Deciphering Rationales that Determine the Resistance of a Lipase Towards Non-Conventional Media

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften genehmigte Dissertation

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Cette thèse est dédiée à mes parents, frères et soeurs ainsi que ma famille entière.
Für meinen Ehemann Christian Frauenkron und meine liebevollen Kinder
Vanessa Frauenkron Mafotsing und Raphael Frauenkron Kouemene

Ntah Pro ka´h fit a kwet a pa´h wo (une seule main ne peut pas fagoter un paquet)
(Adage Fomopea)

I. STATEMENT

Hiermit versichere ich, dass ich die vorliegende Arbeit („Deciphering rationales that determine the resistance of a lipase towards non-conventional media") selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe, dass alle Stellen der Arbeit, die wörtlich oder sinngemäß aus anderen Quellen übernommen wurden, als solche kenntlich gemacht sind und dass die Arbeit in gleicher oder ähnlicher Form noch keiner Prüfungsbehörde vorgelegt wurde.

Alsdorf, den 13. Mai 2015 Victorine Josiane Frauenkron Machedjou

II. PUBLICATIONS

Frauenkron-Machedjou V. J., Fulton A., Zhu L., Anker C., Bocola M., Jaeger KE, Schwaneberg U., (2015), Towards understanding of directed evolution: more than half of all amino acid positions contributed to ionic liquid resistance of *Bacillus subtilis* lipase A. ChemBioChem. 2015, 16(6):937-45.

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Fulton A., Frauenkron-Machedjou V. J., Huesgen K., Bocola M., Schwaneberg U., Jaeger KE. Cysteine-scanning mutagenesis of *Bacillus subtilis* lipase A revealed possible sites for cysteine substitutions with greatly enhanced detergent resistance. In preparation.

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IV. ABSTRACT

The naturally existing enzymes have been evolved to work efficiently in aqueous environment of their hosts. Very often, their activity gets dramatically lost in the presence of non-conventional media (e.g. ionic liquids (ILs) and organic solvents (OS)) which are often present in industrial processes. Therefore, it is crucial to maintain/improve the enzyme resistance towards non-conventional media for the efficient operation of enzymes in industrial process conditions. Despite of the many successful stories of improving enzyme resistance towards many non-conventional media by protein engineering, there have been no geneneral principles for guiding researchers for efficient reengineering of enzymes. The aim of this work was to obtain protein engineering principles for improving IL and OS resistance of enzymes. A systematic study including site saturation mutagenesis (SSM) library generation of the complete sequence of a model enyzme *Bacillus subtilis* LipA (BSLA) and screening in presence of four ILs ([BMIM][Cl], [BMIM][Br], [BMIM][I], [BMIM][TfO]) and three OS (DMSO, TFE, 1,4-dioxane) were performed. The generation of 181 SSM-BSLA libraries was carried out by two PhD fellows in close cooperation (91 SSM-BSLA libraries by Victorine Josiane Frauenkron Machedjou and 90 by Alexander Fulton). Overall, 18547 clones were generated and screened which contained all possible 3620 single mutations as confirmed by sequencing. The number of beneficial positions and substitutions, type of beneficial amino acid exchange and location of positions was investigated. To the best of our knowledge, this is the first study based on such a complete set of experimental data to investigate IL/OS resistance of an enzyme.

In a first study (described in chapter 3), the resistance of SSM-BSLA variants towards four [BMIM]-based ILs (Cl^-, Br^-, I^-, and TfO^-) was investigated. The highest number of improved variants was obtained for [BMIM][Cl]: 69% of all positions and 13% of all substitutions showed improvements. For the four ILs, at 50-69% of all positions at least one substitution led to improvement whereas only 6-13% of all substitutions displayed increased resistance in all four ILs. It was also noteworthy that higher improvements from single substitution (>2.5-fold) were difficult to achieve by screening random mutagenesis library since only 0.3% of all the substitutions resulted in improvement >2.5-fold. Furthermore, amino acid substitutions with polar and charged amino acids were demonstrated to considerably enhance ILs resistance of BSLA. Among the best variants, the highest improvement (11.3-fold) was obtained for [BMIM][Cl] for a substitution from charged acidic to aromatic (Asp91Trp). Two main lessons were obtained: a) resistance improvements were

obtainable at 50-69% of all amino acid positions, explaining the success rate of small sized random mutant libraries; b) in total 6-13% of substitutions led to improved resistance. Among the beneficial substitutions 66-95% were substituted by chemically different amino acids (e.g. aromatic to polar/aliphatic/charged amino acids), indicating that mutagenesis methods introducing transversions should at least for lipases like BSLA be favored to improve IL resistance.

In a second study (described in chapter 4), the resistance pattern of SSM-BSLA variants towards three OS (DMSO, TFE, and 1,4-dioxane) was investigated. For 1,4-dioxane and TFE, 4-5% substitutions at 41% positions led to improved resistance. As observed for IL resistance of BSLA, improvements >2.5-fold were difficult to achieve with single point mutation, only 0.03% and 0.17% variants were obtained for DMSO / 1,4-dioxane and TFE, respectively. In addition, substitutions of BSLA WT amino acid residues with charged amino acids residues predominantly led to improved OS resistance towards TFE and 1,4-dioxane, whereas polar substitutions were preferred for DMSO.

Overall, two features for BSLA resistance in non-conventional media were observed: 1) resistance improvements were obtainable at >41% (in OS) and >50% (in ILs) of all amino acid positions of BSLA, indicating why improved enzyme variants can often be identified from small sized random mutant libraries (1000-2000 clones) and 2) often only few substitutions per amino acid positions led to improved resistance (4-13% of all substitutions) for OS and ILs. Among the beneficial substitutions, 66-95% and 58-95% were exchanged with chemically different amino acids (e.g. aromatic amino acids substituted by polar/aliphatic/charged amino acid) for ILs and OS, respectively. The latter indicates that mutagenesis methods that introduce transversions should, at least for lipases like BSLA be favored to generate variants with improved OS and/or IL resistance. Furthermore, the highest number of improved BSLA variants were obtained for [BMIM][Cl] and DMSO among the four ILs and the three OS, respectively.

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Chapter 1

General Introduction

1 General Introduction

1.1 Non-conventional Media Used in Biocatalysis

Enzymes are powerful biocatalysts, which catalyze a broad range of reactions under mild conditions and have the ability to increase the reaction rates by up to 10^{12} (Carrea and Riva, 2000). For a long time, enzymes have been considered to be active only under mild reaction conditions and in presence of water. Most enzymes have not been designed by nature to work in non-aqueous environments in which many enzymes are destabilized (Arnold, 1988). Water plays a crucial role in the existence of life and its role in enzymatic reactions is indispensable for the production of all organic molecules necessary for life (Ogino and Ishikawa, 2001). In contrast, non-aqueous solvents can affect enzymes performance by: (1) disrupting the hydration shell around enzymes and stripping off essential water from them; (2) penetrating into the water layer to interact with enzymes via electrostatic, hydrophobic, and H-bond interactions and in turn altering the protein dynamics, the conformation, and/or the active site; and (3) interacting directly or indirectly with the substrates and products, changing their concentrations in the aqueous layer (Yang and Pan, 2005; Yang and Russell, 1996). These interactions might cause unfolding and/or aggregation, leading to inactivation of enzymes. Therefore, it is not unexpected that almost all studies in enzymology have been carried out in aqueous media, trying to obtain the highest reaction efficiency. This general idea has impeded the application of biotransformations in synthetic routes that commonly employ organic solvents (OS). Nevertheless, intrinsic properties such as high specificity and selectivity make enzymes attractive biocatalysts for industrial applications (Vermue and Tramper, 1995), especially for the synthesis of biochemical, pharmaceuticals, and chiral intermediates (Ogino and Ishikawa, 2001). These compounds generally display disadvantageous properties such as insolubility or instability in water. Therefore, applying non-conventional media for biocatalysis can be a promising approach to overcoming this problem, enabling the production of compounds that are difficult or expensive to produce by chemical synthesis. It has been demonstrated that biocatalysts are less sensitive to harsh reaction conditions than expected, retaining their activity in all sorts of non-conventional media (Vermue and Tramper, 1995). Non-conventional media include all reaction media different from pure aqueous buffers. The most prominent examples of non-conventional media are OS, ionic liquids, gas and solid phases, supercritical fluids (Cantone *et al.*, 2007; Hari Krishna and Karanth, 2002) or deep eutectic solvents (Abbott *et al.*, 2004; Carriazo *et*

al., 2012; Domínguez de María and Maugeri, 2011; Lehmann *et al.*, 2012; Zhang *et al.*, 2012). Using enzymes in non-conventional media is tantalizing as they enable the production of useful chemicals which are not possible in water and their advantages range from high substrate specificity to higher enzyme activity and stability (Hari Krishna, 2002). Due to these advantages, their scientific as well as industrial applicability is gaining huge interest. Hence, much progress has been made in the fundamental understanding of the phenomena that govern enzyme properties in non-conventional media. Several research groups and institutions, for instance, the graduate school BioNoCo (**Bio**catalysis in **No**n-**Co**nventional media) aim to engineer enzymes as ideal tools for organic chemistry and enhance their stability in different non-conventional media. The non-conventional media investigated in this thesis are ionic liquids (ILs) and organic solvents (OS), which are intensively discussed in the next chapters.

1.1.1 Ionic Liquids (ILs)

1.1.1.1 Definition and History

Ionic liquids (ILs) are low-temperature molten salts composed of a mixture of large organic cations and smaller organic or inorganic anions, which remain liquid at low to moderate temperature (Rogers and Voth, 2007; Wasserscheid and Keim, 2000; Welton, 1999). When in melted state at room temperature, they are also termed room temperature ionic liquids (RTILs). The liquidity of ILs at moderate temperature is a consequence of the inability of such anions and cations to pack well between each other (Keskin *et al.*, 2007). Their low melting points result from the incorporation of bulky asymmetric organic cations into the structure, together with weakly coordinating anions (Domínguez de María, 2012), disturbing the efficient packing of ions in the crystal lattice (Krossing *et al.*, 2006). In contrast to classical molten salts, which are usually highly viscous, high-melting, and very corrosive substances, ILs possess a relatively low viscosity (Wasserscheid and Keim, 2000).

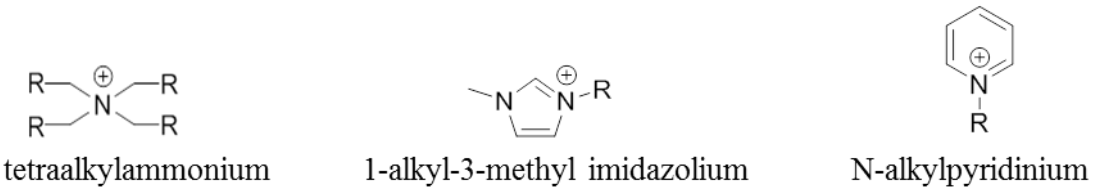
The first ionic liquid, ethanol ammonium nitrate (melting point 52-55°C), was discovered 1888 by Gabriel and Weimer (Gabriel and Weiner, 1888). It was first in 1914, when Paul Walden described the physical properties of ethylammonium nitrate [EtNH3][NO3] as a room temperature ionic liquid (RTIL), with a melting point of 13-14°C (Walden, 1914). Although this report gained less resonance at that time, it is considered to be the starting point of the ILs´ history. The lack of interest in ILs was due to their instability at room temperature. Indeed, the first ILs were found to be sensitive to water and air and these

findings caused a major drawback for their application in chemical processes. ILs started to attract interest in the 1990s, when the second generation of ILs were synthetized with weakly coordinating anions such as BF_4, CH_3CO_2, or NO_3 that were water and air stable (Wilkes and Zaworotko, 1992). In the past years, ILs have been studied intensively and have gained considerable attention as they have been applied as (co)solvents for a wide range of chemical and biochemical reactions. Consequently, more than 8000 papers, including over 900 patents and applications have been reported since 2007 (Rogers and Voth, 2007). Nowadays, these numbers have considerably increased with the endeavors devoted on IL studies. The development of cheaper ILs in particular increases their attractiveness for industrial applications. Deep eutectic solvents (DES) have emerged in early 2000s as a cost-effective alternative to ILs (Domínguez de María, 2012). DES contain biodegradables anions (amino acids, natural bases, and naturally occurring carboxylic acids), derived from renewable resources and are simpler to synthetize compared to ILs (Abbott *et al.*, 2004).

1.1.1.2 Characteristics of Ionic Liquids

ILs generally contain bulky cations such as imidazolium, pyridinium, pyrrolidinium, quaternary ammonium or tetraalkylphosphonium (Figure 1-1A) and small anions (Figure 1-1B). One major interests of applying ILs as reaction media is the possibility to fine-tune their physicochemical properties, allowing them to be designed for task-specific reactions. ILs are also referred to as designer solvents (Freemantle, 1998). The properties of ILs are mainly influenced by the choice of the anion counterpart (influencing chemical properties and reactivity) and/or the cation counterpart (controlling physical properties) (Huddleston *et al.*, 2001). Thus, according to the required process, solubility, viscosity, polarity, density, melting point, hydrophobicity, and hydrophilicity can be finely adjusted (Yang and Pan, 2005). Therefore, an almost infinite number of ILs can be synthesized. Some commonly employed IL-cations and IL-anions are illustrated in Figure 1-1.

A



B

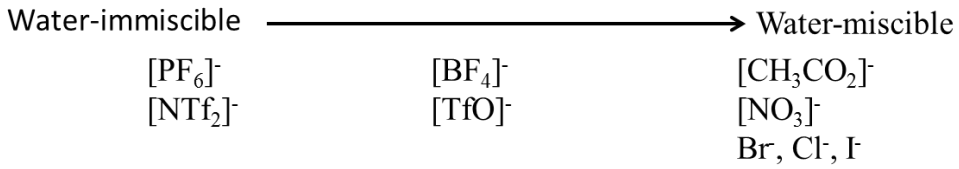


Figure 1-1: Examples of cations and anions used in ionic liquid systems. **A:** common used IL-cations, **B:** common used IL-anions. The anions are classified in increased order of water-miscibility. **$[PF_6]^-$:** hexafluorophosphate, **$[NTf_2]^-$:** bis(trifluoromethylsulfonyl) imide, **$[BF_4]^-$:** tetraflouroborate, **$[TfO]^-$:** trifluoromethanesulfonate, **$[CH_3CO_2]^-$:** acetate, **$[NO_3]^-$** : nitrate, **Br^-:** bromide, **Cl^-:** chloride, **I^-:** iodide. This shema was partly adapted from (Plechkova and Seddon, 2007) with the permission of Royal Society of Chemistry provided by Copyright Clearance Center.

ILs are characterized by high density, high thermal and chemical stability, low viscosity, negligible vapor pressure as well as high polarity (Anderson *et al.*, 2002; Huddleston *et al.*, 2001; Kaar *et al.*, 2003; Park and Kazlauskas, 2003; Sheldon *et al.*, 2002). The high polarity of ILs is one of their most unique features, which increases their attractiveness for chemical and biochemical reactions (Yang and Pan, 2005). In fact, high polarity enables ILs to dissolve a broad range of different substances, for instance polar and non-polar organic, inorganic and polymeric compounds (Madeira Lau *et al.*, 2000). Low vapor pressure and high thermal stability are properties that increase their usage as biocatalysts from an environmental point of view (Anderson *et al.*, 2002). Additionally, the immiscibility of ILs with a range of solvents facilitates their recycling (Murugesan and Linhardt, 2005). Although ILs are used to meet the increasing demand for clean technologies in industrial processes, their toxicology is not yet well studied (Park and Kazlauskas, 2003) and further research is necessary to evaluate their sustainability.

