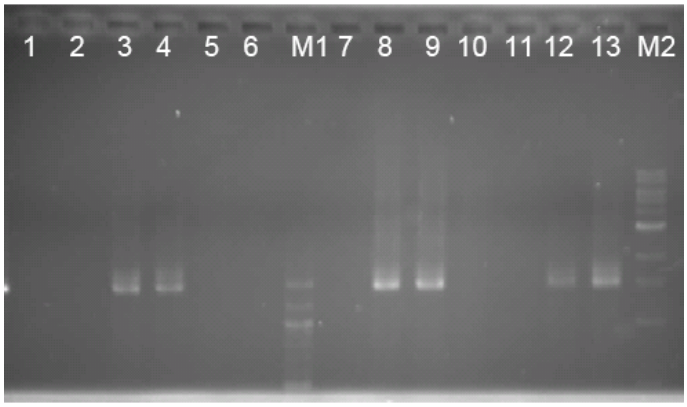


Figure 15 optimization of PCR regarding the DNA template concentration, three different extracted DNA samples were used, the PCR product for the 1st is represented from lanes 1 to 4, 2nd from lanes 6 to 9 and the 3rd from lanes 10 to 13, with different template concentration each, 1X, 10X, 100X and 1000X dilutions, M1 is 100 bp marker and M2 is 1 Kbp marker.

Figure 15 shows that the optimum DNA template concentration, could be obtained after 1000 times dilution of the original soil extracted DNA. This dilution is considered as a compromise between the minimum amount of co-extracted impurities and the maximum possible amount of the extracted DNA. Considering that each dilution step will eliminate the less frequency bacterial species, which may lead in to miss representing the main affecting groups in the high diluted samples.

Based on the previous results, concerning the optimum template concentration, the 16S rRNA PCR reaction for the total 32 different soils plots was carried out based, on the specified PCR protocol mentioned in section 3.9.1.2.3.1. PCR products were evaluated on 1.5 % agarose gel and the resulted gel is shown in Figure 16

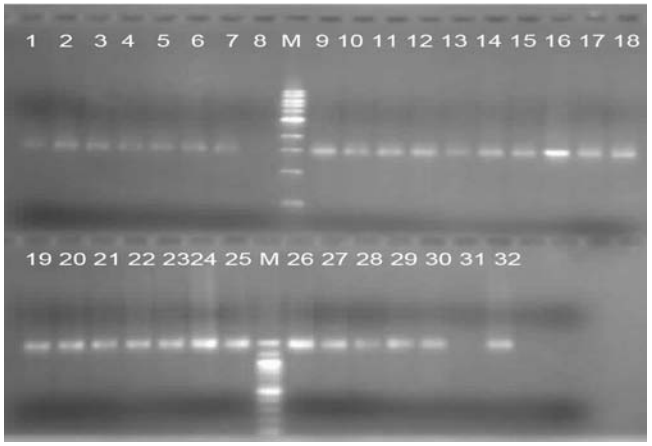


Figure 16 (1.5 %) agarose gel showing the resulted 16sRNA PCR products obtained from 32 different soil samples, each lane represents a different soil sample, M1 is 1.5 kbp marker and M2 is 100 bp marker.

Figure 16 shows the success of the PCR protocol to provide suitable amount of 16S rRNA amplicons for further analysis. Some differences in the intensity between the samples were observed. This could be referred to the differences between the soil samples regarding the biomass quantity and the type of the predominant bacteria in these soil samples.

4.1.2.2.2 PCR for ureC amplification

PCR for *ureC* amplification was carried out to provide a suitable functional-representative amount of the genomic material for T-RFLP analysis.

Screening for the optimum *ureC* PCR regarding the template concentration beside the optimum annealing temperature was carried out. The PCR products obtained from the screening processes were applied on 1.5 % gel electrophoresis, and the resulted stained gel is shown Figure 17.

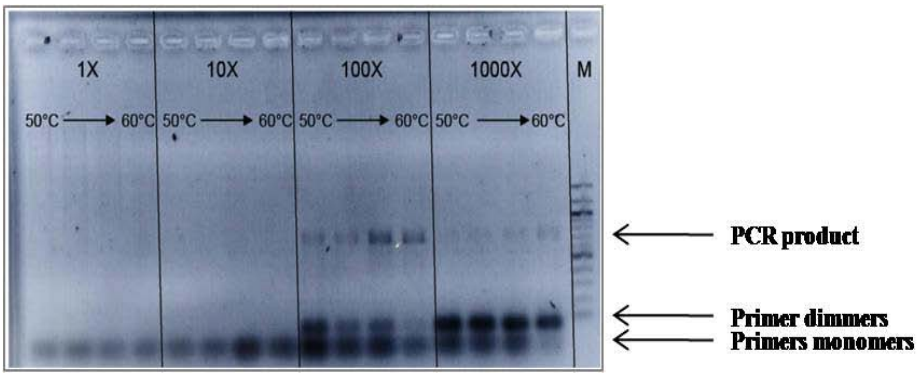


Figure 17 *ureC* PCR product for four different DNA template concentrations and different annealing temperature, each group had a gradient annealing temperature from 50 °C to 60 °C, the groups from left to right are 1X, 10X, 100X and 1000X dilution of the original DNA extraction, the M lane is 100 bp marker.

The optimum extracted DNA concentration for the *ureC* PCR was obtained after 100 times dilutions. The dilution factor in the *ureC* PCR is 10 times lower than the 16S rRNA PCR. This may reflect lower abundance of the *ureC* in the soil relative to 16S rRNA. It is also found that the optimum annealing temperature for the PCR is around 59 °C. The obtained results show a dense primer dimmer formation, which all attempts to minimize their formation, such as altering the annealing temperature, were not successful.

Based on the previous results *ureC* PCR for the total 32 different soils was carried out based on the specified PCR protocol mentioned on section 3.9.1.2.3.2. PCR products were evaluated on 1.5 % agarose gel as shown in Figure 18.

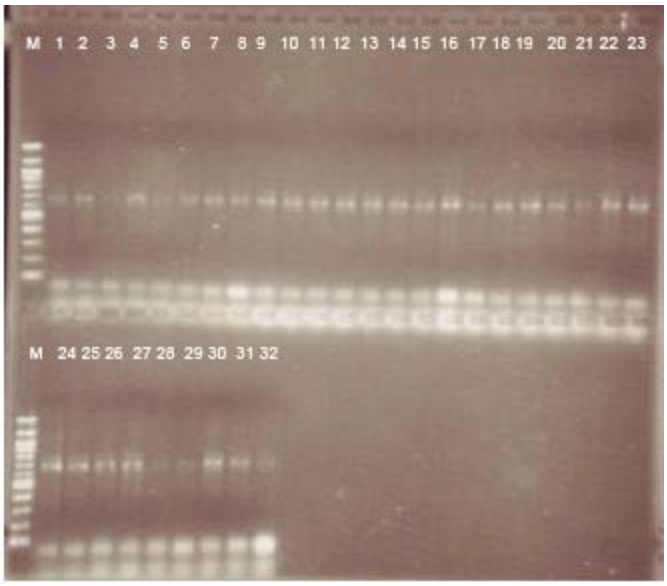


Figure 18 (1.5 %) agarose gel showing the resulted *ureC* PCR products obtained from 32 different soil samples, each lane represents a different soil sample, and M is 100 bp marker.

As shown in Figure 18 it was possible to provide suitable amounts of *ureC* amplicons for further analysis. However dense primer dimmers bands were cofounded, which could not be minimized by any other investigated PCR protocol. To eliminate the influence of the primer dimmers on the final T-RFLP analysis and evaluation data obtained from the T-RFLP regarding the fragments less than 100 bp were be ignored.

4.1.2.3 Purification of the PCR product

The purification of the PCR product was carried out as described previously in the material and methods section 3.9.1.2.3. The purified fragments were evaluated qualitatively and quantify by applying on 1.5 % agarose gel electrophoreses as shown in Figure 19 and Figure 20.

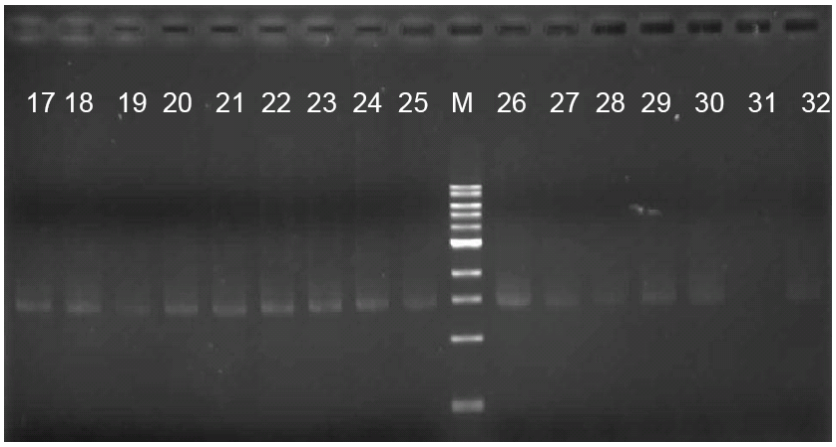


Figure 19 (1.5 %) agarose gel shows the purified 16S rRNA PCR products. each band is representing a different soil sample, this gel is representing only half of the soil samples, and M is 1.5 Kbp marker.

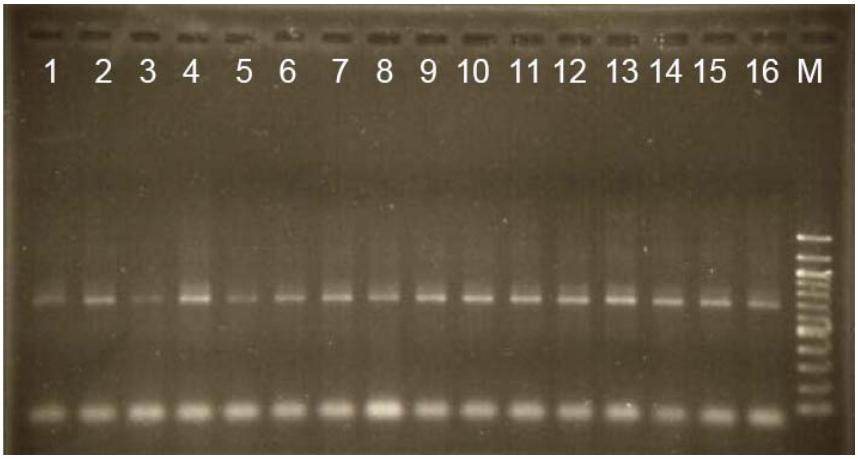


Figure 20 (1.5 %) agarose gel shows the purified *ureC* PCR products. each band is representing a different soil sample, this gel is representing only half of the soil samples, and M is 100 bp markers.

The previous gels show a reduction in the amount of the obtained DNA after purification. In the case of *ureC* PCR product the primer dimmer have been not excluded, which mean that the analysis of the T-RFLP profiles should exclude any data related to fragments less than 100 bp in size.

To avoid any influence of the primer dimmers on the following T-RFLP fingerprinting inter-operated results, an extra agarose gel purification step was carried out to remove these dimmers. The analysis of the T-RFLP profiles avoided the including of any data related to fragments which are less than 100 bp, eliminating any source of primer dimmers possible influence on the final interpretation of the data.

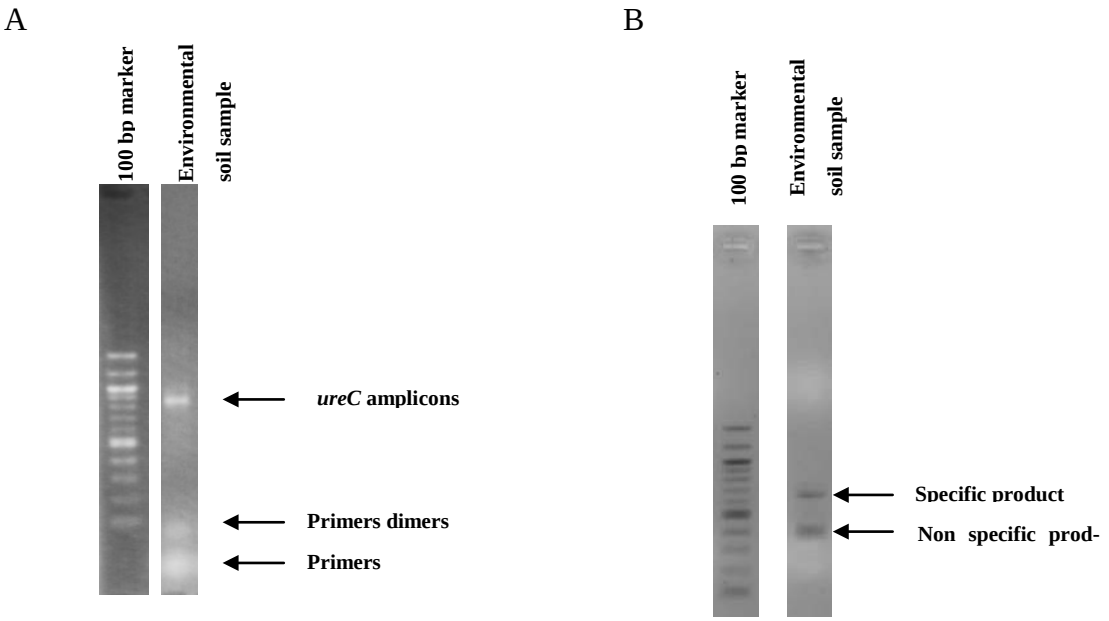


Figure 21 agarose gels show in (A) the heavy primer dimmer developed in the PCR under the specified reaction conditions using the L2F/ureR primer combination and in (B) the non specific PCR development in the case of using L2F/L2R primer combination.

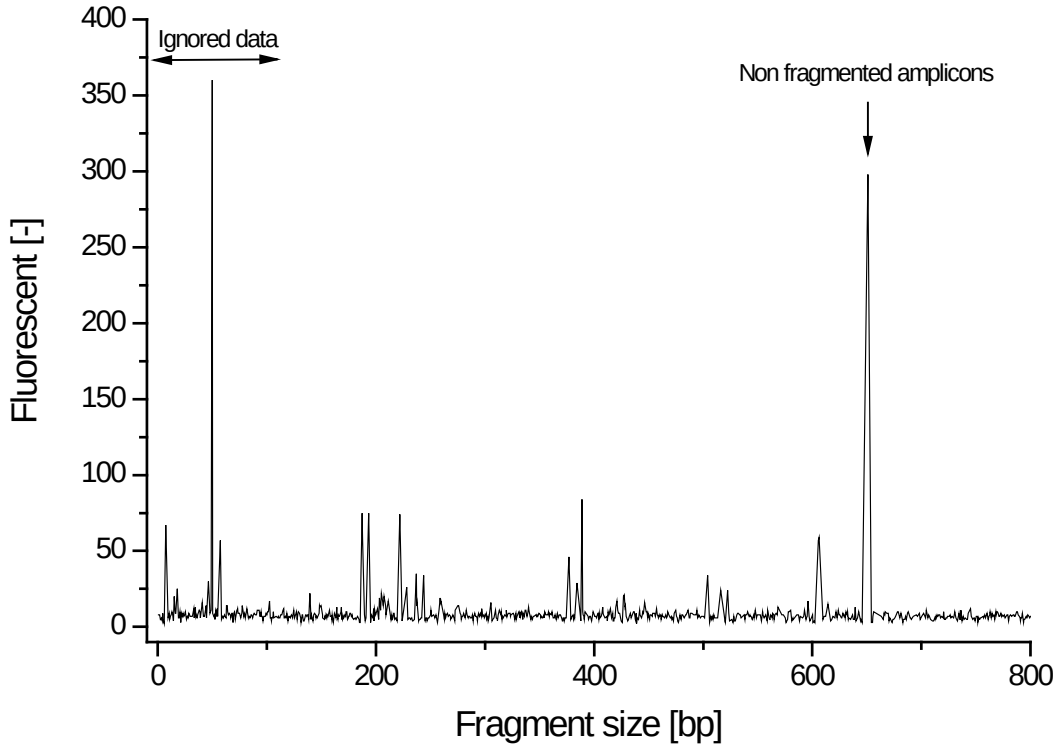


Figure 22 T-RFLP electrogram for the *ureC* restricted fragments by RsaI for a bacterial community in an environmental soil sample. In this electrogram the peak at 650 bp is representing the non fragmented amplicons, by other words the amplicons that the restriction enzyme was not be able to restrict in time course of the reaction. Also all the data obtained below the 100 bp threshold should be ignored to avoid the influence of the primers dimmer on the final analysis.

4.1.2.4 Restriction treatment of the PCR amplicons

As mentioned before in section 3.9.1.2.4 the restriction enzyme treatment was carried out to discriminate the different bacterial communities in the environmental sample. The process of choosing the suitable restriction enzymes passed typically two steps. The first step is *in silico* screening after which eight different enzymes were selected, based on their abilities in discrimination the bacterial communities, to be used in the second step, *in vitro* validation. It is worth to stress the importance of the in vitro validation of these enzymes, as the silico screening for suitable restriction enzymes faces the problematic of misrepresentation of the bacterial genomic databases for the real environmental soil samples. As a result the final proposed restricted enzymes actually represent the best choice for the provided data from the genomic data banks. However the final elected enzymes which provide the most informatics T-RFLP profile was found to be RsaI for the *ureC* amplicons and MspI for 16S rRNA amplicons.

4.1.2.5 Analysis of the T-RFLP data

After the PCR products restriction treatments, T-RFLP analysis for the T-RFs was carried out. The analysis of the obtained T-RFLP profiles was carried out by eliminating the electronic noise signals employing the statistical threshold determination as mentioned in section **3.9.1.2.6**, the minimal cut off peak was 100 bp and the maximum cut off was chosen to be 500 bp. Visualizing the relationship between the bacterial communities based on the processed T-RFLP data was carried out by the aid of nm-MDS as mentioned in section 3.9.1.2.7.

4.1.2.5.1 16S rRNA T-RFLP electropherograms analysis

Non metric multi dimensional scaling (nm- MDS) ordination of the 16s rRNA PCR T-RFLP processed profiles for the two different experimental groups, fertilization and inhibitor treatment, have been analysed separately. The nm-MDS was carried out to evaluate the influence of different fertilization procedures independently on the bacterial biodiversity.

Nm-MDS ordination for the first group, different fertilization treatment experiment show in Figure 23 indicates that the differences within the groups are masking the differences between the groups, i.e. the in-group differences > groups differences. The obtained results show no

significant influence of the fertilization treatment on the bacterial communities within the treated groups. ANOSIM analysis, between the different treatments sub groups based on 1000 permutations shows an R value of - 0.132 with a significance value (P) of 0.934 which indicate a high non significant R value. Lack of differences between subgroups could also be visualized by cluster analysis, shown in Figure 24. These results indicate that the fertilization treatment under the mentioned experimental conditions did not significantly affect the bacterial biodiversity structure in soil.

The carbon and nitrogen content and the pH values of the corresponded soil plots are represented in Table 12. The fitting for these environmental variables onto the nm-MDS ordination was carried. The direction of these vectors show the direction of the gradient and the length of the arrow is proportional of the correlation between the variable and the ordination. Vector fitting for the environmental factors shows a high dependently of the C and N on each other and the non dependency of the pH on them, beside the low goodness of fitting value for each of them, for N it was 0.182 with P = 0.389, for C it was 0.255 with P = 0.260 and for the pH it was 0.025 with an P value of 0.908.

Table 12. Values of the associated environmental variables

Sample	C concentration [%]	N concentration [%]	pH
A1	1.267	0.147	6.04
A2	1.108	0.13	6.07
A3	0.9	0.11	9.01
A4	1.129	0.135	5.98
B1	1.339	0.15	5.9
B2	0.888	0.122	5.92
B3	1.203	0.139	5.93
B4	1.062	0.134	5.91
C1	1.12	0.135	5.85
C2	1.063	0.137	5.83
C3	0.918	0.111	6.07
C4	1.236	0.142	6.1
D1	1.087	0.132	6.11
D2	1.175	0.141	6.18
D3	1.013	0.122	6.22
D4	1.224	0.141	6.28

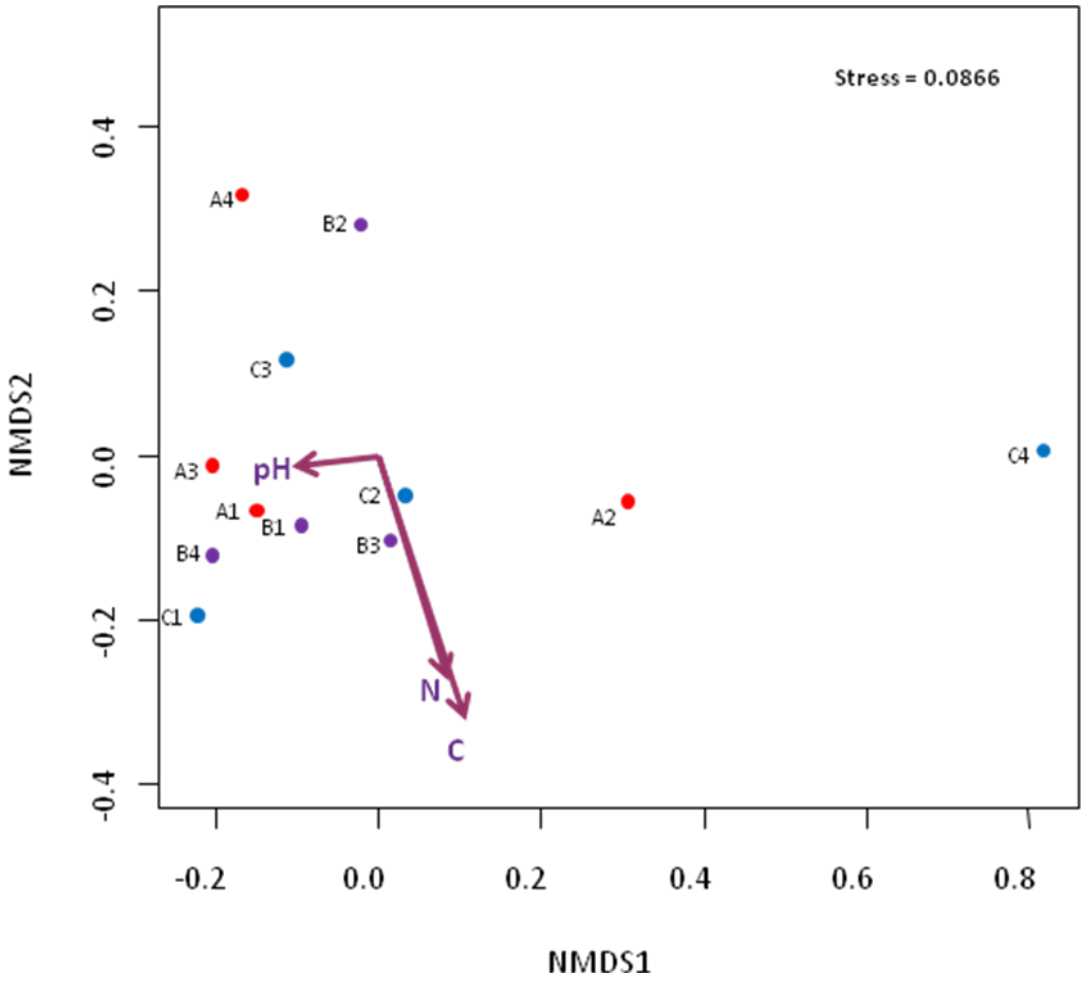


Figure 23 Non-metric multidimensional scaling (nm-MDS) using Bray–Curtis similarity distance index of 16S rRNA, for the data obtained from T-RFLP profiles processing (fertilization experimental groups) with most constraining factors, P values based on 1000 permutations as vectors superimposed. The relative similarity distances between samples of T-RFLP profiles are graphically represented here. The closer two sample are plotted the more similar their T-RFLP profiles are. With *A*= without nitrogen fertilizer added, *B*= KSA fertilizer added, *C*= PIAGRAN 46 added, the number associated with the symbol refer to the number of the treatment replicated sample The vectors show the direction of increase of the three associated environmental factors C=carbon content, N= nitrogen content and the pH.

