Expression of an Indolicidin Analogue in *Pichia Pastoris*

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ABSTRACT

This study deals with the expression of a single tryptophan analogue to indolicidin, IL4 (ILPWKLPLLPLRR), using *P.pastoris* as expression host. The cloning strategy that was developed, centered around the secretion of IL4 into the medium. A pPICZαA/IL4 vector was constructed that coded for IL4, using the pPICZαA vector. Transformation of *P.pastoris* with *Sac*I linearized pPICZαA/IL4 was done using electroporation. Recombinant IL4 was produced and secreted into the medium. SPPS was used to chemically synthesis IL4. Recombinant IL4 and synthetic IL4 was purified using size exclusion chromatography and analyzed using MALDI–TOF MS and LC–ECI MS–MS. Disk diffusion growth inhibition was used to test the antimicrobial activity of the synthesized IL4 and two commercial detergents against gram positive *B.subtilis* and gram negative *P.putida*.

Preface

This thesis has been composed by Stig Castberg at the Technical-Scientific Faculty at Aalborg University.
Book- and article sources are referred to in the text by [author, year of publication]. Websites are denoted by a number in square parenthesis, which refers to the source in the Bibliography. All sources of figures are mentioned under the figures or in the appropriate paragraph. When no source is mentioned, the author of this report have made the figures.
I would like to thank the following people for help during the project period:

- Bo Højen Justesen for good corporation on the experimental part of this project.

In loving memory of
my father, Svend,
and
god–father, Jens Erik.

Contents

1 Introduction

The need for new antibiotics is ever rising. The number of death from hospital infections in the United Stats of America is more then 100,000 and 70% of these deaths are contributed to multi–resistant bacterias. According to the American Journal of Infection Control nearly 2.4% of all patients in the American hospitals are infected with methicillin–resistant *Staphylococcus aureus* or in raw numbers around 880,000 people a year. [1, Payne, 2008] In comparison the number of deaths from HIV and AIDS are around 15000 each year and it is estimated that in 2006 that around 56,300 Americans became newly infected with HIV. [2]
There is a real need for new antibiotics that can combat these infections. Since the 1960's there has only been introduced two new classes of antibiotics into the clinic and the number of new antibiotics waiting to be approved by the US Food and Drug Administration is 60% lower than in the 1980's. [Von Bubnoff, 2006] A new class of possible antibiotics is the antimicrobial peptides (AMPs), which exhibits a broad spectrum of antimicrobial activity against bacteria, viruses, and fungi. AMPs are distributed throughout the animal and plant kingdom. From species such as bacteria, fungi, insects, and mammals more than 1400 different AMPs have been found to date. [3] The distribution of AMPs suggests that they have served in the evolution of complex multicellular organisms. Despite their ancient linage AMPs continue to function as a defensive barrier, thus confounding the common belief that microbes will build up a resistance to any substance. It is probable that the lack of acquired resistance is due to the fact that AMPs target the bacterial membrane and the large diversity of the primary structure of the AMPs. [Zasloff, 2002] Categorizing AMPs is very difficult as they are very diverse. The categorizing system that is currently used is based broadly on their secondary structure and four groups have been devised. β–sheet molecules stabilized by two or three disulfide bridges amphiphilic α–helices, linear peptides, and loops stabilized by a single disulfide bridge. [Hancock, 2001, Zasloff, 2002] The sequences of AMPs indicate that the number of amino acids vary from 11 to 50, contains about 50% hydrophobic residues and that AMPs for the majority are positively charged. [Hancock, 2001]
The preparation of peptide antibodies at a large scale poses significant challenges. Among them is the amount of produced peptides. [Rao et al., 2004] Commercial production of peptides is primarily done through peptide synthesis and the number of produced peptides containing more then 30 amino acids is very low. One of these peptides is Enfuvirtide, a 36 amino acid peptide, that is used is in the inhibition of HIV–1 membrane fusion. It is estimated that every patient needs 80g of Enfuvirtide every year, so it was estimated that the production of Enfuvirtide should exceed 3 tonnes per year to satisfy the US market. In 2002 Roche (www.roche.com) were capable of producing several tonnes of commercial approved Enfuvirtide a year by means of chemical synthesis. [Bray, 2003]
Using bacteria is the simplest and most inexpensive means to produce heterologous proteins. However, when trying to express peptides at a high concentration the peptides can become toxic the host. Systems have been developed that have focused on fusing the antibiotic peptide to a protein that has anionic properties. This fusion diminishes the toxicity of the peptide towards the host. There are difficulties surrounding the use of fusion proteins among those are the subsequent isolation and a relative low yield of peptide is obtained. [Rao et al., 2004] Another possibility is the use of yeast as expression host. Yeast has several advantages over bacteria. It allows for post translation processing and secretion into the media. Also the yeast expression systems have a high cost efficiency. [Cereghino et al., 2000, Daly and Hearn, 2005] There has been reports of AMPs being produced using the expression system of *P.pastoris*. [Zhang et al., 2006, Jin et al., 2009]

1.1 Yeast as an Expression Organism

1.1.1 Methylotrophic Yeasts.

Methylotrophic Yeasts was first discovered in the 1970's and was isolated from rotten fruit, soil, and the gut of insects. [Faber et al., 1995, Jungo et al., 2006] Methylotrophic yeasts can utilize methanol as a sole source of carbon and energy. All strains with this ability have been found to belong to four genera, namely; *Canadia*, *Hansenula*, *Pichia*, and *Torulopsis*. [Faber et al., 1995] The first proposed use of methylotrophic Yeast was as a product for animal feed, though due to the oil crisis of the 70's, methanol was too expensive when compared to growing e.g. soybeans. [Macauley-Patrick et al. 2005] Phillips Petroleum Company was the first to develop protocols for *Pichia pastoris* for the application of heterologous protein expression and licensed the Invitrogen Corporation to market the components. [Cereghino et al., 2000]

1.1.2 *Pichia Pastoris*

As stated previously *P.pastoris* is a methylotrophic yeast, which means that it can metabolize methanol to gain carbon and energy. The first step of the methanol–utilizing pathway is the oxidation of methanol to formaldehyde and hydrogen peroxide by the enzyme alcohol oxidase (AOX). The hydrogen peroxide is degraded into water and oxygen by the enzyme catalase. These reactions takes place in the peroxisome as hydrogen peroxide is toxic the yeast cells. The last enzyme of the methanol–utilizing pathway that is located inside the peroxisome is the dihydroxyacetone synthase (DAS). This enzyme catalyzes formaldehyde into glyceraldehyde 3-phosphate and dihydroxyacetone. [Cereghino et al., 2000] The methanol–utilizing pathway can be seen in figure 1.1.

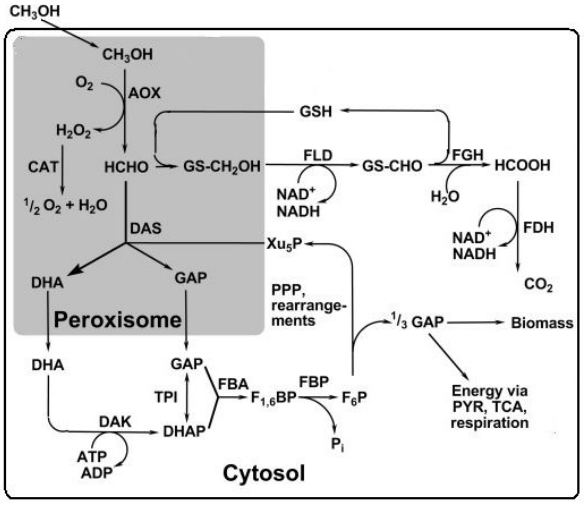

Figure 1.1: The methanol–utilizing pathway in methylotrophic yeasts: The main pathways and the respective enzymes working in the methanol metabolism in methylotrophic yeasts are shown. AOX: alcohol oxidase, CAT: catalase, FLD: formaldehyde dehydrogenase, FGH: S-formylglutathione hydrolase, FDH: formate dehydrogenase, DAS: dihydroxyacetone synthase, TPI: triosephosphate isomerase, DAK: dihydroxyacetone kinase, FBA: fructose 1,6-bisphosphate aldolase, FBP: fructose 1,6-bisphosphatase, DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate, F1,6BP: fructose 1,6-bisphosphate, F6P: fructose 6-phosphate, Pi: phosphate, Xu5P: xylulose 5-phosphate, GSH: glutathione, PYR: pyruvate; PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle [4].

AOX has a low catalytic affinity for oxygen and as a result the cells produce a high amount of the enzyme to compensate. [Koutz et al., 1989] It is possible for AOX to reach 35% of

the total cellular protein when grown solely on methanol. [Sreekrishna et al., 1997] When grown on other carbon scources e.g. ethanol, glycerol, or glycose the enzyme is undetectable. [Larentis et al., 2004] The identification of the genes coding for AOX were done by a cDNA screening of the gene libary for genes, that are active when the cells are grown on methanol. Several sequences were found and corolated with the amino acid sequence of AOX. [Ellis et al., 1985, Koutz et al., 1989] Two genes were found that encode AOX and these were named AOX1 and AOX2, where AOX1 is the main contributor for the production of AOX and AOX2 only contributes with a minor fraction, around 5 to 10% of the total volume of AOX. [Cregg et al., 1993, Macauley-Patrick et al. 2005] Nothern blot was used to analyse the AOX1 mRNA levels in order to study the expression level of the AOX1 gene. When grown on methanol the AOX1 mRNA could represent approximately 5% of the total poly(A)$^+$ RNA, though when grown on glycerol the mRNA is undetectable. [Cregg et al., 1993] Several expression vectors have been designed for *P.pastoris* to utilize the promoter P_{AOX1} for the AOX1 gene. P_{AOX1} is highly inducible and tightly controlled. The regulation of the promoter is done on the transcriptional level and is controlled by a repression and induction mechanism. The promoter represses expression when carbon sources such as glucose, glycerol, or ethanol are present and it induces expression when methanol is present. [Ohi et al., 1994] This requirement of the AOX1 promoter for methanol in order to induce expression is very useful in large–volume high–density fermentation cultures. Other systems would require a high concentration of the repressing carbon substrate, that is also required to be removed before induction. For *P.pastoris* cultures it is enough to grow the culture on a concentration of glycerol that meets the growth requirements. Thus, to allow for induction, the cells can simply utilize the remaining glycerol and then methanol can be added starting the induction. [Cregg et al., 1993]
Integration of the expression cassette into the genome by use of recombination events is a commonly used technique in order to create a stable clone. The target of these recombination events are preferred at the AOX1 locus. [Sreekrishna et al., 1997, Glick et al., 2003] To control the site for homologous integration the vector used, needs a sequence of the genomic locus of the required integration site. Two types of integration are possible in *P.pastoris*, either single or double recombination, see figure 1.2. [Glick et al., 2003]

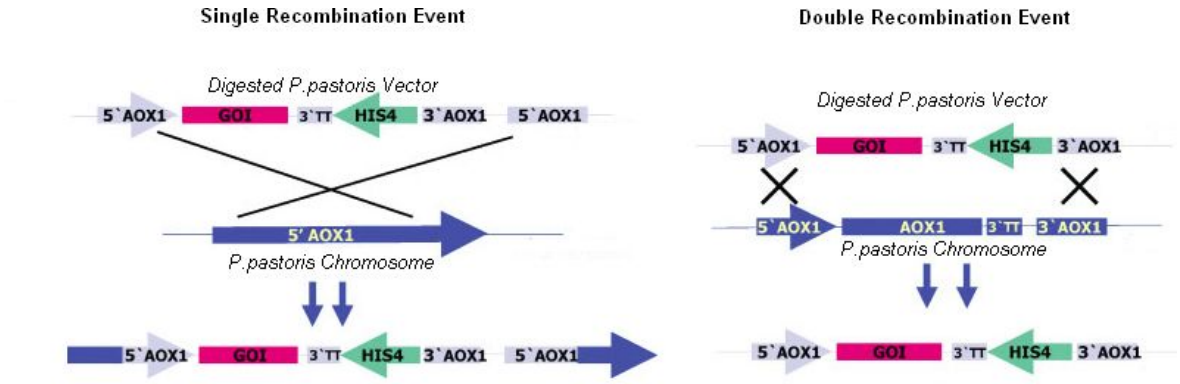

Figure 1.2: An illustration of a single and double recombination event located at the AOX1 locus. During the single recombination event the entire vector is inserted into the AOX1 locus without any disruption of the gene. The double recombination event exchanges the AOX1 gene with the expression cassette of the vector. Figure modified from [Daly and Hearn, 2005]

During a single recombination event the vector is integrated into the locus without disrupting the gene. Linearizing the vector allows for an increase in the frequency of single recombination events. At a frequency of 50–80% the single recombination event is the most frequent of the crossover events. [Cereghino et al., 2000] During the double recombination

event the AOX1 gene is replaced with the expression cassette. Digesting the vector such that the expression cassette is flanked by 5´ and 3´ regions of the AOX1 gene can result in double recombination. This recombination even occurs at a frequency of 5–25%. [Cereghino et al., 2000, Daly and Hearn, 2005] A reduction of *P.pastoris* ability to utilize methanol can occur after a double recombination that has replaced the AOX1 gene and thereby prompting the cell to use the less efficient AOX2 gene instead. [Ohi et al., 1994] There are several advantages of integrating the expression cassette into the genome. In the absence of selective pressure the clone will still have a high level of stability. Thus, it is possible to run an expression without the use of selective pressure. [Cereghino et al., 2000] Also it is possible to integrate multiple expression cassettes into the chromosome, resulting in the creation of a multicopy strain. Multicopy strains are a spontaneously occurring event that happen with a frequency of 1–10%. [Romanos et al., 1995] It was observed by [Hohenblum et al. 2003] that there is a positive correlation between copy number and expression level, in the increase from a copy number of 1 to 2. When high copy numbers were reached a negative correlation was observed and even a lowering of the cell viability, thus releasing cellular protein into the medium.
The presence of proteins secreted by native *P.pastoris* is quite low this presents an advantage when secreting the recombinant protein into the medium. It is possible to reach up to 80–90% recombinant protein of the total amount of secreted protein in the medium.. [Faber et al., 1995] In order to facilitate the secreting a signal sequence is needed. The signal sequence directs the recombinant protein into the secretory pathway. The alpha mating factor that has been derived from *S.cerevisiae* is the most commonly used signal sequence, due to its success. [Brake et al., 1984] The alpha mating factor prepro peptide is made up by a 19 amino acid signal (pre) sequence followed by a 66 amino acid (pro) sequence. The pro region contains the Kex2 endopeptidase site and the Ste13 dipeptidyl aminopeptidase site. [Brake et al., 1984, Cereghino et al., 2000] The recombinant protein is fused to the alpha mating factor carrier after the Kex2 cleavage site. Upon post translational translocation into the endoplasmic reticulum the pre region of the carrier is removed by the signal peptidase. In the separation of the pro region two processes have to occur. First a cleavage of the Kex2 site by the Kex2 peptidase that is conducted in the Golgi and then just before secretion, the Glu–Ala repeats are cleaved by the Ste13 dipeptidyl aminopeptidase. These cleavages results in the separation of the recombinant protein from the carrier and the recombinant protein is the released into the extracellular medium. [Kurjan and Herskowitz, 1982, Sievi, 2002]

1.2 Activity Study of AMPs

In the view of AMPs potential use as a novel antimicrobial therapeutic agent, their specificity and potency toward microbes, and not mammals, are of key interest for further improvements. Before improvement of these properties can be done in an efficient manner, it is necessary to understand the mechanisms behind the antimicrobial activity and the selectivity toward microbes. [Epand and Vogel, 1999]
An unambiguous description of the mechanisms by which AMPs exhibit their antimicrobial activity has not yet been established. Though, ongoing research and experiments have identified several factors which may have an influence on the antimicrobial activity, thereby further increasing the understanding of the mechanism of action. One of the main indications from the research on the mechanisms behind the antimicrobial activity of AMPs is that the membrane of the bacteria is the target for the peptides. This is also supported by the fact that almost all AMPs are amphipathic, containing hydrophobic and hydrophilic areas, allowing them both to interact with the negative charged surface of the bacterial membrane and to enter into the membrane interior [Wu et al., 1999].