1,3-Dialkylimidazolium-based ILs are among the most widely used ILs. Their anions and cations have been found to be associated in part by electrostatic, hydrophobic interactions and mostly by hydrogen bonds resulting in a polymeric supramolecular structure or IL network (Domínguez de María, 2012; Dupont, 2004), which is similar to the three-dimensional network of water. In this structure, one imidazolium cation is surrounded by at least three anions or vice versa (Figure 1-2A). However, the number of anions surrounding the cations (and vice-versa) can change according to the anion size and the type of N-alkyl

imidazolium substituents (Dupont, 2004). Introduction of molecules such as water and macromolecules in the IL network can modify their physicochemical properties resulting occasionally in the formation of polar and non-polar regions (Dupont, 2004). The mesh of ILs (Figure 1-2B) enables the maintenance of enzyme´s active structural conformation with the ability to prevent enzyme from classical thermal unfolding (Fehér *et al.*, 2007).

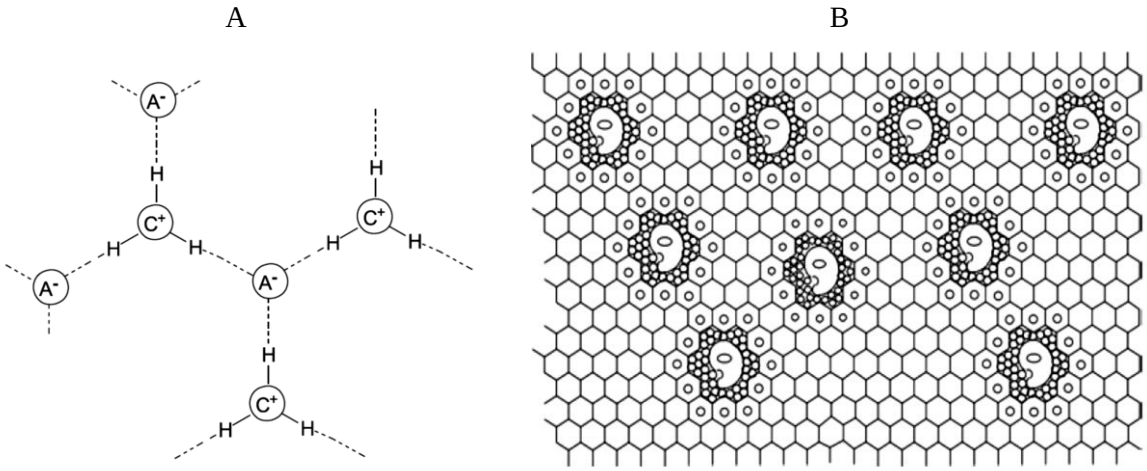


Figure 1-2: Two-dimensional simplified solid-state model of the polymeric supramolecular structure of 1,3-dialkyl imidazolium ILs and ILs´s network. A: Two-dimensional simplified solid-state model of the polymeric supramolecular structure of 1,3-dialkyl imidazolium ILs illustrating the hydrogen bonds between the imidazolium cation (C) and the anions (A) (one cation is surrounded by three anions and vice-versa). B: Enzymes with a small amount of water are firmly trapped in the network of ILs. Figure A reproduced from (Dupont, 2004) under a creative common license and figure B from (Fehér *et al.*, 2007) with the permission of the Biochemical Society.

1.1.1.3 Applications of Ionic Liquids

ILs are tunable, multi-purpose materials for a variety of applications, rather than being just solvents. The main fields for the application of ILs are summarized in Figure 1-3. ILs are used as sensors, solar cells, solid-state photocells and batteries and also as thermal fluids (lubricants, hydraulic fluids, and ionogels) (Rogers and Voth, 2007). Due to their unique properties, ILs have been intensively investigated and are gaining an astonishing interest in industries and particularly in academia. Several reviews (Patel *et al.*, 2014; van Rantwijk and Sheldon, 2007; Wasserscheid and Keim, 2000; Yang and Pan, 2005; Zhao, 2010) deal with this topic, and in particular the one written by Plechkova and Seddon listed the companies working with ILs at an industrial level (Plechkova and Seddon, 2007). Using ILs instead of classical materials offers several advantages such as high conversion rates, high selectivity, better enzyme stability, as well as recoverability and recyclability (Moniruzzaman *et al.*, 2010). Additionally, ILs can reduce the risks associated with the use of volatile and highly flammable organic compounds (Krossing *et al.*, 2006). In the area of protein research, ILs

have been used for stabilization, crystallization, solubilization, separation, extraction of proteins, and performance additives in enzymatic reactions (Patel *et al.*, 2014).

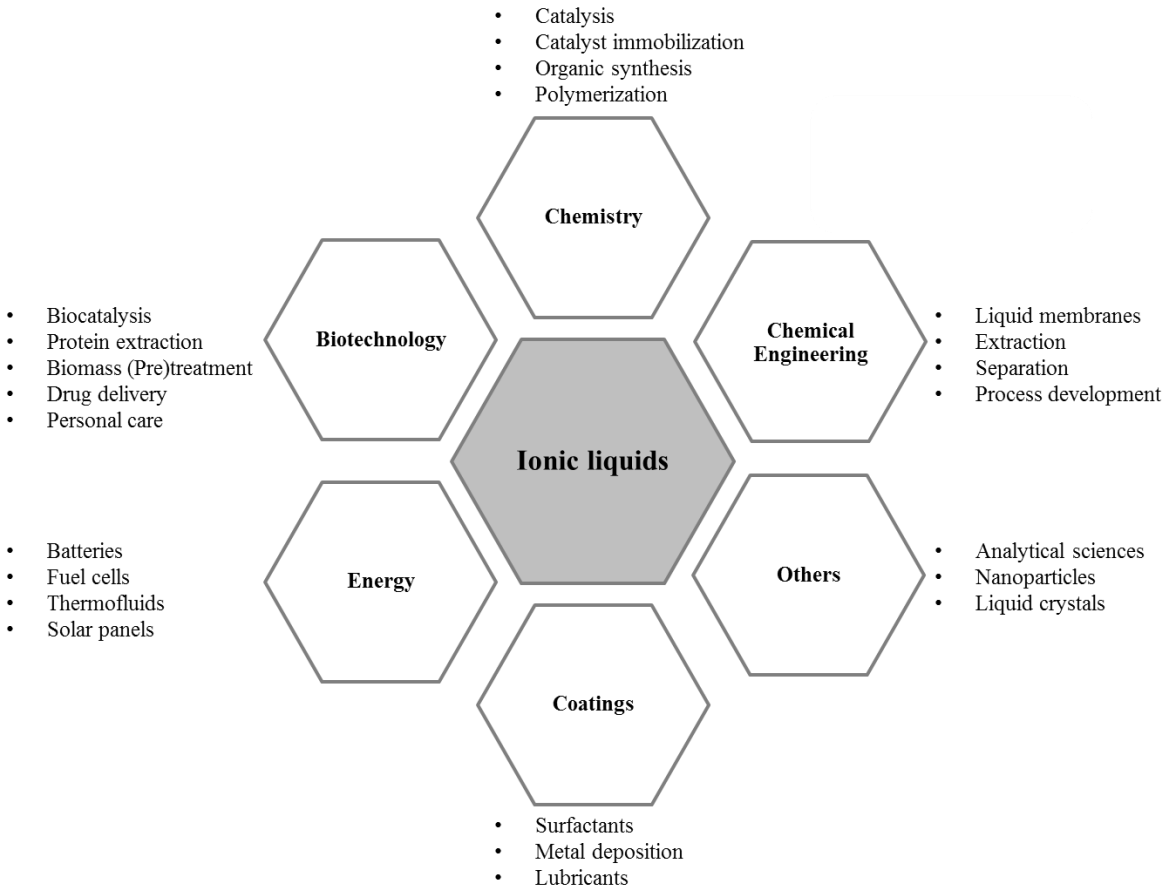


Figure 1-3: Overview of possible applications of ILs in different areas. This figure was adapted from (Domínguez de María, 2012; Patel *et al.*, 2014; Plechkova and Seddon, 2007).

1.1.2 Organic Solvent (OS)

Although water is a crucial constituent of life, it is unfortunately a poor solvent for most synthetic reactions (Serdakowski and Dordick, 2008). Many organic compounds (enzymes and substrates) are insoluble in water and rather soluble in OS. OS efficiently enable an increased solubility of hydrophobic substrates, enhancing reaction rates and product formation (Vermue and Tramper, 1995; Zaks and Klibanov, 1985). Therefore, enzymatic reactions in the presence of OS possess a great potential for academic and industrial applications. From this point of view, researchers have struggled in the last decades to develop methods to enhance the stability of enzymes in the presence of OS. Two different solutions have been proposed to handle the insufficient enzyme tolerance toward OS: **1)** Optimization of the reaction conditions to the available catalysts and **2)** design of biocatalysts, able to withstand ideal process conditions (Luetz *et al.*, 2008). OS are the most used non-conventional media and success stories of increased stability and activity as well as interesting catalytic properties

of enzymes in such solvents have been reported (Carrea and Riva, 2000; Klibanov, 2001; Staahl *et al.*, 1991; Zaks and Klibanov, 1985). Despite that, wild type enzymes generally display low activity and/ or stability in presence of OS and also apt to denaturation or inhibition. Several advantages and disadvantages of using OS instead of water have been listed in Table 1-1.

Table 1-1: Advantages and disadvantages of enzymatic reactions in presence of OS (Castro and Knubovets, 2003; Doukyu and Ogino, 2010; Serdakowski and Dordick, 2008; Zaks and Klibanov, 1985).

Advantages	▪ Increased solubility of most organic compounds in non-aqueous media
	▪ Ability to perform new reactions impossible in water because of kinetic or thermodynamic restrictions
	▪ Outstanding stability of enzymes
	▪ Relative easy product recovery from OS compared to water
	▪ Easy recovery and reuse, elimination of immobilization because of insolubility of enzymes in organic media
	▪ Suppression of water-dependent side reactions
	▪ Elimination of microbial contamination
	▪ Alteration of substrate specificity
Disadvantages	▪ Inactivation of enzymes
	▪ Labor and cost-intensive preparation of biocatalysts in covalently modified systems
	▪ Mass-transfer limitations for viscous solvents or heterogeneous systems
	▪ Necessity to control water activity for processes requiring condensation reactions
	▪ Problems with recycling biocatalysts in non-covalently modified systems

Organic solvent reaction media can be divided into four groups (Figure 1-4): **A)** water/organic-solvent mixtures, **B)** two-phase systems (B1, B2, and B3), **C)** micro-aqueous organic-solvent mixtures and **D)** reversed micelles. Water/organic-solvent mixtures usually contain water with a small amount of water-miscible solvent. In contrast, a two-phase system consists in a water-immiscible OS in an aqueous buffer. Micro-aqueous organic mixtures are those OS in which dry enzymes are suspended and the available water is mainly located on the solid enzyme particles. Reversed micelles involve miniscule droplets, which are stabilized by a surfactant in a bulk of water-immiscible OS (Vermue and Tramper, 1995).

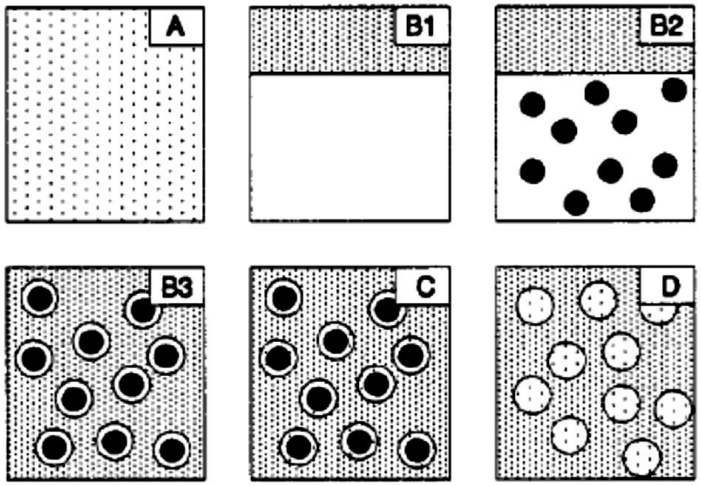


Figure 1-4: Groups of organic solvent reation media. A) water-miscible solvent, **B1**) two-phase system with low volume organic solvent and solubilized biocatylyst, **B2)** two-phase system with low volume organic solvent and immobilized biocatylyst, **B3)** two-phase system with high volume organic solvent and immobilized biocatylyst, **C)** micro-aqueous system, **D)** reverse micelles. White area represents water, small black dots represent organic solvent, big black dots represent biocatalyst, and white dots represent micelles. This figure was taken from (Vermue and Tramper, 1995) under a free common license.

Within all these categories of OS, research effort in this thesis focusses on the first group concerning water-miscible OS. Indeed, the screening system used in this work is based on the lipase catalyzed hydrolysis reaction which is favored in reaction mixtures with high water content. In contrast, lipase catalyzed esterification reaction is favored in pure OS. It is noteworthy, that lipases are the first reported and most studied enzymes in OS (Gupta, 1992; Sharma and Kanwar, 2014; Zadlo *et al.*, 2015; Zaks and Klibanov, 1984, 1988).

1.1.2.1 Characteristics of OS

Volatility, flammability, deleterious effects on health, safety and environment are few reasons why alternative solvents as ILs are sometimes favored to OS (Huddleston *et al.*, 2001). OS harbor a range of properties that should be considered for their utilization in synthesis reactions and processes. Hildebrandt solubility parameter (δ), dielectric constants (ε), dipole moments (μ), and LogP are properties that are associated with the prediction of enzyme activity in OS (Khmelnitsky *et al.*, 1991; Laane *et al.*, 1987). A high dielectric constant (ε) of the solvent commonly enhance the dissolution of ions while the dipole moment (μ) allows the determination of the solvent-solute molecules orientation (Chernyak, 2006). Hildebrandt solubility parameters (δ) describes the miscibility behavior of solvents (Belmares *et al.*, 2004). LogP is the logarithm of the partition coefficient of the solvent in a two-phase system of 1-octanol and water and is used as a measure of the hydrophobicity/polarity of a solvent (Laane *et al.*, 1987; Liu *et al.*, 2009). Among these parameters, logP meets all the

requirements as good indicator since it is a direct measure of polarity unlike other parameters (δ, μ, ε), can be easily calculated, and is very sensitive for polarity differences in wide range (Laane *et al.*, 1987). Thus, the polarity of OS can be classified in three categories using LogP: low polarity (logP < 2); moderate polarity (2 ≥ logP < 4); high polarity (logP > 4) (Laane *et al.*, 1987). LogP has been reported to correlate with activity and stability of various enzymes at least a decreased lipase activity has been observed with decreased logP. For instance, no toxic effects were observed at high logP, unpredictable toxic effects were reported at intermediate logP and inactivation was found at low logP (Liu *et al.*, 2009). Nonetheless, few examples have shown that logP does not always correlate with enzyme activity and stability. It seems to be applicable only for solvents of the same functionality such as polyols and alcohols (Khmelnitsky *et al.*, 1991).