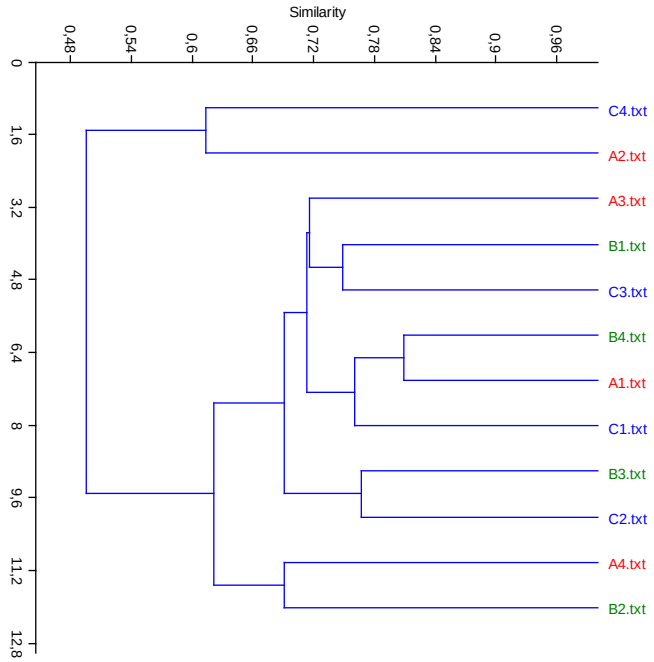


Figure 24 Cluster analysis based on Bray–Curtis similarity distance index of 16S rRNA, for the data obtained from T-RFLP profiles processing (fertilization experimental groups), showing the lack of separation between the experimental subgroups.

On the other hand the nm-MDS ordination of the second group, the inhibitor treatment group, shown in Figure 25, shows a tendency for the different treated groups to separate into four different groups. The separation pattern is illustrated in the figure series from Figure 26 to Figure 29. It is not necessarily that only the inhibitor types have a direct impact on the bacterial community structure and thus this tendency for differentiation, but also the resulted conditions of their action such as different pH pattern or even as a result of altering the nitrogen content after urea degradation inhibition. Nevertheless ANOSIM analysis, between the different treatments sub groups based on 1000 permutations shows an R value of 0.077, which indicate fairly differentiation between the treated subgroups, with a significant value of P = 0.204. These results show that inhibitor type of action is marginally influencing the bacterial community in soil with fairly significant impact.

Vector fitting for the environmental factors shows a high independency of the three difference environmental factors C, N and the pH. Goodness of fitting value for C was 0.02010 with P=0.223, for N it was 0.1583 with P= 0.320 and for the pH was 0.056 with P=0.693.

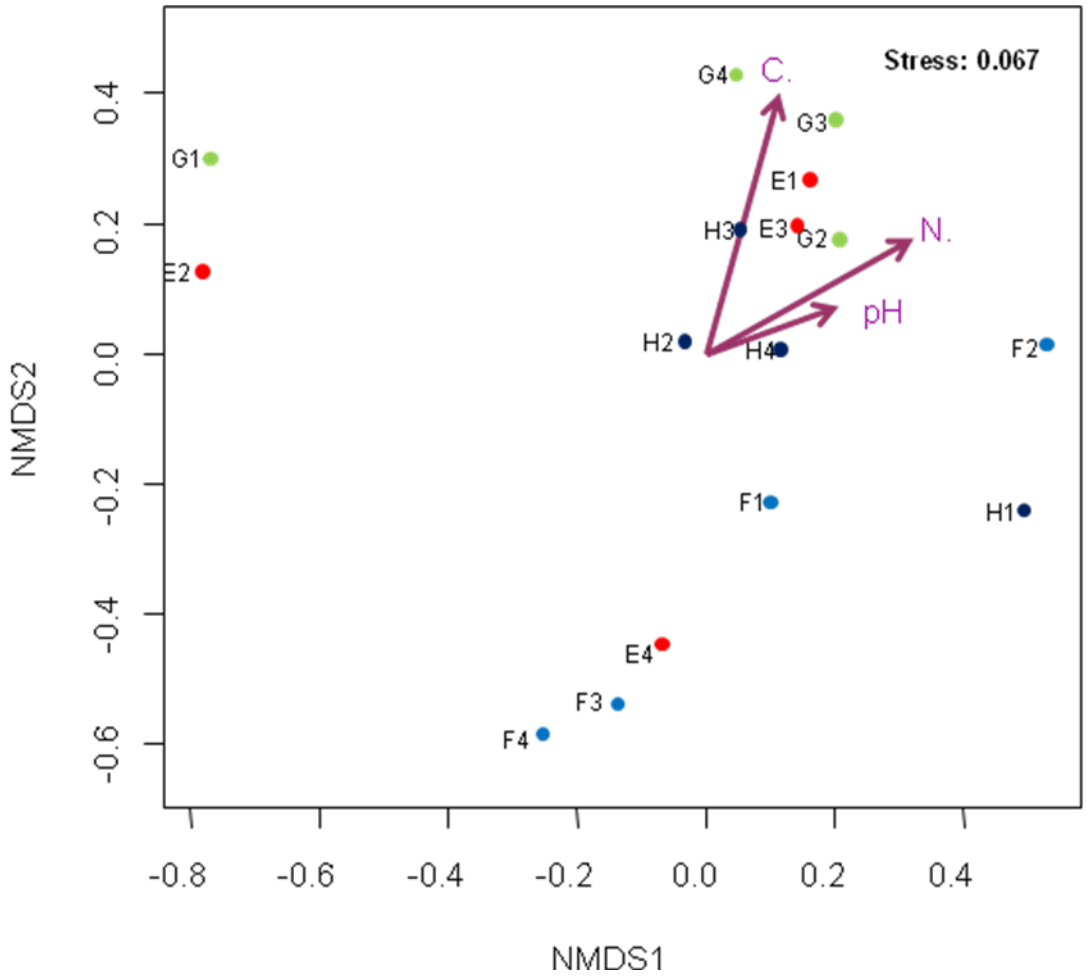


Figure 25 Non-metric multidimensional scaling (nm-MDS) using Bray–Curtis similarity distance index of 16S rRNA, for the data obtained from T-RFLP profiles processing (inhibitor experimental groups) with most constraining factors, P values based on 1000 permutations as vectors superimposed. The relative similarity distances between samples of T-RFLP profiles are graphically represented here. The closer two sample are plotted the more similar their T-RFLP profiles are. With, *E*= no nitrogen fertilizer added (for the inhibitors experiments), *F*= PIAGRAN 46 added (inhibitors experiment), *G*= P 33/06/F-3 fertilizer added and *H*= P 03/06/F-3 added, the number associated with the symbol refer to the number of the treatment replicated sample The vectors show the direction of increase of the three associated environmental factors C=carbon content, N= nitrogen content and pH.

In Figure 26 only the non treated (blank) soil samples were represented. In Figure 27 the urea treated plots were introduced, in which a tendency to shift away from the non treated plots is observed. In Figure 28 the plots which have been treated with the first inhibitor treatment, 0.05 % P/06/F-3, were introduced, in which a shift-back to the non treated plots position was observed. By other words the inhibitor action provides environmental conditions that influence the bacterial communities in soil in a similar way the non- treated plots did. The last figure is representing the plots which have been treated with the second inhibitor treatment 0.15 % P/06/F-3, in these plots the backward-shift toward the non treated plots and away from the urea treated plots was observed, but the magnitude of the shift was not as the first inhibitor

treatment case, This indicate that the different concentration of the same applied inhibitor type may influence the bacterial community structure in a different ways.

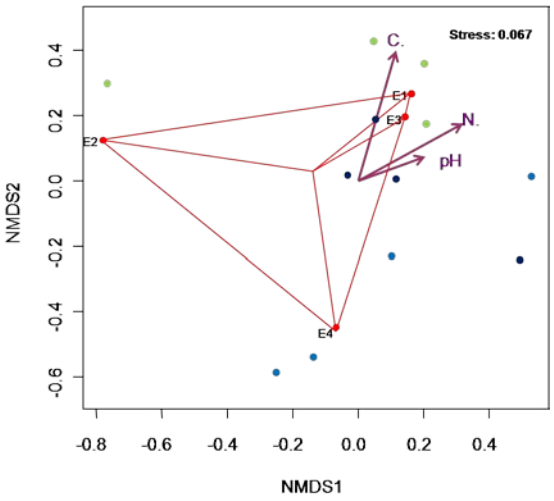


Figure 26 nm - (MDS) of 16S rRNA T-RFLP plots profiles, showing the internal relation between the non fertilized plots.

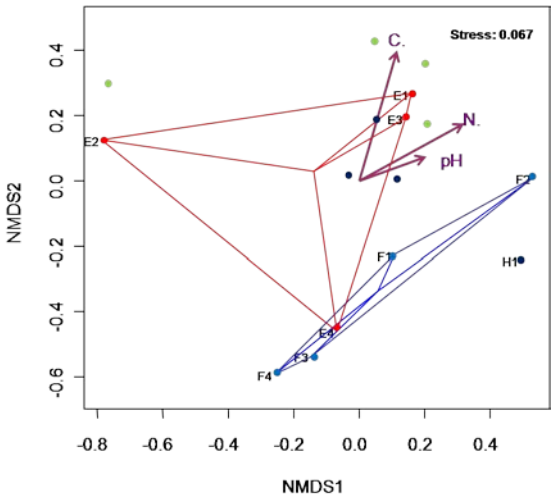


Figure 27 nm - (MDS) of 16S rRNA T-RFLP plots profiles, showing the internal relation between the non fertilized plots and the PIAGRAN 46® treated plots.

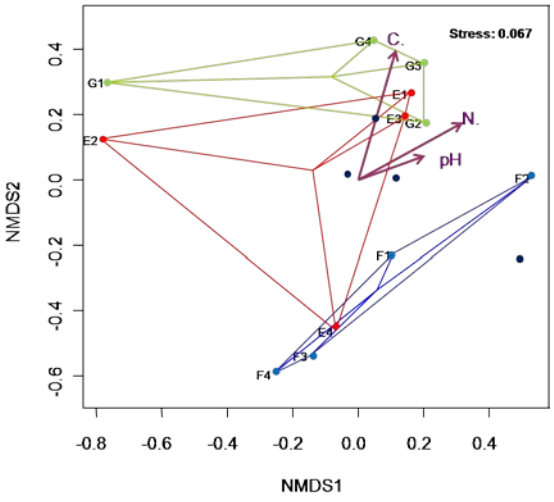


Figure 28 nm - (MDS) of 16S rRNA T-RFLP plots profiles, showing the internal relation between the non fertilized plots, the PIAGRAN 46® treated plots and the P 33/06/F-3 treated plot.

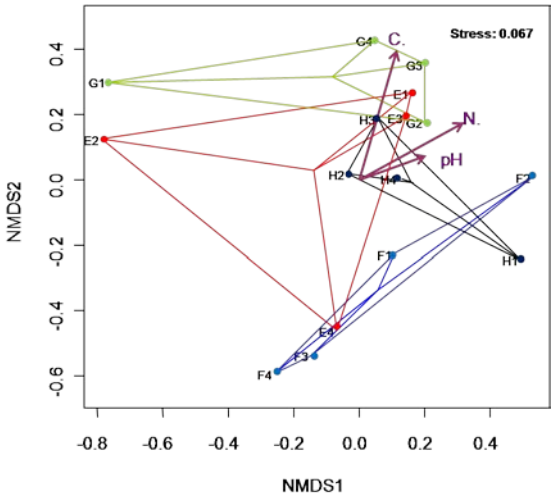


Figure 29 nm - (MDS) of 16S rRNA T-RFLP plots profiles, showing the internal relation between the non fertilized plots, the PIAGRAN 46® treated plots, the P 33/06/F-3 treated plots and the P 03/06/F-3 treated plots.

4.1.2.5.2 *ureC T-RFLP electropherograms analysis*

Non metric multi dimensional scaling (nm- MDS) ordination of the *ureC* PCR T-RFLP processed profiles for the two different experimental groups, fertilization and inhibitor treatment, have been analysed separately. The nm-MDS was carried out to evaluate the influence of different fertilization procedures independently on the bacterial biodiversity.

Nm-MDS ordination for the first group, different fertilization treatment shown in Figure 30 indicates that the differences within the groups are big enough to mask the differences between the main groups, similar to what have been shown previously in the case of the 16s rRNA PCR T-RFLP profiles analysis. The obtained results show no significant influence of the fertilization treatment on the bacterial communities within the treated groups. ANOSIM analysis, between the sub groups based on 1000 permutations shows an R value of - 0.074, which indicate the lack of differences between the gropes although the P = 0.847. Lack of differences between subgroups could also be visualized Cluster analysis, shown in Figure 31. These results indicate that the fertilization treatment under the mentioned experimental conditions did not play a significant role is structuring the bacterial biodiversity in soil.

The carbon and nitrogen content and the pH values of the corresponded soil plots are represented in Table 13. Vector fitting for the environmental factors shows a high dependently of the C, N and pH on each other, the goodness of fitting values for N was 0.005 with P = 0.974, for C was 0.047 with P = 0.820 and for the pH it was 0.0337 with an P value of 0.517.

Table 13. Values of the associated environmental variables

Sample	C concentration [%]	N concentration [%]	pH
E2	1.167	0.126	6.32
E3	1.068	0.126	6.33
E4	1.093	0.142	6.34
F1	1.079	0.128	6.52
F2	1.039	0.118	6.57
F3	1.014	0.129	6.59
F4	0.935	0.113	6.59
G1	1.015	0.116	6.64
G2	0.983	0.131	6.65

G3	1.311	0.15	6.64
G4	1.105	0.134	6.64
H1	1.077	0.136	6.68
H2	1.14	0.128	6.7
H3	1.011	0.119	6.71
H4	1.234	0.139	6.7

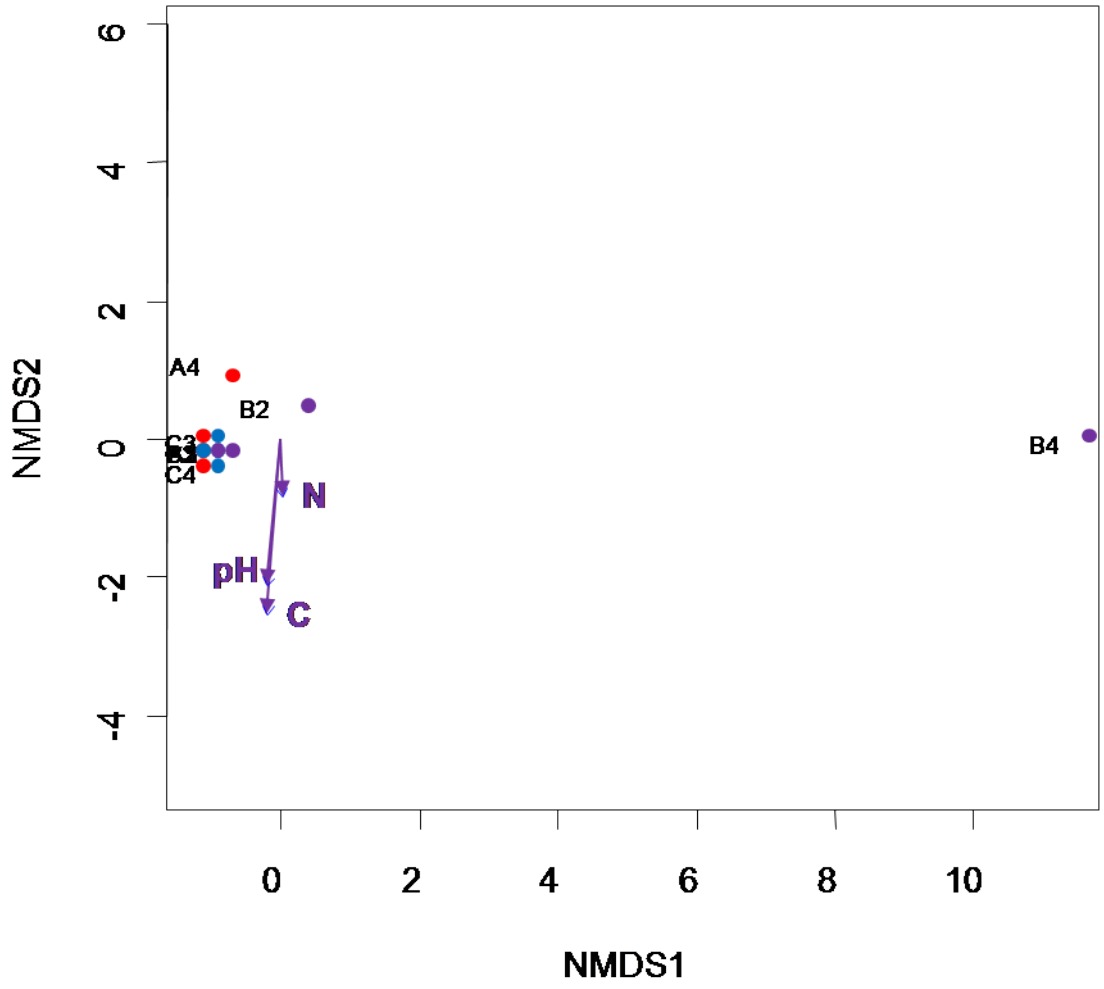


Figure 30. Non-metric multidimensional scaling (nm-MDS) using Bray–Curtis similarity distance index of *ureC*, for the data obtained from T-RFLP profiles processing (fertilization experimental groups) with most constraining factors, P values based on 1000 permutations as vectors superimposed. The relative similarity distances between samples of T-RFLP profiles are graphically represented here. The closer two sample are plotted the more similar their T-RFLP profiles are. With *A*= without nitrogen fertilizer added, *B*= KSA fertilizer added, *C*= PIAGRAN 46 added, the number associated with the symbol refer to the number of the treatment replicated sample The vectors show the direction of increase of the three associated environmental factors C=carbon content, N= nitrogen content and the pH.

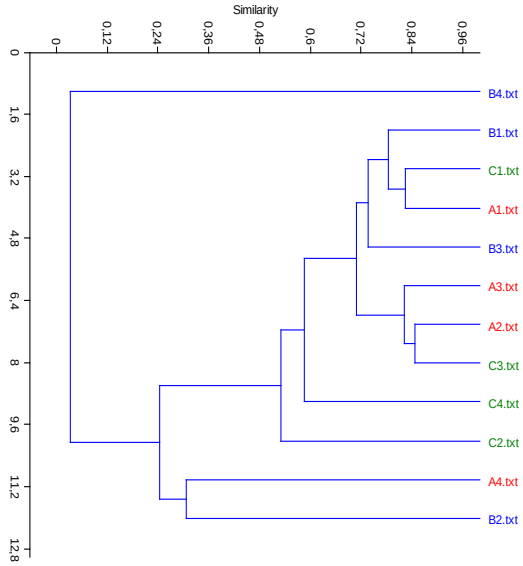


Figure 31. Cluster analysis based on Bray–Curtis similarity distance index of *ureC*, of the data obtained from T-RFLP profiles processing (fertilization experimental groups), showing the lack of separation between the experimental subgroups.

The nm-MDS ordination of the second group, the inhibitor treated group, shown in Figure 32 shows some tendency for the subgroups to differentiate into four different groups. The separation pattern is illustrated in the figures series from Figure 33 to Figure 36. ANOSIM analysis, between the different treatments sub groups based on 1000 permutations shows an R value of 0.037, which indicate the fairly differentiation between the treated subgroups, in the significant value of P=0.296. These results show that inhibitor type of action, as shown before, is marginally influencing the bacterial community structure in soil with fairly significant impact.

Vector fitting for the environmental factors shows an independency of the three difference environmental factors C, N and the pH. Goodness of fitting value for C was 0.18 with P=0.265, for N it was 0.289 with P= 0.101 and for the pH was 0.0199 with P=0.88.

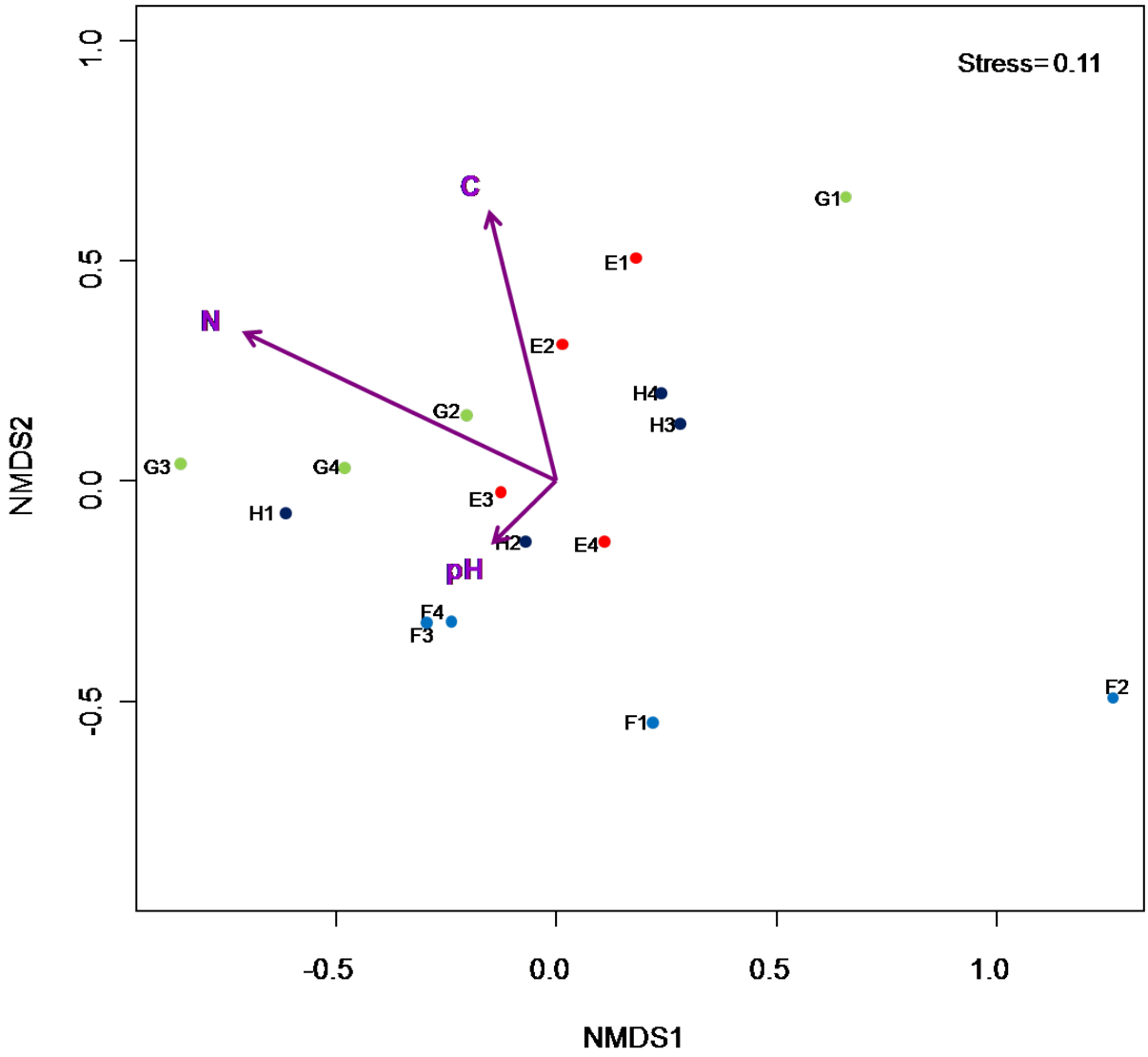


Figure 32. Non-metric multidimensional scaling (nm-MDS) using Bray–Curtis similarity distance index of *ureC*, for the data obtained from T-RFLP profiles processing (inhibitor experimental groups) with most constraining factors, P values based on 1000 permutations as vectors superimposed. The relative similarity distances between samples of T-RFLP profiles are graphically represented here. The closer two sample are plotted the more similar their T-RFLP profiles are. With, *E*= no nitrogen fertilizer added (for the inhibitors experiments), *F*= PIAGRAN 46 added (inhibitors experiment), *G*= P 33/06/F-3 fertilizer added and *H*= P 03/06/F-3 added, the number associated with the symbol refer to the number of the treatment replicated sample The vectors show the direction of increase of the three associated environmental factors C=carbon content, N= nitrogen content and pH.

Analogue to the 16S rRNA case discussed previously, in Figure 33 only the non treated plots were represented. In Figure 34 the urea treated plots were introduced, in which a tendency to shift away from the non treated plots is observed. In Figure 35 the plots which have been treated with the first inhibitor treatment, 0.05 % P/06/F-3, were introduced, in which a shifting-back to the non treated plots position was observed. The inhibitor action may provide environmental conditions that influence the bacterial communities in soil in a similar way the non treated plots did. The last figure is representing the plots which have been treated with the second inhibitor treatment, 0.15 % P/06/F-3, in these plots the backward-shift toward the non

treated plots and away from the urea treated plots was observed, but the magnitude of the shift was not as the first inhibitor treatment case, this indicate that the different concentration of the same applied inhibitor type may influence the bacterial community structure in a different ways.