The interaction of the AMPs with the negative charged bacterial membrane also provides an explanation for the selectivity toward bacterial membranes, rather then mammalian membranes. This is due to the fact that the membranes of gram–negative bacteria contain anionic molecules, contrary to the membrane of eukaryotes as in the case of mammalians, which contain zwitterionic amphiphiles. [Epand and Vogel, 1999]
Experiments using model membranes of planar bilayers or liposomes show that cationic peptides are generally able to interact with the membranes inserting themselves into the membranes resulting in the formation of transmembrane channels. In the case of peptides containing α–helices, the creation of transmembrane channels can be described by a barrel–stave model, where the α–helices form bundles arranging their hydrophobic surfaces toward the lipid core of the membrane, and their hydrophilic surfaces into the channel. This arrangement is mainly driven by hydrophobic interactions, and thus these peptides can bind to both zwitterionic and charged phospholipid membranes. The barrel–stave mechanism is illustrated in figure 1.3. [Oren and Shai, 1998]
Based on the assumption that this also happens in biological membranes it has been proposed that the formation of such channels by AMPs leads to the leakage of cell components, the loss of membrane potential, and eventually cell death [Wu et al., 1999]. Though the observation in model membrane systems, that a very high peptide to lipid ratio is required for membrane disruption, led [Shai, 1995] to propose another model to explain the permeability of the membrane. This model, called the carpet model, proposes that the peptides initially binds to the surface of the membrane, covering it in a carpet–like manner. When a threshold concentration value of the peptides are reached they collapse inward, the membrane ruptures, and the cell undergoes lysis. The carpet model is illustrated in figure 1.3. [Oren and Shai, 1998]

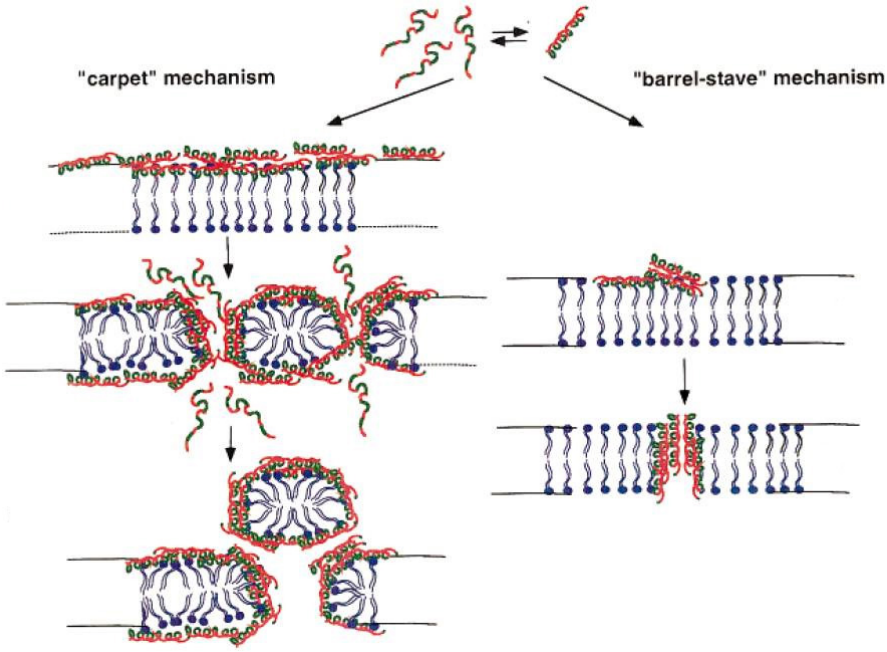

Figure 1.3: Illustration of the barrel–stave mechanisms(right), and the carpet model(left). The barrel–stave mechanism involves three steps: (a) binding of the peptides to the membrane, (b) molecular recognition between the membrane–bound peptides which leads to their assembly, and (c) insertion of the assembled monomers into the membrane, which initiates the formation of a channel. The carpet model involves three steps: (a) preferential binding of the positively charged peptides to the membrane, (b) rotation of the peptides so that the hydrophobic areas are oriented towards the hydrophobic core of the membrane, and (c) disintegration of the membrane by the disruption of the curvature of the bilayer, leading to the formation of micelles. [Oren and Shai, 1998]

Experiments on the membrane of an *Escherichia coli* strain, measuring the channel formation, using 12 different peptides covering all the structural classes of AMPs were performed by [Wu et al., 1999]. These experiments showed that the different peptides varied substantially in their ability to depolarize the membrane potential. Also there was no absolute correlation between a peptides ability to depolarize the membrane potential and its antimicrobial activity. This indicated, at least for these 12 peptides, that the mechanism of action does not include the formation of channels in the membrane. Furthermore, the data were not consistent with the carpet model proposed by [Shai, 1995]. Also [Wu et al., 1999] observed that small peptides (10–14 amino acids), which are not able to span the membrane, show similar effect on the membrane potential as the larger ones. This effect can not be explained using the barrel–stave mechanism described above, as the peptides are not large enough to create channels through the membrane. [Matsuzaki et al., 1998] proposed a model wherein the peptides form an amphipathic helix in the lipid bilayers, oriented parallel to the membrane surface. This will result in the formation of a dynamic supramolecular complex, consisting on an average of five helices and the surrounding lipids. The peptides are able to flip inward, carrying lipids with them, resulting in short term disruptions in the permeability of the membrane. Also when the supramolecular complex disintegrates, some of the peptides will be dislocated into the inner leaflet of the membrane. [Wu et al., 1999] proposed that this model can be used to describe the ability of the smaller peptides to influence the membrane potential.
Based on these experiments [Wu et al., 1999] suggested that the disruption of the bacterial membrane is not the primary mechanism of action for the AMPs. Rather, the ability of the peptides to interact with the membranes reflects their mechanism of passage across the membrane, and their actual targets are in the cytoplasm of the bacteria e.g. the AMPs have shown to interact readily with nucleic acids [Zhang et al., 1999].
A wide range of other mechanisms of action has been proposed including; stimulation of autolytic enzymes [Wu et al., 1999], interference with bacterial DNA [Boman et al., 1993], binding to and inhibition of cellular nucleic acids [Park et al., 1998].

1.3 Indolicidin, an Antimicrobial Peptide

From the cytoplasmic granules of bovine neutrophils [Selsted et al., 1992] obtained a mixture of bactericidal molecules and from this mixture they isolated a novel peptide with bactericidal properties. The peptide consisted of 13 amino acids and of these 13 residues 5 were tryptophan. This unusual large concentration (39%) of indole side chain–containing tryptophan and the peptide's potent antimicrobial activity prompted [Selsted et al., 1992] to name the peptide indolicidin. The amino acid sequence and structure of indolicidin can be seen in figure 1.4. The native indolicdin is amidated at the C–terminus and with a single Lys and two Arg residues, the net positive charge at physiological pH is +4. [Subbalakshmi et al., 2000]
Indolicidin belongs to the Cathelicidin family of AMPs and it exhibits a broad spectrum of antimicrobial activity that includes Gram positive and Gram negative bacteria, protozoa, fungi, and the enveloped virus HIV–1. [Sitaram and Nagaraj, 2002, Muñoz et al., 2007] Against Gram positive and Gram negative bacteria the MIC of indolicidin ranges from 4–64μg/ml and the antifungal activities are located in a similar range. [Falla et al., 1996, Sitaram and Nagaraj, 2002] In addition to indolicidins antimicrobial activities, it has been shown to exhibit hemolytic activity as well as cytotoxicity towards the human T–lymphocytes. These properties complicate the use of indolicidin as a therapeutic agent. Though the MIC against *E.coli* is 0.4μM which is almost 40 times smaller than the minimal hemolytic concentration of 15.6μM that results in the lysis of erythrocytes of rats. [Schluesener et al., 1993, Ahmad et al., 1995] Thus, attempts to alter the structure of indolicidin has been conducted in an effort to modulate its activity and thus creating a therapeutic agent. Of particu-

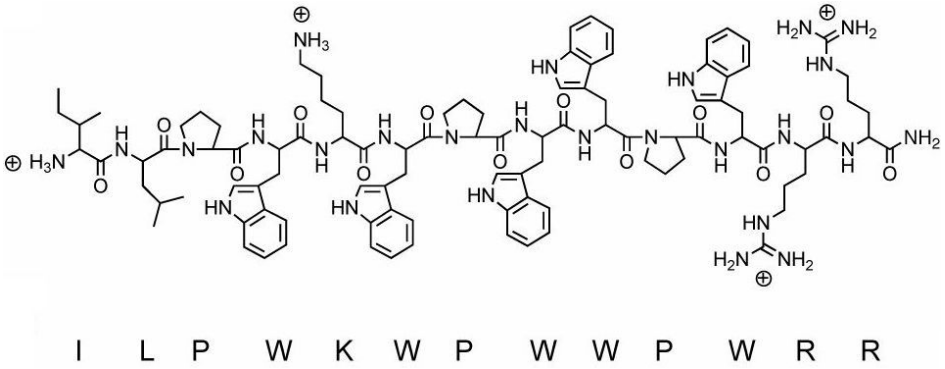

Figure 1.4: The amino acids sequence and structure of indolicidin. [Marchand et al., 2006]

lar interest has been the tryptophan and proline residues. [Subbalakshmi et al., 1996] and [Friedrich et al., 2000] both synthesized an indolicidin analogue where the 3 proline residues had been substituted with alanine, the analogue was named CP10A. It exhibited similar activity towards gram negative bacteria as native indolicidin and increased activity against gram positive and CP10A became more efficient at disrupting the membrane potential of bacteria, though lysis of the membrane was not observed. Additionally the hemolytic activity against rat erythrocytes was also shown to increase. Structural studies of CP10A revealed an α–helical conformation that may be the reason for the increased activity. [Friedrich et al., 2000] An indolicidin analogue, named ILA, was synthesized by [Subbalakshmi et al., 1996] in which the 5 tryptophan residues were substituted with phenylalanine. The activity of ILA was similar to native indolicidin against gram negative and gram positive bacteria. The hemolytic activity against rat erythrocytes had been decreased. At a peptide concentration of 30μM ILA exhibited 20% hemolysis whereas native indolicidin at this concentration exhibited 100% hemolysis. [Subbalakshmi et al., 1996] Single tryptophan analogues of indolicidin were synthesized by [Subbalakshmi et al., 2000]. The tryptophan residues were replaced with leusine, except for the residues 4, 8, or, 11, thus, creating the analogues named IL4, IL8, and IL11, respectively. These analogues showed lower antimicrobial activity than native indolicidin but no hemolytic activity against rat erythrocytes were seen for of any 3 analogues up to a concentration of 100μg/ml. Table 1.1 shows the minimal inhibition concentration and hemolytic activity of indolicidin and the 3 analogues, IL4, IL8, and IL11. [Subbalakshmi et al., 2000]

Peptide	*E.coli* (MIC μg/ml)	*S.aureus* (MIC μg/ml)	% hemolytic activity at 100μg/ml
IL	5	2	100
IL4	15	10	0
IL8	20	10	0
IL11	20	5	0

Table 1.1: The minimal inhibition concentration and hemolytic activity of indolicidin and the 3 analogues, IL4, IL8, and IL11. [Subbalakshmi et al., 2000]

As can be seen from [Subbalakshmi et al., 1996] and [Subbalakshmi et al., 2000], tryptophane only exhibits a minor effect on the bactericidal activity of indolicidin whereas it plays an important part in the hemolytic activity. This reduction in hemolytic activity makes the indolicidin analogues interesting for further study and in the development of a therapeutic agent.

1.4 Cloning Strategy

The indolicidin analogue, IL4, containing a single Trp [Subbalakshmi et al., 2000] was used in this project. Figure 1.5 shows the primary structure of IL4.

ILPWKLPLLPLRR-amide

Figure 1.5: The primary sequence of indolicidin IL4

Codon optimization for *S.cerevisiae* was used to translate the amino acid sequence into the DNA sequence that can be seen in figure 1.6.

1	2	3	4	5	6	7	8	9	10	11	12	13	
Ile	Leu	Pro	Trp	Lys	Lue	Pro	Lue	Lue	Pro	Lue	Arg	Arg	
5'- ATT	TTG	CCA	TGG	AAG	TTG	CAA	TTG	TTG	CCA	TTG	AGA	AGA	-3'
3'- TAA	AAC	GGT	ACC	TTC	AAC	GTT	AAC	AAC	GGT	AAC	TCT	TCT	-5'

Figure 1.6: DNA sequence of IL4 using codon optimization for *S.cerevisiae*.

The shuttle vector pPICZαA, as seen in figure 1.7, was chosen for cloning and expression of the recombinant IL4 in *P.pastoris*.

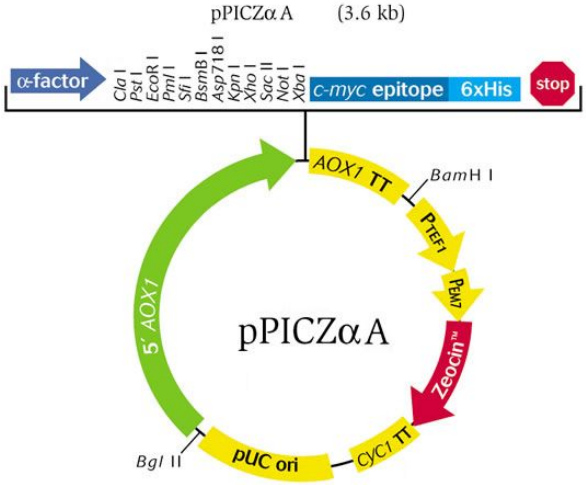

Figure 1.7: Vector map of pPICZαA. The vector has a 5´AOX1 promoter and a AOX1 transcription termination region obtained from the AOX1 gene. Replication in *E.coli* is controled by the pUC origin. The *Sh ble* gene (marked zeocin^{TM}) confers resistance to the antibiotic Zeocin^{TM} and the expression of this gene is driven by the P_{TEF1} and P_{EM7}. The expression cassette consists of an α–factor secretion signal followed by the MCS, a c–myc epitope, and a C–terminal His–Tag. [Invitrogen, 2008]

For pPICZαA the selection marker is the *Sh ble* gene that conveys resistance against Zeocin^{TM}. The structure of Zeocin^{TM} can be seen in figure 1.8. Zeocin^{TM} is a water-soluble, copper–chelated glycopeptide originally isolated from *Streptomyces verticillus* and it is part of the bleomycin family. In the copper–chelated form Zeocin^{TM} is inactive, even though bleomycin antibiotics perturb the plasma membrane. The antimicrobial activity stems from the reduction of the copper cation from Cu^{2+} to Cu^{1+} and subsequent removal by sulfhydryl compounds in the cell. When the copper is removed, Zeocin^{TM} becomes active, binds to the DNA and starts to cleave it, thus resulting in cell death. [Invitrogen, 2001] P_{EM7} is the promotor that drives the production of Zeocin^{TM} resistance in *E.coli*. In *P.pastoris* the promotor is P_{TEF1}.

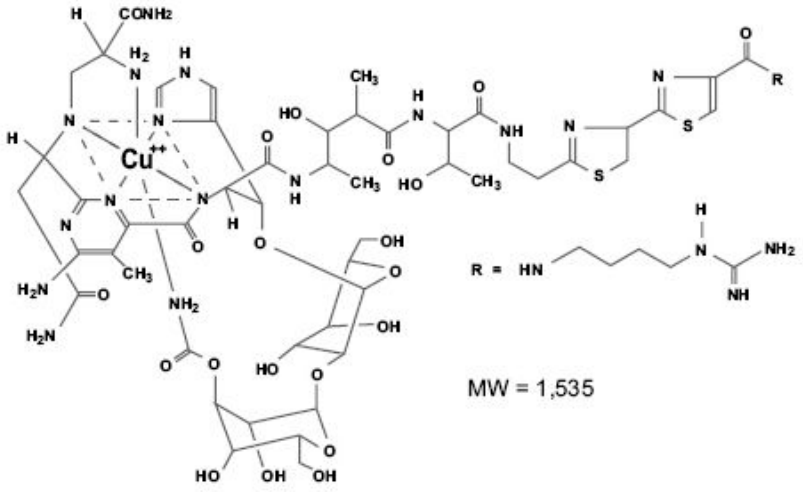

Figure 1.8: The structure of Zeocin^{TM}. [Invitrogen, 2008]

Linearizing the pPICZαA vector in the promoter region allows for homologous recombination events to occur. These events are targeted at the AOX1 gene and thus, it is possible to integrate the pPICZαA vector into the genome, creating a stable clone even in the absence of Zeocin^{TM}. The crossover recombination will result in the gain of the P_{AOX1} promoter that is highly inducible by methanol, but repressed in the presence of glycerol. As can be seen from figure 1.7 the vector contains a α–factor secretion signal upstream from the MCS. The α–factor secretion signal allows for secretion of the peptide into the medium.
The design of the insert allows for the secretion of IL4 from the cell into the medium by use of the α–factor signal sequence. The pPICZαA vector is digested at the two *Xho*I sites and the *Not*I site allowing for direct access to the α–factor signal sequence. The C–terminus of the α–factor secretion signal is added to the insert, excluding the Glu–Ala repeats normally processed by the Ste13 dipeptidyl aminopeptidase. Since IL4 is secreted into the medium no His–tag or c–myc epitope is required and a double stop codon (TAATAA) is placed at the c–terminus of the insert to terminate the translation. A naturally occurring *Nco*I site is located within the insert, in addition to a *Nco*I site located at position 2162 on the pPICZαA vector. This allows for verification of correct cloning by digestion using the *Nco*I restriction enzyme. The digestion of the vector without insert results in a single fragment of 3.6 kb and the digestion of the vector with insert results in two fragments of 959 bp and 2634 bp. In figure 1.9 a sequence of the pPICZαA/Il4 vector in the region containing α–factor signal sequence and the multiple cloning site (MCS), can be seen.
For cloning and propagation of the pPICZαA vector *E.coli* DH5α was chosen. *Sac*I is used to linearize pPICZαA/IL4. The *P.pastoris* X–33 cells are then transformed with the linearized pPICZαA/IL4 by electroporation. By use of PCR the insertion of the expression cassette is verified. From the primers α–factor and 3´AOX1 a PCR product of 281bp is expected for the pPICZαA/IL4 vector. From the primers IL4p and 3´AOX1 a PCR product of 223bp is expected for the pPICZαA/IL4 vector. The IL4p primer is located in the expression cassette within the coding area of IL4, thus preventing the use of false positives with only a partial integration of the expression cassette.