1.1.2.2 Applications of OS

One of the main advantages of applying enzymatic reactions in OS relies on the possibility to perform reactions which are not possible in water because of kinetic or thermodynamic restrictions and insolubility of most organic compounds (Arnold, 1990; Castro and Knubovets, 2003). New alternatives are possible and biotechnological applicability of enzymes is therefore increased. As a result, enzymatic reactions in OS can be applied for, 1) kinetic resolution of racemates in pharmaceutical and agrochemical companies, 2) synthesis of chiral intermediates in chemistry, 3) modification of fats and oils 4) synthesis of sugar-based polymers, 5) gas phase biocatalysis, 6) biopolymers, 7) production of flavors and fragrances (Carrea and Riva, 2000; Choi and Yoo, 2012; Hari Krishna and Karanth, 2002; Schmid *et al.*, 2001; Zumárraga *et al.*, 2007a).

1.1.3 Organic vs Ionic Liquids

Several reports have clearly demonstrated that the catalytic mechanism observed for enzymatic reactions in ILs is similar to the one obtained in water and OS. Accordingly, De Diego and co-workers reported that stabilization of enzymes by ILs as well as by hydrophobic OS correlates to the preservation of their compact native structure, being associated with the preservation of the essential water shell (De Diego *et al.*, 2004). As in OS, enzymes in ILs are presumed to require a microaqueous phase surrounding its structure (Ventura *et al.*, 2012). Both affect enzyme performances in a similar manner. Although the effect of OS on enzymes performance has been reported to be similar to the effect of ILs, the latter however, dissolve and dissociate into individual anions and cations rather than being intact molecules (Yang *et*

al., 2009). Therefore, particular attention has to be paid to the effect of IL-ions on enzyme properties. Because of their excellent properties, better enzyme performance is sometimes achieved in ILs compared to OS. In Table 1-2, properties of ILs and OS are compared revealing the advantages and disadvantages of both non-conventional media.

Table 1-2: Comparison between OS and ILs. This table was adapted from (Plechkova and Seddon, 2007) with the permission of Royal Society of Chemistry provided by Copyright Clearance Center.

Properties	OS	Ionic liquids
Number of solvents	≥1,000	≥1,000,000
Applicability	Single function	Multifunction
Catalytic ability	Rare	Common and tunable
Vapor pressure	Mostly high	Negligible under normal conditions
Flammability	Usually flammable	Usually nonflammable
Solvation	Weakly solvating	Strongly solvating
Tunability	Limited range of solvents available	Virtually unlimited range (designer solvents)
Polarity	Conventional polarity concepts apply	Polarity concept questionable
Recyclability	Green imperative	Economic imperative
Cost	Normally cheap	Typically between 2 and 100 times the cost of OS

1.2 Protein Stability

Proteins are linear polymer chains that consist of a combination of the 20 canonical amino acids. The sequence and arrangement of the amino acids is crucial since small changes in the order of amino acids affect the function. Proteins are essential in all aspects of cell function and as enzymes they catalyze biological processes under physiological conditions of the host organism (Illanes *et al.*, 2012). The intact structure of a protein is essential for achieving a proper function. Four distinct categories of protein structure are known: primary, secondary, tertiary, and quaternary structure. The primary structure consists of the unique linear sequence of the amino acid residues in the polypeptide chain that is held together by covalent peptide bonds made during the process of protein biosynthesis. The secondary structure describes regions of periodic conformations (e.g. α-helix and β-sheet) resulting from hydrogen bonding along the peptide backbone. Alterations in α-helices and β-sheets can lead to denaturation and irreversible aggregation of proteins. Both primary and secondary structures of a protein contribute to its tertiary structure. The tertiary structure consists of the three-dimensional shape that is stabilized by hydrogen bonding, salt bridges, π-π stacking of

aromatic amino acids, as well as by covalent bonds (disulfide bridges) as illustrated in Figure 1-5. The quaternary structure is an aggregation of two or more polypeptide subunits.

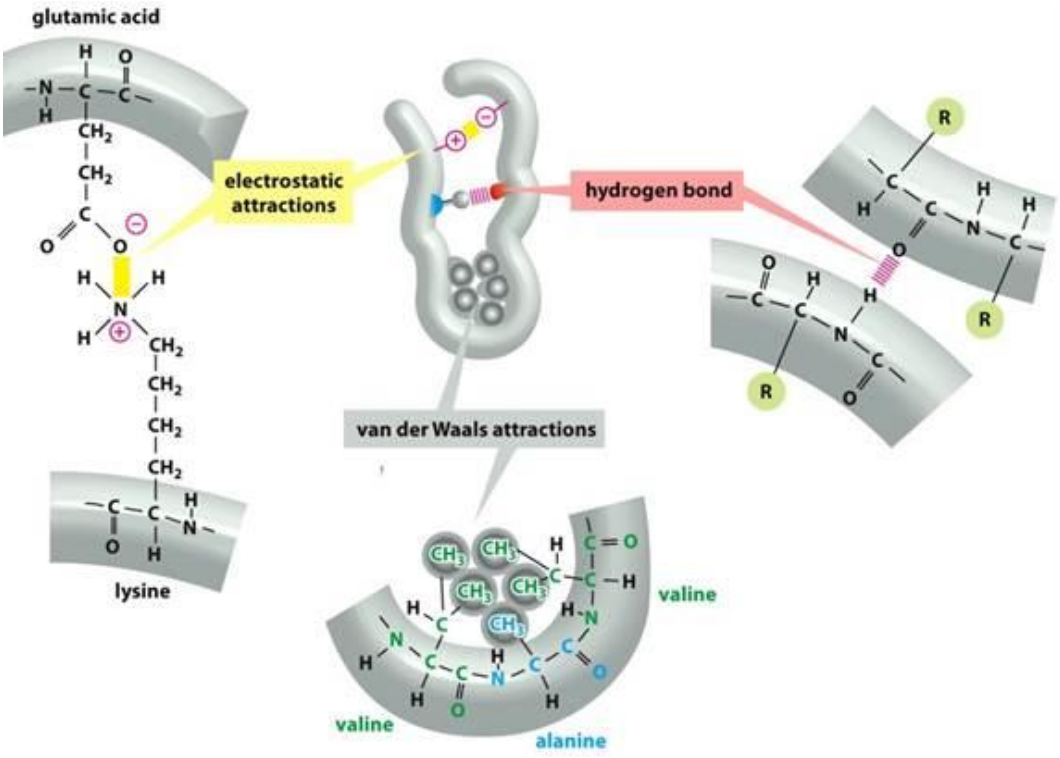


Figure 1-5: Various types of interactions stabilizing proteins. Some examples between residue chains are shown. R= amino acid side chain, yellow represents electrostatic interactions, red represents hydrogen bonds and grey represents van der Waals interactions. This figure was reproduced from Essential Cell Biology, 4th Edition by Alberts *et al.* with the permission of Garland Science/Taylor & Francis LLC.

1.2.1 Strategies for Stabilization of Enzymes

Utilization of proteins in industrial processes requires high stability under process conditions. Indeed, enzymatic reactions in industries are sometimes performed with novel substrates or under extreme conditions of salt content, temperature, pH, or in the presence of inhibiting or denaturing chemicals (Brissos *et al.*, 2008; Eijsink *et al.*, 2005). Under such non physiological conditions, the native structure of proteins is destroyed and followed by the loss of its biological activity, which is closely connected with their native, three-dimensional (3D) structure. This influences the stability and strongly limits the biotechnological applicability of enzymes. In fact, enhanced stability has been reported to be a prerequisite for altering enzymatic activity (Bommarius *et al.*, 2006).

Protein stability can be defined as the preservation of the unique chemical and 3D structure of a polypeptide chain under aqueous conditions. The quantification of the stability can be achieved by breaking the native structure of a protein (Jaenicke, 1991). Denaturation or deactivation induces irreversible protein aggregation, which was proposed by Vagenende

and co-workers as a major manifestation of instability affecting protein functionality *in vivo* and *in vitro* (Vagenende *et al.*, 2009). Protein stability results from a balance between destabilizing and stabilizing forces. The destabilizing forces are mainly due to the large increase in entropy of unfolding, and the stabilizing forces are provided by several types of non-covalent interactions. Disruption of any of these interactions may shift the balance and destabilize the protein (Wang, 1999). The thermodynamical definition of protein stability gives the difference in Gibbs free energy, ΔG, between the folded and the unfolded states. The unique factors affecting stability in this case are the relative free energies of the folded (G_f) and the unfolded (G_u) states. A larger and more positive ΔG_u results in a higher stability of the protein (Pace, 1990). Since the information for the folding is coded in the amino acid sequence and folding is the primary function of the nascent polypeptide chain, biologically active proteins have to be properly folded. Proteins fold to minimize their free energy, leading to well-packed hydrophobic interiors and hydrophilic exteriors. Hydrophobic forces are major driving forces during the folding process (Dill, 1990). Maximizing function leads to active site clefts where charged and polar groups are secluded from water and where hydrophobic pockets are exposed to solvent (Sadana, 1987; Shoichet *et al.*, 1995). Initially, it was demonstrated that folded conformations of proteins occur spontaneously from their linear chain in the appropriate environment, in most cases in milliseconds or less (Kubelka et al., 2004). Isolation of new enzymes, medium engineering and enzyme engineering have been used as strategies to improve enzyme stability in non-aqueous solutions (Carrea and Riva, 2000; Illanes *et al.*, 2012; Stepankova *et al.*, 2013). Few methods of these strategies are listed as well as their advantages and disadvantages are summarized in Table 1-3.

Table 1-3: **Advantages and disadvantages of stabilization strategies.** This table was adapted from (Stepankova *et al.*, 2013) with the permission of American Chemical Society provided by Copyright Clearance Center.

Strategy	Advantages	Disadvantages
• Intrinsically stable enzyme	• applicability in a variety of harsh environments	• laborious and time-consuming screening, possibly problematic recombinant expression, • risk of altered stability and activity of enzymes produced in different hosts
• immobilization	• biocatalyst reuse, continuous mode of operation, *in situ* product recovery facilitated, • definition of biocatalyst microenvironment, enhanced reaction rates, high volumetric productivities, • generally applicable procedures, enzyme activity and enantioselectivity can be tailored	• mostly empirical, high cost, mass transfer limitations, loss of enzyme activity, loss of the enzyme due to leakage, • operational restraints, requirement for additional material and equipment, • immobility of enzyme molecules inside carriers, particle erosion
• chemical modification	• increased solubility of the enzyme in OS, • decreased substrate diffusion limitations, nontoxic solvents, • fast and simple procedure, knowledge of the enzyme structure not necessary, not limited to natural amino acids	• differences between various batches, • enzyme needs to be modified each time
• genetic modification	• enzymes tailored for a target application, • "green" technology, no additional additives required	• time-consuming, high cost, risk of improper protein folding, • detailed knowledge of protein structure often required
• propanol-rinsed enzyme preparation	• drying of the enzyme without denaturation	• only a few reported examples, • risk of enzyme deactivation
• ionic liquid coating	• reduction of ionic liquid consumption, biocatalyst reuse, • ionic liquid properties tailored for target application	• only a few reported examples, uncertain toxicity • high price of ionic liquids, risk of enzyme deactivation
• additives	• simple procedure, enhancement of the enzyme flexibility, • wide choice of additives available	• complicated separation of additives from the product, • incompatibility with the reaction system
• microemulsions	• the enzyme is present in the aqueous phase, • increased solubility of substrates	• risk of enzyme inactivation by the surfactant or • large interface between the two phases

Protein stability is affected differently by ILs and OS. Given the industrial significance of enzymes, unravelling the molecular basis for protein stability has been the focus of numerous studies. Therefore, structural features relevant for improvement of protein stability were summarized including surface regions, the hydrophobic core, access tunnels, binding pockets, the interface of the enzyme's subunits disulfide bridges, surface charges, and isolated charges (Stepankova *et al.*, 2013). The integrity of a protein structure is determined by intramolecular interactions (salt bridges, hydrogen bonds, and hydrophobic interactions) and its interactions with the surrounding solutes and solvents (Burley and Petsko, 1988; Dill, 1990; Pace *et al.*, 1996; Prabhu and Sharp, 2006).

1.2.2 Factors Influencing Enzyme Activity and Stability/Resistance in Ionic Liquids (ILs)

Due to their structure and diversity, ILs are able to participate in most types of interactions (hydrogen bonding, dipolar, ionic or charge-charge) (Anderson *et al.*, 2002). Nevertheless, hydrogen bonding has been reported to be the most important factor that affects the interactions between enzymes and ILs. Therefore, this also has an impact on protein activity and stability as well as on maintenance of its native structure (Anderson *et al.*, 2002; Huddleston *et al.*, 2001; Kaar *et al.*, 2003; Lou *et al.*, 2006).

Effects on activity, stability, and selectivity observed in ILs have been reported to correlate with several factors, such as anion nucleophilicity, hydrogen-bond basicity, excipients, impurities, pH, the overall enzyme–substrate–medium relationship (Fehér *et al.*, 2007) as well as IL polarity characterized by Reichart´s polarity (Park and Kazlauskas, 2003). Better stability was observed in solvents with higher polarity as they increase the solubility of polar substrates, resulting in faster reactions and changes in selectivity (Fehér *et al.*, 2007). Nucleophilicity (defined as the ability of a nucleophile to accept an electrophilic C atom) and hydrogen bonding (defined as the ability of a base to accept an electrophilic H atom) are usually used to demonstrate the foremost importance of the IL-anion on enzyme properties (Anderson *et al.*, 2002; Huddleston *et al.*, 2001; Kaar *et al.*, 2003; Lou *et al.*, 2006; Yang and Pan, 2005). Hydrogen bond capacity increases with basicity, which also increases with nucleophilicity. A higher nucleophilicity leads to higher H-bond basicity, destabilizing the enzyme further. Moreover, decreased enzyme activity and stability have been demonstrated to correlate with increasing nucleophilicity (Sheldon *et al.*, 2002; Zhao, 2010). It is noteworthy that ILs containing strong nucleophilic anions were revealed to interact with positively

charged amino acids of enzymes, enabling the breakage of internal hydrogen bonds or the formation of new hydrogen bonds that perturb the structure (Kaar *et al.*, 2003; Lou *et al.*, 2006). Hydrophobicity/hydrophilicity have also been reported to influence the catalytic performance of enzymes (Yu *et al.*, 2014). Hydrophobic ILs generally display low nucleophilicity/H-bond basicity, whereas hydrophilic ILs possess strong H-bond basicity/nucleophilicity. While hydrophobic ILs displayed stabilizing effects on enzymes, pure hydrophilic ones were reported to lower the activity or even deactivate enzymes (De Los Ríos *et al.*, 2007). Indeed, ILs containing hydrophobic anions are inferred to change the secondary structure of enzymes resulting in enhanced interactions between substrate and active site (Dang *et al.*, 2007). This structural change was observed by the stabilization of *Candida antarctica* lipase B (CALB) in hydrophobic ILs and was attributed to the evolution of α-helix to β-sheet secondary structure of the enzyme, resulting in a more compact enzyme conformation able to exhibit catalytic activity (De Diego *et al.*, 2005). In addition, the authors assigned the increase in β-sheet content to the loss of hydrogen-bonding interactions between water molecules and α-helices. In contrast, ILs with hydrophilic anions have been reported to establish strong interactions via hydrogen bonding with enzymes causing enzyme deactivation (Ventura *et al.*, 2012). Most hydrophilic ILs are reported to remove the crucial enzyme bound water molecules, which maintain enzyme structure and function (Moniruzzaman *et al.*, 2010). However, addition of water to hydrophilic ILs can reduce the negative impact of ILs on the enzyme and can stabilize or even activate an enzyme when an appropriate amount of the mixture is used (Yu *et al.*, 2014). Although some hydrophilic ILs are able to solubilize enzymes through weak hydrogen bonding interactions, most of them cause conformational changes in enzymes leading to deactivation (Yu *et al.*, 2014).