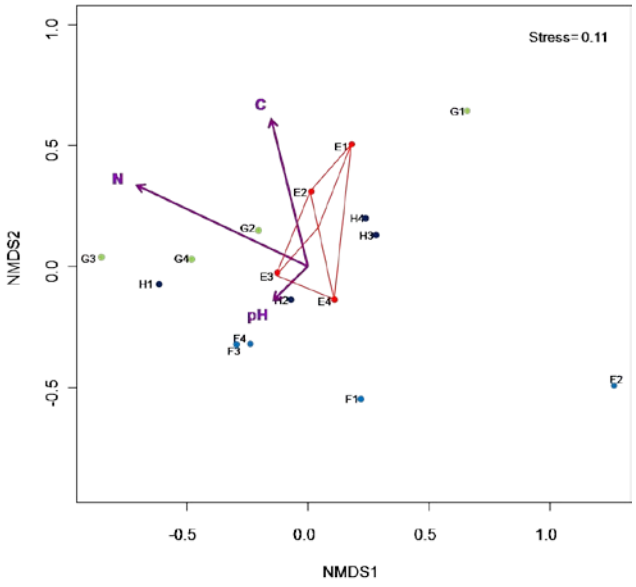


Figure 33 nm - (MDS) of *ureC* T-RFLP plots profiles, showing the internal relation between the non fertilized plots.

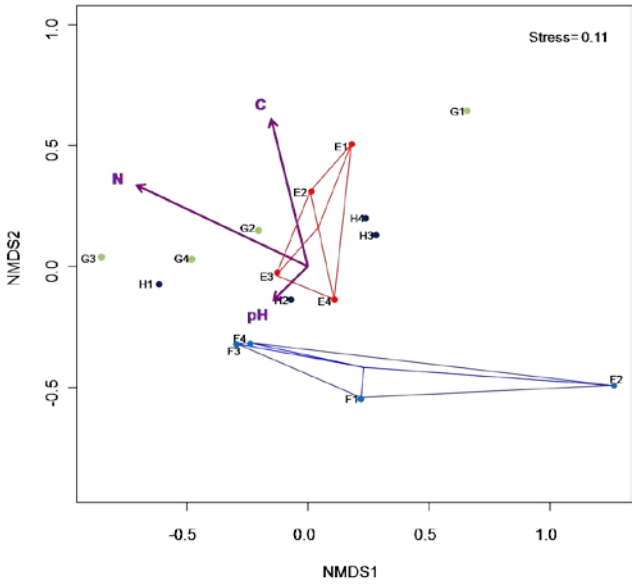


Figure 34 nm - (MDS) of *ureC* T-RFLP plots profiles, showing the internal relation between the non fertilized plots and the PIAGRAN 46 treated plots.

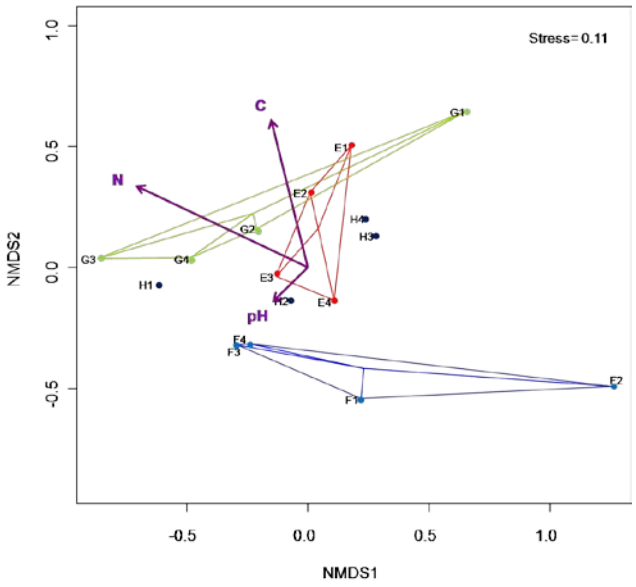


Figure 35 nm - (MDS) of *ureC* T-RFLP plots profiles, showing the internal relation between the non fertilized plots, the PIAGRAN 46® treated plots and the P 33/06/F-3 treated plots.

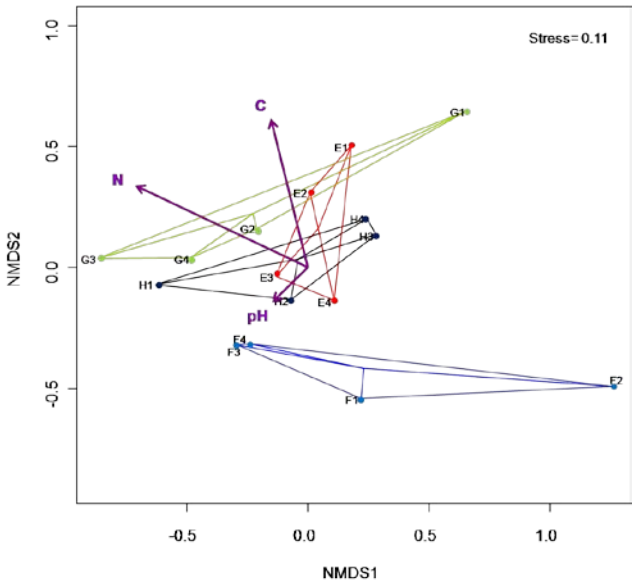


Figure 36 nm - (MDS) of *ureC* T-RFLP plots profiles, showing the internal relation between the non fertilized plots, the PIAGRAN 46® treated plots, the P 33/06/F-3 treated plots and the P 03/06/F-3 treated plots.

4.2 Active urea uptake by *B. megaterium* DSM90

The following experiments were carried out to investigate the potentiality of the active urea uptake systems in *B. megaterium* to be inhibited by the effect of pre-selected phosphoric amide derivatives of *B. megaterium* and the uptake experiments were carried out as mentioned in section 3.9.2.

4.2.1 Cultivation of *Bacillus megaterium* DSM90

In order to obtain sufficient biomass in active-urea-transportation state the optimization of growth conditions for *B. megaterium* DSM90 was investigated. The growth behavior under the specified conditions was investigated as described in material and methods sections 3.9.2.1.4 and 3.9.2.1.5.

4.2.1.1 Growth behavior of *B. megaterium* DSM90 in DSMZ1 liquid nutrient media

The growth curve of *Bacillus megaterium* DSM90 in 500 mL liquid nutrient media, after cultivation at 30 °C at 100 rpm in 2 L Erlenmeyer shaking flask, inoculated by 10 mL over night grown pre-culture OD_{600} = 1.45, is shown in Figure .

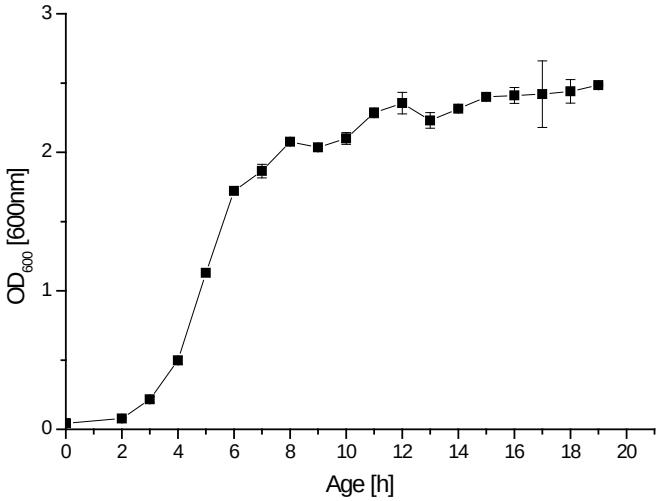


Figure Growth curve of *B. megaterium* DSM90 in 2 L Erlenmeyer flask, 500 mL DSMZ1 liquid nutrient medium, at 30 °C, 100 rpm inoculated by 10 mL of overnight grown bacteria.

The growth behaviour of *B. megaterium* DSM90 followed a typical form of other bacterial species growth curves. The duration of the lag phase was approximately two hours. After the exponential phase, 6 hours, the cells undergo the stationary phase of growth. Under these cultivation conditions the cells were harvested after 8 hours, 2 hours after the stationary phase had begun.

4.2.1.2 Growth behavior of *B. megaterium* DSM90 in Liquid mineral media with different nitrogen sources

To investigate the growth behaviour of *B. megaterium* DSM90 in liquid nutrient media 10 mL of bacterial suspension of approx. OD_{600} = 25 were inoculated into 490 mL of liquid nutrient media. The cultivation took place at 26 °C and 100 rpm, as mentioned in section 3.9.2.1.5. The nitrogen source which has been used in the cultivation media was either KNO_3 or glutamine. The outcome results are shown in Figure 37.

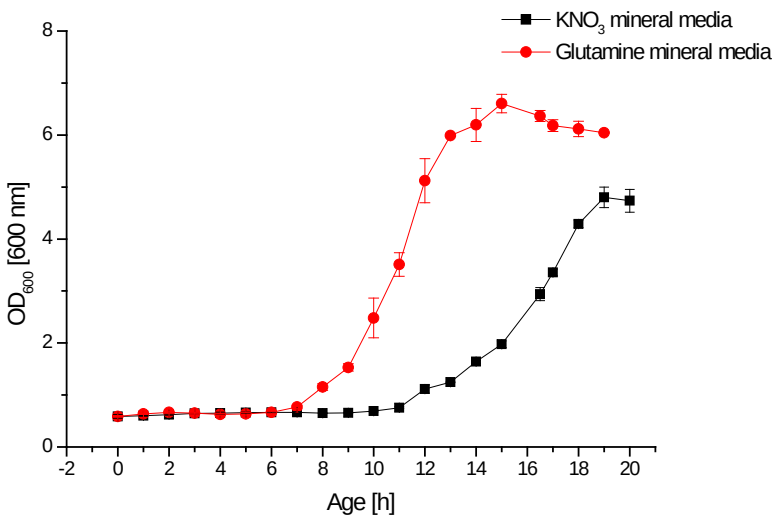


Figure 37. The growth behavior of *Bacillus megaterium* DSM90 in Schlegel mineral media with glutamine or KNO_3. the cultivations were carried out at 26 °C, 100 rpm in 500 mL Erlenmeyer shaking flask, inoculated by 10 mL 10 hours aged culture in DSMZ1 media, OD_{600} 25.

Under the mentioned growth conditions, the lag growth phase in both cases, KNO_3 and glutamine, relative to the nutrient media was remarkably long, 10 hours in the KNO_3 and 7 hours in the glutamine mineral media. This long lag phases could be due to the time needed by the bacteria to adapt the new growth conditions, regarding the nature of the nutrients and the cultivation temperature. After the lag phase the bacteria underwent 7 hours of exponential

growth phase, rather similar to the DSMZ1 media. Then the bacterial cells stepped into the stationary phase. It was also observed that the nitrogen source glutamine boost the growth of bacteria comparison to KNO_3. The maximum yield of biomass that could be obtained is approx. 46 g / L wet cells biomass in the late exponential phase. The cells which have been employed to investigate the active transportation were harvested in the late exponential phase.

4.2.2 The stability of the urea active transportation system over time

To stand on the optimum window phase for the transportation experiment, the activity and stability of the active urea transportation system over time was investigated. The experiment was carried out by incubation the bacterial cells in the suspension media for prolonged time intervals, then the activity has been measured as mentioned in 3.9.2.2.3. The results of the experiment are represented in Figure 38.

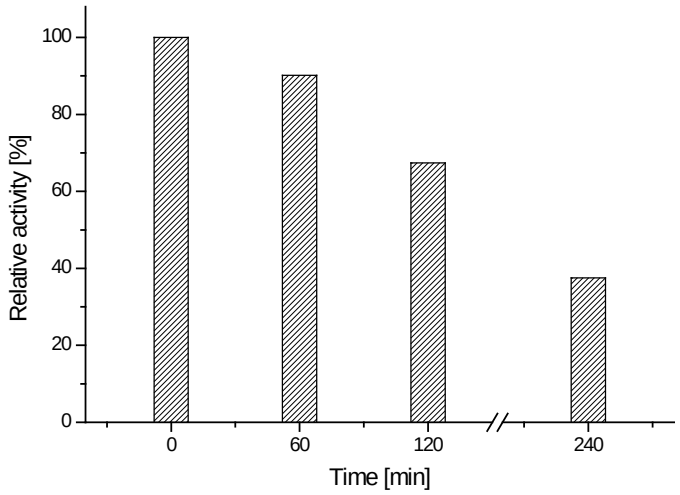


Figure 38 Stability of the urea active transportation in *Bacillus megaterium* DSM90 over time. The bacteria were cultivated in Schlegel mineral media with KNO_3. Urea concentration was 50 µM in 50 mM Na-K-phosphate buffer pH 7.00 Amount of cells in OD_{600} was approx. 30. The final reaction volume was 2970.27 µL. the experiment was carried out at 30 °C and 100 rpm, for storage times of 0.5 min, 60 min, 120 min, 180 min and 240 min at 30 °C before adding the ^{14}C- urea.

The experimental observations show a significant loss in the activity of the urea uptake over time. After 60 min of incubation 10 % of activity was lost and after 120 min of incubation 32.7 % loss of activity was observed. After 240 min 62.4 % loss in activity was observed, which follow the expected track course observed in the previous probes. The lost in activity

over time could be explained due to glucose depletion, which leads in interruption in ATP production in the bacterial cells. These results show the importance of conducting the active urea uptake experiments within the smallest time window as possible. All the experiments which have been conducted in this study were carried out within 60 min of addition of ^{14}C-urea to the reaction suspension.

4.2.3 Active urea uptake in *Bacillus megaterium* DSM90

4.2.3.1 Active urea uptake at 1 mM concentration of urea

This experiment was carried out to investigate the active urea uptake behavior in *B. megaterium* DSM90. The experiment was carried out using ^{14}C urea assay for 1 mM urea concentration. The bacteria were cultivated in Schlegel mineral medial contain KNO_3 as a nitrogen source and was harvested after approx. 16 hours of cultivation. The experiment was carried out for the whole (intact) and the permeabilized form of cells as mentioned in 3.9.2.2.2.

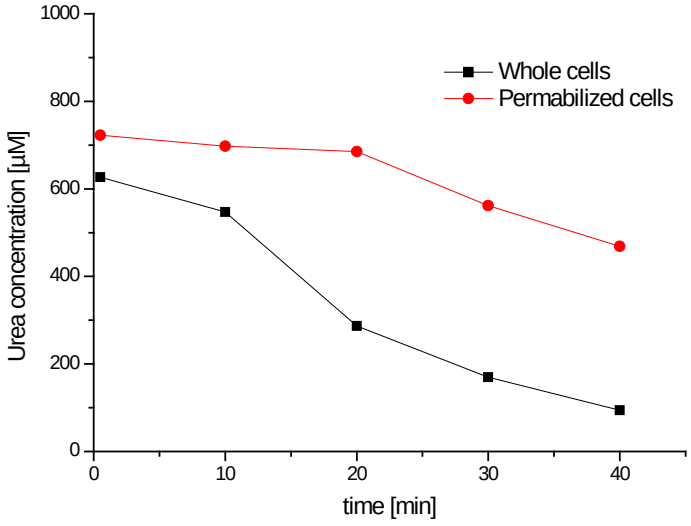


Figure 39 The urea uptake in whole intact cells and permeabilized cells of *Bacillus megaterium* DSM90, urea concentration 1 mM, in 50 mM Na-K-phosphate buffer pH 7.00. amount of cells in OD_{600} approx. 30.the final reaction volume was 2972.27 µL and the experiment was carried out at 30 °C, 100 rpm and for reaction time as indicated in the figure.

The obtained results, Figure 39, show that the initial urea concentration readings, time=0, was far below the applied urea concentration (1 mM of urea). The initial readings for urea concentration were 758 µM for the permeabilized cells and 637µM urea for the whole intact cells. This drop in the urea concentration may represent the amount of urea needed to maintain the concentration equilibrium between extra- and intra- cellular environments achieved by the passive diffusion. Afterwards a non linear decreasing in the urea concentration was observed. The rate of urea uptake in the whole cell, after applying the linear fitting, was 14.5 µM urea / min and for the permeabilized cells 6.5 µM urea/min. the overall urea uptake in the whole intact cells was higher than the case of the permeabilized cells by 87.6 %. Under the mentioned experimental conditions it was found that the active urea transportation system actively transported the urea in higher rates rather than the passive diffusion did.

4.2.3.2 Active urea uptake at 25 µM urea concentration

4.2.3.2.1 Uptake by Bacillus megaterium DSM90 grown in mineral media with KNO_3

This experiment was carried out to investigate the active urea uptake in *B. megaterium* DSM90 at relative low urea concentrations, 25 µM urea. The experiment was carried out using ^{14}C urea assay. The bacteria were cultivated in Schlegel mineral medial contain KNO_3 as a nitrogen source and was harvested after approx. 16 hours of cultivation. The experiment was carried out for the whole (intact) and the permeabilized form of cells as mentioned in 3.9.2.2.2.

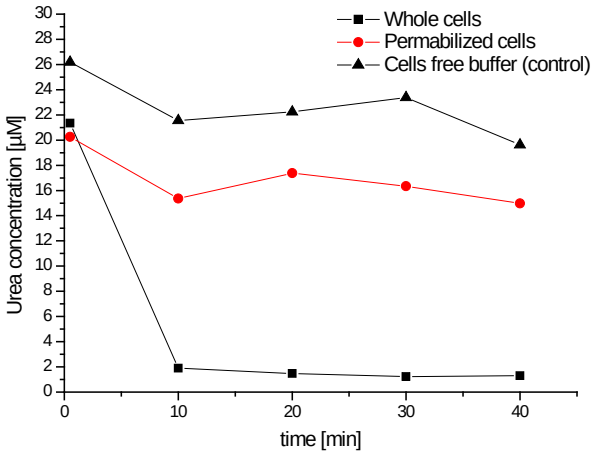


Figure 40 Urea uptake in whole intact cells and permeabilized cells of *Bacillus megaterium* DSM90, urea concentration 25 µM, in 50 mM Na-K-phosphate buffer pH 7.00. Amount of cells in OD_{600} approx. 30.The final reaction volume was 2972.27 µL and the experiment was carried out at 30 °C, 100 rpm and for reaction time as indicated in the figure.

Results shown in Figure 40 indicate a non linear decrease in urea concentration over the time span of the experiment. The total urea uptake in the whole cell case was higher than the permeabilized cells by approx. 330 %. The urea uptake rate in the case of the whole cells, after applying the linear fitting, was 0.4 µM urea / min and 0.096 µM urea / min for the permeabilized cells. These results indicate that under the mentioned experimental conditions the active urea transportation system was actively transport the urea in a higher rate that the passive diffusion, despite the urea concentration used in the experiment.

4.2.3.2.2 The effect of Na azide on the active urea uptake process

To demonstrate the energy dependence nature of the active urea uptake in *B. megaterium* DSM90, thus the role of ABC transporters in the uptake processes, active urea experiment using Na-azide as energy cycle repression was conducted. The experiment was carried out using ^{14}C urea assay. The bacteria were cultivated in Schlegel mineral medial contain glutamine as a nitrogen source and was harvested after approx. 16 hours of cultivation. The experiment was carried out for the whole (intact) form of cells as mentioned in 3.9.2.2.2.

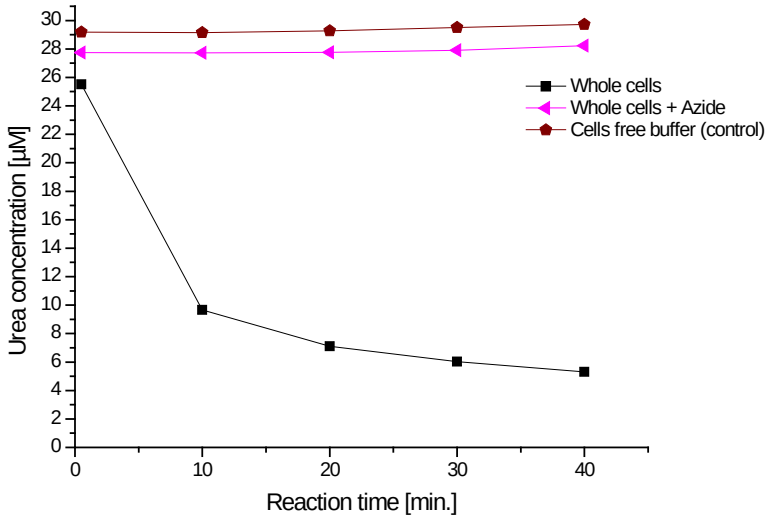


Figure 41 The urea uptake in whole intact cells of *Bacillus megaterium* DSM90, urea concentration 25 µM, in 50 mM Na-K-phosphate buffer pH 7.00. Amount of cells in OD_{600} approx. 30. The final reaction volume was 2972.27 µL and the experiment was carried out at 30 °C, 100 rpm and for reaction time as indicated in the figure. Sample without inhibitor shows the typically predicted behavior over time. The effect of Na azide in the inhibition of urea uptake was observed.

Addition of Na azide to the reaction mixture caused a significant inhibition to the urea uptake processes. These results indicate the energy dependence nature of the active urea transporters. The very limited role of the diffusion in the whole intact cells in the urea uptake processes over the time expanse of the experiment was also observed. This last point emphasise the role of the ABC urea transporter systems in the overall urea uptake procedure under the specified experimental conditions.

4.2.3.3 Active urea uptake in the presence of different types of urea hydrolysis and nitrification inhibitors

To investigate the effect of the selected phosphoric amide derivatives on the urea active uptake processes, urea uptake experiment was carried out for *B. megaterium* DSM90, adopting the ^{14}C urea assay. The bacteria were cultivated in Schlegel mineral medial and were harvested after approx. 16 hours of cultivation. The experiment was carried out for the whole (intact) form of cells as mentioned in 3.9.2.2.2. The results obtained from these experiments are represented in Figure 42 and Figure 43.

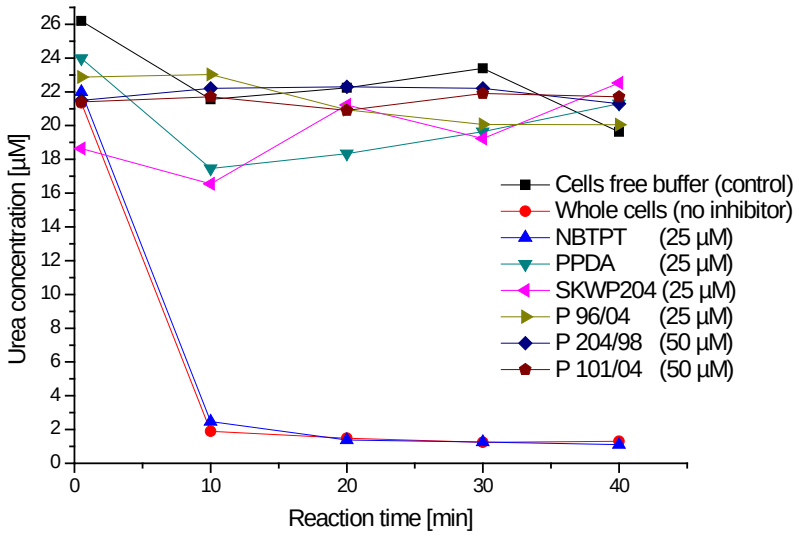


Figure 42 The urea uptake in whole intact cells of *Bacillus megaterium* DSM90, with and without inhibitor treatment. The bacteria were grown in Schlegel mineral media with KNO_3 as a nitrogen source. Urea concentration 25 µM, in 50 mM Na-K-phosphate buffer pH 7.00. Amount of cells in OD_{600} approx. 30.the final reaction volume was 2972.27 µL and the experiment was carried out at 30 °C, 100 rpm and for reaction time as indicated in the figure. Sample without inhibitor shows the predicted behavior over time. The sample without any inhibitor treatment shows the predicted behavior over time. NBTPT shows no significant inhibition effect. On the other hand all the rest of the compounds show a good inhibition effect to urea uptake.