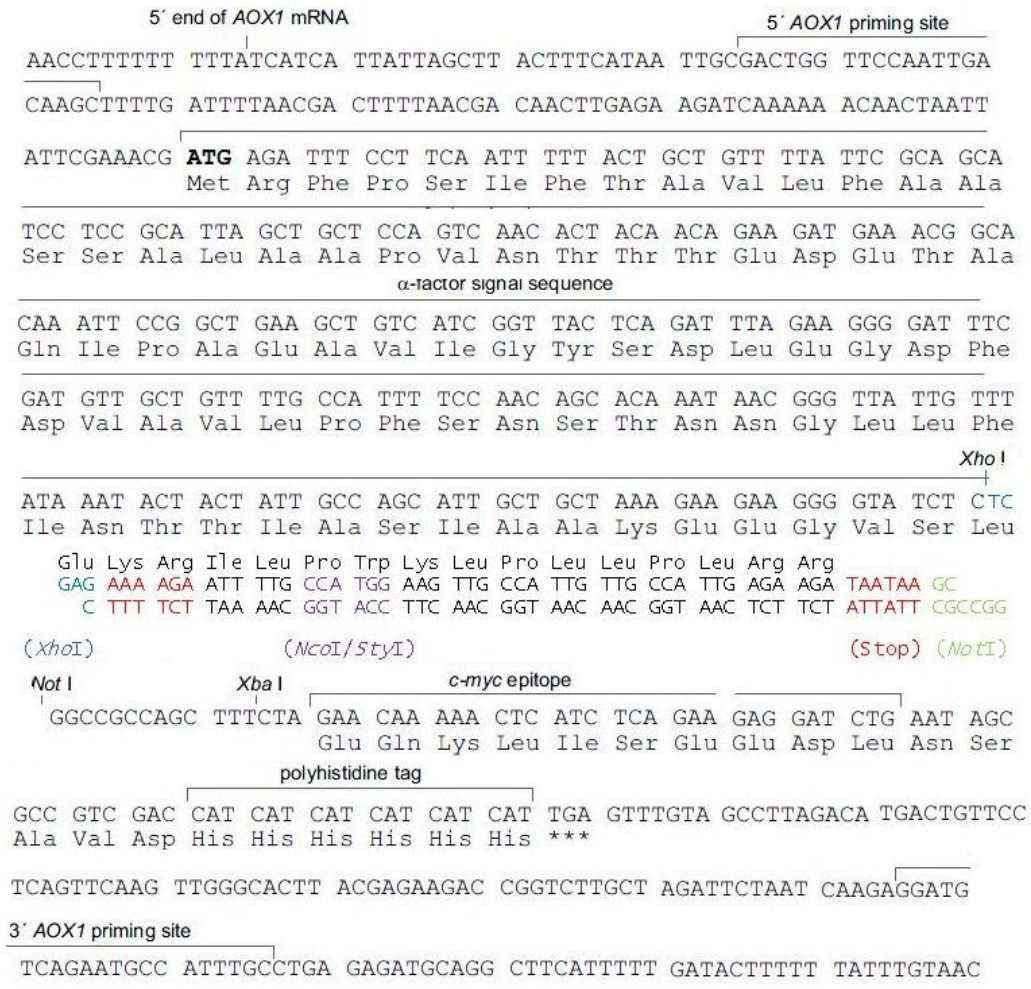

Figure 1.9: Sequence of the region containing α–factor signal sequence and the MCS of the pPICZαA/IL4 vector. The insert is ligated into the area between the *Xho*I restriction site and the *Not*I restriction site.

Materials & Methods 2

2.1 Materials

The materials used are from the companies Sigma–Aldrich, Invitrogen, Fermentas, or New England Biolabs.

2.1.1 Microorganisms and Plasmids

Table 2.1 shows the different bacterial and yeast strains and plasmids used in this work.

Strains and Plasmids	Description	Reference or source
Bacterial strain		
E.coli DH5α	*fhuA2*Δ*(argF–lacZ)U169 proA glnV44* Σ*80*Δ*(lacZ) M15 gyrA96 recA1 relA1 endA1 thi–1 hsdR17*	New England Biolabs
Yeast Strains		
P.pastoris X–33	Wild–type, Mut$^+$	Invitrogen
Plasmids		
pPICZαA	Zeocin resistance through the *Sh ble* gene. Shuttle vector for propagation in *E.coli* and integration in *P.pastoris*	Invitrogen
pPICZα/IL4	pPICZαA containing a *Xho*I and *Not*I gene fragment coding for IL4	This work

Table 2.1: Bacterial and Yeast strains and plasmids used in this work.

2.1.2 Growth Media

Low–Salt Luria-Bertani Broth

Low–salt LB medium was prepared by adding 10g tryptone (Oxoid A/S, Grave, Denmark), 5g yeast extract (Oxoid A/S, Grave, Denmark), and 5g NaCl per liter deionized water. pH was adjusted to 7.5 by adding NaOH and the solution was subsequently autoclaved. To Prepare Low–salt LB plates 15g agar (Merck, Darmstadt, Germany) were added per liter before the medium was autoclaved. The solution was left to cool to 55°C before Zeocin^{TM} (Invitrogen, Inchinnan Buisiness Part, United Kingdom) was added to a final concentration of 25μg/ml.

Yeast Peptone Dextrose (Sorbitol)–Zeocin^{TM}

To prepare Yeast Peptone Dextrose (YPD) medium, 10g yeast extract (Oxoid A/S, Greve, Denmark) and 20g peptone (Oxoid A/S, Greve, Denmark) was dissolved into 900ml deionized water and subsequently the solution was autoclaved. The solution was then left to cool before adding 100ml of filter sterilized 20% dextrose and Zeocin^{TM} is added, if the resistance is needed, to a final concentration of 100μl/ml. Yeast Peptone Dextrose Sorbitol(YPDS) medium was prepared as YPD, though in addition 182.2g sorbitol (Sigma–Aldrich, Brønby, Denmark) was added, for a final concentration of 1M sorbitol, before autoclaving. YPD(S) plates were created by adding 15g agar (Merck, Darmstadt, Germany)) to the solution before

autoclaving. Approximately 20ml were poured into each petri dish and left to cool. The plates and solutions were stored at 4°C and in the dark.

Minimal Glycerol and Minimal Methanol

Minimal Glycerol (MGY) and Minimal Methanol (MM) medium was prepared by mixing 800ml autoclaved deionized water with 100ml filter sterilized 13,4 % Yeast Nitrogen Base (Invitrogen, Inchinnan Business Park, United Kingdom). For MGY 100ml of filter sterilized 10% glycerol was added and for MM 100ml filter sterilized 5% methanol was added. Both solutions were stored at 4°C.

2.2 Methods

2.2.1 DNA Isolation

Two different types of DNA isolations were conducted in this paper, Sigma–Aldrich Mini Prep Kit and Qaigen Plasmid Midi Kit. Both were based on column purification.

Sigma–Aldrich Mimi Prep Kit (Sigma–Aldrich, Brøndby, Denmark) was used to purify plasmid from *E.coli* cultures inoculated in 5ml low–salt LB/Zeocin$^{TM}_{25\mu g/\mu L}$ medium that had grown overnight at 37°C and shaking at 245rpm.

Qaigen Plasmid Midi Kit (Qiagen, Hilden, Germany) was used to purify the plasmid obtained after a production of a 100ml main culture of *E.coli* DH5α.

2.2.2 Gel Electrophoresis

30ml of 1% agarose was mixed with 1μl Ethidium Bromide (Roche Diagnostics Gmbh, Mannheim, Germany) and the gel was then cast. Subsequently the wells were filled with the mixture and was run on the gel electrophoresis at 100V for 60 minutes.

2.2.3 DNA Extraction from Gel

Milipore Ultrafree–DA DNA Extraction Kit (Millipore, Billerica, MA, USA) was used to purify plasmid from agarose gels. The plasmid was run on an 1% agarose gel at 100V against a 1kbp GeneRuler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden). The band containing plasmid was identified using a low intensity UV light, cut out, and subsequently stored overnight at -20°C. The gel was then thawed and the plasmid was purified from the gel using Milipore Ultrafree-DA DNA Extraction Kit (Millipore, Billerica, MA, USA).

2.2.4 Ethanol Precipitation

$\frac{1}{10}$x sample volume 3M Sodium Acetate and 2x sample volume 96% EtOH was added to the solution of DNA. The solution was then incubated at -20°C for 2 hours and subsequently centrigufed at 14000g for 30 minutes at 4°C. The pellet was washed with 70% EtOH and the pellet was allowed to airdry for 2–3 minutes. The pellet was resolvated in DNA/RNAse free water and stored at 4°C.

2.2.5 Preparation of the pPICZαA Plasmid

Transformation of pPICZαA into DH5α

Competent *E.coli* DH5α cells (New England Biolabs, Ipswich, MA, USA) were thawed on ice then 10μl pPICZαA (Invitrogen, Inchinnan Business Park, United Kingdom) plasmid was added and mixed. The mixture was left on ice for 20 minutes and then heat shocked for 90 seconds at 42°C. Immediately after the heat shock 1ml low–salt LB medium was added and then incubate at 37°C for 45 minutes while shaking at 245rpm. After the incubation the cells were centrifuged at 4000rpm for 2 min and subsequently 800μl supernatant is removed. The cell pellet is resuspended in the remaining medium. The solution was plated onto low–salt LB/Zeocin$^{TM}_{25\mu g/\mu l}$ plates and incubated overnight at 37°C.

Digestion of pPICZαA Plasmid

To verify if the plasmid obtained was pPICZαA. Colonies from the plates were tested for successful transformation by purifying the plasmid and digesting it using 10 units of *Nco*I (New England Biolabs, Ipswich, MA, USA) and 10 units *Not*I (New England Biolabs, Ipswich, MA, USA) in a total reaction volume of 20μl and left to incubate for 60 minutes at 37°C.

2.2.6 Construction of the pPICZα/IL4 Plasmid

Digestion of pPICZαA Plasmid

3μl of the purified pPICZαA vector was digested for 1 hour at 37°C in a total volume of 70μl using 20 units *Xho*I (Fermentas, Helsingborg, Sweden). After the digestion an EtOH precipitation was conducted and the precipitate was resuspended in DNA/RNAse free water. Another digestion was conducted using 20 units of *Not*I (New England Biolabs, Ipswich, MA, USA) in a total volume of 69μl at 37°C for 1 hour.

Annealing and Phosphorylation of Oligonucleotides

The two primers IL4–1 and IL4–2rev (TAG Copenhagen A/S, Copenhagen, DK) were dissolved to a concentration of 100pmol/μl. 10μl of each primer were mixed with 20μl DNA/RNAse free water. The solution was heated to 95°C for 5 minutes and slowly cooled to 4°C. After the annealing process 10 units of T4 Polynucleotide Kinase (Fermentas, Helsingborg, Sweden) and 1μl 100mM ATP was added to the solution and left to incubate for 30 minutes at 37°C.

Ligation of IL4 Insert into the Digested Plasmid

A 3:1 insert to vector ratio was used for the ligation. The ligation was run in a total volume of 17μl using 9μl digested plasmid and 3,3μl $\frac{1}{1000}$ diluted IL4 insert, 400 units of T4 DNA ligase (New England Biolabs, Ipswich, MA, USA), and 6μM ATP. The solution was incubated overnight at 8°C.

Transformation of *E.coli* DH5α with pPICZαA/IL4

Competent *E.coli* DH5α cells (New England Biolabs, Ipswich, MA, USA) were thawed on ice and then 13μl ligation mix was added to the cells and mixed. Subsequently, the solution was stored on ice for 30 minutes and then heat shocked for 30 seconds at 42°C. Immediately after the heat shock 500μl low–salt LB medium was added and left to incubate at 37°C and 245rpm. After the incubation the solution was spread over LB/Zeocin$^{TM}_{25\mu g/\mu l}$ plates. The plates were incubated overnight at 37°C.

Digestion of pPICZαA/IL4

To verify if the ligation of the IL4 insert into the pPICZαA vector was successful a digestion of purified plasmid was conducted. Four separate digestions were performed using; 10 units *Xho*I (Fermentas, Helsingborg, Sweden), 10 units *Not*I (New England Biolabs, Ipswich, MA, USA), 10 units *Nco*I (New England Biolabs, Ipswich, MA, USA), or 10 units *Sac*II (New England Biolabs, Ipswich, MA, USA). All digestions were performed in a reaction volume of 20μl. The incubation time used was 60 minutes at 37°C. The digested plasmid was run against a 1kbp GeneRuler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden) on an 1% agarose gel.

Sequencing

pPICZαA/IL4 was sequenced by DNA Technology A/S, Risskov, Denmark, using the two primers α–factor (5´–TATTGCCAGCATTGCTGCTGCT–3´) and 3´AOX1 (5´–GGATGTC AGAATGCCATTTGC–3´). The apparatus used was a 3130xl Genetic Analyzer(Applied Biosystems, CA, USA). The apparatus sequences DNA by capillary electrophoresis utilizing the dideoxy chain terminator. The procedure was developed by [Sanger et al., 1977]. The sequence obtained was analyzed using the program FinchTV (Geospiza, Seattle, USA).

2.2.7 Transformation of *P.pastoris* X–33 with pPICZαA/IL4 by Electroporation

13μg purified plasmid was digested using 100 units of *Sac*I (New England Biolabs, Ipswich, MA, USA) at 37°C for 120 minutes. A main culture of *P.pastoris* X–33 cells were grown at 30°C with shaking at 275rpm until an OD_{600} of 1.3 was reached. The X–33 cells were centrifuged at 1500g for 5 minutes at 4°C and the pellet was resuspended in 500ml ice cold, sterile milli–Q water. This step was repeated 4 times though the volume used in the resuspension changed to 250ml ice cold sterile milli–Q water, then media and volume changed to 20ml ice cold sterile 1M sorbitol, and then two times 1ml ice cold sterile 1M sorbitol. 80μl X–33 cells were mixed with approximately 5μg *Sac*I linearized pPICZαA/IL4 plasmid and subsequently transfered to an ice cold 0.2cm electroporation cuvette(Bio–Rad Laboratories, Symbion Science Park, Copenhagen, Denmark) and left to incubate on ice for 5 minutes. For the electroporation a MicroPulser Electroporator(Bio–Rad Laboratories, Symbion Science Park, Copenhagen, Denmark) was used and the manufactures settings for *S.cerevisiae* were used. The pulse was 13.6ms long and at 1.5kV. Immediately after the pulse 1ml 1M ice cold sterile sorbitol was added to the curvette and the solution was transfered to a 15ml tube and left to incubate for 60 minutes at 30°C. The cells were plated onto YPDS–Zeocin$^{TM}_{100\mu g/\mu l}$ plates in 5 different quantities, 10μl, 25μl, 50μl, 100μl, and 200μl. The plates were left to incubate at 30°C for 4–10 days.

Purification of Chromosomal DNA from Transformants *P.pastoris* X-33/IL4

The method used to purify the chromosomal DNA from the yeast was based on the smash and grab DNA miniprep method from [5]. Colonies that were formed from the transformation were replated onto YPD/Zeocin$^{TM}_{100\mu g/\mu l}$ and left to incubate for 2 days at 30°C. Glass beads with a 5mm diameter were washed in 30% HCl and subsequently milli–q water, and autoclaved. A lysis buffer composing of 10mM TRIS buffer at pH 8.0, 1mM EDTA, 100mM NaCl, 1% SDS, and 2% Triton X–100 was prepared. A 5ml miniprep of X–33/IL4 was grown overnight at 30°C and then the cells were harvested at 1500g. The cells were placed in an eppendorf tube and resuspended in 0.2ml lysis buffer and 0.2ml of a 1:1 mix of phenol and chloroform, and then 4-5 glasbeads were added. The tube was then vortexed at top speed for 2 minutes. 0.2ml TE

buffer (10mM Tris, ph 8.0 and 1mM EDTA, pH 8.0) was added and the tube was vortexed for 10 seconds. The tube was then centrifuged at 14000rpm for 5 minutes at room temperature. The aqueous phase was added to a fresh eppendorf tube and the DNA was precipitated using ethanol precipitation method. The pellet obtained through the precipitation was resuspended in 100μl DNA/RNAse free water.