The Hofmeister series is commonly used to show the specific ions effects: inorganic salts and ions displayed different patterns of precipitating proteins according to a recurring sequence called the Hofmeister series (Hofmeister, 1888; Yang, 2009). The effect of ILs on enzyme properties (activity, stability, enantioselectivity) has also been interpreted in some cases based on the Hofmeister series (Constantinescu *et al.*, 2007; Yang, 2009; Yang *et al.*, 2009; Zhao *et al.*, 2006), according to the kosmotropic (structure-maker) and chaotropic (structure-breaker) properties of the ILs. However, several studies are not in accordance with this theory, which sometimes better correlates with the IL-cations rather than IL-anions. Impurities in ILs such as traces of water, residual solvents, halides, acids, unreacted starting materials, or metal salts considerably affect the enzyme performance (Seddon *et al.*, 2009; Wasserscheid and Keim, 2000) and also have to be taken into consideration. Several attempts

to accurately predict enzymes stability in ILs have been made by studying enzyme classes such as hydrolases, and particularly lipases in ILs (De Diego *et al.*, 2005; Madeira Lau *et al.*, 2000; Ventura *et al.*, 2012). However, there is still a significant knowledge gap in understanding the chemistry of the interactions between enzymes and ILs.

1.2.3 Factors Influencing Enzyme Activity and Stability/Resistance in OS

Water activity, pH control, substrate-product solvation, enzyme form (e.g lyophilisate, immobilisate) and nature of the solvent are parameters, which can dramatically affect enzyme activity in OS (Carrea and Riva, 2000). Water activity plays a crucial role in the behavior of enzymes in non-conventional media as it acts as lubricant to enhance the conformational mobility of enzymes. In fact, water presence around and within an enzyme promotes its flexibility, which enables better substrate binding (Yang and Russell, 1996; Zaks and Klibanov, 1988). Structure and function of enzymes are also affected by the pH of the essential water layer around enzymes. The pH of a solution determines in which state amino acids are, due to the ionization/protonation of their characteristic groups (-COOH and -NH_2) (Tomé *et al.*, 2009). The protonation state of the different groups of an enzyme influence enzyme activity in aqueous solution as well as in OS. However, protonation states in OS cannot be as easily controlled as in water (Carrea and Riva, 2000). "Organic phase buffers" have been proposed to surmount the effect of pre-adjustment of pH in aqueous media before drying. Such buffers allow control of activity by adjusting pH/and or the ionizable state of groups on the enzyme molecules (Blackwood *et al.*, 1994). Enzyme activity and specificity can also be influenced by varying the partition of substrates and products between the bulk organic phase and the microaqueous phase around the active site (Halling, 1994).

Adding small to moderate concentrations of a water-miscible solvent has been shown to slightly affect enzymes properties resulting in an increase activity (Dordick, 1992). However, inhibitory effects on the enzyme have also been observed at higher concentrations (Vermue and Tramper, 1995). Reduced substrate binding and catalytic turnover are observed when non-conventional forces that maintain the active enzyme conformation are disrupted (Dordick, 1992). Additionally, higher conformational stability followed by denaturation can be observed when the water content of an organic solvent exceeds a certain concentration (total inactivation of enzymes mostly occurs at 60-70% v/v organic solvent) (Stepankova *et al.*, 2013). An explanation for these findings is hypothesized to be the crucial role of water in enzymatic reactions. As already mentioned, enzymes are surrounded by a water layer, which

can be disrupted by the addition of OS. Removal of this water dramatically deforms the catalytically active enzyme conformation leading to enzyme inactivation (Natarajan, 1991; Zaks and Klibanov, 1985). As long as the water monolayer surrounding the enzyme remains intact, OS can replace the rest water without affecting its activity (Natarajan, 1991). Based on their results, Klibanov and Zaks assumed that the main factor in the effect of OS on enzymes is the interactions of the enzyme with the bound water and not with itself (Zaks and Klibanov, 1985). The authors showed in case of the porcine pancreatic lipase that the water molecules were bound to the enzyme and could not be removed even by hydrophilic solvents (Zaks and Klibanov, 1985). This state is comparable to a nearly anhydrous organic medium, since the absolute water amount present in this monolayer is minimal (Natarajan, 1991).

Avoiding inactivation of enzymes in OS is a crucial issue, which can be easily solved by a proper selection of the organic solvent (Natarajan, 1991). In the ideal case, the enzyme should be thermodynamically stable as well as catalytically active in the selected organic solvent (Natarajan, 1991). Depending on the polarity, solvents can modify the enzyme conformation, and thereby influence enzyme properties (Carrea and Riva, 2000) as enzymes behave differently in polar and non-polar OS. For instance, deformation of the enzyme surface by the solvent penetration effect was shown to induce a decreased enzyme stability in hydrophilic solvents (Gorman and Dordick, 1992; Yang *et al.*, 2004) but not in hydrophobic ones. Decreased enzyme activity was reported to increase with solvent polarity reflecting the tendency of OS to strip water molecules from the enzyme surface (Yang *et al.*, 2004). Although polar solvents offer a better reaction medium for numerous synthetic applications, they are unfortunately strong denaturants and rapidly cause enzyme deactivation (Martinez *et al.*, 1992). Therefore, hydrophobic water miscible OS are preferentially used for enzymatic reactions. In contrast to hydrophilic OS, hydrophobic OS are unable to strip the essential water away from the enzymes thus avoiding inactivation (Klibanov, 2001; Serdakowski and Dordick, 2008). Only for those enzymes, in which the water is bound so tightly that they cannot be replaced by hydrophilic solvents, enzyme activity can be retained in hydrophilic OS (Natarajan, 1991).

1.3 Protein Engineering

Protein engineering has been used to obtain enhanced protein stability in OS and ionic liquids. Numerous solvent-resistant enzymes have been achieved through protein engineering

(Figure 1-6) and a good summary is available in the supplementary part of a publication reviewed by Stepankova and co-workers (Stepankova *et al.*, 2013).

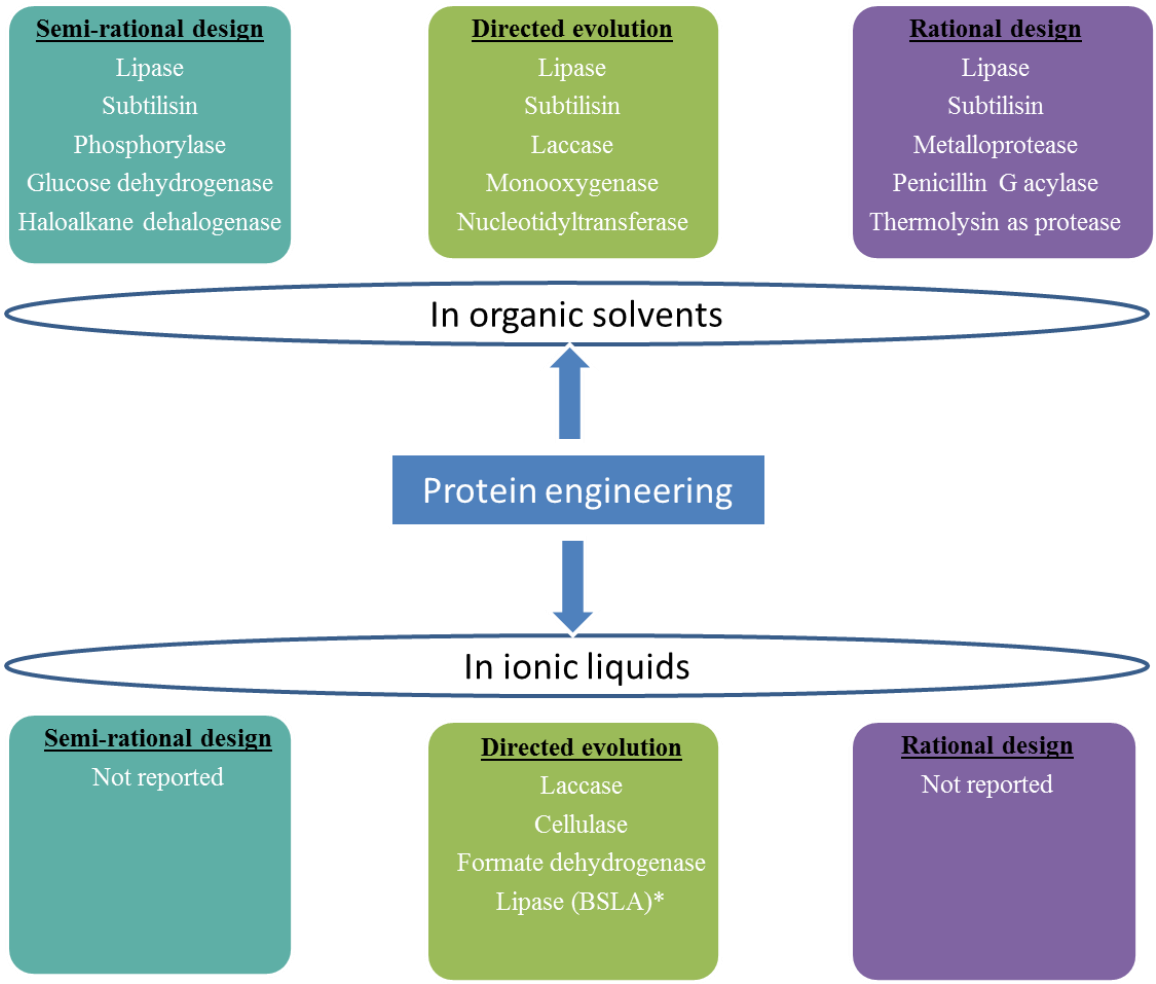


Figure 1-6: Examples of solvent-resistant enzymes achieved by protein engineering. This figure was partly adapted from (Stepankova *et al.*, 2013) with the permission of American Chemical Society provided by Copyright Clearance Center.*****: First report in this work (chapter 3).

Native enzyme properties are difficult to maintain under harsh reactions conditions (high pH, high temperature, presence of OS or ionic liquids), which usually destabilize and/or deactivate enzymes. Therefore, an approach, which can alter proteins to adapt them or even to enhance their properties in non-physiological conditions, is necessary. Protein engineering is a powerful approach based on genetic modification, which enables the generation of tailor-made enzymes meeting the requirements of enzymes for application in industrial processes. Lipases for instance, have been intensively studied for biotechnological applications (Hasan *et al.*, 2006). However, despite its good solvent stability, the wild type enzyme is not evolved for industrial processes since it generally fails to function under other non-physiological conditions. Protein engineering can be used to increase suitability of enzymes for large-scale

industrial applications through rational design, semi-rational design or directed evolution. Table 1-4 compares the approaches used in protein engineering.

Table 1-4: **Classification of protein engineering methods.** This table was adapted from (Stepankova *et al.*, 2013) with the permission of American Chemical Society provided by Copyright Clearance Center.

Methods	Prerequisites	Techniques	Descriptions
Directed evolution	Gene template A functional assay for selection or screening of improved variants	Random mutagenesis	Mutations are randomly generated alongside the whole gene with a preferred mutation rate; however, statistically only one nucleotide in a codon can be changed per round
		Recombination techniques	Fragments from different variants of the gene are combined into a novel DNA molecule; beneficial mutations can be combined, while deleterious mutationsone are eliminated
		Saturation mutagenesis	A degenerate oligonucleotide, which is randomized at a specific position , is incorporated within the coding sequence (e.g. at the hot spot identified by random mutagenesis)
Rational design	Knowledge of enzyme structure or a homology model, knowledge of structure-function relationships, computational design	Site-directed mutagenesis	Mutations are predicted computationally using enzymes structures, homology models, or sequence comparisons; a desired mutant is prepared using mutant synthetic oligonucleotides, which carry with the desired mutation at certain point
Semi-rational design	Knowledge of enzyme structure or a homology model, knowledge of structure-function relationships, computational design. Functional assay for selection or screening of improved variants or direct characterization of a number of mutants	Saturation mutagenesis	A degenerate oligonucleotide randomized at a specific position is incorporated within the coding sequence according to computational design

1.3.1 Rational Design

Rational design is an information-intensive protein engineering approach that is based on in-depth knowledge of the enzyme structure and structure-function relationship. When enzyme's crystal structure is not available, the structure of a homologous enzyme can be alternatively used to identify important residues. Computational biology plays a crucial role in the identification of such residues. **S**ite-**D**irected **M**utagenesis (SDM) is then subsequently applied to introduce site specific mutations in the selected positions to improve the targeted properties. Thus, small libraries are generated and the screening effort is reduced. Properties such as activity and selectivity are easily targeted by rational design as summarized in Table 1-5 for engineering of of lipases.

Table 1-5: Examples of lipases engineered by rational design. EG = Ethylenglycol, **SDM**: site directed mutagenesis, epPCR: error-prone polymerase chain reaction, RMSD: root-mean-square deviation.

Organism of origin	Improved properties	Methods	Mutations	References
Candida Antarctica	1.5-1.8-fold longer half-life in 80% methanol	Rational design; lowering RMSD of the four solvent-affected sites, redesign using RosettaDesign algorithm	4 mutations harboring hydrophilic or charged residues on the surface	(Park *et al.*, 2013)
	1.3-1.5-fold longer half-life in 80% DMSO	Rational design 5 mutants designed using Insight 2	3 mutations (polar and charged residues at the surface) increasing H-bond interactions between surface side-chains and water molecules	(Park *et al.*, 2012)
	Increased thermostability (4.5-fold higher half-life at 50°C)	Molecular dynamics (MD) simulation and SDM	1 double mutation allowing new hydrophobic interactions and formation of a salt bridge and hydrogen bond	(Le *et al.*, 2012)
Bacillus pumilus	Enhanced thermostability and increased kcat (4-fold)	SDM	1 mutation producing local conformation changes that possibly change the flexibility and mobility of amino acids	(Bustos-Jaimes *et al.*, 2010)
Pseudomonas aeruginosa LST-03	Half-lives >100 days for 25% *n*-heptane, *n*-hexane, *n*-octane, *n*-decane, cyclohexane, toluene, and EG	Rational design – SDM on mutants obtained in a previous study by epPCR (Kawata and Ogino, 2009)	4-9 mutations contributing to the formation of new salt bridges, new hydrogen bonds, filling the hydrophobic core and increasing pI of the side chains	(Kawata and Ogino, 2010)
Rhizomucor miehei	Increased thermostability in neat n-heptane	Rational design - introduction of disulfide bonds by SDM	2 substitutions forming disulfide bond	(Han *et al.*, 2009)
Geobacillus stearothermophilus	Improved thermostability, 2-fold improved methanolysis yield and 66-fold improved stability in methanol	Structure-guide consensus	1 mutation facilitating a closed lid conformation	(Dror *et al.*, 2014)

1.3.2 Directed Evolution

Directed evolution, which mimics Darwinian evolution in a test tube, is a powerful technique for altering the protein properties according to the one's needs (Shivange *et al.*, 2009; Wong *et al.*, 2006a). To achieve this goal, large mutant libraries are generated and desired mutants are identified via high-throughput screening (see section 1.4). Directed evolution enables improvement and understanding of important biocatalyst properties such as

activity towards non-natural substrates, resistance towards OS, tolerance towards molar concentrations of substrate and products, as well as pH and temperature stability (Wong *et al.*, 2007a). The advantage of directed evolution over rational protein design is that it does not require knowledge of the 3D structure of enzymes or the relationship among structure, sequence, and mechanism (Schmidt *et al.*, 2004). Basic conditions for a successful campaign of directed evolution are an adequate mutation strategy for the improvement of the protein, the functional expression of the protein in a suitable microbial host as well, as a fast and reliable screening assay system for identification of improved enzyme variants (Bornscheuer *et al.*, 1999). The process of directed evolution is usually repeated in iterative cycles of random mutagenesis and screening until the desired properties of the enzyme is reached. Schwaneberg and co-workers reviewed random mutagenesis methods, focused library generation methods and computational tools to achieve high-quality mutants (Wong *et al.*, 2006a, 2007b). They also proposed the *KISS* principle (***K**eep **I**t **S**imple and **S**mart*) as the best guideline to design a directed evolution campaign. This principle consists in **1)** finding an appropriate point for directed evolution, **2)** choosing a screening system and **3)** selecting a strategy for the diversity generation at the gene level (Wong *et al.*, 2006a).