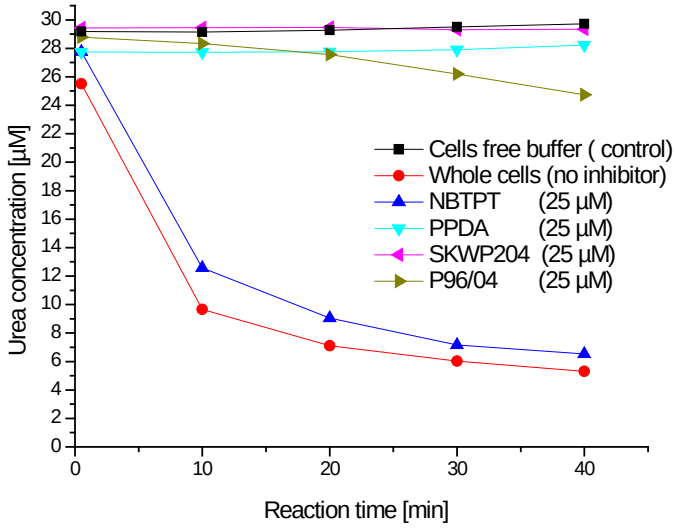


Figure 43 The urea uptake in whole intact cells of *Bacillus megaterium* DSM90, with and without inhibitor treatment. The bacteria were grown in Schlegel mineral media with glutamine as a nitrogen source. Urea concentration 25 µM, in 50 mM Na-K-phosphate buffer pH 7.00. Amount of cells in OD_{600} approx. 30.the final reaction volume was 2972.27 µL and the experiment was carried out at 30 °C, 100 rpm and for reaction time as indicated in the figure. The sample without any inhibitor treatment shows the predicted behavior over time. NBTPT shows no significant inhibition effect. On the other hand inhibitors PPDA, SKWP204 and P96/04 show a good inhibition effect of the urea uptake.

In the case of inhibitor free reaction mixture a non linear decreasing in urea concentration was observed with acceptance with the results obtained before. On the other hand the rate of urea uptake in case of inhibitor existence varies depends on the nature of the applied inhibitor. In the case of NBTPT no inhibition in urea uptake was observed. Applying of PPDA, SKWP204, P96/04, P204/98 or P 101/04 significantly suppressed the observed urea uptake. These observations show also that there is no significant effect of the nitrogen source, KNO_3 or glutamine, positively or negatively on the urea active uptake processes.

4.3 Isolation and purification of *B. megaterium* DMS90 urease

Since the pre-selected phosphoric amide derivatives which have been used in the previous experiments was originally designed as urea hydrolysis inhibitors, the inhibition effect of these compounds on *B. megaterium* DSM90 urease was investigated. Therefore isolation purification and inhibition kinetics of the urease were carried out as mentioned in section 3.9.3.

4.3.1 Cell disruption and enzyme liberation

To isolate and liberate the cytoplamic urease from the obtained cell biomass, section 4.2.1, a high pressure homogenizer treatment was used to disintegrate the cells, break the bacterial cell wall and set the target enzyme free into the suspension buffer, as mentioned in section 3.9.3.1.

Figure 43 shows a SDS-PAGE representing the total protein released and urease activity corresponded to four HPH cycles. From the activity histogram it is shown that the urease liberation from the cells took place after two successful HPH cycles. The maximum amount of the protein concentration was achieved after three cycles of HPH treatments, approx. 3 mg/mL. The volumetric activity increased from the first cycle from 0.82 U / mL to a maximum of 0.94 U / mL after the third and the fourth cycle, while the specific activity ranged from 0.31 to 0.32 U/mg respectively. After two differential centrifugation steps at 10 000 g and 100000 g respectively the final obtained protein concentration was found to be 2.84 mg / mL, with a volumetric urease activity of 0.98 U / mL and specific activity of 0.35 U / mg. No remarkable protein content could be further liberated from the cell debris via any further treatment such as ultrasonic treatment, which indicates that the HPH treatment was successful enough in liberating most of the cytoplasmic proteins.

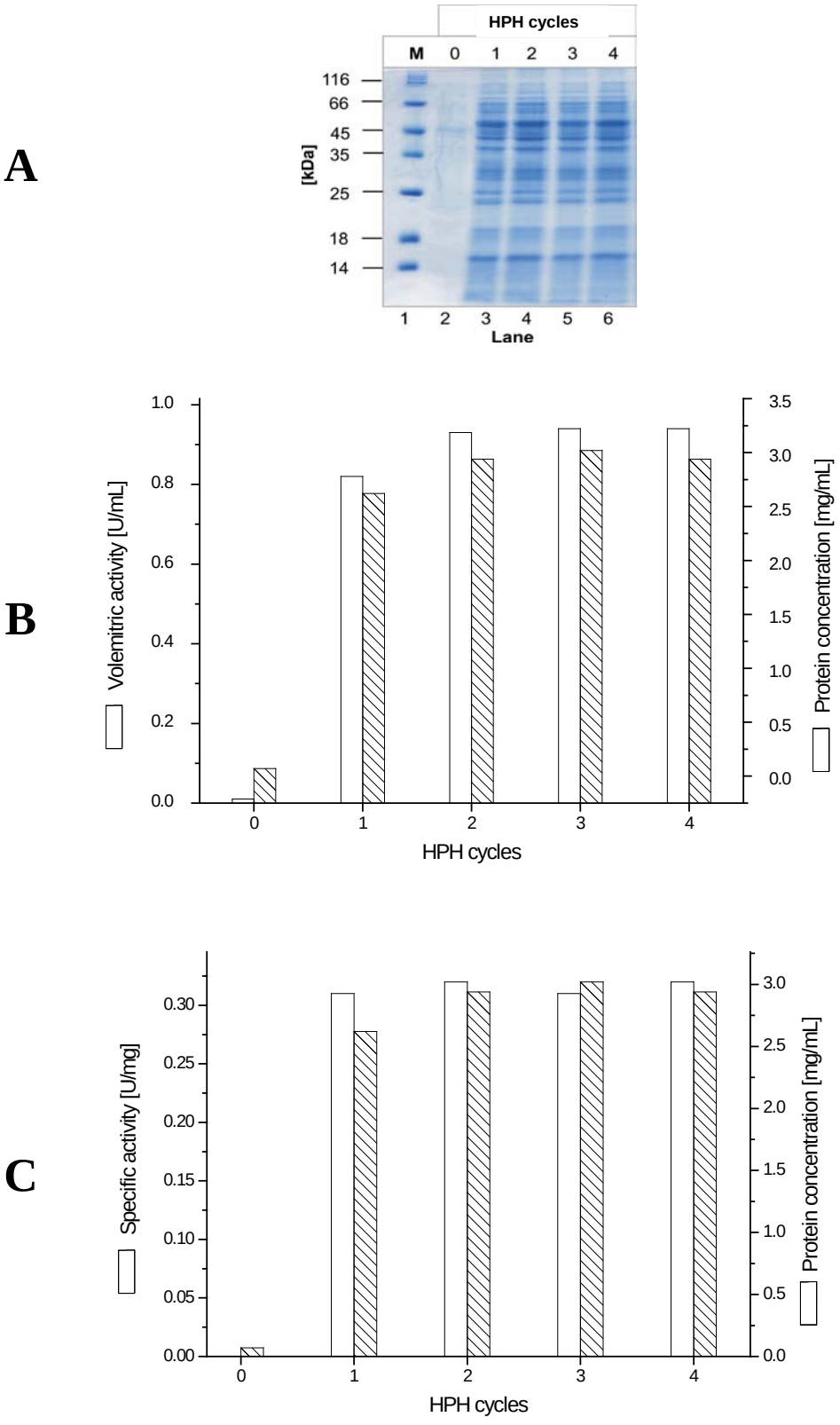


Figure 44 Disruption of *B. megaterium* DSM90 by high pressure homogenizer to liberate the cytoplasmic urease. After incubation with Benzonase ® at 37 °C the cell disruption by successful cycles of HPH treatment at 750 bar. After each round protein concentration and urease activity were measured to analyze the disruption efficiency. A: SDS-PAGE gel, to increase the protein concentration all samples were undergoes acetone perception (2.5-fold concentration), Lane 1: molecular weight marker, Lanes 2. to 6: samples after HPH treatment after different successful cycles. B: Total protein concentration and the volumetric urease activity for the resulted cell lystae after successful HPH treatment cycles. C: Total protein concentration and specific Urease activity for the resulted cell lysate after successful HPH treatment rounds.

4.3.2 Chromatographic purification of *B. megaterium* DSM90 urease

4.3.2.1 Ion exchange chromatography (IEC)

After cell disruption and liberation of urease via HPH treatment, the cell walls and debris were removed by the means of differential centrifugation at 10000 and 100000 g respectively. The obtained supernatant was applied to a Q Sepharose™ Fast Flow (weak Anion exchanger) and was eluted by a linear salt gradient, 0 to 1 M NaCl in 50 mM K-Na phosphate buffer pH 7.5, as mentioned in 3.9.3.2.1.

In Figure 45 the chromatogram obtained from the chromatography and the related SDS-PAGE gel for the eluted fractions which show significant urease activity are represented. In SDS-PAGE gel the first lane is representing the crude protein extract which have been introduced to the column the other lanes from 3 -15 is the eluted fraction which show a significant urease activity, the green-remarked fractions were pooled together afterward.

As shown in the obtained SDS PAGE gel, the purification procedure which has been carried out by IEC was not sufficient enough to discriminate the urease from the other undesired proteins. The presence of dense bands in the range of 30.86 kDa and 49.63 kDa were observed. The molecular mass of these bands may indicate close similarity with the urease obtained from *H. pylori*. Nevertheless the eluted fractions still have a big amount of undesired proteins and further purification step have to be done. From the chromatogram the fragments which show significant urease activity were eluted at approx. 40-50 % elution buffer, which corresponding to 400 mM to 500 mM of NaCl.

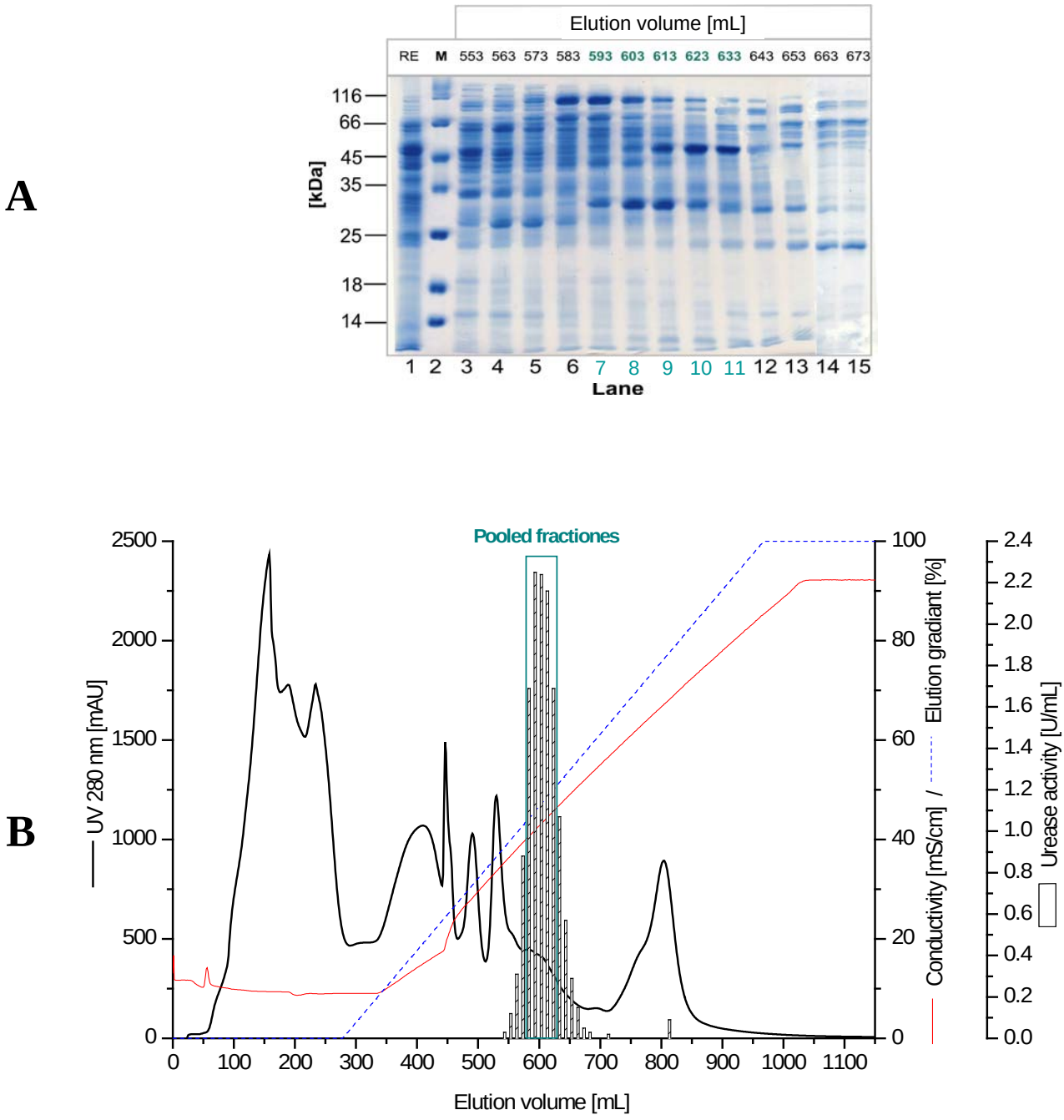


Figure 45. Purification of urease from *B. megaterium* DSM90 using Ion Exchange Chromatography. The supernatant obtained from the differential centrifugation of the crud cell extract was applied on Q-Sepharose™ fast flow column. Fractions with maximum significant urease activity were pooled. A: SDS-PAGE gel, all samples were 1:2 diluted with sample buffer, Lane 1: crude protein extract, Lane 2: molecular weight marker, Lane 3-15: elution fractions, B: chromatogram (column material: Q Sepharose™ Fast Flow column : XK 26/20, CV: 69 mL, flow rate for sample task: 3.0 mL/min, flow speed for washing steps and elution: 3.0 mL / min, the binding buffer: 50 mM potassium phosphate buffer pH 7.5, elution buffer : 50 mM potassium phosphate buffer containing 1 M NaCl, pH 7.5, Elution was carried out by linear gradient salt concentration from 0M to 1 M NaCl. The blue box corresponds to the pooled fractions.

The fractions which show a significant urease activity were pooled together. The volumetric activity was found to be 1.89 U/mL which is approx. the double by comparison with the crude protein extract obtained by the HPH treatment. The specific activity was found to be 1.87 U/mg which is approx. 6 times more than the one obtained after the HPH treatment. Since the SDS-PAGE gel showed that the eluted urease was still heavily contaminated with other undesired proteins, further attempts for purification is to be done including hydrophobic interaction chromatography.

4.3.2.2 Hydrophobic interaction chromatography [HIC]

For further urease purification, the fractions, obtained from the IEC step, which show a significant urease activity, were pooled together. The pooled fractions were dialyzed against 50 mM K-Na phosphate buffer to remove any excess NaCl. After the dialysis the ion strength of the pooled fractions was raised to 1M KCl and has been loaded into Phenyl Sepharose ® resin XK 16/20 column. The protein fractions were eluted with linear gradient concentration of KCL (from 1 M to 0 M KCl). Figure 46 B shows the chromatogram obtained from the HIC step and the corresponding urease activity for each fraction. Figure 46 A is SDS-PAGE gel representing the fractions with highest urease activity after 15 fold concentration by acetone precipitation. From the chromatogram it is clear that the urease has not interact with the chromatographic material, despite this fact the specific activity for urease was approx. doubled compared to the obtained one from the IEC, from 1.82 U/mg to 3.17 U/mg. The volumetric activity was decreased to the half and the total protein concentration is decreased from 1.05 mg/mL to 0.3 mg/mL.

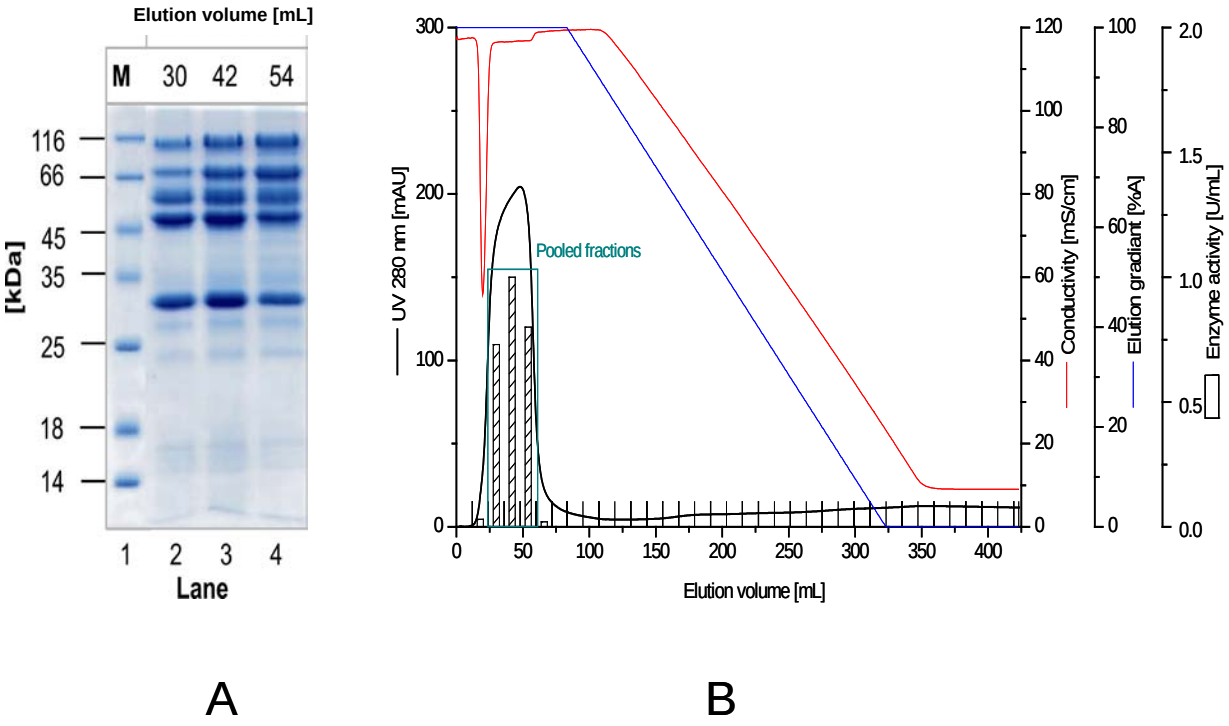


Figure 46. Purification of *B. megaterium* DSM90 urease via hydrophobic interaction chromatography. A: SDS-PAGE gel, all samples were concentrated 15 folds by acetone precipitation, Lane 1: molecular weight markers; Lane 2-4: elution fractions. B: chromatogram of the HIC purification procedure column material: Phenyl Sepharose ® 6 Fast Flow column: XK 16/20, CV: 24 mL, flow speed for washing steps and elution: 3.0 mL / min, Binding and equilibration buffer 50 mM potassium phosphate buffer containing 1 M KCl pH 7.5, elution buffer: 50 mM potassium phosphate buffer pH 7.5 the blue box corresponds to the pooled fractions.

4.3.2.3 Overview about the purification procedure of the *Bacillus megaterium* DSM90

The main steps which have been carried out to isolate and purify *Bacillus megaterium* DSM90 urease could be summarized as following: After cultivation, the obtained wet cells biomass was treated by high pressure homogenizer (HPH) to liberate the cytoplasmic urease. The crude lysate was digested by Benzonase ® to reduce the suspension viscosity and to enhance the liberation of the residual urease from the cell debris. The low viscosity cell extract was differentially centrifuged at 10000 g and 100000 g respectively. The obtained supernatant was chromatographic treated by Ion Exchange Chromatography (IEC) and Hydrophobic Interaction Chromatography (HIC) respectively to isolate and purify the urease from the co-existence contaminants. After each step the fractions which showed maximum urease activity were pooled together. The protein concentration, adopting Bradford test, and the urease activity, via Nessler test, were measured after each purification step. Table 14 is showing the key

resulted parameters form the purification procedures. With the described cleaning procedure, a yield of 36 % and a specific activity of 3.17 U / mg were obtained. From 87.5 g *B. megaterium* DSM90 BWM, 51 mL enzyme solution with a total activity of 48.86 U could be gained. SDS PAGE gel was prepared to show the efficiency of the purification procedure after each step. The SDS PAGE gel in Figure 47 shows a successful reduction of the undesired impurities after each successful purification step. After the last purification step, Hydrophobic Interaction Chromatography, five predominant bands were observed. The identity of urease-representing bands could not be verified in this stage without any further supporting investigations such as native functional PAGE.

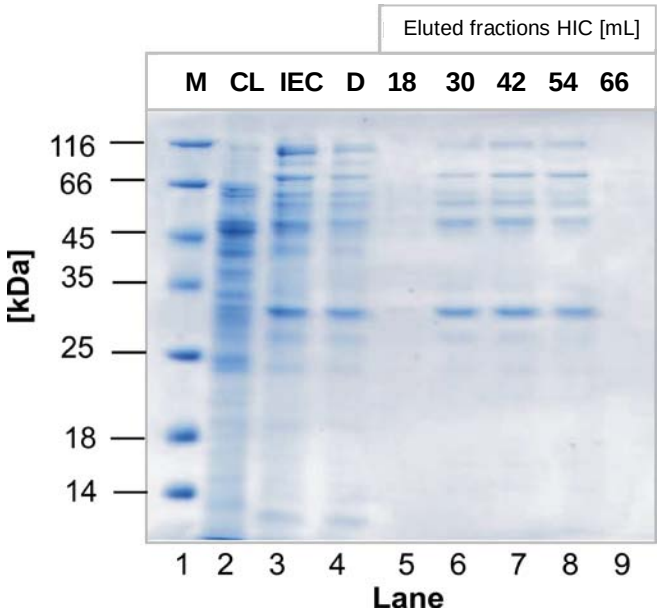


Figure 47. SDS-PAGE gel representing an overview about the successful purification steps for the microbial urease from *B. megaterium*. Lane 1: molecular weight marker, lane 2: the crude protein extract after HPH treatment, Lane 3: urease active pooled fractions after IEC purification step, lane 4 urease active pooled fractions after dialysis against 50 mM potassium phosphate buffer pH 7.5 at 4 °C for 2 successful rounds, lane 5-9: the eluted fractions after HIC purification step.

Table 14. The purification table of microbial urease from *B. megaterium* DSMZ90.

Purification step	Total volume [mL]	Protein concentration [mg/mL]	Volumetric activity [U/mL]	Specific activity [U/mg]	Total activity [U]	Yield %	Purification Folds [-]
HPH and dif. Centrifugation	140	2.84	0.98	0.35	137.2	100	1
IEC	50	1.01	1.89	1.87	94.5	69	5.3
HIC	51	0.3	0.95	3.17	48.9	36	9

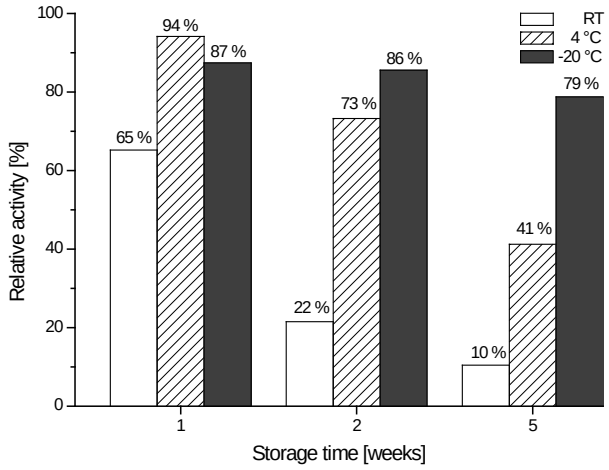
4.3.3 Stability of the purified urease

The stability of the purified urease at different temperatures over time was investigated. After each successful purification step a small portions of the purifies urease was kept in room temperature, 4 °C or -20 °C up to 5 weeks and the enzymatic activity were investigated in fix time intervals.