2.2.8 Test for Integration of pPICZαA/IL4 by PCR

For the following PCRs two primers were used, namely α–factor (5´–TATTGCCAGCATTGC TGCTGCT–3´) (TAG Copenhagen A/S, Copenhagen, DK) and 3´AOX1 (5´–GGATGTCAG AATGCCATTTGC–3´) (TAG Copenhagen A/S, Copenhagen, DK) and for chromosomal DNA IL4p (5‘–AAGTTGCCATTGTTGCCATTG–3‘) (TAG Copenhagen A/S, Copenhagen, DK) was also used.

PCR Analysis on pPICZαA/IL4

The PCR was run on a Geneamp PCR System 9700 (Applied Biosystems, CA, USA) using the cycles; 95°C for 2 minutes, 35 repeats of (95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute), 72°C for 7 minutes and cool to 4°C, a total reaction volume of 100μl, 2.5 units of Taq DNA Polymerase (Fermentas, Helsingborg, Sweden), 2mM MgCl2, 100mM dNTPs, 1pmol 3´AOX1 primer, 1pmol α–factor, and the template. The product obtained was run against a low–range Massruler^{TM} DNA ladder(Fermentas, Helsingborg, Sweden) on a 2% agarose gel.

PCR Analysis on Chromosomal DNA

The PCR was run on an Eppendorf Mastercycler Gradient (Eppendorf, NY, USA) using the cycles; 95°C for 4 minutes, 40 repeats of (95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 1 minute), 72°C for 7 minutes, and cool to 4°C. The reaction was run in a total volume of 100μl using 2.5 units of Taq DNA Polymerase (Fermentas, Helsingborg, Sweden), 2mM MgCl2, 100mM dNTPs, (1pmol 3´AOX1 primer or 1pmol α–factor), 1pmol IL4p, and 20μl chromosomal DNA obtained through the Smash and Grab method. The product obtained was run against a low–range Massruler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden) on a 2% agarose gel.

2.2.9 Expression of IL4 in *P.pastoris*

Single colonies of recombinant X–33/IL4 were picked from the replated YPD/Zeocin$^{TM}_{100\mu g/\mu l}$ plates and inoculated in 25ml of MGY medium and allowed to incubate at 275rpm and 30°C. OD_{600} was continuously measured until an OD_{600} in the interval of 2–6 was reached. The cells were harvested by centrifuging at 3000g for 5 minutes at room temperature. The cells were then resuspended in MM to a final OD_{600} of 1. The culture was shaken at 275rpm and 20°C. Every 12 hours OD_{600} was measured, 1ml medium was extracted, and methanol was added to a final concentration of 0.5%. The medium that was extracted was centrifuged at 1400rpm at 4°C and the supernatant, named Medium fraction, was transfered to a clean eppendorf tube. The two eppendorf tubes containing the medium fraction and the cell fraction were placed in the -80°C freezer. After 83 hours the cultures were centrifuged at 14000g for 10 minutes and the medium fraction and cell fraction were stored seperately at 4°C.

2.2.10 Chemical Synthesis of IL4

Fmoc solid–phase peptide synthesis was used to chemically synthesize the indolicidin analogue IL4 with the primary sequence ILPWKLPLLPLRR. The synthesis was carried out on an automatic peptide synthesizer Active–P11 (Activotec, Cambridge, UK).
100mg of Fmoc protected arginine was loaded into the reaction vessel (RV) and for each subsequent amino acid used a greiner tube with the correct amount was needed, for Arginine, Proline, Tryptophan, and Isoleucine a double coupling was performed and as such two tubes were needed.
In the synthesis cycle the first step was the removal of the Fmoc protection group, this was done by adding 2ml 25% piperidine in N-Methyl-2-pyrrolidone (NMP) to RV and subsequent shaking for 10 minutes. The deprotection solution was remove and the RV was then washed with 5x2ml NMP while shaking. To the greiner tubes, containing the amino acids, 0.125M HOBt/HBTU was added to dissolve the animo acids and 100μl DIPEA was added and allowed to react for 1 minute. The solution was then added to the RV while the RV was shaking for 30 minutes. After the coupling the RV was washed with 5x2ml NMP under shaking for 1 minute. This cycle was repeated for every coupling.
When the final amino acid was coupled and the RV was washed with 5x2ml NMP and subsequent with 5x2ml dicholoromethane. The RV was then placed into a vacuum dessicator for 120 minutes. The 2ml cleavage mixture (95% TFA, 2.5% water, and 2.5% triisopropylsilane) was added and the RV was left at room temperature for 90 minutes. The resin was then washed with 1ml TFA and the peptide was precipitated by the addition of 10ml diethylether and then centrifuged at 1000g for 10 minutes. The supernatant was discarded, 2x10ml diethylether was used to wash the peptide and was then freeze dried.

2.2.11 Size Exclusion Chromatography

Freeze dried Cell and Medium fraction and 1.5mg of the freeze dried IL4 obtained through the chemical synthesis were resuspended in 1ml 5% Trifluoroacetic acid (TFA) and were then analyzed on a fast protein liquid chromatography (FPLC) ÄKTA purifier 10/100 (Amersham Biosciences, CT, USA). A Superdex Peptide 10/300 column (Amersham Biosciences, CT, USA) was equilibrated using 0.1% formic acid and a flow of 0.5ml/min. Absorbance was measured at three wavelengths, namely, 280nm, 245nm, and 215nm. The flow through was fractioned and freeze dried.

2.2.12 Mass Spectrometry

MALDI–TOF

The freeze dried fractions obtained using the FPLC were resuspended in 100μl 5% formic acid. A ReflexIII (Bruker Daltonik, Bremen, Germany) using Matrix assisted laser desorption ionization (MALDI–TOF) mass spectrometry was used to determine the distribution of masses in the samples. 0.5μl sample was applied to the target disk along with the matrix (0.5μl of 2,5–Dihydroxybenzoic acid (DHB) dissolved in 66% (0.1%) trifluoracetic acid and 33% acetonitrile). 100pmol/μl pep mix (containing angiotensin II, bombesin, ACTH 18-39, and somatostatin in equal amounts in 66% (0.1%)trifluoracetic acid and 33% acetonitrile) was used to calibrate the MALDI–TOF. After the samples has been allowed to dry the target disk was loaded into the apparatus and analyzed.

Liquid Chromatography Electrospray Ionisation Tandem Mass Spectrometry

Liquid Chromatography Electrospray Ionisation Tandem Mass Spectrometry (LC–ECI MS–MS) was conducted on chemically synthesized IL4 and the medium fraction of colony 3. The samples were diluted in 50% methanol/1% formic acid and were analyzed using a hybrid quadrupole time-of-flight mass analyzer mass spectrometer (MicroTOFq, Bruker Daltronics, Bremen, DE) using offline nanoelectrospray emitters (Proxeon Biosystems,Odense, Denmark).

2.2.13 Growth Inhibition Assay

B.subtilis and *P.putida* were grown in 5ml nutrient (5g peptone and 3g Yeast extract pr liter and adjusted to pH 7.0), overnight at 37°C and 275rpm. 12ml of nutrient medium was added to a 15ml greiner tube along with 0.12g of agarose. The tube was heated in a boiling water bath until the agarose was melted and then the tube was allowed to cool to 45°C. 100μl of either *B.subtilis* or *P.putida* was added to the media. The medium was gently mixed and poured out into 9cm petri dishes and left to solidify. 5mm wells were made using 10–100μl pipette tips. Different solutions were filled into the wells; Crude IL4 obtained through the synthesis (3.36μM and 2.32μM) dissolved in 240μl HEPES buffer, HEPES buffer, 0.5% A–Skum dissolved in HEPES buffer, 0.5% A–Skum plus IL4 (same concentration as the crude IL4), 0.5% ARCO–DESI dissolved in HEPES buffer, 0.5% ARCO–DESI plus IL4 (same concentration as the crude IL4), Medium fractions from X–33 at 72h and 59h freeze dried and resolvated in HEPES buffer, and Medium fraction from Col. 3 at 72h and 59h freeze dried and resolvated in HEPES buffer. The plates were then allowed to incubate overnight at 37°C.

Results

3

3.1 Construction of Expression Plasmid pPICZαA/IL4

E.coli DH5α was transformed with the vector pPICZαA and using the restriction enzymes *Not*I and *Nco*I positive transformations were found. A main culture was prepared and pPICZα was purified and digested using *Not*I and *Xho*I. Gel electrophoresis was used to analyze the digested vector as seen in figure 3.1.

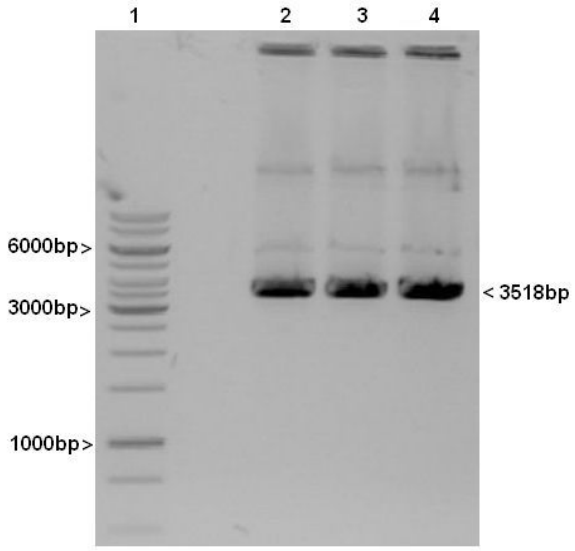

Figure 3.1: 1% agarose gel of pPICZαA digested with *Not*I and *Xho*I and run against a 1kbp GeneRuler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden). Lane 1 contains the ladder. Lane 2, 3, and 4 contains the *Not*I and *Xho*I digested pPICZαA vector

The digestion of pPICZαA by *Xho*I and *Not*I should result in a fragment of 3518bp, as the digestion with the two restriction enzymes removes a 75bp fragment. From figure 3.1 it is evident that a band can be seen that corresponds to the expected size of digested pPICZαA. In addition two bands, besides the band containing the digested pPICZαA fragment, can be seen. These bands are likely different conformations of circular undigested pPICZαA.
In figure 3.2 a gel electrophoresis was used to analyze the annealing of the IL4–1 and IL4–2rev. The size of the annealed insert should be 58bp and the bands in figure 3.2 corresponds to the theoretical size. A band can be seen to the right of well 3 this band is likely overflow from loading the wells.

*Xho*I and *Not*I digested pPICZαA and insert were ligated. This ligation mix was used in the transformation of *E.coli* DH5α. 40 colonies were picked, replated, and the plasmid was purified. The plasmid was then tested for the presence of the insert coding for IL4 by use of the restriction enzymes *Nco*I and *Sac*II. The only positive match out of the 40 colonies was colony 29, as seen in figure 3.3.
From figure 3.3 it can be seen that only colony 29 showed any sign of the expected confirmation. The *Nco*I digestion of colony 29 showed 3 bands at approximately 900bp, 2600bp, and 3600bp. These bands corresponds to a digested pPICZαA/IL4 and a supercoiled pPICZαA plasmid. The *Sac*II digestion of colony 29 resulted in two bands at approximately 3600bp and 2500bp. These bands corresponds to a single digested pPICZαA plasmid and super-

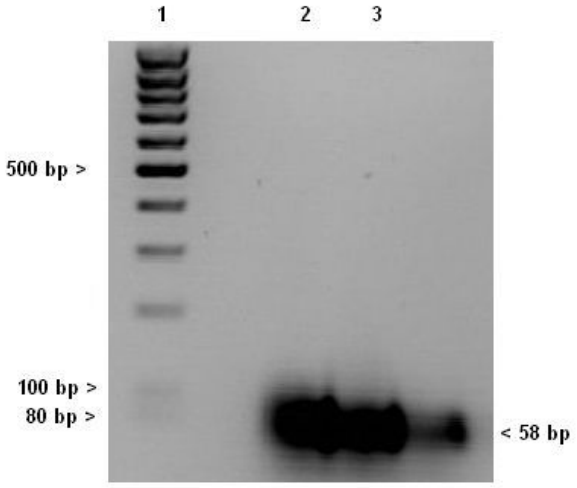

Figure 3.2: A 2% agarose gel of the insert that was obtained through the annealing of the two primers IL4–1 and IL4–2rev. The gel was run against a low–range Massruler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden). Lane 1 contains the DNA ladder, lane 2 contains 1μl anealing mixture, and lane 3 dcontains 0.5μl anealing mixture

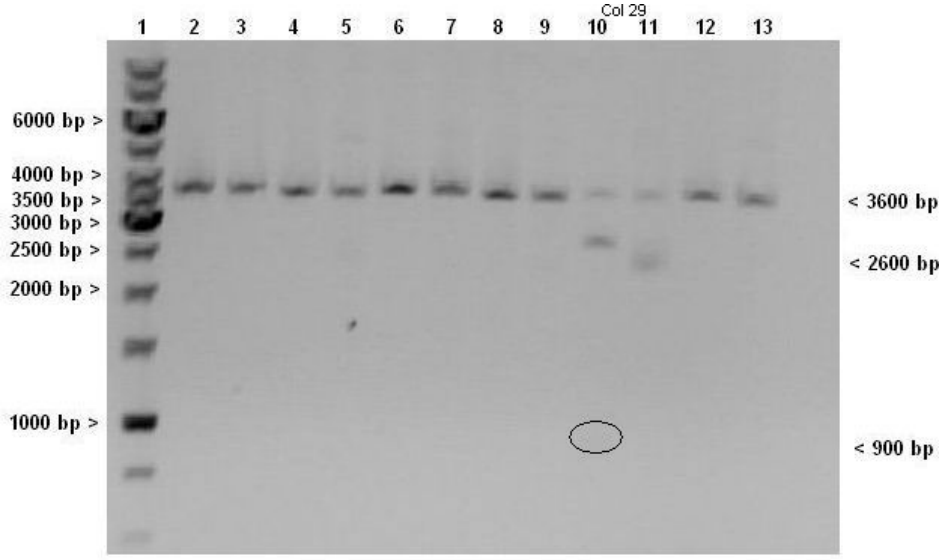

Figure 3.3: 1% agarose gel of plasmid pPICZαA/IL4 digested with *Nco*I and *Sac*II and run against a 1kbp GeneRuler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden). Lane 1 contains the ladder. Lane 2 and 3 contains plasmid obtained from colony 25 and digested with *Nco*I and *Sac*II, respectively. Lane 4 and 5 contains plasmid obtained from colony 26 and digested with *Nco*I and *Sac*II, respectively. Lane 6 and 7 contains plasmid obtained from colony 27 and digested with *Nco*I and *Sac*II, respectively. Lane 8 and 9 contains plasmid obtained from colony 28 and digested with *Nco*I and *Sac*II, respectively. Lane 10 and 11 contains plasmid obtained from colony 29 and digested with *Nco*I and *Sac*II, respectively. Lane 12 and 13 contains plasmid obtained from colony 30 and digested with *Nco*I and *Sac*II, respectively.

coiled pPICZαA/IL4 plasmid. Thus, the results indicate that the transformant contains both pPICZαA and pPICZαA/IL4 and to verify this assumption a PCR was conducted on plasmid obtained from colony 29, as can be seen in figure 3.4

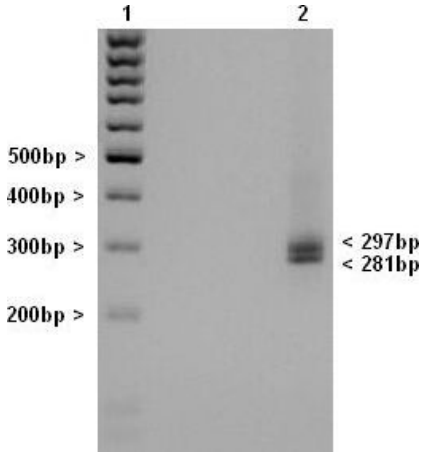

Figure 3.4: 2% agarose gel of the PCR product from plasmid purified from colony 29, using the primers α–factor and 3´AOX1, and run against a low–range Massruler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden). Lane 1 contains the ladder. Lane 2 contains the PCR product.