Error-prone PCR (epPCR) and Sequence Saturation Mutagenesis (SeSaM) are examples of techniques used to introduce random mutations into DNA. Due to its versatility and simplicity, epPCR is one of the most successful used random mutagenesis methods (Wong et al., 2004a). It is a fast and robust method using either unbalanced dNTPs or varying concentrations of Mn2+ and allowing mutant library generation with a limited diversity (Tee and Schwaneberg, 2007). However, owing to the degeneracy of the genetic code and the biased mutational spectra of DNA polymerases, epPCR is not truly random (Cline and Hogrefe, 2000) and has the disadvantage to favor transitions (C vs. T or G vs. A) leading to conservative amino acid exchanges (Wong et al., 2004a, 2005, 2008). Therefore, site saturation mutagenesis (SSM) is often additionally applied in directed evolution experiments at mutated positions identified via epPCR to generate subsequent mutations, which explore more diverse sequence space. In this way, further improved variants can be yielded by SSM (Wong et al., 2007a) enabling generation of substitutions, that would likely never have been found by routine procedures, such as epPCR (Kretz et al., 2004). An upgraded random mutagenesis method is SeSaM having the advantage to overcome limitations deriving from biased polymerases. SeSaM can target the sequence at every single nucleotide and regulate the mutational spectra through a universal base (Wong et al., 2004a, 2005). DNA Shuffling (Stemmer, 1994) and StEP (Zhao et al., 1998) are recombinant methods, which enable

accumulation of beneficial mutations in a separate gene and removal of deleterious ones. Directed evolution has been successfully used to improve some lipase properties (Table 1-6).

Table 1-6: Examples of lipases engineered by directed evolution. epPCR: error-prone polymerase chain reaction**; SSM:** site-saturation mutagenesis.

Organism of origin	Improved properties	Methods	Mutations	References
Candida antarctica	20-fold increase in half-life at 70 °C	epPCR	2 mutations in hydrophobic sequence with additive effects (1 in α-helix of the substrate-binding pocket) disrupting the hydrophobic stretch or decreasing the hydrophobicity	(Zhang *et al.*, 2003)
P. aeruginosa	1.7-2-fold increase in amidase activity	epPCR	3 mutations located near to the calcium binding site (α-helix8 and loops near to the surface→ no interactions with the catalytic triad and the oxyanion hole), cumulative effect in a double mutant	(Fujii *et al.*, 2005)
Geobacillus sp. NTU 03	79.4-fold higher activity at 55°C; 6.3–79-fold enhanced thermoactivity	ep PCR and SSM	1-5 mutations located on the lid of α6-helix increasing the hydrophobicity and electrostatic properties of the lid	(Shih and Pan, 2011)
Pseudomonas aeruginosa LST-03	3, 9, 5 and 11 times; longer half-life in 25% DMSO, cyclohexane, *n*-octane, and *n*-decane	epPCR	1-6 mutations hindering penetration of organic solvents into the protein molecule, neutral/polar residues changed to basic residues	(Kawata and Ogino, 2009, 2010)
Pycnoporus cinnabarinus	144-fold enhanced thermostability and 20-fold k_{cat}/K_m	epPCR	1 acidic mutation resulting in extensive H-bond	(Sharma *et al.*, 2012)
Geobacillus stearothermo-philus	23-fold-improved stability in methanol	epPCR	1 mutation facilitating a closed lid conformation	(Dror *et al.*, 2014)

1.3.3 Semi-rational Design

Semi-rational design is an approach combining directed evolution and rational design to generate small, functionally enriched mutant libraries using rationally preselected target sites and limited amino acid diversity (Stepankova *et al.*, 2013). To achieve this, semi-rational design uses information on protein sequence, structure and function, as well as computational predictive algorithms (Lutz, 2010). Semi-rational approaches include computer-based methods (Chica *et al.*, 2005) as well as efficient experimental methods such as OmniChange (Dennig *et al.*, 2011), **C**ombinatorial **A**ctive-site **S**aturation **T**est (CAST) (Reetz et al., 2005), **G**ene **S**ite **S**aturation **M**utagenesis (GSSM) (Gray *et al.*, 2001; Kretz *et al.*, 2004), **S**tructure-based **CO**mbinatorial **P**rotein **E**ngineering (SCOPE) (O'Maille *et al.*, 2002), and **I**terative

Saturation **M**utagenesis (ISM) (Reetz and Carballeira, 2007). Among these methods, GSSM also referred as complete or **S**ite **S**aturation **M**utagenesis (SSM) is most widely applied. SSM allows the focused exploration of defined segments of the vast protein sequence space (Kille *et al.*, 2013; Romero and Arnold, 2009) and offers the advantage to deeply study (Fowler and Fields, 2014) the effect on each single mutation on the protein. With other random mutagenesis methods, mutations are randomly targeted leading to loss of possibly important mutations. The main challenge in the generation of site saturation libraries is to overcome limitations due to the redundancy of the genetic code. A small high quality mutant library can easily allow the identification of hotspots, with a reduced screening effort (Quin and Schmidt-Dannert, 2011). Therefore, strategies have been developed to reduce the size of the library using mutagenic primers. The commonly used ones are NNN (64 codons) (Wong *et al.*, 2006a), NDT (12 codons), NNK (32 codons) (Reetz *et al.*, 2008); and NNS (32 codons) (Funke *et al.*, 2005) covering all 20 canonical amino acids, with exception of NDT covering only 12 amino acids. N represents all 4 bases (A, C, T, G); K represents only two bases (G and T) as well as S (G and C), and D represents 3 bases (A, G, T). Compared to rational design and directed evolution, semi-rational design of lipases is not well studied. Examples of applying semi-rational approach to improve few lipases properties are illustrated in Table 1-7.

Table 1-7: Examples of lipases engineered by semi-rational design. **ACN:** acetonitrile; **DMF**: dimethyl formamide; **DMSO**: dimethyl sulfoxide: **ISM**: iterative saturation mutagenesis.

Organism of rigin	Improved properties	Methods	Mutations	References
Bacillus subtilis	Enhanced tolerance in 50% organic co-solvents (ACN; DMF, DMSO)	Semi-rational design of thermostability; B-FIT method with ISM at 6 positions	5 mutations stabilizing flexible surface loops	(Reetz *et al.*, 2006, 2010)
	8 times higher catalytic turnover in 60% DMSO	Semi-rational design-saturation mutagenesis of 91 residues in loops	Cumulative effect in a mutant with 6 mutations improving surface polarity	(Yedavalli and Rao, 2013)

1.4 Protein Engineering for Improving IL and OS Resistance

Oxidoreductases (e.g. laccase (Liu *et al.*, 2013), formate dehydrogenase (Carter *et al.*, 2014)) and hydrolases (e.g. celullase (Chen *et al.*, 2013; Lehmann *et al.*, 2014; Pottkämper *et al.*, 2009) were reported to have been successfully reengineered by protein engineering for improved IL resistance/tolerance. Very few reports (only six reports to the best of our knowledge) on improving IL resistance of enzymes are described (Carter *et al.*, 2014; Chen *et al.*, 2013; Lehmann *et al.*, 2014; Liu *et al.*, 2013; Nordwald *et al.*, 2014b; Pottkämper *et al.*,

2009). In contrast, organic solvent tolerance of numerous enzymes have been improved by protein engineering (Carrea and Riva, 2000; Klibanov, 2001; Stepankova *et al.*, 2013) mostly by using directed evolution (Chen and Arnold, 1991, 1993; Dror *et al.*, 2014; Kawata and Ogino, 2009; Kumar *et al.*, 2006; Seng Wong *et al.*, 2004; Song and Rhee, 2001; Takahashi *et al.*, 2005; Zumárraga *et al.*, 2007a, 2007b). Few cases for enhanced organic solvent stability by rational design have been reported (Dror *et al.*, 2014; Kawata and Ogino, 2010; Martinez *et al.*, 1992; Park *et al.*, 2012, 2013; Takagi *et al.*, 2000) and semi-rational design yielded in few cases organic solvent tolerant variants (Reetz *et al.*, 2006, 2010; Yedavalli and Rao, 2013). Despite first success stories on improving IL and organic solvent resistance, there is still lack of basic knowledge indispensable to understanding and improving enzyme towards ILs and OS. General design principles to guide protein engineering campaigns have still not been discovered and are therefore, deeply investigated in chapter 3 (ILs) and chapter 4 (OS) of this thesis.

1.5 High-throughput Screening

Directed evolution allows the generation of large mutant libraries consisting of innumerable protein variants. However, its success depends heavily on the efficiency of the screening strategy employed for the identification of target enzymes with desired properties. Therefore, the main challenge of a successful directed evolution experiment after generation of diversity is to develop a reliable, accurate, highly sensitive, and robust high throughput screening (HTS) assay (Yang and Withers, 2009; Zhang *et al.*, 1999). The screening system has to be developed and optimized for each specific enzyme and reaction (Aharoni *et al.*, 2005). Various screening methods have been employed in protein evolution: solid phase screening, display technologies, and liquid screening systems (Wong *et al.*, 2006a). Common methods for screening enzymes are based on microtiter plate or agar plate assays, which typically require the screening of up to 10^6 variants using robotic automation (Leemhuis *et al.*, 2009; Yang and Withers, 2009). Solid phase screening on agar plates, filter papers or membranes are also often used (Lin and Cornish, 2002). Display technologies such as mRNA, ribosome, or phage-display can be applied to screen huge protein libraries (He and Khan, 2005). Fluorescence-activated cell sorting (FACS) allows the analysis of multiple fluorescent parameters of individual particles (cells, microbeads, or emulsions) at rates of approximately 10^7 cells per hour, and isolate those with the targeted properties (Yang and Withers, 2009). FACS enables direct analysis of single cells or proteins and, thus, gain (ultra)high throughput by avoiding the transfer of individuals (Brakmann, 2001). Table 1-8 summarizes throughput,

principles and main advantages and disadvantages of various screening platforms. The most used assays in microtiter format are often spectroscopic assays based on chromogenic or fluorogenic substrates that have functional groups bearing a highly acidic phenol or aniline leaving group (Cohen *et al.*, 2001). Widely used examples are nitrophenyl and umbelliferyl esters.

Table 1-8: Summary of throughput, principles; and main advantages and disadvantages of screening technologies and formats. GC: gas chromatography, **LS-MS**: liquid chromatography–mass spectrometry, **NMR**: nuclear magnetic resonance, **HPLC**: high performance liquid chromatography. This table is taken from (Tee and Schwaneberg, 2007).

Strategies	Throughput	Principles	Main advantages	Main disadvantages
GC/LC-MS NMR, HPLC	10^2 - 10^4	Increased throughput of "classical" analytic methods; often adapted to sampling in 96-well plate format	Enantioselective analytics	Significant investments in equipment and comparably high running costs; low throughput
Microtiter plate	10^3 - 10^5	Colorimetric or fluorometric reaction performed for each individual clone in each well of a microtiter plate	Quantitative information derived for individual variant; accurate	High expenses; medium throughput; laborious
Solid phase	10^4 - 10^6	Screening on a solid surface (agar plates, filter papers, membranes)	High throughput; low costs	Comparably low accuracy; often used as pre-screen for qualitative/semi-quantification of activity
Flow cytometry	> 10^7	Sorting of individual cells based on the generated fluorescence	Ultra High throughput; cells are directly isolated after screening (no replica required)	Mode of detection limited to fluorescence; comparably low accuracy due to dye diffusion and variation in catalyst expression per cell
Phage/cell display	> 10^7	Proteins are displayed on the phage/cell surface providing phenotype and genotype linkage	Ultra High throughput; powerful for improving protein affinities	No general use as many enzymes cannot be displayed in active form; limited applicability for improving enzyme activity

1.6 Lipases

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) belong to the enzyme family of hydrolases, which are the most important and studied enzyme class for biotechnological applications so far (Jaeger and Eggert, 2002). First reports on the lypolitic activity of lipases originate from the microbiologist Eijkmann around 1901 (Hasan *et al.*, 2006). Since then, the number of available lipases has considerably increased, making them one of the most important groups of biotechnological relevant enzymes, with immense industrial applications. Lipase-catalyzed reactions are required in several industries for the production of important intermediates. Their ability to efficiently work in neat OS is an advantage exploited in industrial processes, as lipase-catalyzed reactions involve the use of water insoluble substrates

and products (Ogino and Ishikawa, 2001). Lipases find commercial and biotechnological applications in the production of food and dairy products, textiles, biodiesel, detergents, enantiopure pharmaceuticals, perfumes flavor enhancers and more. Lipases catalyze with high regio- and/or enantioselectivity the hydrolysis of triacylglycerols (Figure 1-7) under aqueous conditions whereby fatty acid and glycerol are released. They also have the unique ability to carry out the reverse reaction in OS, leading to esterification, alcoholysis and acidolysis (Gupta *et al.*, 2004).

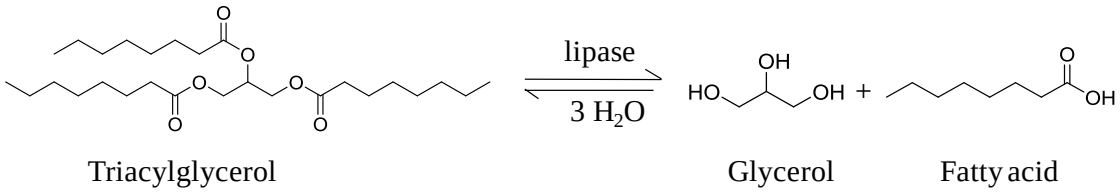


Figure 1-7: Hydrolysis and synthesis of a triacylglycerol on the carboxyl ester bond. The forward reaction is a hydrolysis and the reverse reaction is an esterification.