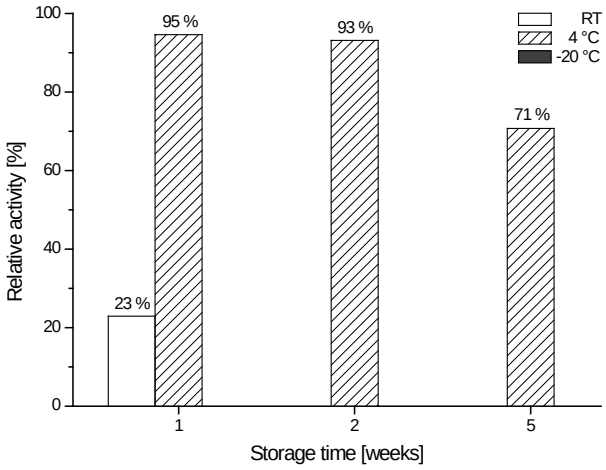
Figure 48 is representing the outputs of this experiment, it was found that the storage temperature coupled the purity state of the enzyme Influence its activity. Regarding the crude cell lystae, after the HPH treatment, the stability of urease activity was found to be inversely proportional with the storage temperature. After 5 weeks at room temperature 20 ± 2 °C the urease significantly lost its activity, only 10 % of the original activity. The urease showed better stability after storing at 4 °C or -20 °C, for the same period of time, 40 % and 80 % of the original activity respectively. On the other hand the stability of urease after IEC and HIC purification treatments was found to be directly proportional to the storage temperature. Total lost of activity after 2 weeks storage at -20 °C and relatively stable urease after storage at 4 °C for the same period of time were observed. Although that addition of glycerol enhance the stability of HIC purified urease over time but the significance in stability of the purified urease at 4 °C is overwhelming the glycerol-stability-enhancement magnitude.

Generally conclusion enzyme fractions obtained after the HPH treatment should be kept at -20 °C for more stability for urease. On the other hand urease-active fractions obtained after IEC and HIC treatment should be kept at 4 °C.

A: HPH



B: IEC



C: HIC

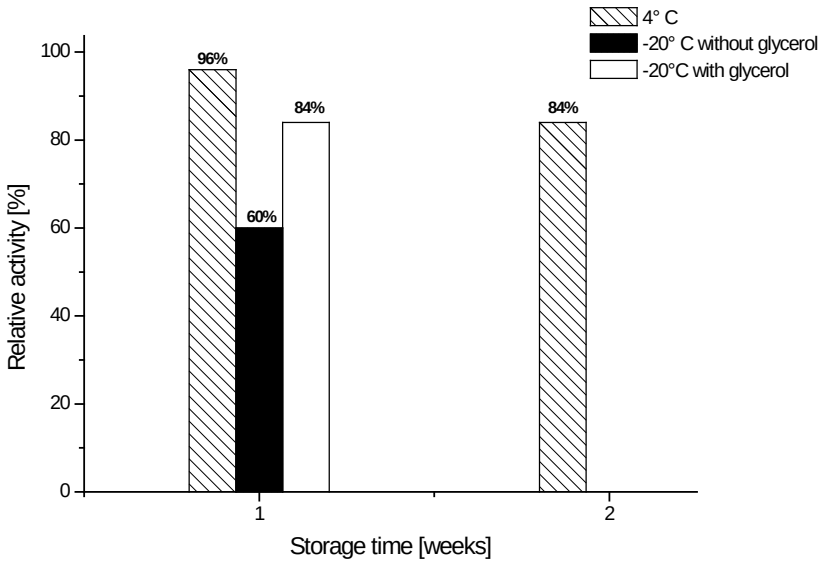


Figure 48. Stability of the purified microbial urease from *B. megaterium* DSM90. After different successful purification steps over time after preservation in different temperatures. A: Storage stability of urease obtained after HPH treatment after 1, 2 and 5 weeks at room temperature, 4 ° C, -20 °C, B: storage stability of the urease-active-pooled fractions obtained from the IEC purification after 1, 2 and 5 weeks at room temperature, 4 °C and -20 °C and C: the stability of the urease-active-pooled fractions obtained from HIC treatment after 1 and 2 weeks stored at 4 °C and -20 °C with and without glycerol.(50 % (v/v)).

4.3.4 Kinetic studies for the *B. megaterium* DSMZ90 purified urease

4.3.4.1 Enzyme kinetics characterization for the purified enzyme

To determine the enzyme kinetics parameters V_{max} and K_m for the purified urease from *B. megaterium* DSM90 purified urease, the enzymatic reaction velocity under different substrate concentrations were determined using steady state analytical assay, indophenol assay. The data obtained from these experiments were processed in MATLAB to fit the data into the Michaels-Menten equation and thus determining V_{max} and K_m as mentioned in section 3.9.4.

The resulted curves indicate that *B. megaterium* DSM90 urease is following simple Michaels-Menten type kinetic behaviour with K_M value of 0.466 [mM] and V_{max} of 0.039 [mM/min]. **Figure 49** is representing the interpretation of the obtained data points from this experiment.

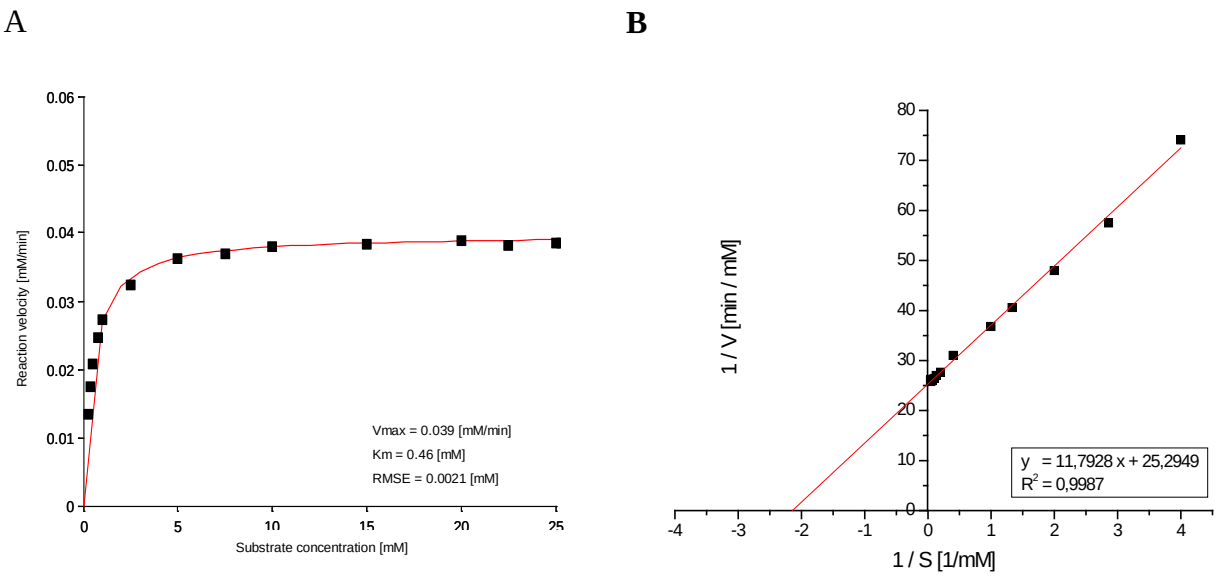


Figure 49. Kinetic behaviour of microbial urease from *B. megaterium* 16 dys after the HIC purification. A: fitting the obtained data, the reaction velocity against different substrate concentrations, to the Michaels-Menten equation. B: double reciprocal representation of the obtained data in the Lineweaver-Burk plot.

4.3.4.2 *B. megaterium* DSM90 urease inhibition kinetics

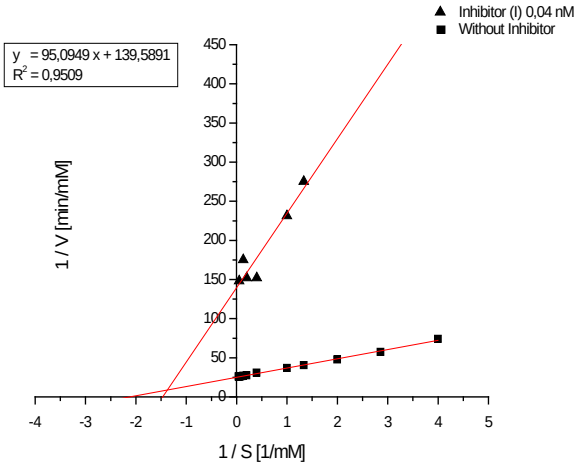
To characterize the inhibition kinetics and parameters, for different pre-selected phosphoric amide derivativeson the *B. megaterium* DSM90 urease activity, the enzymatic kinetics in the existences of six different inhibitors was performed. Inhibitors were added to the reaction mixture 30 min. prior the urea of the substrate and the liberation of ammonia was determined using steady state assay, indophenol assay.

Table 15 represents a short summary for the obtained results, as it could observed the interoperated data show that all the pre-selected inhibitors follow a non competitive inhibition mechanism, Figure 50and Figure 51summarizing the inhibition kinetics parameters obtained for the different tested inhibitors.

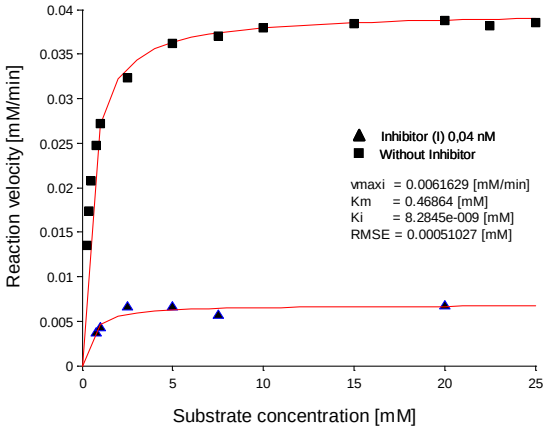
Table 15. Overview about the inhibition kinetics characterization outcome of different pre-selected inhibitors

Inhibitor	concentration [nM]	K_I [nM]	V_{max} [mM/min]
I	0.04	0.006	0.0072
II	0.04	0.010	0.0086
III	0.04	0.017	0.0131
IV	0.04	0.009	0.0079
V	0.04	0.011	0.0100
VI	0.04	0.011	0.0099

A1



B1



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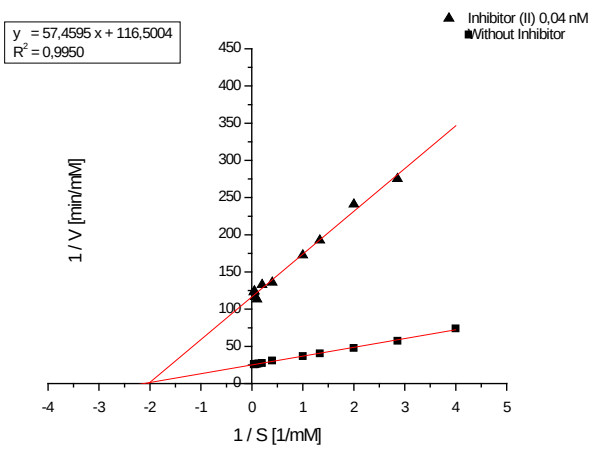

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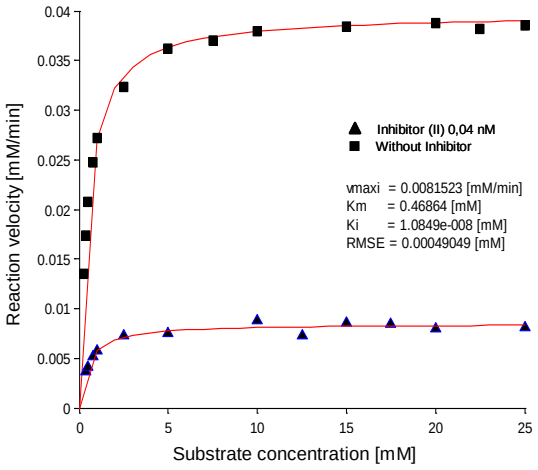
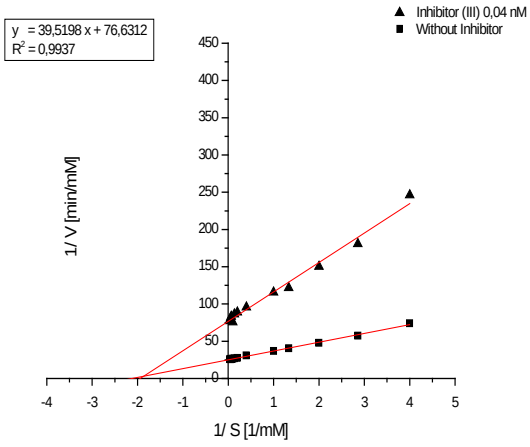


A3



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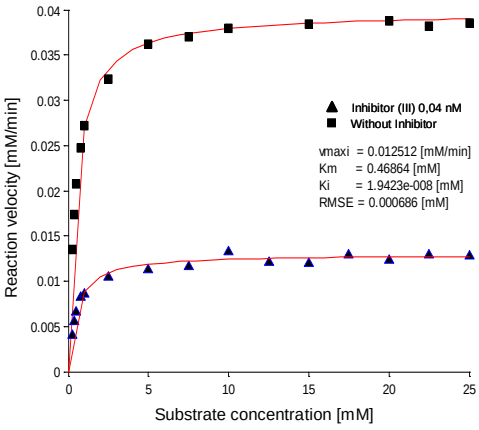
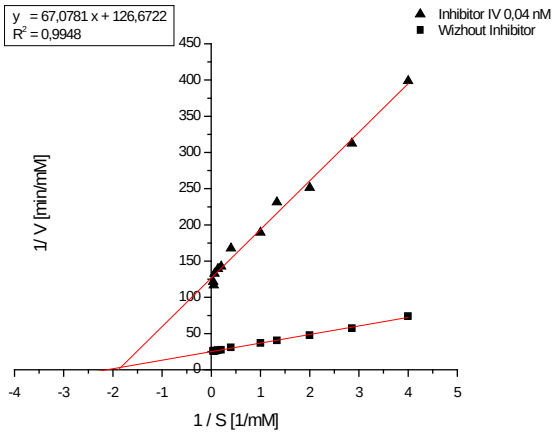
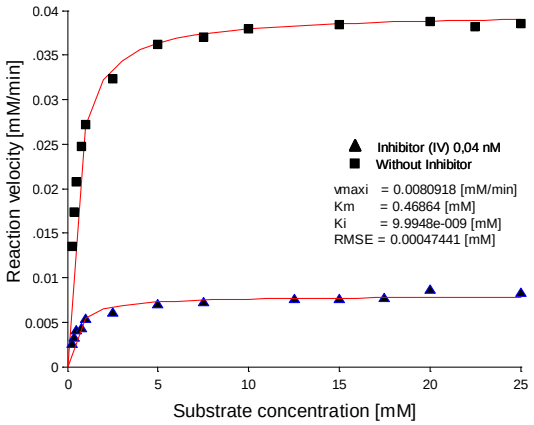


Figure 50. Inhibition kinetics of *B. megaterium* urease inhibited by inhibitors I, II and III after pre-incubation with 0.04 nM of these inhibitors 30 min. prior to substrate addition. The enzymatic reaction for each point last 10 min and the liberation of ammonia was measured by steady state indophenol assay: A1-3 are representing the double reciprocal interpretation of the data and the B 1-3 are representing the data fitting to Michaels-Menten equation.

A4

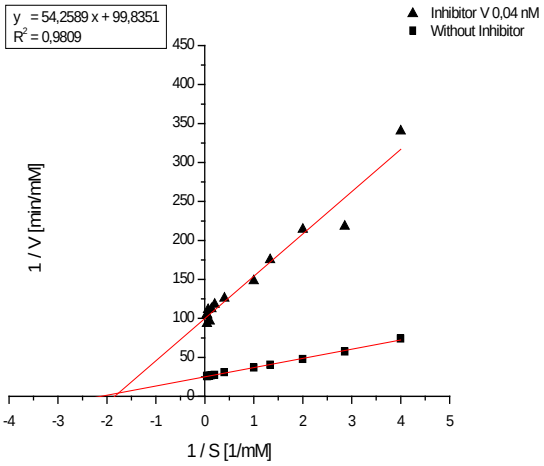


B4

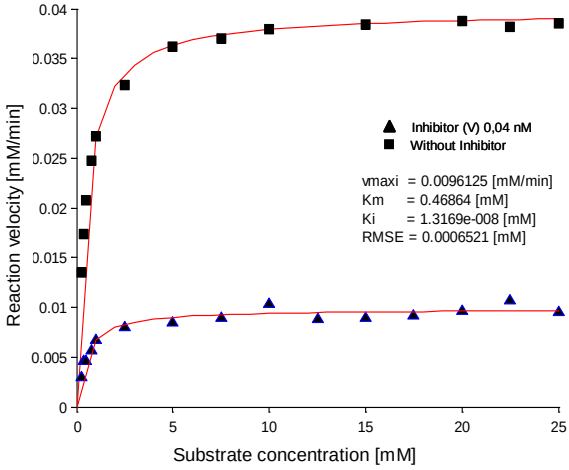


A4

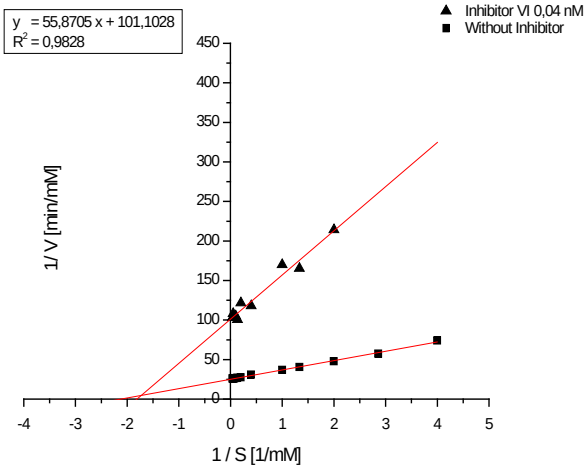
A5



B5



A6



B6

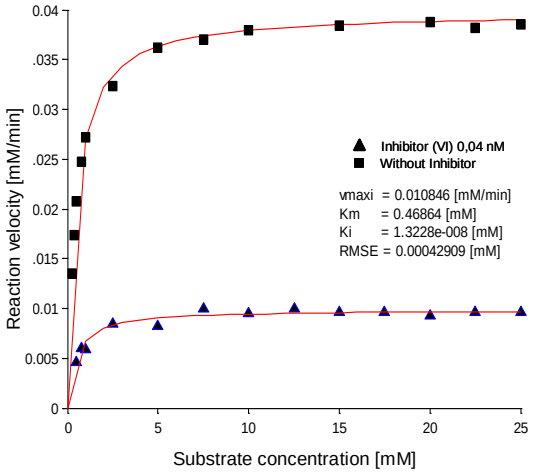


Figure 51. Inhibition kinetics of *B. megaterium* urease inhibited by inhibitors I, II and III after pre-incubation with 0.04 nM of these inhibitors 30 min. prior to substrate addition. The enzymatic reaction for each point last 10 min and the liberation of ammonia was measured by steady state indophenol assay: A1-3 are representing the double reciprocal interpretation of the data and the B 1-3 are representing the data fitting to Michaels-Menten equation.

5 Discussion

To minimize the negative impacts of uncontrolled urea hydrolysis, three aspects related to the urea management in agriculture were investigated in this study. First one concerned about investigating the urease-producing bacterial communities in soil and their dynamic changing as a response to different fertilization managements. The second aspect was investigating the active urea uptake by a soil bacteria model, *B. megaterium* DSM90, via the ABC urea transportation system and the effect of some phosphoric amide derivatives on the active uptake processes. The last aspect was concerned about investigating the effect of these inhibitors on the purified form of *B. megaterium* DSM90 urease.

5.1 Urease-producing bacterial communities in soil

To gain an access to the dynamic response of urease producing bacteria in soil toward different agricultural management two main approaches have been adopted. The first approach is the classical culturable-dependent approach based on isolation and culturing the microorganisms under different cultivation conditions. The second one is the culturable-independent approach based on different nucleic acid surveys.

5.1.1 Culturable-dependent analysis of urease-producing soil bacteria

Few number of studies provide brief comments on the taxonomy of culturable soil bacteria [Elsas, 1997]. Regarding the urease-producing bacteria, some studies conduct a cultivation-based method for enumerating ureolytic cells in groundwater [Tyler, 2004]. A most-probable-number (MPN) technique was performed using a broth growth medium containing urea and the pH indicator phenol red. In the present study the results show the possibility to discriminate the soil urease-producing bacteria by adopting a media initially designed for detecting urea hydrolysis in clinical bacterial species [Christensen, 1946]. Numerous urease producing isolates were tested using this medium, but many isolates were unable to grow, and those that could grow did not hydrolyze detectable amounts of urea after several days of incubation. The inability of this medium to university grow wide varieties of bacterial species could be due to the incompatibility with the slow growth and relatively low urea hydrolysis rates of environ-

mental ureolytic bacteria. Nevertheless it was found that applying KAS to the soil as a fertilizer lead to an increase in the detectable number of urease producing bacteria. KAS treatment would probably enhance the microbial biomass output and the nitrification potential of the treated soils. The previous observation was similar with the ones discussed in a previous studies in which applying a mineral fertilizer to the soils enhanced the bacterial biomass output [Chu et al., 2007]. The enhanced ureolytic microbial biomass output and activity could be observed indirectly using the selective Christensen media. In the present study it was found that the incubation of the testing plates in relative high temperature, 30 °C, caused in some cases an excessive urease activity. The high concentration of ammonia liberated from the urease action caused the complete testing plates to turn red-violet color, which cause a technical difficulty to discriminate the urease producing bacteria. In such cases further verification of the suspected single colonies was carried out using even a new testing plates or a cap sealed micro titer plates.

Identification of the culturable bacterial isolates shows that most of them belong to the family Bacillaceae. This point could be explained by considering the long storage in unfavorable environmental conditions, lack of nutrients, aeration...etc. This unfavourable environmental conditions provocate the bacterial cells from this family to undergo the sporulation process, transforming the vegetative cells to more resistance form [Driks, 2004]. After reinitiating the favourable environmental conditions, only the spore formation bacteria could grow and dominate the bacterial biodiversity in the experimental sample [Cheun et al., 2003].

Among the isolated bacterial species *B. megaterium* has been identified at least in 20 % of the isolates. *B. megaterium* is a common soil bacteria [Vary et al., 2007], and have been used previously as a soil inoculums in agriculture and horticulture [Huang, 2008]. The isolation and identification of *B. megaterium* in the tested soil samples indicates its suitability as a bacterial model for further investigations related to urea active uptake and metabolism.

Phylogenetic analysis of the identified isolates shows that they are sharing the same hypothetical taxonomic unit node. That means that all of them are relatively related to each other more closely than the other uncultured bacteria included in the analysis. The fact that all of the identified isolates were form the Bacillacea family could further support this conclusion. It was also found that the phylogenetic analysis based on the *ureC* was in congress with the one based on the 16s rRNA analysis. These findings are in agreement with previous conducted

study [Tyler, 2004]. This is pinpointing the possibility to use the *ureC* as a phylogenetic marker analogue to the previously verified genes such as *tuf* [Mignard and Flandrois, 2007] and β-tubulin gene [Huang et al., 2009].

Although the *ureC* gene is not a robust phylogenetic marker for bacteria, identifications of bacterial species could be inferred from *ureC* sequences in environmental samples. This could be achieved when they form statistically relevant groupings with *ureC* sequences from organisms of known phylogenetic identity. Beside that they do not group with *ureC* sequences that appear to have been transferred horizontally [Taylor et al., 2002]. Similar conclusions were drawn from comparative phylogenetic analyses of 16S rRNA and enzyme-coding genes such as the dissimilatory sulfite reductase [Klein et al., 2001] and nitrate and nitrous oxide reductases [Delorme et al., 2003]. Partial congruence with the 16S rRNA phylogeny was observed in these comparative studies; however, evidence for lateral transfers among these genes was revealed as well [Taylor et al., 2002].

5.1.2 Culturable-independent analysis of urease-producing soil bacteria

5.1.2.1 Extraction of the genetic material from soil

The bacterial genetic material, which is representing the actual bacterial phyla existence, was extracted from the soil via different methods as mentioned in section 3.9.1.2.1.The extraction of DNA yield a sufficient amount of genomic material in an acceptable quality and purity of for further fingerprinting analysis.

The analysis of the extracted genomic material showed a significant contamination with co extracted humic material compounds. It is common for soil extracted nucleic acid to be contaminated with other molecules such as protein, phenol, or humic materials [Chandler et al., 1997]. Thus the ratio of absorptions at 260 nm : 280 nm is used to assess the purity of the extracted nucleic acid with respect to protein contamination and 260 nm: 230 nm ratio for purity estimation regarding the humic materials impurities [Yeates et al., 1997].