Two bands are obtained though the PCR and are located around the 300bp marker. These bands are in good agreement with the expect size of the fragments from pPCIZαA and pPCIZαA/IL4, 297bp and 281bp. These results indicate the presence of both pPCIZαA and pPCIZαA/IL4. In conjunction with the results from figure 3.3 it is quite evident that both are present in the transformant.
Plasmid from colony 29 was digested using *Sac*II since it only digests pPICZαA and a sample of the digested plasmid was analyzed by gel electrophoresis as can be seen in 3.5. The figure shows two bands, one above the 3500bp marker and one between the 2000bp and 2500bp marker. This corresponds to the desired results of the digested pPICZαA of 3593bp and the undigested pPICZαA/IL4, that is supercoiled.

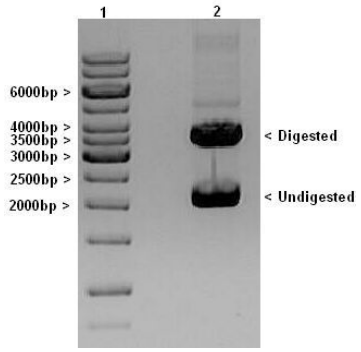

Figure 3.5: 1% agarose gel of *Sac*II digested plasmid from colony 29 run on a against a 1kbp GeneRuler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden). Lane 1 contains the ladder. Lane 2 contains the *Sac*II digested plasmid.

E.coli DH5α was transformed with the mixture of plasmid DNA from colony 29 and 10 colonies, named A–J, were picked and analyzed using the *Nco*I and *Sac*II restriction enzymes. The digested plasmid was analyzed using gel electrophoresis as can be seen in figure 3.6.

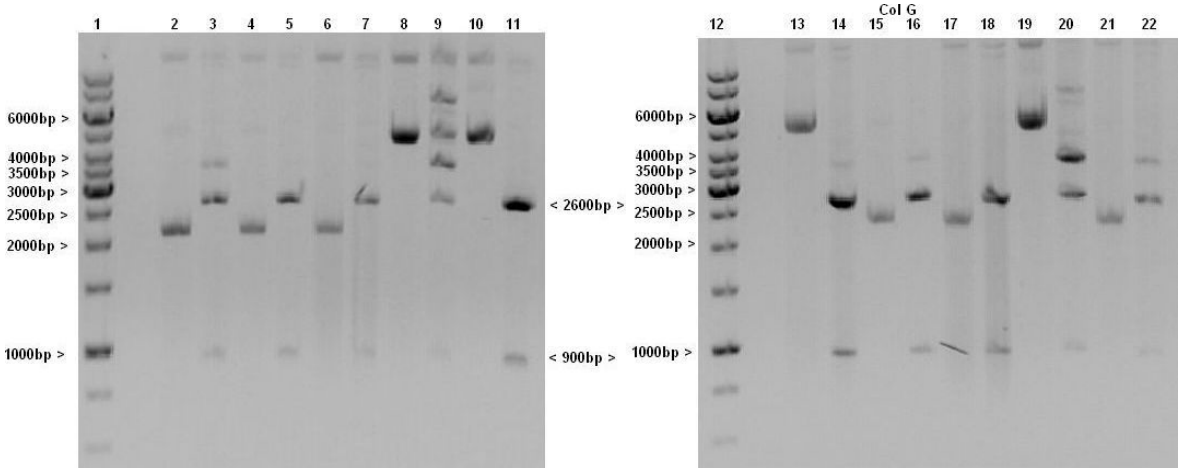

Figure 3.6: 1% agarose gel of *Sac*II and *Nco*I digested plasmid obtained from the 10 colonies, A–J, run against a 1kb GeneRuler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden). Lane 1 and 12 contains the ladder. Lane 2 and 3 contains plasmid from colony A digested using *Sac*II and *Nco*I, respectively. Lane 4 and 5 contains plasmid from colony B digested using *Sac*II and *Nco*I, respectively. Lane 6 and 7 contains plasmid from colony C digested using *Sac*II and *Nco*I, respectively. Lane 8 and 9 contains plasmid from colony D digested using *Sac*II and *Nco*I, respectively. Lane 10 and 11 contains plasmid from colony E digested using *Sac*II and *Nco*I, respectively. Lane 13 and 14 contains plasmid from colony F digested using *Sac*II and *Nco*I, respectively. Lane 15 and 16 contains plasmid from colony G digested using *Sac*II and *Nco*I, respectively. Lane 17 and 18 contains plasmid from colony H digested using *Sac*II and *Nco*I, respectively. Lane 19 and 20 contains plasmid from colony I digested using *Sac*II and *Nco*I, respectively. Lane 21 and 22 contains plasmid from colony J digested using *Sac*II and *Nco*I, respectively.

From the figure 3.6 none of the *Sac*II digested plasmid have a band around 3600bp, thus indicating that there is no pure pPICZαA plasmid present. Instead all of the *Sac*II digested plasmid is located around 6000bp or between 2500bp and 2000bp, indicating that the plasmid is in a supercoiled state. When looking at the *Nco*I digested plasmid it can be expected that the bands are located around 959bp and 2634bp. This pattern is present in all colonies. Though there is also the presence of other bands indicating that not all plasmid has been digested by the *Nco*I restriction enzyme.
Colony G was chosen to prepare a main culture from and subsequent purify the plasmid. A PCR was run on the purified plasmid using the primers α–factor and 3'AOX1 and the PCR product was analyzed by gel electrophoresis, as can be seen in figure 3.7.
As can be seen the PCR product obtained is located just below the 300bp marker, this is in accordance with the expect size for the PCR product of pPICZαA/IL4 that is 281bp.
As an extra verification of the purified plasmid from colony G, samples were digested using the restriction enzymes *Xho*I, *Not*I, *Nco*I, and *Sac*II, and then analyzed by gel electrophoresis. The gel can be seen in figure 3.8
The undigested plasmid and the *Sac*II digested plasmid both give a band between 2500bp and 2000bp. This implies that the *Sac*II treated plasmid is undigested as would be expect of pPICZαA/IL4, since the *Sac*II restriction site has been removed, and thus *Sac*II does not cut the plasmid anymore. The *Xho*I and *Not*I digested plasmid both give a band around 3600bp and this corresponds to single digested pPICZαA/IL4. *Nco*I digested plasmid shows two bands at 2600bp and 900bp and this corresponds with the expected double digest of pPICZαA/IL4.
A DNA sequencing was conducted to verify that the sequence was correct. Two primers were

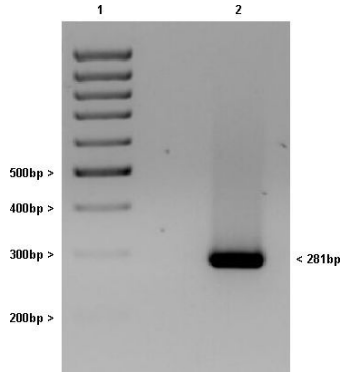

Figure 3.7: 2% agarose gel of the PCR product from colony G, using the primers α–factor and 3'AOX1, run against a low–range Massruler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden). Lane 1 contains the ladder. Lane 2 contains the PCR product of colony G.

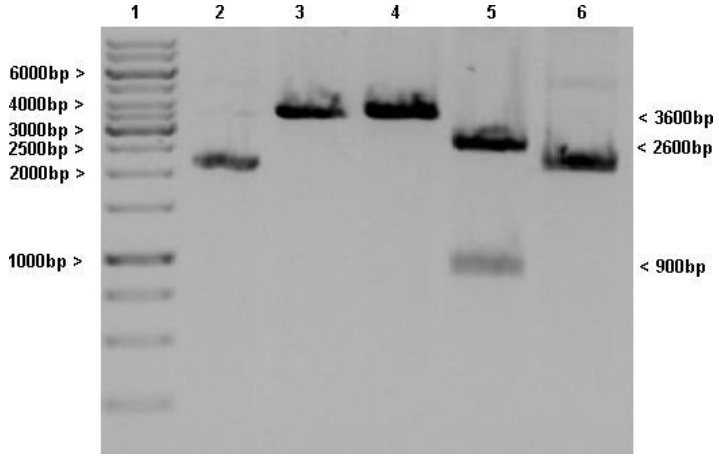

Figure 3.8: 1% agarose gel of plasmid obtained from colony G and digested with *Xho*I, *Not*I, *Nco*I, and *Sac*II run against a 1kb GeneRuler^{TM} DNA ladder (Fermentas, Helsingborg, Sweden). Lane 1 contains the ladder. Lane 2 contains undigested pPICZαA/IL4. Lane 3 contains *Xho*I digested pPICZαA/IL4. Lane 4 contains *Not*I digested pPICZαA/IL4. Lane 5 contains *Nco*I digested pPICZαA/IL4. Lane 6 contains *Sac*II digested pPICZαA/IL4.

used in the sequencing, namely α–factor and 3‘AOX1. In figure 3.9 a part of the 3‘AOX1 sequence obtained, can be seen. The full DNA sequence and chromatogram obtained though the sequencing can be seen in appendix A. As can be seen from this figure the expected sequence for IL4 was obtained.

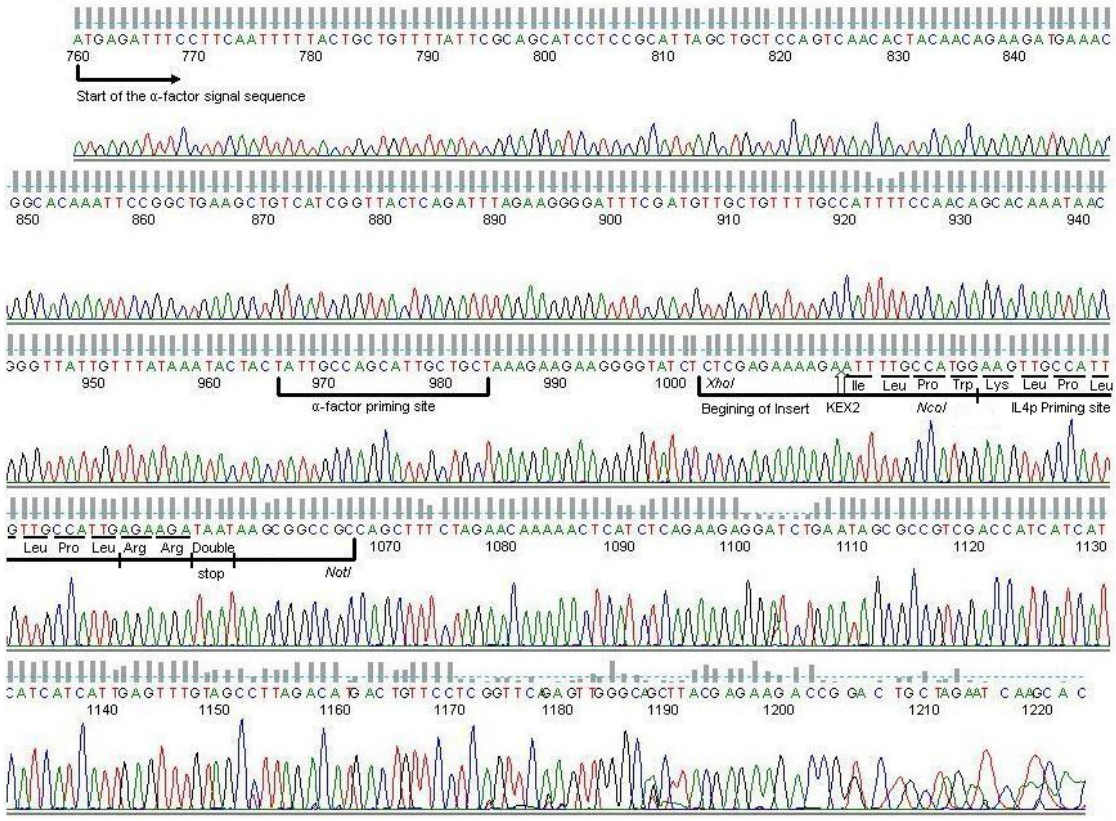

Figure 3.9: A cutout of the DNA sequence and chromatogram obtained through with sequencing of pPICZαA/IL4 plasmid by using the 3‘AOX1 primer. The sequence that is displayed is the reverse complement of the DNA sequence.

3.2 Transformation of *P.pastoris* X–33 with pPICZαA/IL4

Plasmid from colony G was linearized using the *Sac*I restriction enzyme and *P.pastoris* X–33 was then transformed by electroporation using the linearized pPICZαA/IL4. The cells were then plated onto YPDS–Zeocin$^{TM}_{100\mu g/\mu l}$ plates. After 4–10 days colonies were picked and replated. Chromosomal DNA was extracted from the colonies and PCRs were conducted on the DNA. In figure 3.10 a gel electrophoresis analysis of PCR products using the α–factor and 3‘AOX1 primers can be seen.
In figure 3.10 a band between the markers 300bp and 200bp can be seen for the colonies 3, 4, 7, 8, 10, 12, and 14. This fits with the expect value of a PCR using the α–factor and 3‘AOX1 primers, namely 281bp. The band indicates that the colonies have been transformed with the expression cassette of pPICZαA/IL4 vector. In figure 3.11 a gel electrophoresis analysis of PCR products using the IL4p and 3‘AOX1 primers can be seen.
The expected value of a PCR product using the IL4p and 3‘AOX1 primers would be 223bp. In figure 3.11 a band between the 250bp and 200bp marker can be seen for the colonies 3, 4, 6, 7, 8, 9, 10, 12, 13, and 14. Thus for the IL4p and 3‘AOX1 primers this indicates that all but colony 1 have been transformed with the expression cassette of pPICZαA/IL4 vector.

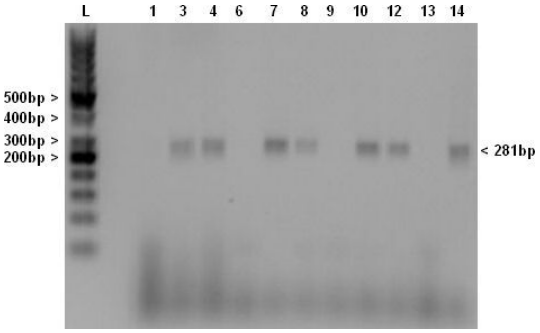

Figure 3.10: 2% agarose gel of the PCR product, using the α–factor and 3'AOX1 primers, from the chromosomal DNA of 11 colonies of transformed *P.pastoris* X–33 run against a 50bp GeneRuler^{TM} (Fermentas, Helsingborg, Sweden). Lane L contains the ladder. Lane 1, 3, 4, 6, 7, 8, 9, 10, 12, 13, and 14 contains the PCR product from the selected *P.pastoris* X–33 colonies.

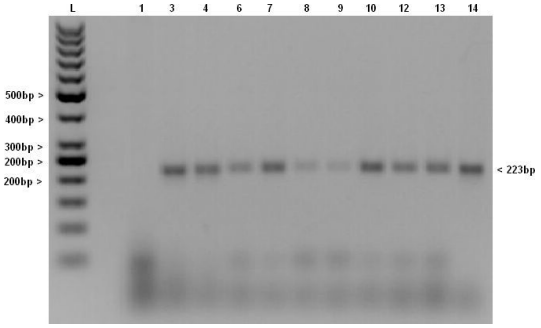

Figure 3.11: 2% agarose gel of the PCR product, using the IL4p and 3'AOX1 primers, from the chromosomal DNA of 11 colonies of transformed *P.pastoris* X–33 run against a 50bp GeneRuler^{TM} (Fermentas, Helsingborg, Sweden). Lane L contains the ladder. Lane 1, 3, 4, 6, 7, 8, 9, 10, 12, 13, and 14 contains the PCR product from the selected *P.pastoris* X–33 colonies.

Positive results from both PCRs were obtained for colonies 3, 4, 7, 8, 10, 12, and 14. The colonies 3, 4, 12, and 14 were chosen for test expression.