Lipase reactions usually take place at the lipid–water interface in a phenomenon known as interphase activation. This means that the rate of a lipase catalyzed reaction drastically increases, as soon as the critical micellar concentration (CMC) of the substrate is exceeded and an interface between the hydrophobic substrate and the aqueous environment is formed (Sarda and Desnuelle, 1958). High catalytic activity is detected only in the presence of a hydrophobic phase dispersed in an organic solvent or water. This property was used in 1958 by Sarda and Desnuelle to classify and distinguish lipases from esterases, which display rather a classic Michaelis-Menten kinetic. The structural reason for this phenomenon was identified some years later, with the elucidation of the first 3D structures of two lipases showing the presence of a mobile α-helical domain termed "lid" covering the entrance to the active site cavity (Brady *et al.*, 1990; Winkler *et al.*, 1990). This lid is amphiphilic, meaning that it possesses hydrophobic amino acid residues on the inside and hydrophilic amino acid residues facing the aqueous phase. The reorientation of the lid takes place at the water – lipid interface (Verger, 1997). Due to the hydrophobic interactions between the outside of the lid and the lipophilic organic solvent, the lid moves enabling the interphase activation (Louwrier *et al.*, 1996). The active center is then consequently exposed and the substrate can enter the binding pocket (Verger, 1997).

Some years later, lipases were described as having the ability to hydrolyze long chain fatty acids without an interphase activation or possessing a lid (van Pouderoyen *et al.*, 2001).

Based on these observations, a new definition of lipases relative to their substrate spectrum was suggested (Verger, 1997) and is still the mostly used criteria to distinguish lipases from esterases. According to this criterion, the natural substrates of lipases are long chain triacylglycerols with low or no solubility in water whereas esterases prefer short chain fatty acids as substrate. In OS, lipases exhibit very good stability and remain enzymatically active. Additionally, some lipases display a remarkable chemo- and stereoselectivity and do not require cofactors. The availability of crystal structures of many lipases is a further attractive criterion facilitating the design of rational engineering strategies (Jaeger and Reetz, 1998). Although lipases are ubiquitously produced in the nature (plants, animals, bacteria and fungi), microbial lipases are more attractive for industrial purposes (Akbulut *et al.*, 2013; Jaeger and Eggert, 2002; Saxena *et al.*, 1999). One of the main reasons is their ability to operate in extreme environments, the wide diversity of their properties, and the substrate specificity (Patameswaran *et al.*, 2010). This specificity allows reduction and complete elimination of unwanted side products that are normally formed in chemically catalyzed reactions (Ahmed *et al.*, 2009). Subsequently, microbial lipases are good targets for genetic manipulations and possess promiscuous catalytic activity. High product yields and rapid growth (independent of seasonal fluctuations) of microbial lipases is achieved on inexpensive cultivation media (Hasan *et al.*, 2006). Some important lipase-producing bacterial genera include *Bacillus, Pseudomonas, Burkholderia, Thermomyces* and *Staphylococcus* (Gupta *et al.*, 2004). Bacterial lipases are generally secreted in extracellular medium and are significantly influenced by factors, such as temperature, pH, inorganic salts, nitrogen and carbon sources (Gupta *et al.*, 2004; Jaeger *et al.*, 1994) for example the lipase A (LipA) from *Bacillus subilis* (BSLA), which was employed as model enzyme in this thesis. Based on the general conclusion that lipases remain enzymatically active in certain non-conventional media, much progress has been made in the fundamental understanding of the phenomena that governs this property.

1.6.1 The Alpha/Beta Hydrolase Fold of Lipases

Since the first elucidation of the crystal structure of two eukaryotic lipases around 1990 many other lipase structures have been solved allowing a better understanding of the relationship between protein structure and its catalytic activity. A common characteristic of all lipases is the canonical α/β-hydrolase fold (Figure 1-8:A), which is also found in esterases, proteases and haloperoxidases. It constitutes a central β-sheet surrounded by six α-helices (αA to αF). The β-sheet consists of seven parallel (β1 and β3-β8) and an antiparallel (β2) strand.

The strands β3 to β8 are connected to each other by flexible loops and alternate with the six α-helices that arrange themselves on both sides of the folded sheet (Figure 1-8A). An exception to this model is found in the lipase A and B from *Bacillus subtilis* (BSL). The structure of these lipases comprises a six-stranded β-sheet, flanked by 5 α-helices that are arranged asymmetrically around the sheets (two on the one side, three on the other side). A comparison between the secondary structure elements of BSL and the canonical α/β hydrolase fold leads to the conclusion that BSL can be regarded as a minimal α/β hydrolase fold enzyme (Figure 1-8B) lacking a lid domain (van Pouderoyen *et al.*, 2001). Aside from BSL, two other α/β hydrolase fold enzymes (from *Fusarium solani* (Longhi and Cambillau, 1999) and *Penicillium purpurogenum* (Ghosh *et al.*, 1999)) are known to lack lid domains. The small size of BSL is due to the absence of the two β strands (β1 and β2) of the canonical fold and the replacement of the αD helix by a small 3_{10} helix in the crystal structure of BSL.

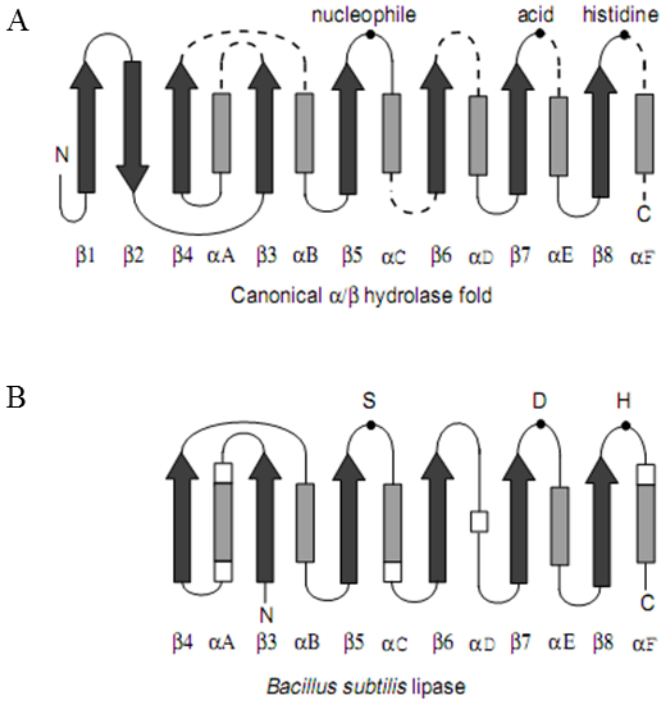


Figure 1-8: General folding model of the α/β hydrolase. A: The canonic α/β hydrolase fold; B**:** *Bacillus subtilis* lipase (BSL). The position of the catalytic triad is marked with bold points. This figure was taken from (van Pouderoyen *et al.*, 2001) with the permission of Elsevier provided by Copyright Clearance Center.

1.6.2 The Catalytic Mechanism of Lipases

The catalytic triad of lipases is composed of Ser, Asp/Glu, His and also of a highly-conserved consensus sequence (Gly-x-Ser-x-Gly) around the active site serine (Jaeger *et al.*, 1999). The nucleophile residue (serine) is located behind the β5-strand, the acidic residue

(aspartate/glutamate) usually behind the β7-strand, and the histidine residue behind the β8-strand as illustrated in Figure 1-8: (van Pouderoyen *et al.*, 2001). These residues are responsible for the substrate binding and are found on the C-terminal end of the β-sheet (Nardini and Dijkstra, 1999). The structure of this catalytic triad is similar to the one observed in serine proteases, although it differs in the α/β hydrolase arrangement as follows: nucleophile, acid and histidine (Ollis *et al.*, 1992).

The reaction mechanism of lipases consists of an acylation (Figure 1-9A/B) and a deacylation step (Figure 1-9C/D). The catalytic cycle is initiated with the binding of the substrate to the catalytic center of the free enzyme (Kamal *et al.*, 2012). The transfer of a proton occurs during a nucleophilic attack of the serine residue, via the oxygen atom of the hydroxyl group on the activated carbonyl carbon of the susceptible lipid ester bond (Figure 1-9A). This proton transfer is considerably affected by the catalytic acid. This acidic residue is responsible for the correct orientation of the histidine by interaction with the imidazole ring, which partially neutralizes the generated charge (Jaeger *et al.*, 1999). A tetrahedral intermediate, which is energetically and structurally most similar to the transient state is formed (Figure 1-9B). This is characterized by a negative charge on the carbonyl oxygen atom of the scissile ester bond and the four atoms bonded to the carbonyl group arranged as a tetrahedron. The stabilization of the formed intermediate occurs by the formation of hydrogen bonds between the negatively charged carbonyl oxygen atom and the amide function of the oxyanion hole (Jaeger *et al.*, 1999).

The next reaction step also called deacylation is characterized by the hydrolysis of the covalent intermediate by a water molecule. The active site histidine activates the water molecule by drawing a proton from it (Figure 1-9C) and the resulting OH^- ion attacks the carbonyl carbon atom of the acyl group, which is covalently attached to the serine (Figure 1-9D). Once more, a transient negatively charged tetrahedral intermediate is formed and stabilized in the same way as the first tetrahedral intermediate. As soon as the proton at the histidine is transferred to the catalytic serine, the acyl group separates from the active serine. Consequently, a second reaction product is liberated by release of a fatty acid molecule (hydrolysis) or fatty acid ester (alcoholysis), which in turn drives the regeneration of the catalytic triad to its original state (free enzyme) able to enter a new reaction cycle (Jaeger *et al.*, 1999).

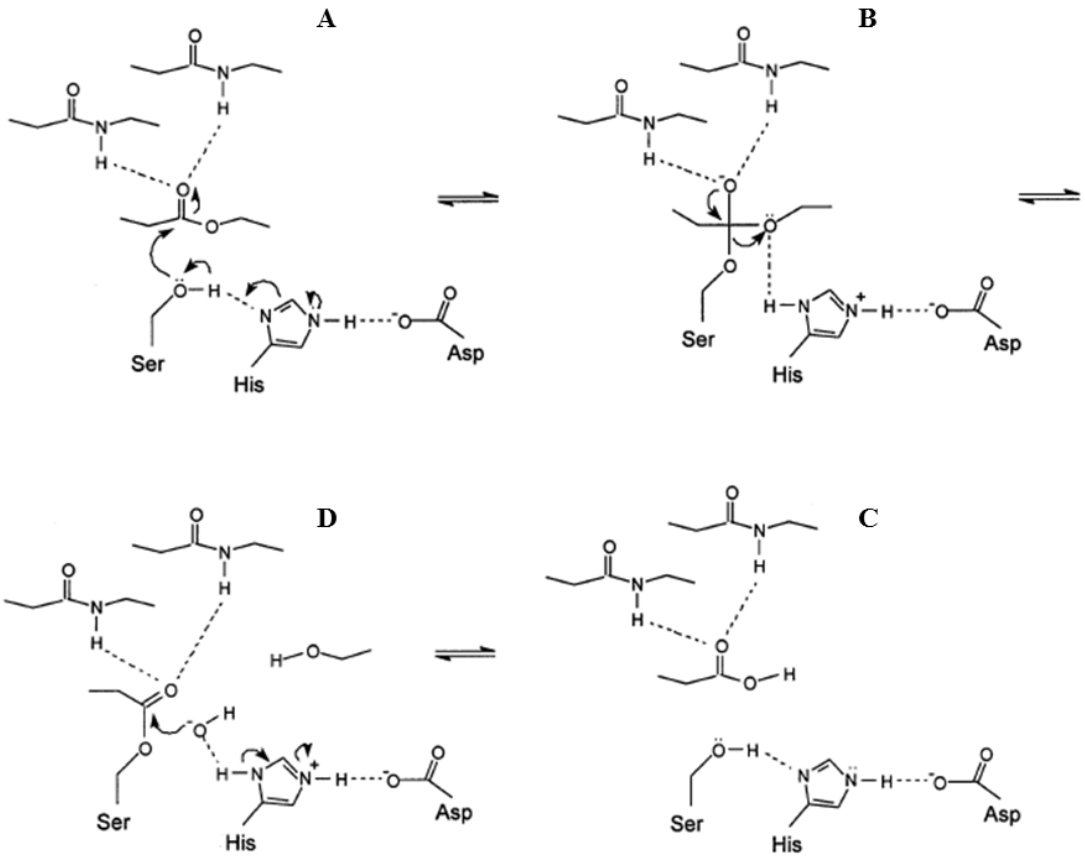


Figure 1-9: Catalytic mechanism of lipases. Ser, His and Asp represent the catalytic active amino acids. A: nucleophile attack, B: transient tetrahedral intermediate, C: covalent intermediate (acyl enzyme), D second transient tetrahedral intermediate. This figure was taken from (Jaeger *et al.*, 1999) under a free common licence.

1.6.3 Lipases from *Bacillus subtilis* (BSL)

Bacillus subtilis is a Gram-positive, aerobic, endospore-forming bacterium that produces and excretes lipases (*lipA* and *lipB*) and several other extracellular enzymes (van Pouderoyen *et al.*, 2001). *Bacillus subtilis* is widespread in nature and is besides the Gram-negative *Escherichia coli*, one of the best studied microorganisms (Juhas *et al.*, 2014; Westers *et al.*, 2004). Additionally, it is a non-pathogenic microorganism without lipopolysaccharide (LPS)-layer and classified as GRAS (generally regarded as safe) organism (Singh *et al.*, 2009). The extracellular lipolytic activity of *B. subtilis* was first observed around 1979 (Kennedy and Lennarz, 1979). However, intensive molecular research did not start more than one decade later (1992) with the cloning and sequencing of a lipase gene, *lipA* (Dartois *et al.*, 1992). Afterwards, this lipase was recombinantly expressed, purified and characterized (Lesuisse *et al.*, 1993). A second gene *lipB* was later identified as being 68% identical to *lipA* (Eggert *et al.*, 2000) followed by its cloning, recombinant expression, purification and characterization. *B. subtilis* lipases have several biotechnologically interesting characteristics. They are the smallest lipases known with molecular masses of 19.4 kDa or 19.5 kDa and

chain lengths of only 181 and 182 amino acids, respectively. As previously mentioned, *B. subtilis* lipases possesses in contrast to most other lipases, no lid and do not show any activation in the presence of an oil-water interface. The catalytic serine is located on the surface (Figure 1-10:) and freely accessible to solvents. In addition, both enzymes do not contain cysteine residues and are stable without disulfide bridges (Franken *et al.*, 2011; Nthangeni *et al.*, 2001).