Co-extracted humic acids are the major contaminant when DNA or RNA is extracted from soil, as the humic materials in soil have similar size and charge characteristics to DNA resulting in their co-purification [Holben et al., 1988]. As little as 1 μL of undiluted humic-acid-like

extract, regardless of the amount of DNA present [Tsai and Olson, 1992], may interfere the efficiency of some subsequence molecular biology manipulation such as PCR [Yeates et al., 1997]. Humic contaminants also interfere in nucleic acid quantification since they exhibit absorbance at both 230nm and at 260nm, the later used to quantify DNA. This characteristic can be used to determine the contamination degree by humic material by examining absorbance ratios. A high 260/230 ratio (>2) is indicative of pure nucleic acid material and a low ratio is indication of humic acid contamination. The protein impurities may interfere with the readings in the range of 280 nm and the phenol, which is used routinely in the nucleic acid extraction, is interfering the reading at 270 nm. Thus a high 260/280 ratio (>1.7) is indicative of pure nucleic acid preparation, while a low ratio is indicative of protein or phenol contamination [Warburg and Christian, 1942].

5.1.2.2 PCR for 16S rRNA and *ureC*

PCR for 16S rRNA and *ureC* was carried out to amplify the represented amplicons from the soil extracted genomic material. The aim was to provide a sufficient suitable amount of the represented amplicon, 16S rRNA or *ureC*, for further genomic fingerprinting analysis.

Due to vast varieties of Taq polymerase inhibitors in soil, an optimization of the genomic template concentration was carried out in a way compromising between the inhibitors and the targeted amplicons concentrations. The outcomes of the optimization process varied from sample to another depending on the nature of soil and the localized micro environmental conditions beside the naturally frequent existing of the targeted genomic amplicon species, 16s rRNA or *ureC*. These outcomes gone along with the observations and recommendations stated previously in several studies [Mizuno, 2004; Wallenstein and Vilgalys, 2005].

In the case of *ureC* PCR an intense primer dimmers were observed in the final product. These non specific PCR products could not be practically predicted due to the nature of their parental primers, degenerate primers, which leads to a huge number of probabilities regarding the primer structure and thus the compatibility chances. Nevertheless these primer combination, compared with the ones used in other studies [Taylor et al., 2002], yielded practically better quality and quantity of the represented *ureC* amplicons. Considerable amount of primers have been consumed in the PCR due to primer dimmers formation bias, which may indirectly influence the identification of low frequency species of bacteria in soil.

5.1.2.3 Bacterial communities structure analysis

In most of the obtained soils T-RFLP profiles a significant peak which show a similarity to the one obtained from a pure *B. megaterium* culture was observed. This observation indicates the presence of *B. megaterium*, or at least a very closely related species, in the tested soil samples. Identification of the genetic fingerprints of *B. megaterium* in the obtained T-RFLP profiles, beside the direct isolation and identification of this bacterium as mentioned in the previous section, gives a good hint about its suitability to be a good bacterial model regarding further investigations related to active urea uptake systems and urea metabolism.

The data obtained from the analysis of the 16S rRNA T-RFLP profiles show a tendency for the non-treated plots to gather in more related cluster separated away from the inhibitors-treated plots. This indicates the influence of the inhibitors on the bacterial biodiversity. Actually this influence on the bacterial community structure is not necessarily due to the direct act of these inhibitors on the bacteria. The changing could refer to side effect of altering the surrounded micro environmental conditions. The inhibitors may inhibit the utilization of the urea which leads to minimize the elaborating ammonia, thus the changing in the pH toward more basic conditions is kept to its minimum. This may provide new environmental states with a different effect on the bacterial community structure.

A detailed analysis for the T-RFLP profiles of the non-treated plots, as an individual separate unit, shows that the differences between the members of the same plot is bigger or equal the differences between the members of different groups. This effect may mask the differences between the different groups. The T-RFLP analysis in this case failed to define any significant differences between the different soil plots based on the fertilization treatment. Thus the effect of different fertilization treatment on the bacterial communities in soil could not be considered significant. The statistical analysis of the obtained data supports the previous statements regarding the lack of significant differences between the soil plots.

The previous conclusion is supported by similar studies [Ogilvie et al., 2008]. In these studies the bacterial diversity of the broadbalk classical winter wheat experiment in relation to long term fertilization input was investigated. The effects of long term nitrogen application has shown that different nitrogen inputs forms does not have a significant influence on the biodiversity of bacteria. These observations were true at the species and the gene level, even when

the chosen genes are directly involved in nitrogen nutrition. The hypothesis which evolve from these observations that the soil characteristics such as particle size [Girvan et al., 2003] or type [Sessitsch et al., 2001] is providing the dominating structural influence on microbial communities. The lack of detectable differences in distribution of 16S rRNA and other nitrogen utilization related genes within the samples may indeed be a product of the heterogeneous nature of the soil with relatively low or high fixed N available in microsites within the soil.

On the other hand analysis of the T-RFLP profiles of the inhibitor treated plots shows some tendency for the bacterial community structure to be shaped and influenced by the inhibitor treatment. As mentioned before, it is not necessarily to be the direct effect of the inhibitor on the bacteria which produce this pattern but the possible consecutive effects of their action such as altering the pH or nitrogen content in the system. Nevertheless the statistical analysis, regarding this observation, show that despite the visual tendency pattern formation in the MDS plots the differences between the plots was not so high to be considered significant. Traditionally in the statistics when the value of the obtained significant measurement (P value) is more than 5 % it is preferred to be considered a non significant result [Hinton, 2004]. In the present study the obtained P values were ranged around 20 %. The previous statement did not eliminate any existence of differences between the plots but it states the absence of any handy evidence of this differences.

5.2 Active urea uptake in *B. megaterium* DSM90

5.2.1 Cultivation of *B. megaterium* DSM90

The presence of *Bacillus megaterium* in the tested soil samples has been confirmed previously in this study by employing the cultural dependent and independent approaches. Beside that *B megaterium* was shown to be a suitable model to investigate the active urea transportation in soil bacteria. *B megaterium* is a common soil bacteria [Vary et al., 2007], acquire an ABC urea active transportation system [Jahns and Kaltwasser, 1989] and have been used as a soil inoculants in agriculture and horticulture [Huang, 2008]. To investigate the active transportation of urea, *B. megaterium* DSMZ90 biomass has to be obtained in a condition that demonstrate this phenomena, typically under urea deficiency growth conditions. However in the work of Jahns and Kaltwasser [Jahns and Kaltwasser, 1989] it was shown that cultivation of

B. megaterium DSM90 in Schlegel mineral media with KNO_3 as a nitrogen source will produce a biomass with high active urease transportation capabilities. The cultivation of *B. megaterium* DSM90 directly on the defined mineral media faced some difficulties, including the low quantity and quality of the final obtained biomass yield and the long time of the cultivation process. Therefore some optimization of the cultivation protocol was approached by introducing a high amount of biomass to the mineral media, in the form of high density inoculums, to boost the cultivation process and gain higher yield of the biomass. Beside that any source of nitrogen beside the KNO3 or glutamine was eliminated from the cultivation media. The introducing of high biomass inoculums was achieved by cultivation the bacteria in a complex nutrient media and harvesting the cells before the end of the log phase of growth and introducing the resulted biomass after washing into the mineral media.

After introducing the bacterial biomass into the mineral media an adaptation period before the log phase of growth was observed. This period may be needed by the bacteria to adapt their physiological system to the new growth conditions. The relative high density of the bacterial cells may mimic the starvation mode and provoke the physiological system responsible of utilization of the nutrients in the defined mineral media. This may be explaining why low concentrations of the bacterial cells were no able to grow directly on the mineral media. Cultivation of *B. megaterium* DSM90 in mineral media, which exclusively contain glutamine as the only nitrogen source instead of KNO_3, yields higher biomass and shows shorter cultivation cycle. This may be indicating that glutamine is more suitable, easier to utilize, source of nitrogen for the bacteria rather than the KNO_3 under the mentioned cultivation conditions.

5.2.2 Active uptake of urea

Although the physiological functions of ABC transporters are not well known, their wide distribution through all kingdoms in nature strongly suggest that they must be crucial for cell and organism survival [Ponte-Sucre, 2007]. ABC transporters possess transmembrane domains, which anchor the protein to the membrane, and form a pore through which the transport of substrates occurs. They also posses ATP binding domains, which provide the molecular compartment where the ATP energy is released [McKeegan et al., 2004].

Structural analysis of the inhibitor used in this study shows that they are sharing a significant structure features. All of them include at least one protonable nitrogen; show a highly lipo-

philic behavior and some of them accommodate a planar aromatic domain. These structural signatures show some similarity to compounds that act as substrates or blockers of ABC transporters [Ponte-Sucre, 2007]. Theoretically these compounds should be able to form numerous and strong H-bond interactions with the ABC transporter [Seelig, 1998].

ABC transporters have a strongly conserved primary sequence; however, few orthologous pairs of transporters are shared between phyla [Ponte-Sucre, 2007]. This characteristic beside the presence of multiple binding sites complicates the understanding of an ABP-substrate interaction and hinders the development and speculations of new inhibitors [Boumendjel et al., 2005; Wiese and Pajeva, 2001].

The central paradigm of inhibitor / transporter interaction is that compounds with similar structures act at the same site and with the same mechanism [Wiese and Pajeva, 2001]. Unfortunately inhibition of the ABC transporters in general can occur via different mechanisms as well as at a number of different sites [Ponte-Sucre, 2007]. As a consequence, generalizing the inhibitor/transporter interaction mechanism or kinetics has been difficult to achieve [Ponte-Sucre, 2007].

The experiments which have been conducted to investigate the active ABC urea transportation show that the action of active transportation overcame the passive diffusion even when the urea concentration gradient ≥ 1 mM of urea. Deactivation of the ABC transporter by applying Na-azide or permeabilizing the cells membrane by CTAB show significant suppression in the observed urea uptake. These results are in acceptance with the ones observed in previous studies [Jahns and Kaltwasser, 1989]. Although using glutamine as a nitrogen source in the growth media lead to a slight suppression for the urea uptake, the suppression magnitude was not as expected. According to Jahns et. al. adopting glutamine in the growth media leads to suppress the active uptake up to 98 % comparison to the using KNO_3 as a nitrogen source in the cultivation media.

As mentioned before addition of sodium azide to the reaction mixture leads to total suppression in observed uptake process. This indicates the dependence of these urea active transporters on ATP, in which the sodium azide alter and inhibit their synthesis. The related experiments show the limited role of passive diffusion in the case of whole intact cells in the urea up

taking process and at the same time emphasize the role of ABC urea transporters in the active urea uptake processes under the specified experimental conditions.

It was not known previously if any of the compounds which have been designed for the inhibition of urea hydrolysis are able to interact with or inhibit the active urea uptake systems. The effect of the pre-selected phosphoric amide derivatives on the transportation system varied depending on the nature of the inhibitor. From one side Inh.I shows no significant effect on the rate of urea uptake process, on the other hand applying Inh (II – VI) show a significant inhibition for the uptake process. Due to the deficiency in the supporting information, such as the crystal structure of the urea ABC transportation system, the active transportation inhibition mechanism is still an open question. The high resolution structural data are available for only few of the ABC transporters [Higgins, 2007; Holland and Blight, 1999]. The three-dimensional structures that are known only providing static view of the transporter [McKeegan et al., 2004]. For a full understanding of solute-ABC transporter interaction mechanisms and biology, the structure of many transporters, with and without substrates, is thus required [McKeegan et al., 2004].

A key answer hypothesis regarding this issue could be based on the information gained about the inhibitors chemical, geometrical and special structures in which a particular domain is mimicking the actual structure of the urea. This domain may somehow interact with the active site of the ABC transporter in a way similar to the urea-transporter interaction, but the relative big special structure hindrance the complete transferring of the molecule into the cell. This may lead to block the path toward the cells cytoplasm in the face of the urea molecules. The whole picture regarding this particular issue could only be established with further structural analysis and inhibition kinetics investigations.

5.3 *Bacillus megaterium* DSM90 urease isolation and purification

Previously in this study, the active transportation of urea through the bacterial cell membrane was investigated employing *B. megaterium* DSM90 as model organism. It was found that some preselected phosphoric amide derivatives inhibit the active uptake of urea by the ABC transporters. In order to emphasize the actual inhibition mechanism of these compounds wherever their effect is only toward the transportation systems or there is another companion

effect on the cytoplasmic urease, the bacterial urease from *B. megaterium* DSM90 was isolated and purified. Unfortunately there is a lack of information in the literature about the purification of *B. megaterium* urease; however other isolation and purification protocols for ureases from other bacterial strains have been comprehensively discussed. Table 16 is representing an overview about the previous conducted studies related to bacterial urease purification. To elect a suitable down streaming protocol, urease encoding genes homology investigation was carried out. It was found that the best match, regarding the bacterial strains used in the purification protocols, was with the urease originated from *B. pasteurii*. The comparison was carried out between the genomic data obtained from the first draft of the *B. megaterium* QMB1551 genomic sequence project, established in the institute of genome science, university of Maryland*B. pasteurii* obtained from NCBI data bank Acc N°. X78411.

Table 16 Overview about the conducted studies related to bacterial urease purification

Host strain	Purification protocol *	Spec. Act. [U/mg]	Buffer	Refrence
Bacillus pasteurii	S, AC, Hydroxyapatit	3800	3 mM Na-Phosphate pH 7.0	[Vassiliou et al., 2008]
Staphylococcus leei	GBM,F,F,IEC, HIC,SEC	1460	1 mM tris-HCl pH 6.8	[Jin et al., 2004]
recombinant *B. pasteurii* Urease, in *E. coli*	FPC, IEC,SEC, IEC	3682	50 mM HEPES, pH 7.5	[Shin and Lee, 1999]
Staphylococcus sp.	S, IEC, SEC, IEC, SEC	2240	30 mM Tris-HCl, pH 8.00	[Lee and Calhoun, 1997]
Bacillus pasteurii	FPC, IEC,HIC,SEC	4935.6	0.002 M phosphate pH7.5	[Benini et al., 1996]
Sporosarcina ureae	FPC,IEC,HIC,IEC,IEC	18680	50 mM HEPES, 0.5 mM EDTA pH 7.5	[McCoy et al., 1992]
Morganella morganii	FPC. IEC, HIC, IEC, SEC	4260	3 mM Na-Phosphate pH 6,8	[Hu et al., 1990]
Lactobac. Reuteri	EP, SEC. IEC, AC, CF,AC	350	0.1 m Na-citrate pH 4	[Kakimoto et al., 1990]
Bacillus pasteurii	S, IEC, AP, SEC	1993	0,05 M Phosphate pH 7.5	[Christians and Kaltwasser, 1986]

*** Legends: Ep: ethanol perspiration, AP: ammonium m sulfate presentation, S: sonication, AC: affinity chromatography, GMB: glass beads milling, F : filtration, IEC: ion exchange chromatography, HIC: hydrophobic interaction, SEC: size exclusion chromatography and FPC: French press cell.**

The *B. megaterium* QMB1551 genome was undergoing a Clustalw analysis against *ure* operons obtained from several *Bacillus sp*. The amplicon which scored the highest value of similarity was analyzed by ORF, open reading frame finder [Stothard, 2000]. The three urease structural genes *ureABC* were proposed beside 5 possible accessory genes *ureEFGDH*. The homology between *B. megaterium* and *B. Pasteurii* urease was found to be 64 % for *ureA*, 71 % for *ureB* and 47 % for *ureC*. Despite the low homology between the two ureases, *B. Pasteurii* urease was showing the best match to *B. megaterium* urease rather than the other compared bacterial strains. Based on that, the *B. pasteurii* urease purification protocol men-

tioned in the work of Benini et. al. [Benini et al., 1996] was adopted as the bases for the purification processes of *B. megaterium* urease.

Generally the purification process mentioned in the related article was found to be a combination of three main down streaming landmarks: ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and gel filtration (GF). The amount of urease in the crude protein extract after the HPH treatment was found to be 100 times less than in the case of *B. Pasteurii*. Nevertheless the magnitude of the purification was found to be similar. The concentration of NaCl needed to elute the *B. megaterium* urease in the (IEC) found to be approx. 10 times higher than in the case of *B. pasteurii*. High salt concentration needed for protein elution indicate the high overall net charge of the *B. megaterium* urease, since strong bounding with the chromatographic material will be formed and high salt concentration is needed for elution. The specific activity achieved after the (IEC) was 1.87 U / mg, well below the literature values of 316.6 U / mg for *B. pasteurii* urease [Benini et al., 1996] and 126 U / mg for *Morganella morganii* urease [Hu et al., 1990]. However the purification magnitude (5.3) showed a comparable values with which have been obtained for *B. Pasteurii* urease (7) [Benini et al., 1996] and purification *Morganella morganii* urease (5.3) [Hu et al., 1990].

The SDS PAGE analysis of the urease active fractions obtained from the (IEC) indicate the need of further purification to remove the non desired proteins, thus the (HIC) was conducted. In Benini et. al. ammonium sulfate was used in (HIC) to elute the urease from the binding material, but due to the interfering effect of the ammonium sulfate with the urease activity assay, it was replaced in this study with sodium sulfate. It was found that urease did not bind to the chromatographic material. Despite that, a significant amount of the non desirable contaminants and protein was removed. Similar case has been described in previous study [Hu et al., 1990] in which the urease did not bind to the chromatographic material and almost 80 % of the non desired contaminates did, therefore a significant purification magnitude has been achieved. After (HIC) 9 folds of purification magnitude of *B. megaterium* urease has been achieved. This magnitude of purification is far below the achieved ones in previous studies for BMU (70 folds) [Benini et al., 1996] and 23 fold for MMU [Hu et al., 1990].

With the described cleaning procedure, a yield of 36 % and a specific activity of 3.17 U / mg were obtained. From 87.5 g B. megaterium DSM90 BWM, 51 mL enzyme solution with a total activity of 48.86 U could be gained. The SDS PAGE analysis showed a successful re-

duction of the undesired impurities after each successful purification step. After the last purification step, Hydrophobic Interaction Chromatography, five predominant bands were observed. Although there were no unique bans that could be referred directly to the urease, dense bands at the range of 30.86 kDa and 49.63 kDa were observed. The molecular mass of these bands is showing some similarities with the bands obtained from purified urease of *H. Pylori* [Mobley et al., 1995]. Urease from *H. pylori* is characterized by its two distinct subunits instead of three in other bacterial species. The molecular mass of these subunits is in the range of 26.3 kDa and 60.3 kDa respectively. The identity of urease-representing bands could not be verified in this stage without any further supporting investigations such as native functional PAGE.

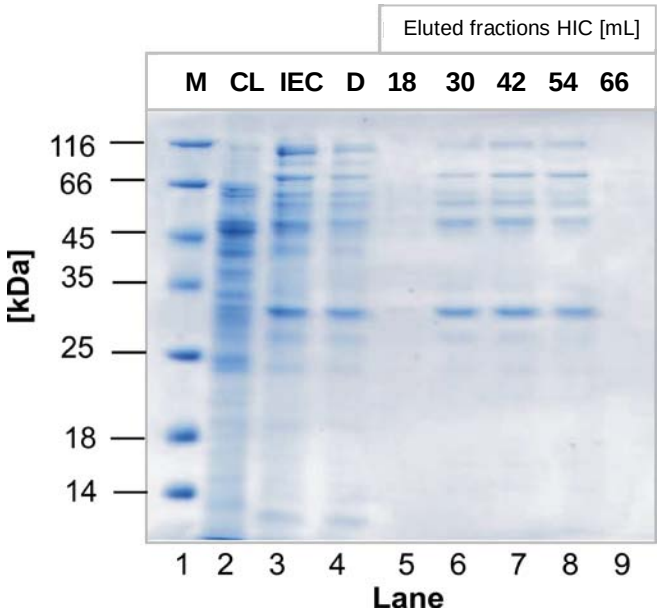


Figure 52. SDS-PAGE gel representing an overview about the successful purification steps for the microbial urease from *B. megaterium*. Lane 1: molecular weight marker, lane 2: the crude protein extract after HPH treatment, Lane 3: urease active pooled fractions after IEC purification step, lane 4 urease active pooled fractions after dialysis against 50 mM potassium phosphate buffer pH 7.5 at 4 °C for 2 successful rounds, lane 5-9: the eluted fractions after HIC purification step.

Plasmid borne ureases have been described for at least two species, *Clostridium perfringens* [Dupuy et al., 1997] and *Escherichia coli* [D'Orazio and Collins, 1993]. The presence of two different copies of *ureC* has been identified by genome sequencing of at least four bacterial species (*Streptomyces avermitilis*, *Streptomyces coelicolor*, *Brucella suis*, and *Brucella melitensis*). Prior to these genome-sequencing efforts, multiple copies of urease genes within a single genome had not been described, although native gel electrophoresis and activity stain-

ing of urease enzymes had revealed that multiple urease isoforms commonly exist in bacteria [Burne and Chen, 2000].

5.4 Urease kinetic characterization

5.4.1 Purified enzyme kinetic characterization

The data obtained from the kinetic characterization of the purified urease was undergone non linear fitting to the Michaelis-Menten's equation to form the ordinary hyperbolic saturation curve by the aid of MATHLAB®, MathWorks, Inc. Beside that double reciprocal curve, Lineweaver-Burk was plotted. The results obtained from the both methods were processed to minimize the differences between them and to overcome the backdrops of each of them [Bisswanger, 2000].

Analysis of the obtained data show that *B. megaterium* DSM90 urease show a simple Michaelis-Menten's type kinetic behavior, which is in agreement with other types of bacterial ureases investigated before [Mobley et al., 1995]. The K_m value which has been obtained, at 50 mM K-Na phosphate buffer pH 7 and 30 °C, was found to be 485 ± 15 µM which lays on the lower end of the K_m values spectrum obtained for other microbial urease species (0.1 - 100 mM) [Mobley and Hausinger, 1989]. Nevertheless the K_m value is slightly higher than the values obtained for *B. megaterium* DSM90 cell lysate in Jahns et. al (400 µM) [Jahns and Kaltwasser, 1989] This K_m value demonstrate a high affinity of *B. megaterium* DSM90 urease toward urea. Vassiliou et. al. found the K_m value for urease from *B. pasteuri* to be in the value of 28 mM (in 3 mM phosphate buffer, pH 7 at 30 °C) [Vassiliou et al., 2008]. Conducting the kinetic characterization, under the equivalent experimental conditions, lead to *B. megaterium* DSM90 urease K_m value of 940 µM. Thus, the affinity of *B. pasteurii* urease toward urea is showed to be significantly lower than the affinity of the *B. megaterium* DSM90 urease. Larson and Kallio showed that the affinity of the *B. Pasteurii* is propositional to the pH value [Larson and Kallio, 1954]. This was also noted for the *B. megaterium* DSM90 urease; the affinity for urea which have been obtained at pH 7.5 was higher than that at pH 7, which confirming the dependence of the K_m on the pH value.

The obtained *B. megaterium* DSM90 V_{max}, in 3 mM phosphate buffer pH 7, was found to be 0.067 mM / min, approx. 63 times lower than the corresponded V_{max} value of *B. pasteurii* urease, 3.78 mM / min, in 3 mM phosphate buffer, pH 7 at 30 °C [Vassiliou et al., 2008]. The maximum reaction velocity of the purified *B. megaterium* DSM90 urease after 7 days of HIC treatment, at 50 mM K-Na phosphate buffer pH 7 and 30 °C, was found to be 0.06 mM / min. Prolonging the storage period cause a significant loss in the in the V_{max} value. After additional 8 days of storage the V_{max} was found to be 0.04 mM / min. The conducted stability experiments suggest that the V_{max} value directly after the HIC treatment suppose to be higher than 0.06 mM / min. However these presumptions could not be validated due to lack of information in the literature related to the *B. megaterium* urease stability over time.

5.4.2 Enzyme inhibition kinetics characterization

The inhibition mechanism and constants of six different pre selected phosphoric amide urease inhibitors on the purified *B. megaterium* DSM90 urease were investigated to characterize their inhibition effect. The inhibition characterization was carried out as described in the work of Vassiliou et. al. [Vassiliou et al., 2008]. The purified urease was incubated at for 30 min with the investigated inhibitor before introducing the urea to the reaction mixture. Afterwards, the steady-state kinetic measurement was performed. A strong inhibition effect of the preselected inhibitors on the purified *B. megaterium* DSM90 urease was observed with K_I values less than 0.02 nM. No significant differences were found between K_I values of the introduced inhibitors, even when a very low inhibitor concentrations, as low as 0.04 nM was introduced.