3.3 Chemical Synthesis and Characterization of IL4

IL4 was chemically synthesized using Fmoc–SPPS on the peptide synthesizer Activo–P11. Synthetic IL4 was analyzed using size exclusion chromatography to remove salts and divide the contents of the samples into fractions. Figure 3.12 shows a size exclusion chromatogram of chemical synthesized IL4.
From figure 3.12 it can be seen that three peaks of absorption at 215nm, 254nm, and 280nm are located at 10.5ml, 16ml, and 21.5ml. For the absorption of 215nm an additional peak can be seen at 18.5ml. The conductivity is continuously rising until 13ml after which it plateaus with the exception of a peak located around 21.5ml. The fluctuations of absorption at 215nm and conductivity could be the result of the FPLC not being fully equilibrated. The samples 2, 5, and 7 obtained though the size exclusion chromatography on chemically synthesized IL4 were analyzed on the MALDI–TOF MS. Samples 2 and 7 contained no protein in the range of IL4. Figure 3.13 shows a MALDI–TOF MS spectra conducted on sample 5.
The expected size for IL4 is 1614.039Da. For a MALDI–TOF MS the expected size of IL4 should be 1615.3Da, due to the ionization charge obtained in the process ($[M_{IL4}+H]^+$). As can be seen in figure 3.13 there is a peak at 1615.3Da, but there are also peaks at 1616.3Da, 1617.3Da, 1618.3Da, and 1619.3Da. These four peaks corresponds to IL4 where a ^{12}C has been exchange with a ^{13}C one, two, three, or four times respectively.
A new solution of chemically synthesized IL4 was measured in the FPLC by size exclusion chromatograph as can be seen in figure 3.14. From this figure it can be seen that the four peaks for the absorption of 215nm, seen in figure 3.12, are also present in this chromatogram.
A LC–ESI MS–MS was conducted on chemically synthesized IL4, sample 1 from figure 3.14 and the chromatogram can be seen in figure 3.15. IL4 should be located at 1615.3DA as seen in the MALDI–TOF MS, figure 3.13. The peak for IL4 is located at 808.02Da, this is at the expected as it corresponds to a double charged IL4. From the full length of IL4 the fragments can be found.

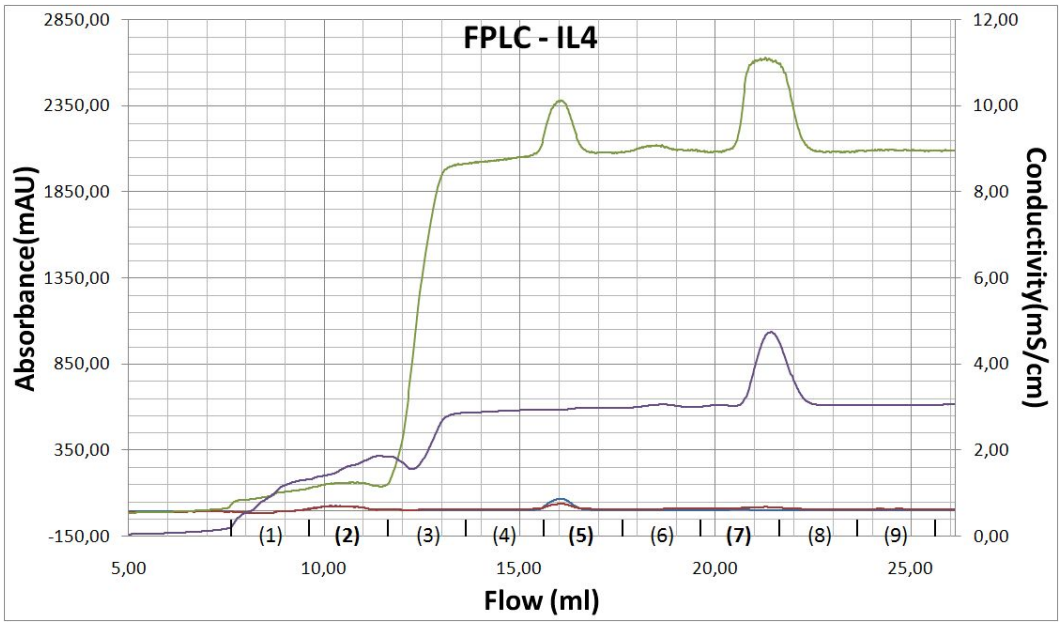

Figure 3.12: Size exclusion chromatogram of chemical synthesized IL4. 100μl of the chemically synthesized IL4 was loaded. The sample was fractionated into 9 samples by size exclusion chromatography using a Superdex Peptide 10/300 column (Amersham Biosciences, CT, USA). The pressure was kept constant with an average of 0.6MPa and a flow of 0.5ml/min. The green line is the absorption of light at 215nm, the red line is absorption of light at 254nm, the blue line is the absorption of light at 280nm, and the purple line is the conductivity. The fractionated samples are numbered on the x–axis.The eluted samples are numbered on the x–axis with the samples selected for further analysis in bold.

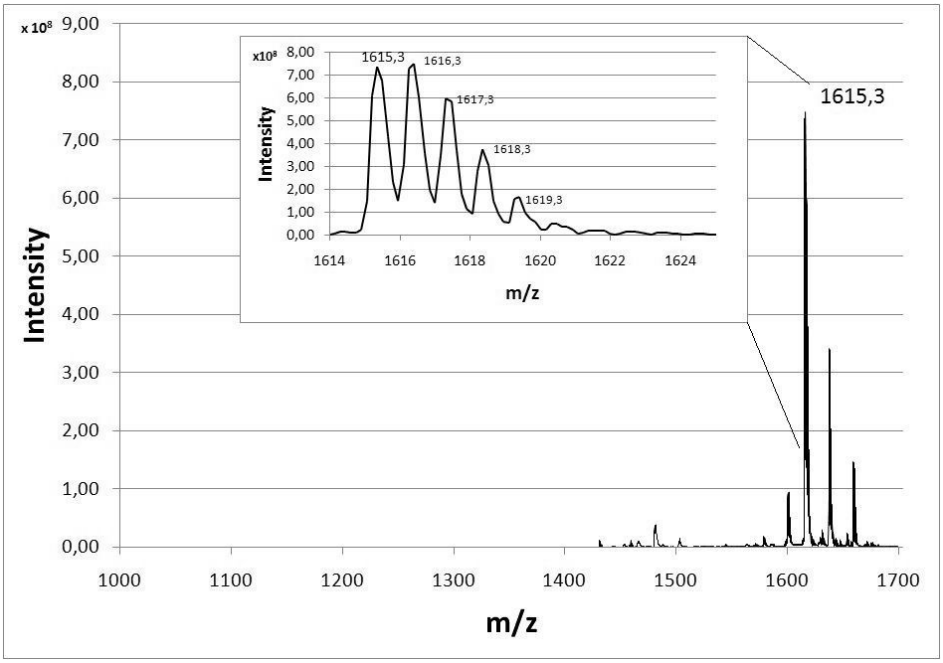

Figure 3.13: MALDI–TOF MS spectra of sample 5, obtained from the size exclusion chromatography on chemically synthesized IL4. An enlargement of the m/z area of 1615 is included.

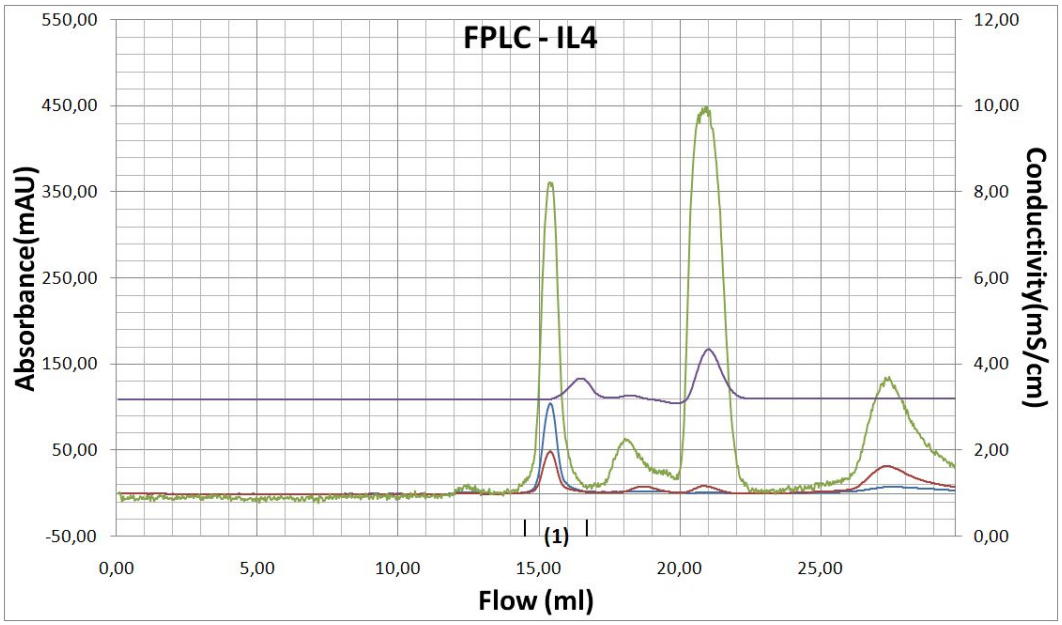

Figure 3.14: Size exclusion chromatogram of chemical synthesized IL4. 500μl of the chemically synthesized IL4 was loaded. The sample was fractionated by size exclusion chromatography using a Superdex Peptide 10/300 column (Amersham Biosciences, CT, USA). The pressure was kept constant with an average of 0.72MPa and a flow of 0.5ml/min. The green line is the absorption of light at 215nm, the red line is absorption of light at 254nm, the blue line is the absorption of light at 280nm, and the purple line is the conductivity. The fractionated samples are numbered on the x–axis. The fractionated samples are numbered on the x–axis.The eluted samples are numbered on the x–axis with the samples selected for further analysis in bold.

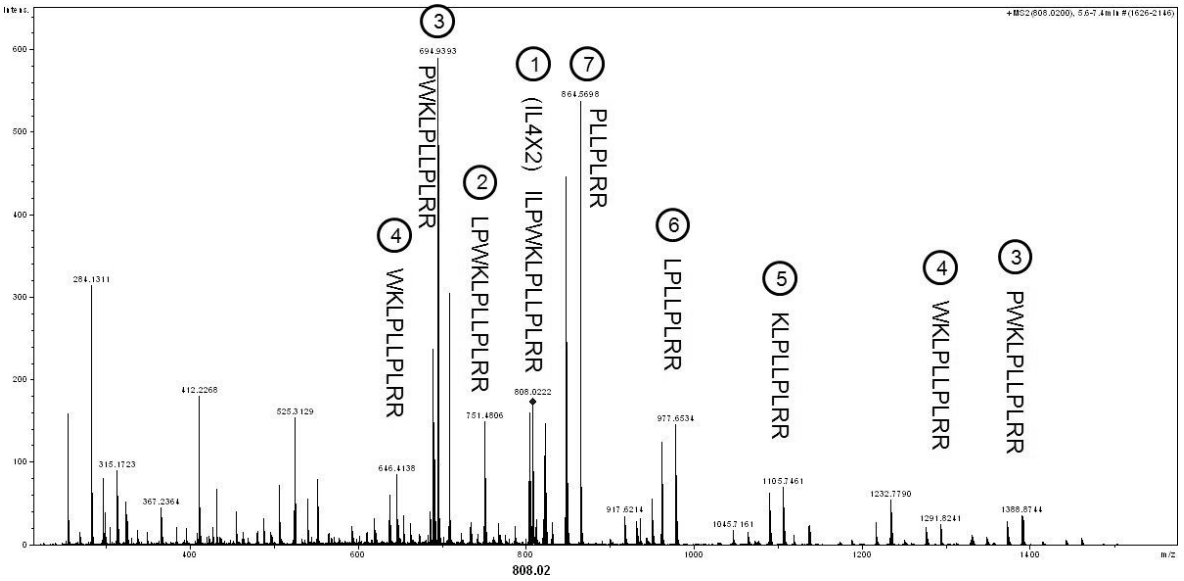

Figure 3.15: A LC–ESI MS–MS chromatogram of chemically synthesized IL4. 1 denotes the full size of IL4. 2 denotes the m/z of IL4 minus Ile. 3 denotes the m/z of IL4 minus Ile and Leu. 4 denotes the m/z of IL4 minus Ile, Leu, and Pro. 5 denotes the m/z of IL4 minus Ile, Leu, Pro, and Trp. 6 denotes the m/z of IL4 minus Ile, Leu, Pro, Trp, and Lys. 7 denotes the m/z of IL4 minus Ile, Leu, Pro, Trp, Lys, and Leu.

3.4 Expression Study

An expression was conducted for the colonies 3, 4, 12, and 14 using the *P.pastoris* X–33 as a reference. Samples were extracted at 12 hour intervals, though for the first 12 hours a 6 hour interval was used, and OD_{600} was measured. Every 12 hours methanol was added after the OD_{600} measurement. Figure 3.16 displays the measured OD_{600} for the colonies 3 and 4 and *P.pastoris* X–33.

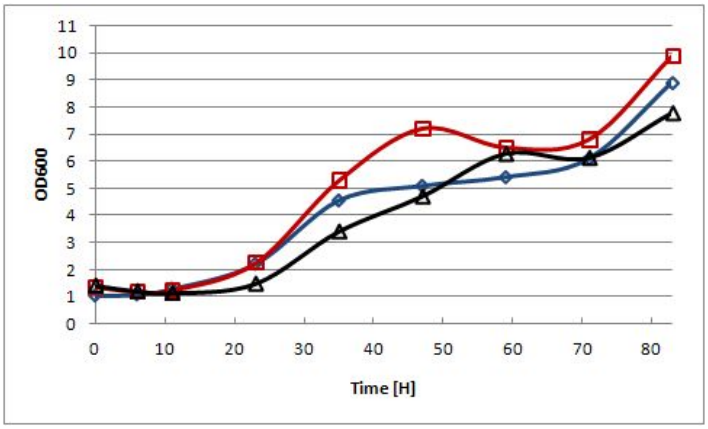

Figure 3.16: Measured OD_{600} during the expression of *P.pastoris* X–33 and the colonies 3 and 4. The expression was conducted at 20°C and in minimal medium. Induction with methanol was done every 12 hours after the OD_{600} measurement. The black line with the triangle is *P.pastoris* X-33. The red line with the square is colony 4. The blue line with the diamond is colony 3. The OD was measured at 0h, 6h, 11h, 23h, 35h, 47h, 59h, 71h, and 83h.

From the time of t=0 to the time t=83 an increase in the OD_{600} is evident for all expressions. Colony 4 shows the highest OD followed by colony 3 and *P.pastoris* X–33 exhibits the lowest OD. For both colony 4 and *P.pastoris* X–33 exhibits a drop in their OD at 47h and 69h, respectively. Whereas colony 3 is the only colony with a continuous increase in the OD.
The medium fraction from 83h for colony 3, and *P.pastoris* X–33, that was obtained during the expression, was freeze dried and resuspended in 5% TFA. Subsequently it was analyzed using size exclusion chromatography using the same parameters as for the synthesized IL4. The chromatogram of the medium fraction of colony 3 can be seen in figure 3.17 and for *P.pastoris* X–33 in figure 3.18.
From the chromatograms a similar conductivity and absorption profile could be seen, though the intensity was higher in the chromatogram for *P.pastoris* X–33. At the interval of 14.25ml to 16.30ml there is a difference in the similarity between the profiles for colony 3 and *P.pastoris* X–33. At this interval it can be seen that there is an increase in the absorption for 215nm, 254nm, and 280nm for colony 3. Based on the size exclusion chromatography done on the chemical synthesis of IL4 where it is expected that IL4 would be eluted around 15.5ml sample 4 from colony 3 was chosen for further analysis on the MALDI–TOF MS. From *P.pastoris* X–33 the samples 1, 4, and 7 was analyzed on MALDI–TOF MS, but these samples contained no protein with the mass of IL4. Figure 3.19 shows a MALDI–TOF MS spectra of sample 4 from colony 3, figure 3.17.
The expected mass of IL4 in its monoisotopic state is 1614.039Da, though in the MALDI–TOF MS IL4 will be ionized. A peak can be seen at 1615.5 that corresponds to $[M_{IL4}+H]^+$. Three peaks are located 1616.5, 1617.5, and 1618.5. These three peaks corresponds to IL4 where a ^{12}C has been exchange with a ^{13}C one, two, or three times, respectively. Sample 4 was also run though a LC–ESI MS–MS and the chromatogram can be seen in figure 3.20.
IL4 should be located at 1615.5DA as seen in the MALDI–TOF MS, figure 3.19. The peak

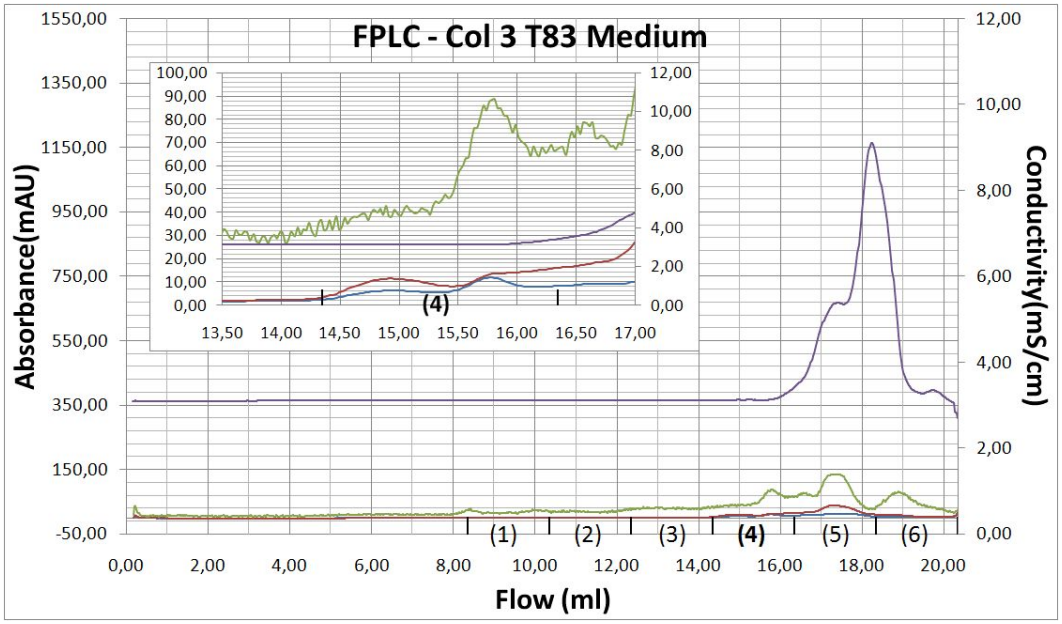

Figure 3.17: Size exclusion chromatogram of the medium fraction from colony 3. A magnification of sample 4 has been included. The sample was fractionated by size exclusion chromatography using a Superdex Peptide 10/300 column (Amersham Biosciences, CT, USA). The pressure was kept constant with an average of 0.59MPa and a flow of 0.5ml/min. The green line is the absorption of light at 215nm, the red line is absorption of light at 254nm, the blue line is the absorption of light at 280nm, and the purple line is the conductivity. The fractionated samples are numbered on the x–axis. The fractionated samples are numbered on the x–axis.The eluted samples are numbered on the x–axis with the samples selected for further analysis in bold.