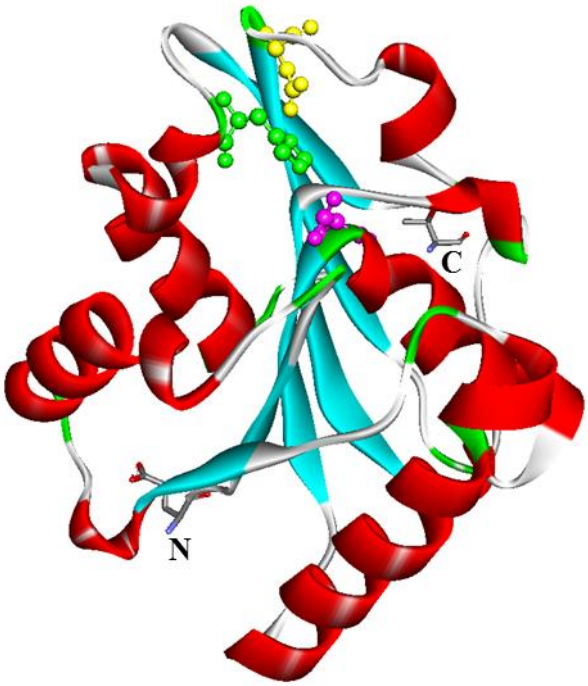


Figure 1-10: Crystal structure of *Bacillus subtilis* LipA (BSLA). PDB ID: 16WP**, c**atalytic triad residues are highlighted: Ser77 (pink), His156 (green) and Asp133 (yellow). N: N-terminal and C: C-terminal. Yasara was used for visualization.

Based on sequence similarities, differences in the conserved domains or motifs, and in the biological properties, bacterial lipolytic enzymes are classified into eight families (families I – VIII) (Arpigny and Jaeger, 1999). True lipases are grouped in family I, the largest group containing six subfamilies. *Bacillus* lipases have been placed in subfamilies 4 and 5, in which alanine replaces the first glycine residue in the conserved G-X-S-X-G pentapeptide around the active site serine residue. Subfamily I4 consists only of five members: LipA and LipB from *B. subtilis*, a lipase from *Bacillus pumilis*, *Bacillus circulans* and *Bacillus licheniformis* which all share altogether 74-80% sequence identity (Arpigny and Jaeger, 1999; Bustos-Jaimes *et al.*, 2010). This high sequence identity denotes a common fold for the whole family with the advantage that mutational effects observed in one member of the subfamily could possibly be reproduced in one of their member (Bustos-Jaimes *et al.*, 2010). Common characteristics of all enzymes of this subfamily are: monomeric, mature

forms with 181 amino acid residues and molecular masses close to 19.5 kDa (Bustos-Jaimes *et al.*, 2010).

The crystal structure of *B. subtilis* lipase A displays a globular protein with dimension 35 Å x 36 Å x 42 Å (Figure 1-10). BSLA is considered as a "minimal"α/β hydrolase, whose catalytic triad consists of the amino acids Ser77, Asp133 and His156. The residues Ser 77 and His156 were reported to be directly involved in catalytic reaction, where they act as nucleophilic attacking groups and general acid-base catalytic elements, respectively. Asp133 behaves as activator of His156 and assists in stabilization of positive charges developed on His156 during the reaction (Kamal *et al.*, 2012). The backbone amide groups of residues Ile12 and Met78 form the oxyanion hole, which is required for stabilization of the transient intermediate (van Pouderoyen *et al.*, 2001). BSLA has an unusually high pI value of 9.9 (Lesuisse *et al.*, 1993) and consists of approximately 34% of hydrophobic amino acids. It shows maximum stability at pH 12. LipA is very tolerant to basic pH and has its optimum enzymatic activity at pH 10 (van Pouderoyen *et al.*, 2001). Lipases generally prefer substrates, which are insoluble in aqueous solutions. Non-aqueous solutions such as OS or organic-aqueous two phase media are advantageous for many lipase catalyzed reactions. Like LipA, LipB has the ability to hydrolyze short- and long-chain glycerol esters, showing highest activity with C8 fatty acid chains. However, LipB does not show activity in the presence of the typical lipase substrate triolein in contrast to LipA. Therefore, LipA is regarded as a true lipase, while LipB is an esterase (Eggert *et al.*, 2000).

1.7 Aim and Objectives

The aim of the project was to systematically investigate and identify single amino acid substitutions and positions that contribute to the IL and organic solvent resistance of a model hydrolase, and to elucidate the underlying molecular principles governing this resistance. For this purpose, site saturation mutagenesis (SSM) libraries targeting the complete sequence of a *Bacillus subtilis* lipase A (BSLA) were generated and screened in presence of four ionic liquids and three OS. In depth analysis of the generated data set allowed for the first time to compare the resistance of BSLA evolution in in ionic liquids and OS. In addition, each used non-conventional media was ranked with regard to suitability of use with BSLA´s resistance. This project comprises the following steps:

- Generation of site saturation mutagenesis libraries targeting the first 91 BSLA amino acid positions.
- Selection of four ionic liquids and three OS and setting up screening system in microtiter plate format in presence of the selected ILs and OS.
- Screening of the SSM-BSLA libraries (1-181) in presence of the selected ILs and OS.
- Systematic analysis of screening results in the following aspects: the number of positions and subtitutions leading to improvement and locations of the variants influencing BSLA resistance in ILs and in OS.

Chapter 2

Optimization of Screening System in MTP format

2 Optimization of Screening System in MTP Format

2.1 Experimental Section

2.1.1 Chemicals and Reagents

All chemicals were of analytical grade or higher and were purchased from Sigma Aldrich (Steinheim, Germany), AppliChem (Darmstadt, Germany), Fluka (Neu-Ulm, Germany), and Carl Roth (Karlsruhe, Germany). All ionic liquids were synthetized by Io.Li.Tec (Ionic Liquids Technologies, Heilbronn, Germany). Enzymes and dNTPs were obtained from New England Biolabs (Frankfurt, Germany) and Sigma Aldrich (Steinheim, Germany). The HPLC-purified oligonucleotides applied for mutagenesis were obtained from GATC (Konstanz; Germany) and Eurofins MWG Operon (Ebersberg, Germany). The used oligonucleotides are summarized in Table 7-1 and Table 7-2.

2.1.2 Media, Buffers and Additives

All components for microbial cultivation media were sterilized by autoclaving at 120 °C for 20 min and were either stored at room temperature or frozen as 1 mL aliquots at -20 °C until further use. The media additives and the antibiotics were prepared as 1000x stock by dissolving individual components in ddH_2O followed by sterilization via filtration through a 0.2 µm sterile PVDF (polyvinylidene difluoride) filter (Carl Roth, Karlsruhe, Germany) and stored at -20 °C if not stated otherwise. Media additives were used in 1:1000 dilution in all media. Trace element solution was stored at 4 °C.

Lysogeny Broth (LB medium): It contained 10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl; pH 7.5 adjusted with 1 M HCl or NaOH. Agar plates were prepared by adding Agar-agar (15 g/L) additionally to the medium. The medium was supplemented with ampicillin to enable selection of resistant *E. coli* colonies after transformation with a vector carrying the ampicillin resistance gene.

Super Optimal Broth with Catabolite Repression (SOC medium): It contained 20 g/L tryptone, 2 g/L NaCl, 5 g/L yeast extract and 2.5 g/L KCl. After autoclaving, the media was supplemented with separately sterilized solutions to achieve a final concentration of 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose.

Modified Auto-Induction Medium (termed as MAIM in the present work) (Studier, 2005). LB-medium was used as pre-culture medium. This modified auto-induction medium consisted of three components, which were separately autoclaved before mixing under sterile conditions. **1. Medium component:** consists of 12 g casein hydrolysate; 24 g yeast extract (better expression was obtained with the yeast extract from Merck); 5 g glycerol, fill up to 800 mL with ddH_2O; **2. Buffer component:** consisted of 90 mL of 1 M Kp_i (Potassium phosphate inorganic) buffer pH 7.0; (K_2HPO_4/KH_2PO_4); **3. Induction component** consisted of 10 mL glucose (50 g/L), 100 mL lactose α-monohydrate (20 g/L).

ZYP Medium (Studier, 2005)**:** The pre-cultures were incubated in ***ZYP-505*** **medium** containing 50 mL 20xNPS (66 g/L $(NH_4)_2SO_4$, 136 g/L KH_2PO_4 and 142 g/L Na_2HPO_4), 20 mL 50x505 (250 g/L glycerol and 25 g/L glucose dissolved in ddH_2O) and 2 mL $MgSO_4$ (1 M) and filled to 1 L with ZY medium (10 g/L tryptone and 5 g/L yeast extract). The main-cultures were grown in ***ZYP-5052*** **medium** containing 50 mL 20xNPS, 20 mL 50x5052 (100 g/L α-lactose, 250 g/L glycerol and 25 g/L glucose dissolved in ddH_2O and 2 mL $MgSO_4$ (1 M), filled to 1 L with ZY medium.

LS-medium (Studier, 2005)**:** It consisted in two media: ***1) LSG-medium*** was used for pre-cultures and consisted in: 20 mL 50xL, 20 mL succinate (1 M), 12.5 mL glucose (40% w/v); 0.2 mL trace elements (1000x) and 2 mL $MgSO_4$ (1 M). Fill to 1 L with ddH_2O. ***2) LS-5052 medium*** was used for main-cultures and contained: 20 mL 50xL, 20 mL succinate (1 M), 12.5 mL glucose (40% w/v); 0.2 mL trace elements (1000x) and 2 mL $MgSO_4$ (1 M), filled to 1 L with ddH_2O.

Trace Element Solution: consisted in 0.5 g/L $CaCl_2{}^*2H_2O$, 0.18 g/L $ZnSO_4{}^*7H_2O$, 0.1 g/L $MnSO_4{}^*H_2O$, 20.1 g/L, Na_2-EDTA, 16.7 g/L $FeCl_3{}^*6H_2O$, 0.16 g/L $CuSO_4{}^*5H_2O$ dissolved in ddH_2O.

Ampicillin: 100 mg/mL antibiotic stock solutions were prepared with ddH_2O and diluted to a final concentration of 100 µg/mL in microbiological cultivation media.

Sørensen-Phosphate buffer: It was prepared through 17:1 mixture of solution A (50 mM $Na_2HPO_4{*}2H_2O$) and solution B (50 mM K_2HPO_4) and pH was adjusted to 8.0 with HCl.

Triethanolamine (TEA) buffer: 50 mM TEA buffer was prepared with ddH_2O and the pH adjusted to 7.4 with 37% (v/v) HCl.

Potassium Phosphate (inorganic) Buffer (KP_i-buffer): 50 mM KP_i (pH 7.2) buffer was prepared using stock solutions of 1 M K_2HPO_4 and 1 M KH_2PO_4 and stored at room temperature until further use.

Tris(hydroxymethyl)aminomethane Buffer (Tris/HCl buffer): 6.05 g/L Tris were dissolved in ddH_2O water to prepare a 50 mM solution and pH values were adjusted to pH 7.5 and 8.0 by titration with 37% (v/v) HCl.

3-(N-morpholino)propansulfonic Acid Buffer (MOPS buffer): 10.47 g/L acid free MOPS were dissolved in ddH_2O water to prepare a 50 mM solution and pH 7.2 was adjusted with 10 M NaOH.

4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid Buffer (HEPES buffer): 11.92 g/L acid free HEPES were dissolved in ddH_2O water to prepare a 50 mM solution and pH 7.09 was adjusted with 10 M NaOH.

2.1.3 Strains and Plasmids

The strains *E. coli* DH5α and *E. coli* BL21-Gold (DE3) (Agilent Technologies; Santa Clara, USA) were used as hosts for DNA manipulation and recombinant protein expression, respectively (Table 2-1). The vector used in this work was pET22(b)+ (Novagen/Merck; Darmstadt, Germany) and is represented in Figure 7-1. Plasmid purification was performed employing the kit "NucleoSpin Plasmid kit" from Macherey-Nagel (Düren, Germany) according to the supplier's recommendations, and DNA concentrations were quantified using the A_{260nm} with a NanoDrop photometer (NanoDrop Technologies, Germany).

Table 2-1: Strains and plasmid.

Strains	Genotypes/ description	Origin
E. coli DH5α	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk^-, mk^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Agilent Technologies
E. coli BL21-Gold (DE3)	F^- *dcm*$^+$ Hte *ompT hsdS*(rB^- mB^-) *gal* λ (DE3) *endA* $Tet^{r\,a}$	Agilent Technologies
pET-22b(+)	Expression vector, Amp^R	Novagen

Amp^R**:** ampicillin resistance, Tet^R: tetracyclin resistance.

2.1.4 Microbiological Methods

2.1.4.1 Preparation of Electrocompetent Cells *E. coli* BL21-Gold (DE3)

Electro competent E. coli cells were prepared by inoculation of a single colony of E. coli BL21-Gold (DE3) from a fresh agar plate containing 10 mL LB media followed by incubation (37 °C, overnight, 250 rpm) in a rotary shaker Certomat S2 (Sartorius, Göttingen, Germany). Two 500 mL flasks containing each 200 mL pre-warmed (37 °C) LB-medium were inoculated with the pre-cultures (1:200) and cultured (37 °C, 250 rpm) until an OD600 of 0.5-0.6 was measured (BioPhotometer Plus, Eppendorf, Hamburg, Germany). The cultures were rapidly transferred to an ice-water bath for 10-15 min and swirled occasionally to ensure fast cooling. For optimal transformation efficiency, the centrifuge bottles were cooled in an ice-water bath and all following steps were performed at 4 °C or on ice under sterile conditions. After cooling, the cells were transferred into the ice cold centrifuge bottles and harvested by centrifugation (4 °C, 3000 rpm, 10 min) in a Eppendorf 5804R/5810R centrifuge (Eppendorf AG, Hamburg, Germany). The cell pellets were resuspended in 250 mL ice cold pure water and centrifuged (4 °C, 3000 rpm, 10 min). This step was repeated once and the obtained cell pellets were further resuspended in 200 mL ice cold 10 % w/v glycerol. After harvesting (4 °C, 3000 rpm, 10 min), the cells were resuspended in 500 µL ice cold 10% w/v glycerol, aliquoted (50 µL/tube) in sterile pre-cooled Eppendorf tubes, frozen in a bath of liquid nitrogen and stored at -80 °C. Before use, the transformation efficiency of each new stock was verified by transforming cells with 1 ng pUC19 plasmid DNA. Transformation efficiency of up to 1 x 107 cfu µg-1 pUC19 was achieved.

2.1.4.2 Preparation of Chemically Competent *E. coli* DH5α

Chemically competent E. coli cells were prepared using a modified standard protocol (Hanahan et al., 1991). To obtain better efficiency, aliquots from cryostock were used to spread agar plates and to obtain single clones. One single colony was picked from the agar plate and used for inoculation of 3 mL LB media, which was then incubated (37 °C, 16 h, 250 rpm) in a rotary shaker Certomat S2 (Sartorius, Göttingen, Germany). Main culture was obtained by inoculation of 200 mL LB medium with 1 mL pre-culture in 1 L flask followed by incubation on a shaker (37 °C, 250 rpm) until an OD600 of 0.4 was measured (BioPhotometer Plus, Eppendorf, Hamburg, Germany). The culture was cooled on ice and subsequently harvested (4 °C, 4000 rpm, 10 min) in a Eppendorf 5804R/5810R centrifuge (Eppendorf AG, Hamburg, Germany). All following steps were carried on ice and under

sterile conditions. The cell pellet was resuspended in 15 mL filter sterilized ice cold TFB-1-solution (30 mM potassium acetate, 50 mM manganese chloride, 100 mM rubidium chloride, 10 mM calcium chloride, 15% w/v glycerol in ddH2O water) and incubated for 10 min on ice. The resuspended cells were centrifuged (4 °C, 3000 rpm, 10 min) and the obtained cell pellet was further resuspended in 2 mL filter sterilized ice cold TFB-2 solution (10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% w/v glycerol in ddH2O). Competent cells were aliquoted (50 µL/tube) in sterile and pre-cooled Eppendorf tubes, frozen in a bath of liquid nitrogen and stored at -80 °C. Before use, the transformation efficiency of each new stock was verified by transforming cells with 1 ng pUC19 plasmid DNA. Transformation efficiency of up to 1 x 107 cfu µg-1 pUC19 was obtained.