In the work of Mc Carty et. al. the inhibition mechanism of PPDA, corresponded to Inh II in the current study, and Inh VI were characterized [McCarty et al., 1990]. The inhibitor concentrations which have been used in mentioned study, to investigate the inhibition effect on *B. pasteurii* urease, were significantly higher, for PPDA 5-40 nM and 200-1600 nM for Inh IV were used. On the other hand In Vassiliou et al. the used inhibitor concentration were ranged between > 1 µM to 100 mM of PPDA to investigate the inhibition effect on *B. pasteurii* urease [Vassiliou et al., 2008]. In Table 17 an overview about the K_I values for selected urease inhibitors on ureases from different origins is represented. The PPDA inhibition parameters conducted in the current study on Jack bean urease show a similar values with the ones mentioned in the literature [McCarty et al., 1990].

Table 17. Overview about the inhibition parameters for different selected inhibitors on ureases from different origins.

Inhibitor	Urease origin	K_I [nM]	Buffer	Reference
PPDA (InhII)	*B. pasteurii*	0.6	Tris, pH 7	[McCarty et al., 1990]
	Jack bean	0.16	Tris, pH 7	[McCarty et al., 1990]
		0.06^{a}-0.12^{b}*	3 mM K-P, pH 7	Current study
		0.16^{b}-0.19^{a}**	3 mM K-P, pH 7	Current study
	B. megaterium	0.01*	50 mM K-P, pH 7,5	Current study
		0.1^{b}-0.36^{a}*	3 mM K-P, pH 7	Current study
		0.22^{b}-0.53^{b}**	3 mM K-P, pH 7	Current study
	K. aerogenes	< 0.1	k. A.	[Mobley and Hausinger, 1989]
N-(4-Nitrophenyl) phosphoric triamide	*B. pasteurii*	42	Tris, pH 7	[McCarty et al., 1990]
	Jack bean	24	Tris, pH 7	[McCarty et al., 1990]
Aminomethyl (P-methyl) phosphin acid	*B. pasteurii*	340000	3 mM K-P, pH 7	[Vassiliou et al., 2008]
N-(N´-Benzyloxy-carbonylglycyl)-aminomethyl(P-methyl)phosphi-nothio acid	*B. pasteurii*	170	3 mM K-P, pH 7	[Vassiliou et al., 2008]

***. Inhibitor concentration 0.04 nM in the reaction assay, for K_I values: MATLAB[a] and Lineweaver-Burk-Plot[b].**
****. Inhibitor concentration 0.4 nM im the reaction assay, for K_I values: MATLAB[a] and Lineweaver-Burk-Plot[b].**
Cbz: Benzyloxycarbonyl
Tris-Puffer: 100 mM, 0.1 mM ß-Mercaptoethanol and 1 mM EDTA.

The kinetics of inhibition of the jack bean and *Bacillus pasteurii* ureases by eight phosphoroamides inhibitors showed that the onset of inhibition was time dependent [McCarty et al., 1990]. Which indicate that the kinetic data obtained from such experiments could not be fitted to the steady-state, Michaelis-Menten kinetic models developed for classical enzyme inhibitors [McCarty et al., 1990]. Moreover, these it was shown that some of the phosphoroamides were extremely potent inhibitors of urease. For example, it was observed that (PPDA) and 4-chlorophenylphosphorodiamidate (4CI-PPD) inhibited urease even when the molar concentrations of these compounds were comparable to that of the urease. Under such conditions, the concentration of free inhibitor decreases significantly, thereby violating an assumption made in kinetic models for classical inhibitors that concentrations of free inhibitor remain constant. The time dependence for the inhibition of ureases by phosphoroamides and the tight-binding properties of these compounds indicated that phosphoroamides have the characteristics of slow, tight-binding inhibitors. However to overcome this problematic equilibrium conditions for mixtures of enzyme and inhibitor with slow-binding characteristics should be achieved by pre-incubation of such mixtures for sufficiently long periods of time prior to the addition of substrate [Cha, 1975]. Preliminary experiments showed that pre-

incubation of mixtures of the plant urease and PPD or 4C1-PPD for 2.5 h permitted good approximation of equilibrium conditions inhibitor [McCarty et al., 1990].

As discussed before in section 2.1.1.4 the proposed urease inhibition mechanism by PPDA and NBTPT could be summarized in three main steps: (I) Transporting the inhibitor toward the active site of the enzyme, (II) The formation of the active inhibitor form of PPDA and NBTPT, DAP formation and (III) the last step could be consider the actual inhibition step [Hanisch, 2008]. Each of the previous mentioned steps has its own rate over time. The rate determining steps may significantly affect the observed "appeared" inhibition mechanism, for example, when the pre-incubation time is too short to achieve the equilibrium between the inhibitor / urease binding and dissociation. Lack of information regarding the enzymatic kinetics of *B. megaterium* urease hindrances any further speculation about this point.

According to the literature most of the investigated urease inhibitors show a competitive inhibition mechanism [Mobley and Hausinger, 1989]. However there was no information regarding the inhibition mechanism of *B. megaterium* urease. In the current study it was found that all the used inhibitors show a non competitive inhibition mechanism. The accuracy of experimental procedures was verified by conducting an inhibition characterization of jack bean urease by PPDA. The obtained results were identical to the ones obtained from the literature.

6 Summary

In the aim of understanding the effect urea based fertilizers and hydrolysis inhibitors on the soil microbial communities and the urease uptake physiology and activity, three aspects in this study were conducted.

The first aspect was investigating the phylogenetic structure of urease producing bacterial in soil and the effect of different fertilization managements on the soil bacterial communities. Different soil samples under different agricultural and fertilization managements in Sachsen-Anhalt were sampled and different conventional and metagenomic approaches were employed to investigate the bacterial communities in these soils. The conventional investigation was carried out by culturing, identifying and isolating the urease-producing bacteria on a selective medium, Christensen media. Changing in the color of the medium was used to discriminate the urease-producing bacteria. Sequencing the 16S rRNA and *ure*C genes amplicons obtained from the urease positive isolates were carried out and a phylogenetic analysis was established. The phylogenetic analysis shows that the isolates obtained from this study are sharing the same hypothetical taxonomic unit node therefore they are relatively closely related to each other. Identification of the culturable bacterial isolates shows that most of them belong to family Bacillaceae.

The non conventional method, metagenomic approach was carried out by extracting the bacterial genomic materials from the corresponded fresh-obtained soil samples. T-RFLP was conducted to analyze the actual bacterial communities in the sampled soils and the effect of different agricultural and fertilization managements on the bacterial communities. In this study it was found that the agricultural management history has an observable influence on altering the bacterial communities in the soil. It was also found that there is no significant effect of the fertilizers nature on bacterial communities' structure. However there is some tendency of these communities to be altered as a response of applying different urea hydrolysis and nitrification inhibitors, in a way mimicking the absence of the associated fertilizers. Further investigation have to be done concerning the dynamic changing of the bacterial community considering the time space and the climate dimensions as influence factors on the bacterial community structure.

Evidence of *B. megaterium* existence in the tested soils has been confirmed in this study by employing the cultural dependent and independent approaches, which show its suitability as a bacterial model regarding further investigations related to the active urea uptake systems and urea metabolism.

The second aspect was evaluating the effect of some selected phosphoric amide derivatives on the active urea transportation in *Bacillus megaterium* DSM90. The urease-producing bacterium, *Bacillus megaterium* DSM90, which also harbors ABC active urea transporters, was grown under various cultivation conditions. The nitrogen source KNO_3 did lead to the highest urease activity and urea up taking rate. The evidence of energy dependence of these active transporters was proved and their role in urea active uptake was demonstrated. The effect of the preselected phosphoric amide derivatives on the active urea uptake varied. NBTPT showed no effect on the transportation process, on the other hand the other five investigated inhibitors showed a significant suppression for the observed urea uptake process.

The last aspect was concerning about the isolation, purification, characterization and investigation of the kinetic inhibition of urease from *Bacillus megaterium* DSM90. The aim was to emphasize the action of the preselected phosphoric amide derivatives on the urease activity without any interference of the urea active uptake systems. Sufficient bacterial biomass was gained after two stages of batch cultivations in complex and mineral media. 87.5 g of BWM, approx. 3.5g BDM, were obtained and the urease was isolated and purified through three different downstream procedures: HPH, IEC and HIC. A purification factor of nine was achieved and the specific activity of the purified urease was found to be 3.17 U / mg with a total obtained amount of 48.86 U. It was found that the enzyme is following a simple Michaelis-Menten kinetic behavior with a K_m value of 485 µM and V_{max} value of 0.06 mM/min. The inhibition characterization shows that all the preselected phosphoric amide derivatives inherit a non competitive inhibition mechanism toward the *B. megaterium* DSM90 urease and the K_I value was shown to be less than 0.02 nM, which indicates a high inhibition potential for these compounds. The storage stability of urease as a function of the respective cleaning condition was investigated. The investigations have shown that after one week of storage at -20 °C of HIC purified urease 40 % of the activity was lost. It was found that adding glycerol enhanced remarkably the stability of the HIC purified urease to 84 % activity after 1 week at -20 °C.

7 Outlook

For an optimum employing of the culturable depended approaches, fresher soil samples have to be obtained. These samples should be more representative for the actual bacterial communities in the time of sampling. Furthermore, investigation have to be done concerning the dynamic changing of the bacterial community versus time, climate and special dimensions as influencing factors on the bacterial community structure. The number of the analyzed samples, in both culturable and unculturable dependence approaches should be increased to enhance the statistical significance of the results. Beside the qualitative analysis, which have been done in this study, quantitative investigation for the main urease-producing bacteria could be considered. The information that may be gained from these sorts of investigations could help in designing more efficient soil inoculums that could be applied to soils with poor nitrogen utilization capacity.

To determine the minimum amount of the proposed inhibitors needed to significantly suppress the active transportation phenomena, the urea active transportation kinetic and inhibition kinetic should be investigated. These investigations could be approached by employing a urease knockout *B. megaterium* DSM90 strain. Introducing this strain will eliminate any interference of the intracellular urease in the kinetic characterization process. However the kinetic characterization could be also achieved using an inhibitor that has an exclusive effect on urease, such as NBTPT.

The purification processes of *Bacillus megaterium* DSM90 urease should be further optimized to get rid of all the associated contaminants. Size exclusion chromatography could be introduced, from one side to enhance the final specific activity and from the other side to determine the actual size of the urease. Native and functional PAGE should be carried out to emphasize the actual size and structure of the *B. megaterium* DSM90 urease subunits. Regarding the inhibition kinetic characterization, the optimum inhibitor/enzyme pre-incubation prior to substrate introduction should be further investigated.

8 References

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9 Academic Résumé

Personal information

Name	Hany Hassounah
Date of birth	27.08.1979
Place of birth	Abu Dhabi, UAE.
Nationality	Palestinian

Education

- **[Martin-Luther-University, Halle (Saale), Germany] 11.2005 to 02.2010**

Doctoral dissertation in institute of pharmaceutical technology and biopharmacy, department of downstream processing, Thesis: "Investigations on the phylogenetic diversity of urease producing bacteria in soil, the inhibition of urea active transportation and metabolizing in *Bacillus megaterium* DSM 90".

- **[Martin-Luther-University, Halle (Saale), Germany] 09.2003 to 10.2005**

Master in natural science (M.Sc.), "Applied polymer science", faculty of engineering, institute of bioengineering. Thesis: Screening for applications of microbial transglutaminase for the production of biodegradable polymers.

- **[Ain-shames University, Cairo, Egypt] 10.1997 to 06.2001**

Bachelor in science (B.Sc.), major microbiology and chemistry

- **[Abu-Dhabi Secondary school, Abu-Dhabi, UAE] 10.1996 to 06.1997**

International Bacheloria (IB)

10 Attachments

Table 18. NBCI association numbers for the clones which have been used in the construction of the *16s rRNA* phylogenetic tree.

Clone suffix	Code name and NCBI association number
Clone 001	gi|225936810|emb|FM946069.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-50
Clone 002	gi|225936809|emb|FM946068.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-48
Clone 003	gi|225936808|emb|FM946067.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-45
Clone 004	gi|225936807|emb|FM946066.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-43
Clone 005	gi|225936806|emb|FM946065.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-42
Clone 006	gi|225936805|emb|FM946064.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-40
Clone 007	gi|225936804|emb|FM946063.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-39
Clone 008	gi|225936803|emb|FM946062.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-38
Clone 009	gi|225936802|emb|FM946061.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-36
Clone 010	gi|225936801|emb|FM946060.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-35
Clone 011	gi|225936800|emb|FM946059.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-34
Clone 012	gi|225936799|emb|FM946058.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-33
Clone 013	gi|225936798|emb|FM946057.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-32
Clone 014	gi|225936797|emb|FM946056.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-31
Clone 015	gi|225936796|emb|FM946055.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-30
Clone 016	gi|225936795|emb|FM946054.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-29
Clone 017	gi|225936794|emb|FM946053.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-28
Clone 018	gi|225936793|emb|FM946052.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-23
Clone 019	gi|225936792|emb|FM946051.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-22
Clone 020	gi|225936791|emb|FM946050.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-21
Clone 021	gi|225936790|emb|FM946049.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-19
Clone 022	gi|225936789|emb|FM946048.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-17
Clone 023	gi|225936788|emb|FM946047.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-16
Clone 024	gi|225936787|emb|FM946046.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-11
Clone 025	gi|225936786|emb|FM946045.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-10
Clone 026	gi|225936785|emb|FM946044.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-9
Clone 027	gi|225936784|emb|FM946043.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-8
Clone 028	gi|225936783|emb|FM946042.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-6
Clone 029	gi|225936782|emb|FM946041.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-5
Clone 030	gi|225936781|emb|FM946040.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-3
Clone 031	gi|225936780|emb|FM946039.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-2
Clone 032	gi|225936779|emb|FM946038.1| Uncultured soil bacterium partial 16S rRNA gene, clone C01-7-7
Clone 033	gi|193783169|emb|FM174320.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPh-7-R
Clone 034	gi|193783168|emb|FM174319.1| Uncultured soil bacterium partial 16S rRNA gene, clone CadS-3
Clone 035	gi|193783167|emb|FM174318.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPh-13-RS
Clone 036	gi|193783166|emb|FM174317.1| Uncultured soil bacterium partial 16S rRNA gene, clone GcPh-4-R
Clone 037	gi|193783165|emb|FM174316.1| Uncultured soil bacterium partial 16S rRNA gene, clone GcPh-13-RS020
Clone 038	gi|193783164|emb|FM174315.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPhMC-5-R
Clone 039	gi|193783163|emb|FM174314.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlR-4-1020
Clone 040	gi|193783162|emb|FM174313.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlR-8-05
Clone 041	gi|193783161|emb|FM174312.1| Uncultured soil bacterium partial 16S rRNA gene, clone LnU-2-RS
Clone 042	gi|193783160|emb|FM174311.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlPh-2-R
Clone 043	gi|193783159|emb|FM174310.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlPh-1-R
Clone 044	gi|193783158|emb|FM174309.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlPh-9-R
Clone 045	gi|193783157|emb|FM174308.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlPh-6-RS
Clone 046	gi|193783156|emb|FM174307.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlPh-1-RS
Clone 047	gi|193783155|emb|FM174306.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlR-13-510
Clone 048	gi|193783154|emb|FM174305.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaSI-5
Clone 049	gi|193783153|emb|FM174304.1| Uncultured soil bacterium partial 16S rRNA gene, clone BoZ-1-RS
Clone 050	gi|193783152|emb|FM174303.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaR4-2
Clone 051	gi|193783151|emb|FM174302.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPh-2-R-BS
Clone 052	gi|193783150|emb|FM174301.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPhMC-16-R
Clone 053	gi|193783149|emb|FM174300.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPhMC-4-R
Clone 054	gi|193783148|emb|FM174299.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaFf-1
Clone 055	gi|193783147|emb|FM174298.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPh-21-R
Clone 056	gi|193783146|emb|FM174297.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPh-2-R-A
Clone 057	gi|193783145|emb|FM174296.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPhsec-16-R
Clone 058	gi|193783144|emb|FM174295.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPhsec-1-R
Clone 059	gi|193783143|emb|FM174294.1| Uncultured soil bacterium partial 16S rRNA gene, clone BoZ-6-RS
Clone 060	gi|193783142|emb|FM174293.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlR-17-510
Clone 061	gi|193783141|emb|FM174292.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaR2-7
Clone 062	gi|193783140|emb|FM174291.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPhMC-4-RS
Clone 063	gi|193783139|emb|FM174290.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlR-5-1020
Clone 064	gi|193783138|emb|FM174289.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPh-1-R
Clone 065	gi|193783137|emb|FM174288.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPh-12-R
Clone 066	gi|193783136|emb|FM174287.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlPh-2-RS
Clone 067	gi|193783135|emb|FM174286.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlPh-4-R
Clone 068	gi|193783134|emb|FM174285.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlR-9-05
Clone 069	gi|193783133|emb|FM174284.1| Uncultured soil bacterium partial 16S rRNA gene, clone GcPh-3-RS
Clone 070	gi|193783132|emb|FM174283.1| Uncultured soil bacterium partial 16S rRNA gene, clone GcPh-4-RS020
Clone 071	gi|193783131|emb|FM174282.1| Uncultured soil bacterium partial 16S rRNA gene, clone GcPh-5-RS2030
Clone 072	gi|193783130|emb|FM174281.1| Uncultured soil bacterium partial 16S rRNA gene, clone GcPh-5-R
Clone 073	gi|193783129|emb|FM174280.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPh-17-R
Clone 074	gi|193783128|emb|FM174279.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPh-16-R

Clone 075	gi|193783127|emb|FM174278.1| Uncultured soil bacterium partial 16S rRNA gene, clone GcPh-17-RS
Clone 076	gi|193783126|emb|FM174277.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaFf-6-BS
Clone 077	gi|193783125|emb|FM174276.1| Uncultured soil bacterium partial 16S rRNA gene, clone LnU-5-RS
Clone 078	gi|193783124|emb|FM174275.1| Uncultured soil bacterium partial 16S rRNA gene, clone CdvP-4
Clone 079	gi|193783123|emb|FM174274.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlR-6-05
Clone 080	gi|193783122|emb|FM174273.1| Uncultured soil bacterium partial 16S rRNA gene, clone LnU-7-RS
Clone 081	gi|193783121|emb|FM174272.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaPhMC-1-RS
Clone 082	gi|193783120|emb|FM174271.1| Uncultured soil bacterium partial 16S rRNA gene, clone LlR-2-RS1020
Clone 083	gi|193783119|emb|FM174270.1| Uncultured soil bacterium partial 16S rRNA gene, clone CaR3-3-RS
Clone 084	gi|193783118|emb|FM174269.1| Uncultured soil bacterium partial 16S rRNA gene, clone GcPh-6-RS020
Clone 085	gi|193783117|emb|FM174268.1| Uncultured soil bacterium partial 16S rRNA gene, clone LnU-4-RS
Clone 086	gi|158148112|emb|AM884741.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-46
Clone 087	gi|158148111|emb|AM884740.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-45
Clone 088	gi|158148110|emb|AM884739.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-43
Clone 089	gi|158148109|emb|AM884738.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-40
Clone 090	gi|158148108|emb|AM884737.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-39
Clone 091	gi|158148107|emb|AM884736.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-38
Clone 092	gi|158148106|emb|AM884735.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-37
Clone 093	gi|158148105|emb|AM884734.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-36
Clone 094	gi|158148104|emb|AM884733.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-35
Clone 095	gi|158148103|emb|AM884732.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-34
Clone 096	gi|158148102|emb|AM884731.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-33
Clone 097	gi|158148101|emb|AM884730.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-32
Clone 098	gi|158148100|emb|AM884729.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-31
Clone 099	gi|158148099|emb|AM884728.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-30
Clone 100	gi|158148098|emb|AM884727.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-29
Clone 101	gi|158148097|emb|AM884726.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-28
Clone 102	gi|158148096|emb|AM884725.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-27
Clone 103	gi|158148095|emb|AM884724.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-26
Clone 104	gi|158148094|emb|AM884723.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-25
Clone 105	gi|158148093|emb|AM884722.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-24
Clone 106	gi|158148092|emb|AM884721.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-18
Clone 107	gi|158148091|emb|AM884720.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-16
Clone 108	gi|158148090|emb|AM884719.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-15
Clone 109	gi|158148089|emb|AM884718.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-14
Clone 110	gi|158148088|emb|AM884717.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-13
Clone 111	gi|158148087|emb|AM884716.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-12
Clone 112	gi|158148086|emb|AM884715.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-11
Clone 113	gi|158148085|emb|AM884714.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-10
Clone 114	gi|158148084|emb|AM884713.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-9
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Clone 116	gi|158148082|emb|AM884711.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-6
Clone 117	gi|158148081|emb|AM884710.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-5
Clone 118	gi|158148080|emb|AM884709.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-4
Clone 119	gi|158148079|emb|AM884708.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-3
Clone 120	gi|158148078|emb|AM884707.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-2
Clone 121	gi|158148077|emb|AM884706.1| Uncultured soil bacterium 16S rRNA gene, clone FL9cT72-1
Clone 122	gi|158148076|emb|AM884705.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X24
Clone 123	gi|158148075|emb|AM884704.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X17
Clone 124	gi|158148074|emb|AM884703.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X15
Clone 125	gi|158148073|emb|AM884702.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X13
Clone 126	gi|158148072|emb|AM884701.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X9
Clone 127	gi|158148071|emb|AM884700.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X8
Clone 128	gi|158148070|emb|AM884699.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X7
Clone 129	gi|158148069|emb|AM884698.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X5
Clone 130	gi|158148068|emb|AM884697.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X4
Clone 131	gi|158148067|emb|AM884696.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-X3
Clone 132	gi|158148066|emb|AM884695.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-25
Clone 133	gi|158148065|emb|AM884694.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-22
Clone 134	gi|158148064|emb|AM884693.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-21
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Clone 136	gi|158148062|emb|AM884691.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-15
Clone 137	gi|158148061|emb|AM884690.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-14
Clone 138	gi|158148060|emb|AM884689.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-11
Clone 139	gi|158148059|emb|AM884688.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-10
Clone 140	gi|158148058|emb|AM884687.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-9
Clone 141	gi|158148057|emb|AM884686.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-6
Clone 142	gi|158148056|emb|AM884685.1| Uncultured soil bacterium 16S rRNA gene, clone FL9_T0-5
Clone 143	gi|158148055|emb|AM884684.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-48
Clone 144	gi|158148054|emb|AM884683.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-46
Clone 145	gi|158148053|emb|AM884682.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-45
Clone 146	gi|158148052|emb|AM884681.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-43
Clone 147	gi|158148051|emb|AM884680.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-42
Clone 148	gi|158148050|emb|AM884679.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-41
Clone 149	gi|158148049|emb|AM884678.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-40
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Clone 162	gi|158148036|emb|AM884665.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-25
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Clone 169	gi|158148029|emb|AM884658.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-15
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Clone 171	gi|158148027|emb|AM884656.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-12
Clone 172	gi|158148026|emb|AM884655.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-11
Clone 173	gi|158148025|emb|AM884654.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-9
Clone 174	gi|158148024|emb|AM884653.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-8
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Clone 176	gi|158148022|emb|AM884651.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-4
Clone 177	gi|158148021|emb|AM884650.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-3
Clone 178	gi|158148020|emb|AM884649.1| Uncultured soil bacterium 16S rRNA gene, clone H4aT14-2
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Clone 180	gi|158148018|emb|AM884647.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-e
Clone 181	gi|158148017|emb|AM884646.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-d
Clone 182	gi|158148016|emb|AM884645.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-b
Clone 183	gi|158148015|emb|AM884644.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-39
Clone 184	gi|158148014|emb|AM884643.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-38
Clone 185	gi|158148013|emb|AM884642.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-37
Clone 186	gi|158148012|emb|AM884641.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-36
Clone 187	gi|158148011|emb|AM884640.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-35
Clone 188	gi|158148010|emb|AM884639.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-34
Clone 189	gi|158148009|emb|AM884638.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-32
Clone 190	gi|158148008|emb|AM884637.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-28
Clone 191	gi|158148007|emb|AM884636.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-27
Clone 192	gi|158148006|emb|AM884635.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-23
Clone 193	gi|158148005|emb|AM884634.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-22
Clone 194	gi|158148004|emb|AM884633.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-21
Clone 195	gi|158148003|emb|AM884632.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-19
Clone 196	gi|158148002|emb|AM884631.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-18
Clone 197	gi|158148001|emb|AM884630.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-16
Clone 198	gi|158148000|emb|AM884629.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-15
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Clone 204	gi|158147994|emb|AM884623.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-4
Clone 205	gi|158147993|emb|AM884622.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-2
Clone 206	gi|158147992|emb|AM884621.1| Uncultured soil bacterium 16S rRNA gene, clone H4_T0-1
Clone 207	gi|124270302|emb|AM412814.1| Uncultured soil bacterium partial 16S rRNA gene, isolate DGGE band L1
Clone 208	gi|124270301|emb|AM412813.1| Uncultured soil bacterium partial 16S rRNA gene, isolate DGGE band H4
Clone 209	gi|124270300|emb|AM412812.1| Uncultured soil bacterium partial 16S rRNA gene, isolate DGGE band H3
Clone 210	gi|57434247|emb|AJ871263.1| Uncultured soil bacterium partial 16S rRNA gene, clone 185-1
Clone 211	gi|57434246|emb|AJ871262.1| Uncultured soil bacterium partial 16S rRNA gene, clone 165-6
Clone 212	gi|57434245|emb|AJ871261.1| Uncultured soil bacterium partial 16S rRNA gene, clone 165-5
Clone 213	gi|57434244|emb|AJ871260.1| Uncultured soil bacterium partial 16S rRNA gene, clone 165-4
Clone 214	gi|57434243|emb|AJ871259.1| Uncultured soil bacterium partial 16S rRNA gene, clone 165-3
Clone 215	gi|57434242|emb|AJ871258.1| Uncultured soil bacterium partial 16S rRNA gene, clone 165-1
Clone 216	gi|57434241|emb|AJ871257.1| Uncultured soil bacterium partial 16S rRNA gene, clone 141-3
Clone 217	gi|50949237|emb|AJ630287.1| Uncultured soil bacterium partial 16S rRNA gene, clone MFC-EB19
Clone 218	gi|50949229|emb|AJ630279.1| Uncultured soil bacterium partial 16S rRNA gene, clone MFC-EB10
Clone 219	gi|79759058|emb|AM039609.1| Uncultured soil bacterium partial 16S rRNA gene, clone 10A
Clone 220	gi|79759055|emb|AM039607.1| Uncultured soil bacterium partial 16S rRNA gene, clone 92A
Clone 221	gi|79759054|emb|AM039606.1| Uncultured soil bacterium partial 16S rRNA gene, clone 90A
Clone 222	gi|79759052|emb|AM039604.1| Uncultured soil bacterium partial 16S rRNA gene, clone 76A
Clone 223	gi|79759051|emb|AM039603.1| Uncultured soil bacterium partial 16S rRNA gene, clone 75A
Clone 224	gi|79759050|emb|AM039602.1| Uncultured soil bacterium partial 16S rRNA gene, clone 66A
Clone 225	gi|79759049|emb|AM039601.1| Uncultured soil bacterium partial 16S rRNA gene, clone 65A
Clone 226	gi|79759047|emb|AM039599.1| Uncultured soil bacterium partial 16S rRNA gene, clone 61A
Clone 227	gi|79759046|emb|AM039598.1| Uncultured soil bacterium partial 16S rRNA gene, clone 60A
Clone 228	gi|79759045|emb|AM039597.1| Uncultured soil bacterium partial 16S rRNA gene, clone 53A
Clone 229	gi|79759044|emb|AM039596.1| Uncultured soil bacterium partial 16S rRNA gene, clone 46A
Clone 230	gi|79759043|emb|AM039595.1| Uncultured soil bacterium partial 16S rRNA gene, clone 43A
Clone 231	gi|79759042|emb|AM039594.1| Uncultured soil bacterium partial 16S rRNA gene, clone 40A
Clone 232	gi|79759041|emb|AM039593.1| Uncultured soil bacterium partial 16S rRNA gene, clone 25A
Clone 233	gi|79759040|emb|AM039592.1| Uncultured soil bacterium partial 16S rRNA gene, clone 23A
Clone 234	gi|79759039|emb|AM039591.1| Uncultured soil bacterium partial 16S rRNA gene, clone 21A
Clone 235	gi|79759037|emb|AM039589.1| Uncultured soil bacterium partial 16S rRNA gene, clone 10A
Clone 236	gi|79759036|emb|AM039588.1| Uncultured soil bacterium partial 16S rRNA gene, clone 9A
Clone 237	gi|79759035|emb|AM039587.1| Uncultured soil bacterium partial 16S rRNA gene, clone 8A
Clone 238	gi|79759034|emb|AM039586.1| Uncultured soil bacterium partial 16S rRNA gene, clone 6A
Clone 239	gi|79759033|emb|AM039585.1| Uncultured soil bacterium partial 16S rRNA gene, clone 96S
Clone 240	gi|79759032|emb|AM039584.1| Uncultured soil bacterium partial 16S rRNA gene, clone 94S
Clone 241	gi|79759031|emb|AM039583.1| Uncultured soil bacterium partial 16S rRNA gene, clone 93S
Clone 242	gi|79759030|emb|AM039582.1| Uncultured soil bacterium partial 16S rRNA gene, clone 83S
Clone 243	gi|79759029|emb|AM039581.1| Uncultured soil bacterium partial 16S rRNA gene, clone 82S
Clone 244	gi|79759028|emb|AM039580.1| Uncultured soil bacterium partial 16S rRNA gene, clone 80S
Clone 245	gi|79759027|emb|AM039579.1| Uncultured soil bacterium partial 16S rRNA gene, clone 79S
Clone 246	gi|79759025|emb|AM039578.1| Uncultured soil bacterium partial 16S rRNA gene, clone 76S