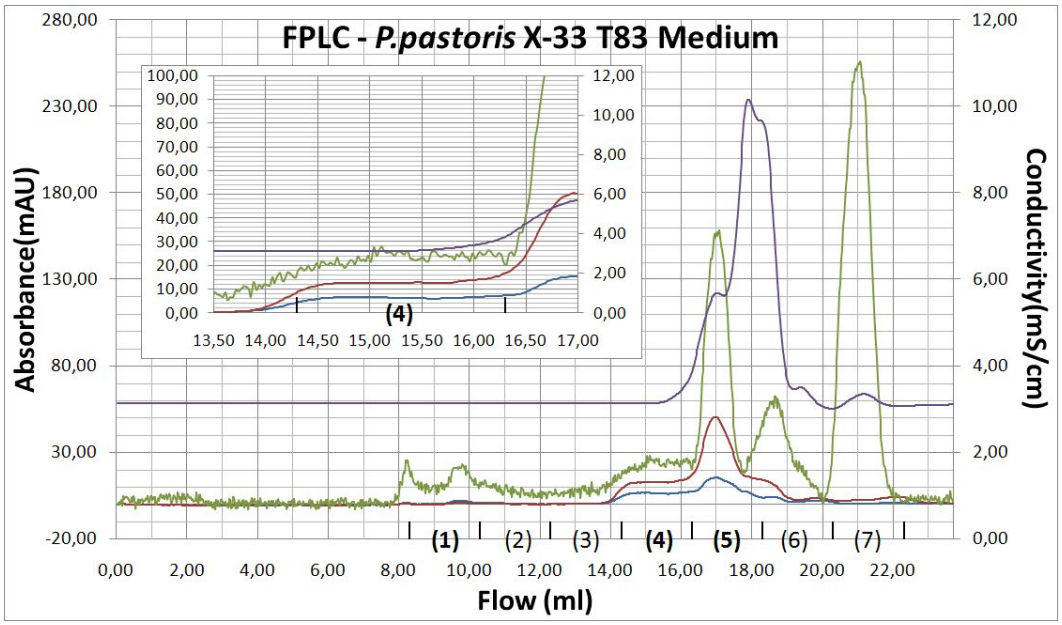

Figure 3.18: Size exclusion chromatogram of the medium fraction from *P.pastoris* X–33. A magnification of sample 4 has been included. The sample was fractionated by size exclusion chromatography using a Superdex Peptide 10/300 column (Amersham Biosciences, CT, USA). The pressure was kept constant with an average of 0.62MPa and a flow of 0.5ml/min. The green line is the absorption of light at 215nm, the red line is absorption of light at 254nm, the blue line is the absorption of light at 280nm, and the purple line is the conductivity. The fractionated samples are numbered on the x–axis. The fractionated samples are numbered on the x–axis.The eluted samples are numbered on the x–axis with the samples selected for further analysis in bold.

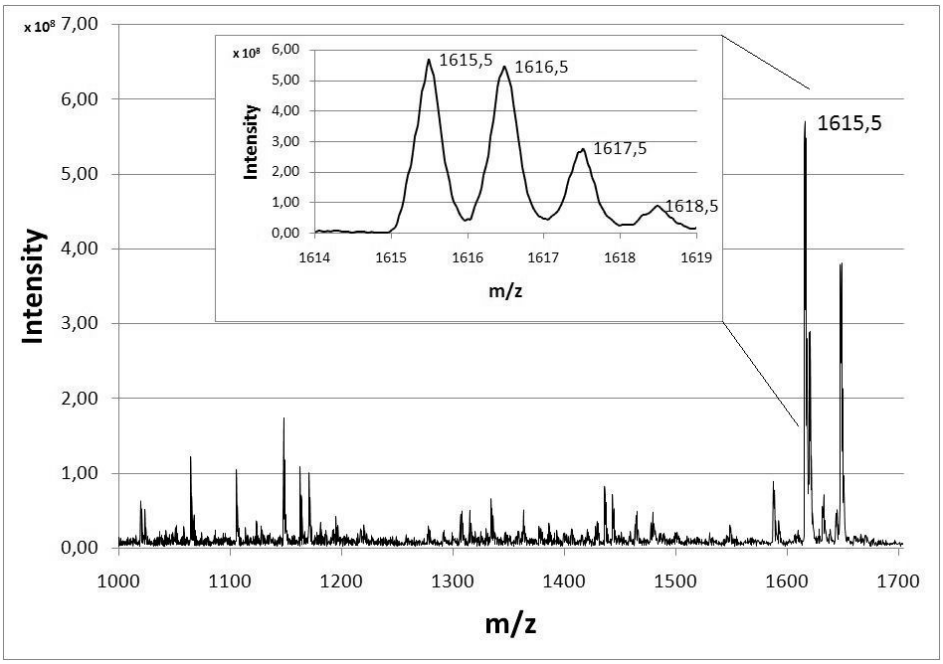

Figure 3.19: MALDI–TOF MS spectra of sample 4, obtained from the size exclusion chromatograph on sample 4 from colony 3, figure 3.17. A magnification of the m/z area of 1615 is included.

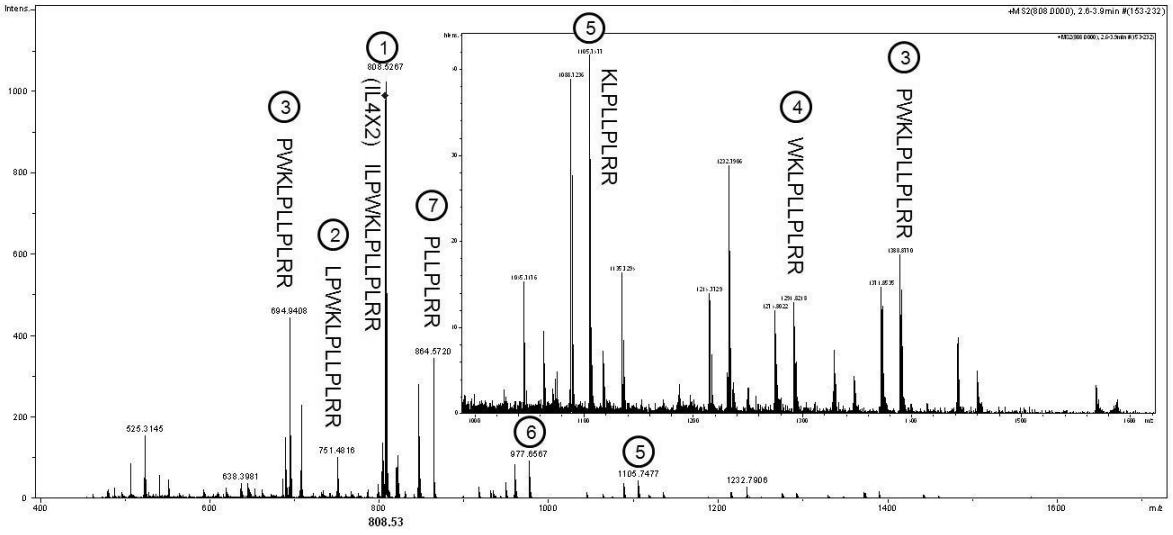

Figure 3.20: A LC–ESI MS–MS chromatogram of sample 4. 1 denotes the full size of IL4. 2 denotes the m/z of IL4 minus Ile. 3 denotes the m/z of IL4 minus Ile and Leu. 4 denotes the m/z of IL4 minus Ile, Leu, and Pro. 5 denotes the m/z of IL4 minus Ile, Leu, Pro, and Trp. 6 denotes the m/z of IL4 minus Ile, Leu, Pro, Trp, and Lys. 7 denotes the m/z of IL4 minus Ile, Leu, Pro, Trp, Lys, and Leu.

for IL4 is located at 808.56Da, this is at the expected as it corresponds to a double charged IL4. From the full length of IL4 the fragments can be found.

3.5 Growth Inhibition

Growth inhibition assays were conducted using 1% agarose nutrient medium that had been inoculated with *B.subtilis* or *P.putida*. Figure 3.21 shows a growth inhibition assay on *B.subtilis*.

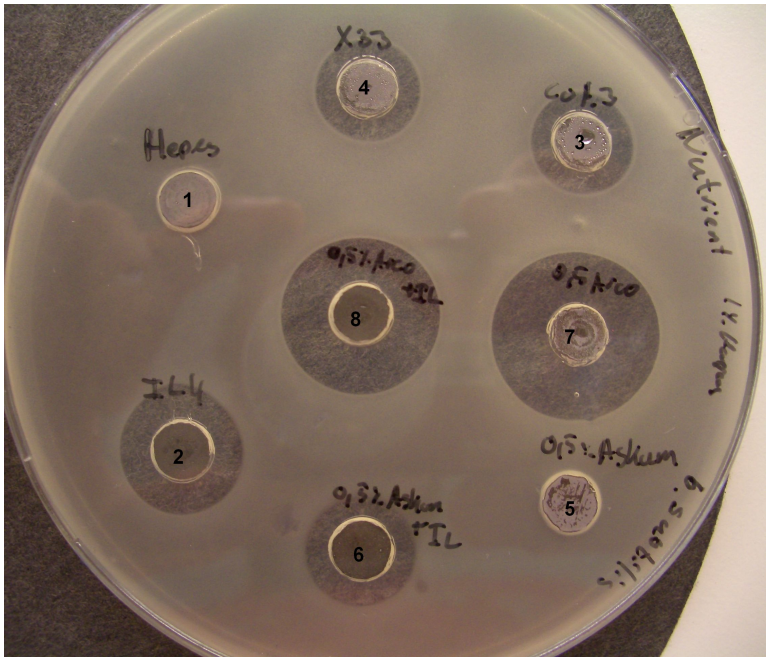

Figure 3.21: Growth inhibition assay conducted on *B.subtilis* using 1% agarose nutrient medium. In well 1 20μl HEPES buffer was placed. In well 2 20μl 2.32μM synthetic IL4 was placed. In well 3 20μl medium fraction from col 3 was placed. In well 4 20μl medium fraction from *P.pastoris* X–33 was placed. In well 5 20μl 0.5% A–Skum was placed. In well 6 20μl 0.5% A–Skum + 2.32μM synthetic IL4 was placed. In well 7 20μl 0.5% ARCO–DESI was placed. In well 8 20μl 0.5% ARCO–DESI + 2.32μM synthetic IL4 was placed.

Hepes buffer well 1 and 0.5% A–skum in well 5 showed no sign of inhibition against *B.subtilis*. The synthetic IL4 in well 2, 0.5% A–skum + 2.32μM synthetic IL4 in well 6, medium fraction from colony 3 in well 3, and medium fraction from X–33 in well 4 showed a good inhibition against *B.subtilis*. 0.5% ARCO–DESI in well 7 and 0.5% ARCO–DESI + 2.32μM synthetic IL4 in well 8 exhibited the largest inhibition area. A growth inhibition assay conducted on *P.putida* can be seen in figure 3.22.
Hepes buffer in well 1 and 0.5% A–skum in well 5 showed no sign of inhibition against *P.putida*. Synthetic IL4 in well 2 and 0.5% A–skum + 2.32mM synthetic IL4 in well 6 showed a small area of inhibition. The medium fraction from colony 3 in well 3 and medium fraction from X–33 in well 4 exhibits a good area of inhibition. 0.5% ARCO–DESI in well 7 and 0.5% ARCO–DESI + 2.32mM synthetic IL4 in well 8 has the largest area of inhibition.
Synthetic IL4 exhibits activity against both gram positive and gram negative bacteria, though it showed higher activity against the gram positive bacteria *B.subtilis*. The medium fractions from X–33 and colony 3 were active against both type of bacteria, but both showed the strongest activity against the gram negative bacteria *P.putida*.
For the commercial detergent ARCI–DESI adding synthetic IL4 to the mixture showed no increased effect at these concentrations against neither *B.subtilis* or *P.putida*. Whereas for the other commercial detergent A–skum an increase in activity was quite evident, against

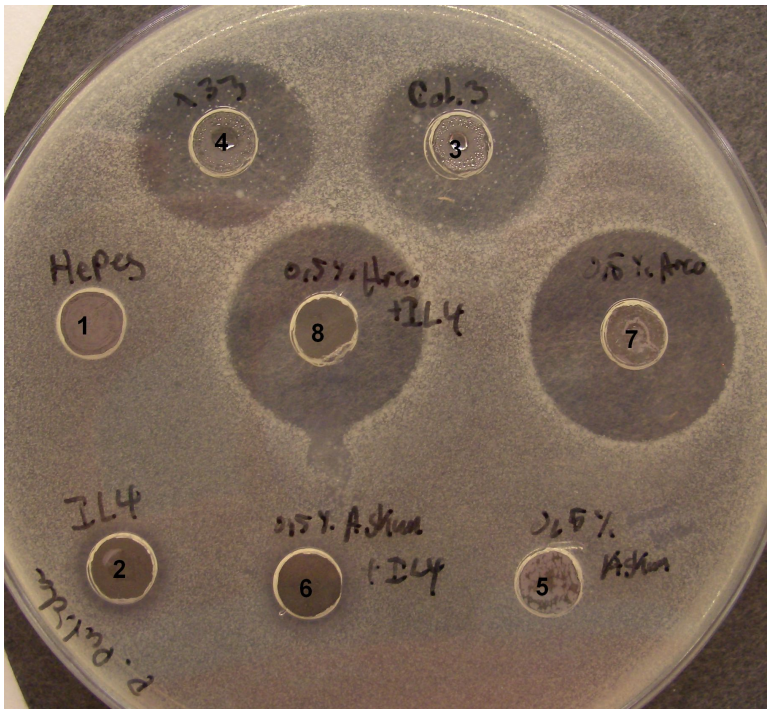

Figure 3.22: Growth inhibition assay conducted on *P.putida* using 1% agarose nutrient medium. In well 1 20μl HEPES buffer was placed. In well 2 20μl 2.32μM synthetic IL4 was placed. In well 3 20μl medium fraction from col 3 was placed. In well 4 20μl medium fraction from *P.pastoris* X–33 was placed. In well 5 20μl 0.5% A–Skum was placed. In well 6 20μl 0.5% A–Skum + 2.32μM synthetic IL4 was placed. In well 7 20μl 0.5% ARCO–DESI was placed. In well 8 20μl 0.5% ARCO–DESI + 2.32μM synthetic IL4 was placed.

both *B.subtilis* and *P.putida*, by the addition of synthetic IL4.