Clone 247	gi|79759024|emb|AM039577.1| Uncultured soil bacterium partial 16S rRNA gene, clone 75S
Clone 248	gi|79759023|emb|AM039576.1| Uncultured soil bacterium partial 16S rRNA gene, clone 72S
Clone 249	gi|79759022|emb|AM039575.1| Uncultured soil bacterium partial 16S rRNA gene, clone 66S
Clone 250	gi|79759021|emb|AM039574.1| Uncultured soil bacterium partial 16S rRNA gene, clone 64S
Clone 251	gi|79759020|emb|AM039573.1| Uncultured soil bacterium partial 16S rRNA gene, clone 47S
Clone 252	gi|79759019|emb|AM039572.1| Uncultured soil bacterium partial 16S rRNA gene, clone 44S
Clone 253	gi|79759018|emb|AM039571.1| Uncultured soil bacterium partial 16S rRNA gene, clone 43S
Clone 254	gi|79759017|emb|AM039570.1| Uncultured soil bacterium partial 16S rRNA gene, clone 42S
Clone 255	gi|79759016|emb|AM039569.1| Uncultured soil bacterium partial 16S rRNA gene, clone 36S
Clone 256	gi|79759015|emb|AM039568.1| Uncultured soil bacterium partial 16S rRNA gene, clone 30S
Clone 257	gi|79759014|emb|AM039567.1| Uncultured soil bacterium partial 16S rRNA gene, clone 26S
Clone 258	gi|79759013|emb|AM039566.1| Uncultured soil bacterium partial 16S rRNA gene, clone 25S
Clone 259	gi|79759012|emb|AM039565.1| Uncultured soil bacterium partial 16S rRNA gene, clone 23S
Clone 260	gi|79759011|emb|AM039564.1| Uncultured soil bacterium partial 16S rRNA gene, clone 20S
Clone 261	gi|79759010|emb|AM039563.1| Uncultured soil bacterium partial 16S rRNA gene, clone 19S
Clone 262	gi|79759009|emb|AM039562.1| Uncultured soil bacterium partial 16S rRNA gene, clone 18S
Clone 263	gi|79759008|emb|AM039561.1| Uncultured soil bacterium partial 16S rRNA gene, clone 17S
Clone 264	gi|79759007|emb|AM039560.1| Uncultured soil bacterium partial 16S rRNA gene, clone 16S
Clone 265	gi|79759006|emb|AM039559.1| Uncultured soil bacterium partial 16S rRNA gene, clone 15S
Clone 266	gi|79759005|emb|AM039558.1| Uncultured soil bacterium partial 16S rRNA gene, clone 11S
Clone 267	gi|79759004|emb|AM039557.1| Uncultured soil bacterium partial 16S rRNA gene, clone 9S
Clone 268	gi|79759002|emb|AM039556.1| Uncultured soil bacterium partial 16S rRNA gene, clone 8S
Clone 269	gi|79759001|emb|AM039555.1| Uncultured soil bacterium partial 16S rRNA gene, clone 6S
Clone 270	gi|79759000|emb|AM039554.1| Uncultured soil bacterium partial 16S rRNA gene, clone 5S
Clone 271	gi|79758998|emb|AM039553.1| Uncultured soil bacterium partial 16S rRNA gene, clone 4S
Clone 272	gi|79758997|emb|AM039552.1| Uncultured soil bacterium partial 16S rRNA gene, clone 2S
Clone 273	gi|79758996|emb|AM039551.1| Uncultured soil bacterium partial 16S rRNA gene, clone 1S
Clone 274	gi|111035894|emb|AM295189.1| Uncultured soil bacterium partial 16S rRNA gene, clone LC2
Clone 275	gi|111035893|emb|AM295188.1| Uncultured soil bacterium partial 16S rRNA gene, clone LC1
Clone 276	gi|109114427|emb|AM265565.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB199
Clone 277	gi|109114426|emb|AM265564.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB188
Clone 278	gi|109114425|emb|AM265563.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB171
Clone 279	gi|109114424|emb|AM265562.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB165
Clone 280	gi|109114423|emb|AM265561.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB155
Clone 281	gi|103421920|emb|AM265550.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB99
Clone 282	gi|103421909|emb|AM265547.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB45
Clone 283	gi|103421906|emb|AM265546.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB24
Clone 284	gi|103421903|emb|AM265545.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB17
Clone 285	gi|103421899|emb|AM265544.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB14
Clone 286	gi|103421896|emb|AM265543.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB10
Clone 287	gi|103421887|emb|AM265540.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB36
Clone 288	gi|103421880|emb|AM265539.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB13
Clone 289	gi|103421876|emb|AM265538.1| Uncultured soil bacterium partial 16S rRNA gene, clone EB07
Clone 290	gi|86604453|emb|AM229078.1| Uncultured soil bacterium partial 16S rRNA gene, clone E23 (S14)
Clone 291	gi|86604452|emb|AM229077.1| Uncultured soil bacterium partial 16S rRNA gene, clone E21 (S14)
Clone 292	gi|86604451|emb|AM229076.1| Uncultured soil bacterium partial 16S rRNA gene, clone E79 (S14)
Clone 293	gi|86604450|emb|AM229075.1| Uncultured soil bacterium partial 16S rRNA gene, clone E78 (S14)
Clone 294	gi|86604449|emb|AM229074.1| Uncultured soil bacterium partial 16S rRNA gene, clone E24 (S14)
Clone 295	gi|86604448|emb|AM229073.1| Uncultured soil bacterium partial 16S rRNA gene, clone E12 (S14)
Clone 296	gi|86604447|emb|AM229072.1| Uncultured soil bacterium partial 16S rRNA gene, clone E11 (S14)
Clone 297	gi|86604446|emb|AM229071.1| Uncultured soil bacterium partial 16S rRNA gene, clone E10 (S14)
Clone 298	gi|84452211|emb|AM180919.1| Uncultured soil bacterium partial 16S rRNA gene, clone E14 (S14)
Clone 299	gi|84452210|emb|AM180918.1| Uncultured soil bacterium partial 16S rRNA gene, clone E15 (S14)
Clone 300	gi|24745515|emb|AJ512670.1| Uncultured soil bacterium partial 16S rRNA gene, isolate E130
Clone 301	gi|24745512|emb|AJ512669.1| Uncultured soil bacterium partial 16S rRNA gene, isolate D96
Clone 302	gi|24745508|emb|AJ512668.1| Uncultured soil bacterium partial 16S rRNA gene, isolate C57
Clone 303	gi|24745505|emb|AJ512667.1| Uncultured soil bacterium partial 16S rRNA gene, isolate B29
Clone 304	gi|24745501|emb|AJ512666.1| Uncultured soil bacterium partial 16S rRNA gene, isolate A0
Clone 305	gi|50344400|emb|AJ748457.1| Uncultured soil bacterium partial 16S rRNA gene, clone 27-7
Clone 306	gi|50344399|emb|AJ748456.1| Uncultured soil bacterium partial 16S rRNA gene, clone 27-6
Clone 307	gi|50344398|emb|AJ748455.1| Uncultured soil bacterium partial 16S rRNA gene, clone 27-5
Clone 308	gi|50344397|emb|AJ748454.1| Uncultured soil bacterium partial 16S rRNA gene, clone 27-2
Clone 309	gi|50344396|emb|AJ748453.1| Uncultured soil bacterium partial 16S rRNA gene, clone 27-1
Clone 310	gi|50344395|emb|AJ748452.1| Uncultured soil bacterium partial 16S rRNA gene, clone 26-6
Clone 311	gi|50344394|emb|AJ748451.1| Uncultured soil bacterium partial 16S rRNA gene, clone 26-2
Clone 312	gi|50344393|emb|AJ748450.1| Uncultured soil bacterium partial 16S rRNA gene, clone 26-1
Clone 313	gi|50344392|emb|AJ748449.1| Uncultured soil bacterium partial 16S rRNA gene, clone 24-4
Clone 314	gi|50344391|emb|AJ748448.1| Uncultured soil bacterium partial 16S rRNA gene, clone 23-5
Clone 315	gi|50344390|emb|AJ748447.1| Uncultured soil bacterium partial 16S rRNA gene, clone 23-4
Clone 316	gi|50344389|emb|AJ748446.1| Uncultured soil bacterium partial 16S rRNA gene, clone 22-8
Clone 317	gi|50344388|emb|AJ748445.1| Uncultured soil bacterium partial 16S rRNA gene, clone 22-7
Clone 318	gi|50344387|emb|AJ748444.1| Uncultured soil bacterium partial 16S rRNA gene, clone 22-6
Clone 319	gi|50344386|emb|AJ748443.1| Uncultured soil bacterium partial 16S rRNA gene, clone 22-5
Clone 320	gi|50344385|emb|AJ748442.1| Uncultured soil bacterium partial 16S rRNA gene, clone 22-4
Clone 321	gi|50344384|emb|AJ748441.1| Uncultured soil bacterium partial 16S rRNA gene, clone 22-3
Clone 322	gi|50344383|emb|AJ748440.1| Uncultured soil bacterium partial 16S rRNA gene, clone 22-1
Clone 323	gi|50344382|emb|AJ748439.1| Uncultured soil bacterium partial 16S rRNA gene, clone 21-10
Clone 324	gi|50344381|emb|AJ748438.1| Uncultured soil bacterium partial 16S rRNA gene, clone 21-9
Clone 325	gi|50344380|emb|AJ748437.1| Uncultured soil bacterium partial 16S rRNA gene, clone 21-8
Clone 326	gi|50344379|emb|AJ748436.1| Uncultured soil bacterium partial 16S rRNA gene, clone 21-7
Clone 327	gi|50344378|emb|AJ748435.1| Uncultured soil bacterium partial 16S rRNA gene, clone 21-6
Clone 328	gi|50344377|emb|AJ748434.1| Uncultured soil bacterium partial 16S rRNA gene, clone 21-4
Clone 329	gi|50344376|emb|AJ748433.1| Uncultured soil bacterium partial 16S rRNA gene, clone 21-3
Clone 330	gi|50344375|emb|AJ748432.1| Uncultured soil bacterium partial 16S rRNA gene, clone 21-1
Clone 331	gi|50344374|emb|AJ748431.1| Uncultured soil bacterium partial 16S rRNA gene, clone 20-7
Clone 332	gi|50344373|emb|AJ748430.1| Uncultured soil bacterium partial 16S rRNA gene, clone 20-6

Clone 333	gi|50344372|emb|AJ748429.1| Uncultured soil bacterium partial 16S rRNA gene, clone 20-5
Clone 334	gi|50344371|emb|AJ748428.1| Uncultured soil bacterium partial 16S rRNA gene, clone 20-4
Clone 335	gi|50344370|emb|AJ748427.1| Uncultured soil bacterium partial 16S rRNA gene, clone 20-3
Clone 336	gi|50344369|emb|AJ748426.1| Uncultured soil bacterium partial 16S rRNA gene, clone 20-2
Clone 337	gi|50344368|emb|AJ746415.1| Uncultured soil bacterium partial 16S rRNA gene, clone 20-10
Clone 338	gi|23504814|emb|AJ509086.1| Uncultured soil bacterium partial 16S rRNA gene, clone DS2
Clone 339	gi|23504813|emb|AJ509085.1| Uncultured soil bacterium partial 16S rRNA gene, clone DS1
Clone 340	gi|15131708|emb|AJ318777.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM1430
Clone 341	gi|15131707|emb|AJ318776.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM730
Clone 342	gi|15131706|emb|AJ318775.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM1563
Clone 343	gi|15131705|emb|AJ318774.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM1161
Clone 344	gi|15131704|emb|AJ318773.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM328
Clone 345	gi|15131703|emb|AJ318772.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM2343
Clone 346	gi|15131702|emb|AJ318771.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM642
Clone 347	gi|15131701|emb|AJ318770.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM541
Clone 348	gi|15131700|emb|AJ318769.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM146
Clone 349	gi|15131699|emb|AJ318768.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM936
Clone 350	gi|15131698|emb|AJ318767.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM1636
Clone 351	gi|15131697|emb|AJ318766.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM2012
Clone 352	gi|15131696|emb|AJ318765.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM1812
Clone 353	gi|15131695|emb|AJ318764.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM412
Clone 354	gi|15131694|emb|AJ318763.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM1034
Clone 355	gi|15131693|emb|AJ318762.1| Uncultured soil bacterium partial 16S rRNA gene, clone FHM2110
A	A current study
B	B current study
C	C current study
G	G current study
H	H current study

Table 19. NBCI association numbers for the clones which have been used in the construction of the *ureC* phylogenetic tree.

Clone Suffix	Code name and NCBI association number
Clone_U001	gi|222112953|emb|FM991842.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U21
Clone_U002	gi|222112951|emb|FM991841.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U19
Clone_U003	gi|222112949|emb|FM991839.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U12
Clone_U004	gi|222112947|emb|FM991838.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U25
Clone_U005	gi|222112945|emb|FM991837.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U24
Clone_U006	gi|222112943|emb|FM991836.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U23
Clone_U007	gi|222112941|emb|FM991835.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U21
Clone_U008	gi|222112939|emb|FM991834.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U20
Clone_U009	gi|222112937|emb|FM991833.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U19
Clone_U010	gi|222112935|emb|FM991832.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U17
Clone_U011	gi|222112933|emb|FM991831.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U14
Clone_U012	gi|222112931|emb|FM991830.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U13
Clone_U013	gi|222112929|emb|FM991829.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U12
Clone_U014	gi|222112927|emb|FM991828.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U09
Clone_U015	gi|222112925|emb|FM991827.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U08
Clone_U016	gi|222112923|emb|FM991826.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U07
Clone_U017	gi|222112921|emb|FM991825.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U06
Clone_U018	gi|222112919|emb|FM991824.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U05
Clone_U019	gi|222112917|emb|FM991823.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U03
Clone_U020	gi|222112915|emb|FM991822.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P9U01
Clone_U021	gi|222112913|emb|FM991821.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U21
Clone_U022	gi|222112911|emb|FM991820.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U19
Clone_U023	gi|222112909|emb|FM991819.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U17
Clone_U024	gi|222112907|emb|FM991818.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U16
Clone_U025	gi|222112905|emb|FM991817.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U15
Clone_U026	gi|222112903|emb|FM991816.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U14
Clone_U027	gi|222112901|emb|FM991815.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U13
Clone_U028	gi|222112899|emb|FM991814.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U11
Clone_U029	gi|222112897|emb|FM991813.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U10
Clone_U030	gi|222112895|emb|FM991812.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U09
Clone_U031	gi|222112893|emb|FM991811.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U08
Clone_U032	gi|222112891|emb|FM991810.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U07
Clone_U033	gi|222112889|emb|FM991809.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U06
Clone_U034	gi|222112887|emb|FM991808.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U05
Clone_U035	gi|222112885|emb|FM991807.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U04
Clone_U036	gi|222112883|emb|FM991806.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U03
Clone_U037	gi|222112881|emb|FM991805.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P8U01
Clone_U038	gi|222112879|emb|FM991804.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P3U21

Clone_U039	gi|222112877|emb|FM991803.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P3U16
Clone_U040	gi|222112875|emb|FM991802.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P3U15
Clone_U041	gi|222112873|emb|FM991801.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P3U14|
Clone_U042	gi|222112871|emb|FM991800.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P3U13
Clone_U043	gi|222112869|emb|FM991799.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P3U08
Clone_U044	gi|222112867|emb|FM991798.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P3U06
Clone_U045	gi|222112865|emb|FM991797.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P3U05
Clone_U046	gi|222112863|emb|FM991796.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P3U04
Clone_U047	gi|222112861|emb|FM991795.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U25
Clone_U048	gi|222112859|emb|FM991794.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U24
Clone_U049	gi|222112857|emb|FM991793.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U21
Clone_U050	gi|222112855|emb|FM991792.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U20
Clone_U051	gi|222112853|emb|FM991791.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U14
Clone_U052	gi|222112851|emb|FM991790.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U13
Clone_U053	gi|222112849|emb|FM991789.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U12
Clone_U054	gi|222112847|emb|FM991788.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U10
Clone_U055	gi|222112845|emb|FM991787.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U09
Clone_U056	gi|222112843|emb|FM991786.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U06
Clone_U057	gi|222112841|emb|FM991785.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U05
Clone_U058	gi|222112839|emb|FM991784.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D15P2U04
Clone_U059	gi|222112837|emb|FM991783.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U23
Clone_U060	gi|222112835|emb|FM991782.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U21
Clone_U061	gi|222112833|emb|FM991781.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U20
Clone_U062	gi|222112831|emb|FM991780.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U19
Clone_U063	gi|222112829|emb|FM991779.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U18
Clone_U064	gi|222112827|emb|FM991778.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U17
Clone_U065	gi|222112825|emb|FM991777.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U13
Clone_U066	gi|222112823|emb|FM991776.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U11
Clone_U067	gi|222112821|emb|FM991775.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U10
Clone_U068	gi|222112819|emb|FM991774.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U08
Clone_U069	gi|222112817|emb|FM991773.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U07
Clone_U070	gi|222112815|emb|FM991772.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U06
Clone_U071	gi|222112813|emb|FM991771.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P9U05
Clone_U072	gi|222112811|emb|FM991770.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U21
Clone_U073	gi|222112809|emb|FM991769.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U19
Clone_U074	gi|222112807|emb|FM991768.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U18
Clone_U075	gi|222112805|emb|FM991767.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U17
Clone_U076	gi|222112803|emb|FM991766.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U15
Clone_U077	gi|222112801|emb|FM991765.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U14
Clone_U078	gi|222112799|emb|FM991764.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U12
Clone_U079	gi|222112797|emb|FM991763.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U10
Clone_U080	gi|222112795|emb|FM991762.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U09
Clone_U081	gi|222112793|emb|FM991761.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U08
Clone_U082	gi|222112791|emb|FM991760.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U07
Clone_U083	gi|222112789|emb|FM991759.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U03
Clone_U084	gi|222112787|emb|FM991758.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P8U01
Clone_U085	gi|222112785|emb|FM991757.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U24
Clone_U086	gi|222112783|emb|FM991756.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U23
Clone_U087	gi|222112781|emb|FM991755.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U16
Clone_U088	gi|222112779|emb|FM991754.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U14
Clone_U089	gi|222112777|emb|FM991753.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U13
Clone_U090	gi|222112775|emb|FM991752.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U11
Clone_U091	gi|222112773|emb|FM991751.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U05
Clone_U092	gi|222112771|emb|FM991750.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U03
Clone_U093	gi|222112769|emb|FM991749.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P3U01
Clone_U094	gi|222112767|emb|FM991748.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U24
Clone_U095	gi|222112765|emb|FM991747.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U19
Clone_U096	gi|222112763|emb|FM991746.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U12
Clone_U097	gi|222112761|emb|FM991745.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U11
Clone_U098	gi|222112759|emb|FM991744.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U10
Clone_U099	gi|222112757|emb|FM991743.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U20
Clone_U100	gi|222112755|emb|FM991742.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U09
Clone_U101	gi|222112753|emb|FM991741.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U06
Clone_U102	gi|222112751|emb|FM991740.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U05
Clone_U103	gi|222112749|emb|FM991739.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U02
Clone_U104	gi|222112747|emb|FM991738.1| Uncultured soil bacterium partial ureC gene for urease subunit alpha, clone D01P2U01
A	A current study
B	B current study

C	C current study
D	D current study
E	E current study