4 Discussion

4.1 Recombinant Expression of Indolicidin

Native indolicidin is known to exhibit a broad spectrum of antifungal and antibacterial activity. [Ahmad et al., 1995] Indolicidin is cytotoxic to mammalian cells as well as hemolytic to red blood cells. [Ahmad et al., 1995, Subbalakshmi et al., 2000] Analogues to indolicidin with single of Trp were shown to displayed no hemolytic activity against red blood cells of rats, while retaining its antibacterial activity. The Trp appeared to be a controlling factor for the hemolytic activity of indolicidin. [Subbalakshmi et al., 2000]
Native indolicidin has been expressed in *E.coli* by [Zhang et al., 1998, Morin et al., 2005], though due to the antibacterial nature of indolicidin, fusion protein was used. Fusion proteins were used to mask the activity of indolicidin during the expression. [Zhang et al., 1998] created a fusion protein using a small replication protein RepA from *E.coli*, cellulose binding domain from the fungus *Trichoderma ressei*, and prepro sequence from the gene for human defensin (HNP–1). HNP–1 was chosen for its anionic sequence to counteract the cationic charge of indolicidin. [Zhang et al., 1998]
[Morin et al., 2005] attempted to use the multimerization of indolicidin by fusing it to a thiorodoxin protein and thereby lowering its activity against *E.coli*. In order to facilitate the release of the active monomeric indolicidin, Met was used as a separator between the individual genes since it allowed for post processing cleavage using cyanogen bromide. [Morin et al., 2005] The expressed thiorodoxin/indolicidin fusion protein exhibited a high degree of insolubility and thus it was not possible to cleave the thioredoxin fusion protein prior to release of the recombinant indolicidin by CNBr digestion, thus several contaminated peptides were generated. [Morin et al., 2005]
In the expression of indolicidin in *E.coli* the use of a fusion protein is needed. Sometimes the formation of inclusion bodies will result in an inactive protein. Also *E.coli* lacks a post–translational machinery. [Shadev et al. 2008] Two of the major reason behind using fusion proteins are indolicidins toxicity towards *E.coli* and the proteolytic degradation of indolicidin. [Piers et al., 1993, Glick et al., 2003] The use of a fusion protein e.g. *E.coli* maltose binding protein (40kDa) or *E.coli* N-utilizing substance-A (54.8kDa) increases the metabolic load on the *E.coli* compared to the expression of indolicidin without a fusion protein. [Shadev et al. 2008] Fusion proteins expressed in *E.coli* require post–processing e.g. disruption of the cell membrane to release the protein and cleavage of the fusion protein to release the peptide.
In this work *P.pastoris* X–33 was used to express a single tryptophan analogue to indolicidin, IL4. The strategy involved the use of homologous recombination events targeted at the promoter region of the AOX1 gene, thus integration the vector pPICZαA/IL4 into the genome. The vector contains the α– factor signal sequence that allows for the secretion of IL4 into the medium. Secreting IL4 into the medium offers several advantages compared with the approaches used during the expression in *E.coli*. *P.pastoris* secretes very few proteins during the expression, thus allowing for easier purification of the active peptide. [Invitrogen, 2008] There is also no need for post–processing since the peptide is secreted in its active form. Though this could also be a disadvantage due to the antifungal activity of IL4. Another factor that might lower the production yield is proteolytic activity. IL4 is susceptible to degradation by proteases in all steps of the expression.

4.1.1 Expression Level

PCR was conducted on the chromosomal DNA of the obtained *P.pastoris* transformants to verify if the expression cassette had been integrated into the AOX1 gene. The colonies 3, 4, 12, and 14 were chosen for the test expression of the recombinant IL4. Expression of the recombinant IL4 was only detected for colony 3, that exhibited a low expression level. A reason that colony 3 was the only colony that showed any sign of recombinant IL4 could be found in the integration of the expression cassette into the genome. Sometimes multiple integration of the expression cassette into the genome will result in a higher production yield of the expressed recombinant protein as compared to a single copy strain. [Cereghino et al., 2000] This could explain why there was a positive result by PCR for the colonies 3, 4, 12, and 14 but in the expression only colony 3 showed any positive result. Though [Hohenblum et al. 2003] observed that integration of the expression cassette into AOX1 promoter results in a sharp optimum that was observed at 2 copies per cell. A decrease in the cell viability was observed when the copy number was 3 or more. [Hohenblum et al. 2003] Thus, it might be that the low expression level that was observed for colony 3 could be due to low cell viability. When the results of the OD_{600} measurements from the expression study are taken into account, figure 3.16, it seems unlikely that there is a decrease in cell viability, since the growth curve of colonies 3 and 4 are similar and *P.pastoris* X–33 exhibited the lowest OD_{600} at the end of the test expression.
Other factors that could contribute to the low production yield of recombinant IL4 is the lack of pH control, inadequate aeration of the cultures, and non optimal feeding of methanol to a growth limiting rate. [Cregg et al., 1993] For this work aeration was conducted by shaking the cultures at 300rpm. As mentioned degradation of IL4 by proteases could be a reason for the low production yield of the recombinant IL4. Several factors have been found to influence proteolysis. The length of induction effects the proteolysis, as expression time increases the number of viable cells decreases. [Daly and Hearn, 2005] The amount of proteolysis can be lowered using some methods. Lowering the temperature during the expression from 30°C to 23°C resulted in an increase in the production of herring anti–freeze protein by 10 fold. Lowering the activity of the proteases and an increase in the cell viability due to a stabilization of the cell membrane should have a positive effect on the production. [Li et al., 2001] The expression conducted in this work was done at 20°C so it can be assumed that the reduction in proteolysis and the increase in cell viability also is present at this temperature. The pH value of the medium can influence the level of proteolysis. If the medium is buffered at a pH value between 3.0 and 6.0, proteolysis of recombinant protein might be reduced. [Daly and Hearn, 2005] The medium used in this work was not buffed and using a buffered medium might lead to an increase in the expression level. Reduction of the proteolysis can also be done with the addition of casamino acids. [Zhang et al., 2006] [Clare et al. 1991] experienced a reduced yield that was contributed to the production of extracellular proteases. The problem was overcome using medium buffering and supplementing with Casamino acids. The casamino acids can possibly act as excess substrates for the proteases. [Cregg et al., 1993] Another option for decreasing the level of proteases is to use protease deficient strains. The protease deficient stains are not as viable as wild type *P.pastoris* and also they are more difficult to transform and have a slower growth rate. [Cereghino et al., 2000]. Sometimes the expression of a high level of foreign protein will overload the secretory pathway and thus be the limiting factor in the secretion of the desired protein. [Glick et al., 2003]
[Invitrogen, 2008] recommends the addition of methanol to a final concentration of 0.5% every 24 hours. In this work the induction was done every 12 hours to obtain a more steady rate of methanol. [Zhang et al., 2006] observed in the expression of the antimicrobial peptide CM in *P.pastoris* that increased concentrations of methanol resulted in a decrease of the expressed

peptide. The growth of the *P.pastoris* X–33 and the colonies 3 and 4 were monitored during the expression, figure 3.16. From these measurements it can be deducted that the colonies exhibit Mut^+ phenotype as their ability to grow on methanol is similar to that of *P.pastoris* X–33. The difference in OD_{600} between *P.pastoris* and the colonies 3 and 4 were small, indicating that the produced amount of IL4 was not toxic for *P.pastoris*.
A mass corresponding to IL4, obtained through both the MALDI–TOF and the Tandem MS, indicates that IL4 was produced without any post translation modifications such as glycosylation. The Glu–Ala repeats that are normally cleaved by the Ste13 were excluded. [Brake et al., 1984] found that keeping the Glu–Ala repeats resulted in a decrease in the pure protein due to an overload by the high amount of expressed protein that needed to be cleaved. Also it is found that the Kex2 endopeptidase did not require the presence of the Glu–Ala repeats. [Brake et al., 1984] It was found that the Glu–Ala repeats assisted in the maturation of the α–factor maturation. It might be that the omission of the Glu–Ala repeats were a mistake, as it may have resulted in a lower level of secretion of IL4. If there is a decrease of the efficiency in the processing of the α–factor, the pro–protein can be secreted into the medium intact. [Daly and Hearn, 2005]

4.2 Growth Inhibition

Growth inhibition essays were conducted on *B.subtilis* and *P.putida*. The method used was disk diffusion susceptibility test. From the results it could be seen that IL4 had a higher activity towards *B.subtilis* then towards *P.putida*. The difference in the cell envelop of gram negative and gram positive bacteria could explain the higher affinity towards gram positive bacteria. Where gram negative have an outer membrane that is not present in gram positive bacterias. [Glick et al., 2003] Thus the higher activity towards *B.subtilis* may be contributed to the ability of IL4 to transverse or disrupt the bacterial membrane. Native indolicidin has an amidation at the C–terminus that is not present in the synthesized IL4. It has been shown that the amidation of the C–terminus results in an increase in the activity of indolicidin against both gram positive and gram negative bacteria. [Falla et al., 1996] It is worth noticing that the medium of both colony 3 and *P.pastoris* X–33 exhibited antimicrobial activity even though only colony 3 have had the expression cassette integrated into its genome. When compared to the synthesized IL4, the medium of colony 3 and *P.pastoris* X–33 displayed a higher degree of activity against *P.putida* then against *B.subtilis*. This indicates the presence of something other then IL4 that displays activity and influences the results.
The size of the inhibited zone in disk diffusion essays is the result of several factors. The toxicity of the substance used against the chosen bacteria and the diffusion through the test medium by the substance. [Mickel and Wright, 1999] The ability of a substance to diffuse through a medium is a function of its hydrophilicity or hydrophobicity, size of the molecule, and the rate of release from the insoluble matrix that the molecule is bound to. [Mickel and Wright, 1999] A comparison between different substances is very difficult since each substance diffuses differently, some more readily than others. [Mickel and Wright, 1999] The difference in diffusion of substances makes it interesting to combine different types of molecules. The combination of different substances might allow for an effect even though one of the substances does not exhibit an effect in some instance. The growth inhibition done with A–skum with and without IL4 is an example of this, figures 3.21 and 3.22. There it can clearly be seen that the addition of IL4 allowed for antimicrobial activity even though A–skum showed no activity at that low dosage, 1/10th of the recommended dosage.

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Appendix

A

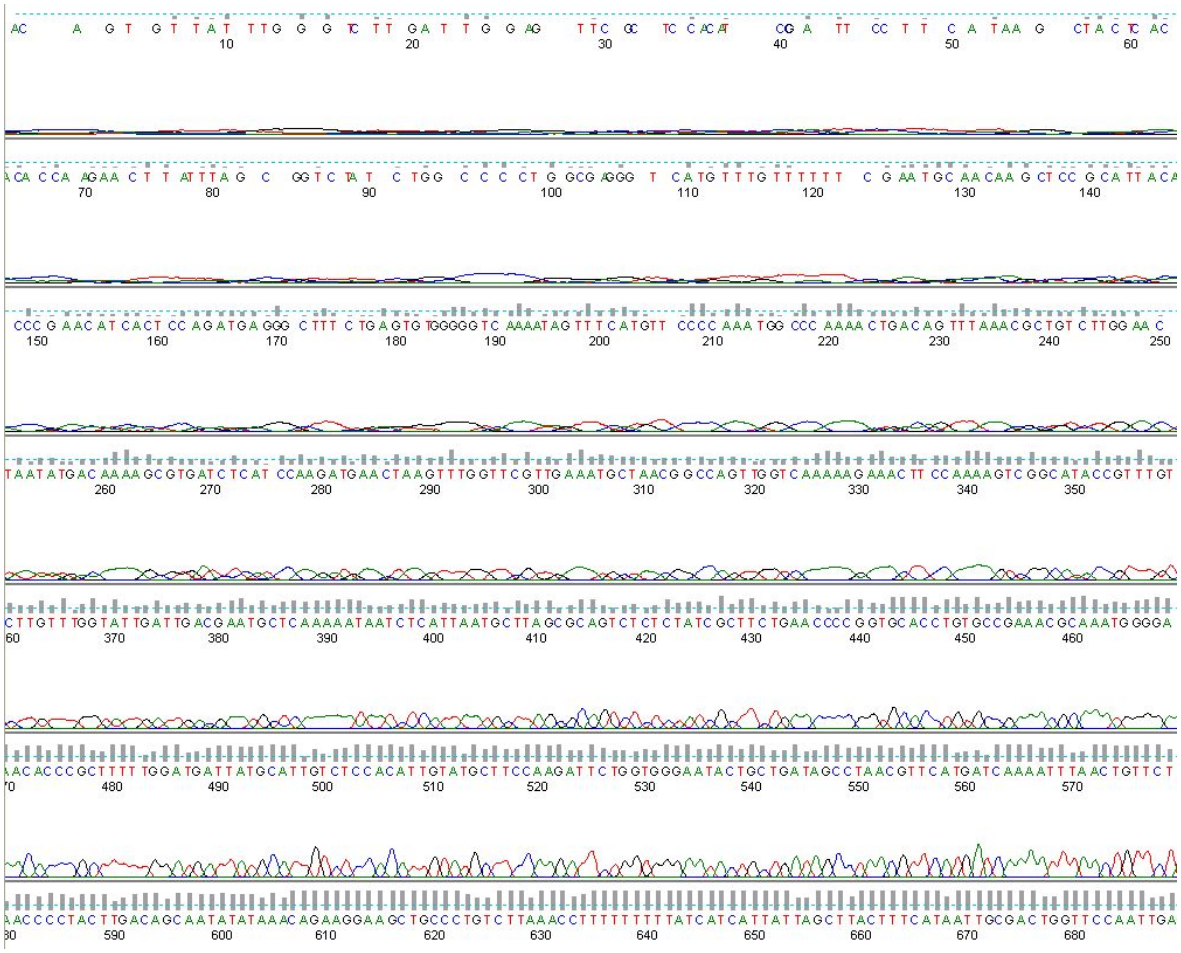

Figure A.1: Chromatogram and DNA sequence from the sequencing of pPICZαA/IL4 using the 3´AOX1 primer. The chromatogram covers codon 1–689

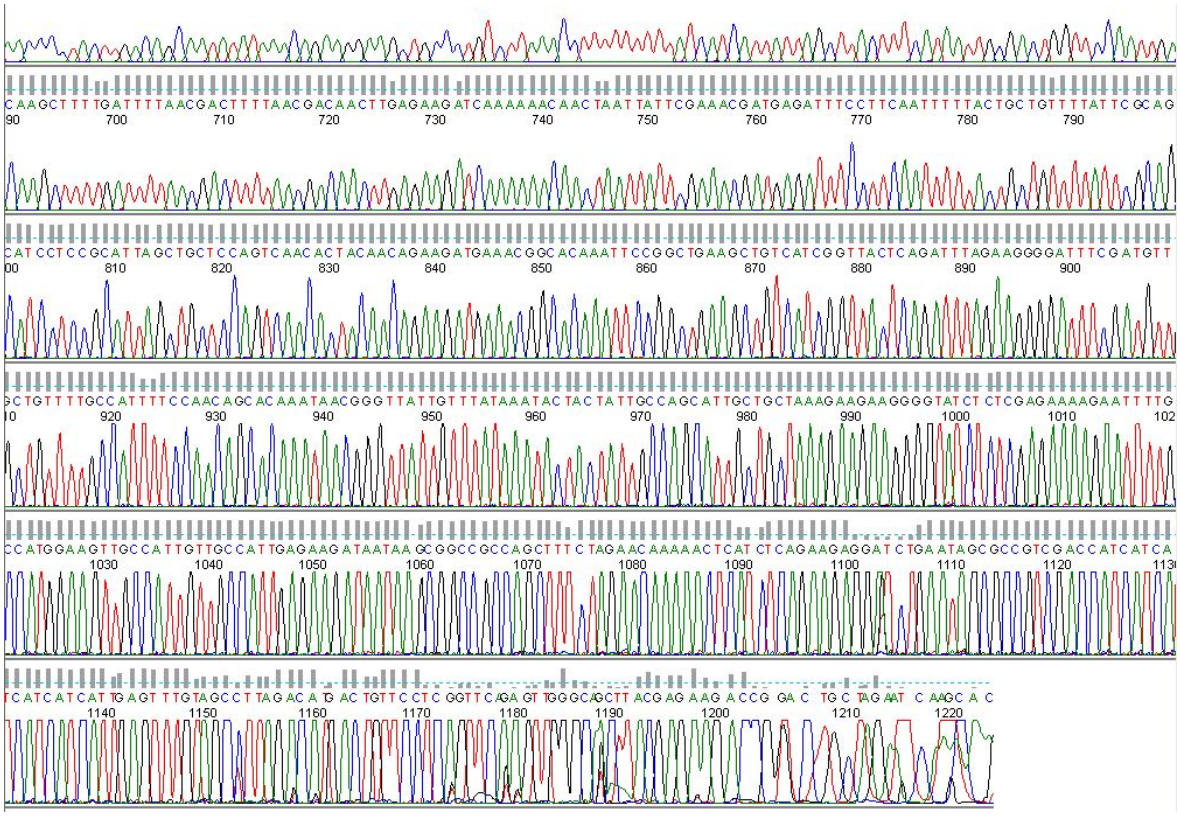

Figure A.2: Chromatogram and DNA sequence from the sequencing of pPICZαA/IL4 using the 3´AOX1 primer. The chromatogram covers codon 690–1224