2.1.5 Molecular Biology Methods

2.1.5.1 Cloning

The template for all PCRs (pET22b(+)-BSLA WT) illustrated in Figure 7-2 was kindly provided by the research group of Prof. Jaeger (Research Center Juelich; Germany) that performed the cloning of *Bacillus subtilis* lipase A (BSLA) in a pET22b(+) vector (Figure 7-1) (Novagen; Darmstadt, Germany) in a previous work (Eggert, 2001). The pET system from Novagen is a powerful system for the cloning and expression of recombinant proteins in *E. coli*, in which the coding sequence for the desired protein is placed in a plasmid under control of a T7 promoter. Expression is most commonly induced by Isopropyl-β-D-thiogalactoside (IPTG) addition to the bacterial culture or using an auto-induction medium. The presence of the pelB signal sequence in pET22b(+) allows for protein secretion and directs proteins to the periplasmic space. From there, BSLA is unspecifically released into the culture medium.

2.1.5.2 Site Saturation Mutagenesis

Site saturation mutagenesis (SSM) (Barettino *et al.*, 1994) libraries targeting the first 91 amino acids of the BSLA sequence were generated using degenerated NNS (N=A/T/G/C and S=C/G) oligonucleotide primers encoding all 20 amino acids with 32 distinct codons (Table 7-1 and Table 7-2). A modified QuikChange PCR protocol (Wang and Malcolm, 1999) using pET22b(+)-BSLA as template was applied. Thermal cycler (Mastercyler gradient; Eppendorf, Hamburg, Germany) and thinwall PCR tubes (Multi-ultra tubes, 0.2 mL; Carl Roth, Germany) were used in all PCRs. The PCR mixture contained sterile water, 0.2 mM

dNTPs, 1x Phusion HF buffer, 17-20 ng/µL template DNA and 1 U of Phusion DNA polymerase (New England Biolabs, Frankfurt, Germany) in a total volume of 50 µL. For each targeted amino acid position, the reaction mixture was split into two tubes and 10 µM of forwards or reverse primers were respectively added in the corresponding tube. The first step of the reaction was performed using the following PCR program: 98 °C for 30 s, 1 cycle; 98 °C, 10 s / 55 °C, 30 s / 72 °C, 130 s, 3 cycles). For the second PCR step, forward and reverse reaction products were mixed together. Following program was employed to obtain the expected products: 98 °C for 30 s, 1 cycle; 98 °C,10 s / 55 °C, 30 s / 72 °C, 130 s, 15 cycles; 72 °C for 10 min, 1 cycle). The PCR products were verified by agarose gel electrophoreses before further use.

The SSM for the last 90 BSLA amino acid positions were performed in the frame of a BioNoCo project cooperation by Alexander Fulton (Institute for Molecular Enzyme Technology (IMET), Research Center Juelich (FZJ), research group of Prof. Jaeger) (Fulton *et al.*, 2015).

2.1.5.3 Agarose Gel Electrophoresis

DNA quality control was performed by agarose gel electrophoresis as described by Sambrook and Russell (Sambrook and Russell, 2001) using a 1.0% (w/v) agarose gel. The gel was prepared by heating agarose powder after dissolution in 1X TAE-buffer (TAE-buffer (50X): 2 M Tris, 1 M glacial acetic acid, 50 mM EDTA dissolved in ddH_2O water pH 8.0) and stored at 65 °C. The gels were cast after pre-mixture with Roti-Safe GelStain (Carl Roth, Karlsruhe, Germany) and used after solidification at room temperature. DNA samples (3 µL) were mixed with 0.5 µL 6x loading dye (10 mM Tris-HCl, pH 7.6; 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol, 60% (w/v) glycerol; 60 mM EDTA) and loaded along with a DNA ladder (GeneRuler 1 kb DNA Ladder, Fermentas, St. Leon-Rot, Germany) for 40 min at 110 V. DNA samples were visualized on agarose gels in an UV illuminator (Gel documentation U: Genius, Syngene, Cambridge, England) connected with a gel scanning system. In case of successful PCR, the methylated template DNA was hydrolyzed by *Dpn*I restriction enzyme (40 U; 37 °C, 16 h) and stored (at 4 or -20 °C) for further use.

2.1.5.4 Transformation of DNA into Electrocompetent *E. coli* BL21-Gold (DE3)

Electroporation was used for the transformation of *DpnI* digested PCR products into electro competent *E. coli* BL21-Gold (DE3) cells. Electroporation is a procedure in which

electric pulses of intensity in kilovolts per centimeter and of duration in microseconds to milliseconds induce a temporary loss of the semipermeability of cell membranes, resulting in ion leakage, escape of metabolites, and increased uptake by cells of drugs, molecular probes, and DNA (Tsong, 1991). The presence of salts in PCR products can inhibit the transfer of the voltage and therefore reduce the efficiency of the transformation. To avoid that, PCR samples were purified by PCR drop dialysis on small membranes (MF Millipore membrane 0.025 µm diameter, Merck KGaA, Darmstadt, Germany) following the manufacturer´s instructions. Each purified sample (5 µL) was added to a tube containing 50 µL electro competent cells which were previously thawed on ice and the mixture was incubated on ice for 5 min. The competent cells were electroporated at 1700 V using electroporator 2510 (1 mm electrode distance, Eppendorf AG, Hamburg, Germany). Subsequently, 450 µL SOC medium were directly added to the transformed cells and the tubes were incubated (45 min, 37 °C, 250 rpm) for cell regeneration. The regenerated cells were spread on LB_{Amp} agar plates following by incubation overnight at 37 °C.

2.1.5.5 Generation of BSLA Mutant Libraries

Single colonies obtained after transformation of the PCR products from 91 SSM were picked from LB_{Amp} agar plates to MTP for master plates preparation. For each SSM library, 92 BSLA clones were picked to 150 µL LB_{Amp} medium in 96-well microtiter plate (MTP) (flat-bottomed, polystyrene plates, Greiner Bio-One GmbH, Frickenhausen, Germany). In addition, three BSLA WT clones and one empty vector clone (EV) were used as control in the same MTP (Figure 2-1). The cultures were incubated at 37 °C (70% humidity, 900 rpm) for 16 h in an Infors microtiter plate shaker (Infors GmbH, Eisenach, Germany). Subsequently, the plates were stored as master plates at -80 °C after addition of 50 µL of 50% (w/v) sterile glycerol to each well.

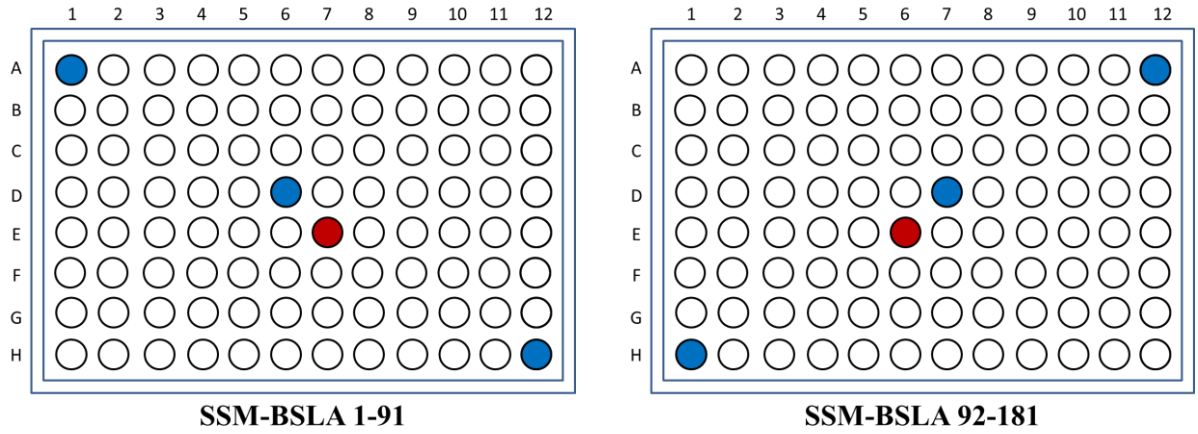


Figure 2-1: Scheme for MTP arrangement of generated SSM libraries. White colored wells contain SSM-BSLA variants, blue colored wells contain the WT (positive control, for SSM-BSLA 1-91: A1; D6 and H12; for SSM-BSLA 92-181: A12, D7 and H1) and red colored wells contain the EV (negative control, E7 for SSM-BSLA 1-91 and D6 for SSM-BSLA 92-181).

2.1.5.6 Quality Control of Generated Libraries

To ensure high diversity in the generated 91 SSM libraries (amino acid position 1-91 in this work) as well as the 90 SSM libraries (amino acid position 92-181) generated in another study (Fulton *et al.*, 2015), quality control was carried out by sending all libraries for sequencing. The libraries were replicated (replicator, EnzyScreen BV, Leiden, Netherlands) from the corresponding master plates and cultured (37 °C, 70% humidity, 900 rpm, 16 h) in an Infors microtiter plate shaker (Infors GmbH, Eisenach, Germany). Each 10 µL culture/well was used to inoculate a LB_{Amp} agar MTP (150 µL/well LB_{Amp} agar medium with 13 g/L agar) using a multi-channel pipette (Eppendorf AG, Hamburg, Germany). The agar MTPs were incubated overnight (37 °C, 16 h) and after sterile sealing with lids, they were sent for sequencing (Eurofins MWG Operon, Ebersberg, Germany).

2.1.6 Site Directed Mutagenesis

Site directed mutagenesis (SDM) was performed after results analysis of sequenced SSM libraries to generate missing substitutions. The same protocol as described for SSM (2.1.5.2) was applied to generate the missing clones however, using SDM primers (Table 7-2). The efficiency of the SDM was verified on agarose gel and the products were digested (2.1.5.3) and stored (4 °C or -20 °C) for further use.

2.1.6.1 Chemical Transformation in *E. coli DH5α*

Transformation of SDM products was performed using chemically competent *E. coli* DH5α cells. DNA (5 µL) was added to 50 µL (on ice) thawed competent *E. coli* cells

followed by 30 min incubation on ice. DNA uptake by *E. coli* cells was achieved by heat-shock treatment in a water bath (42 °C, 45 s), followed by incubation 2 min on ice. Cell regeneration was achieved by addition of 900 µL SOC medium followed by incubation (37 °C, 45 min and 250 rpm). The regenerated cells were spread on LB_{Amp} agar plates and the agar plates were incubated overnight at 37 °C. The obtained clones were cultured (45 min, 37 °C, 250 rpm) in 3 mL LB_{Amp} medium and plasmid extraction and purification was performed using the NucleoSpin Plasmid kit from Macherey Nagel (Düren, Germany) following the supplier´s instructions. DNA Concentrations were determined with the NanoDrop spectrophotometer 1000 (Thermo Scientific, Wilmington, USA). The purified DNA samples were sent for sequencing (Eurofins MWG Operon, Ebersberg, Germany) and by confirmation of the point mutations, the samples were transformed into *E. coli* BL21-Gold (DE3) (2.1.5.4) and proceeded as described before (2.1.5.5) however, with a different repartition of the variants in MTPs (Figure 2-2).

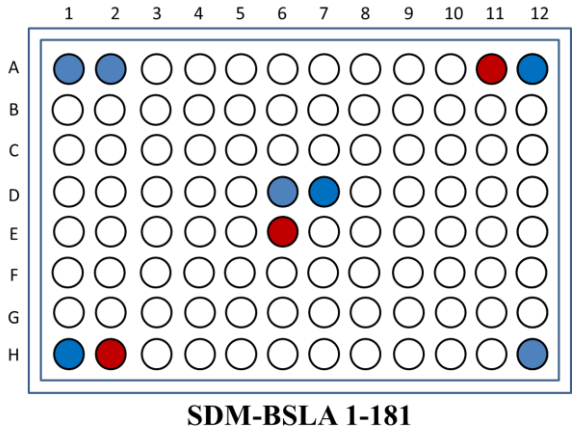


Figure 2-2: Scheme for MTP arrangement of generated SDM libraries. White colored wells contain SDM-BSLA variants, blue colored wells contain the WT (positive control: A1; A2, A12, D6, D7, H1, and H12) and red colored wells contain the EV (negative control, A1, E6, and H2).

2.1.6.2 Selection of BSLA Expression Medium in pET22b(+)

BSLA wild type clones were used to investigate the optimal medium for BSLA expression. Therefore, three different auto-induction media (ZYP-5052, LS-5052, and MAIM, see 2.1.2) were selected and used for flask expression. The strain *E. coli* BL21-Gold (DE3)/ pET22b(+)-BSLA WT was first cultivated in 5 mL pre-culture media (see 2.1.2) inoculated with an aliquot from the cryostocks and cultivated overnight at 37 °C in a rotary shaker Certomat S2 (Sartorius, Göttingen, Germany). A strain with the empty vector was used as negative control. Main cultures were grown in 300 mL flask after inoculation of 50 mL auto-induction media containing lactose as inducer with each 1 mL pre-culture. Samples were

harvested after incubation (37 °C, 12 h, 250 rpm) by centrifugation (Eppendorf 5804R/5810R, 4 °C, 4000 rpm, 20 min) and the supernatants were diluted (1:2) and used for activity measurements with *p*NPP (*p*-nitrophenyl palmitate) as substrate (Gupta *et al.*, 2002). BSLA activities in the three auto-induction media were compared and the best medium was selected for BSLA expression of the generated SSM and SDM libraries.

2.1.6.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Protein samples were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Sambrook and Russell, 2001). 12% SDS-PAGE were obtained by casting stacking gel over the resolving gel. The resolving gel (for 2 gels) consisted in 4.2 mL ddH_2O, 3 mL acrylamide mix (30% v/v), 2.5 mL Tris (1.5 M, pH 8.8), 50 µL SDS (10% w/v), 0.1 mL ammonium persulfate (APS; 10% w/v) and 4 µL TEMED. The stacking gel (for 2 gels) contained 1.44 mL ddH_2O, 0.26 mL acrylamide mix (30% v/v), 0.25 mL Tris (0.5 M, pH 6.8), 20 µL 10% w/v SDS, 20 µL 10% w/v APS and 2 µL TEMED. Samples were collected after expression and prepared by incubating 20 µL of protein sample with 6 µL 4xSDS-PAGE loading buffer in a thermostat (Eppendorf, Hamburg, Germany) for entire denaturation of peptides (95 °C, 10 min). Each 5 µL sample as well as a pre-stained reference protein ladder (10-170 kDa, Fermentas, St. Leon-Rot, Germany) was loaded on the SDS-PAGE gel. The protein fractions were separated by gel electrophoresis in 1 X SDS-buffer (0.3% w/v Tris, 1.5% w/v glycine, 0.1% w/v SDS in ddH_2O) at 30 mA for 75 min using a Mini-Protean Tetra Cell system (Bio-Rad, München, Germany). For visualization of protein bands, the gels were stained in Coomassie Brilliant Blue solution (0.25% v/v coomasie, 45.5% v/v methanol, 9.2% v/v acetic acid) for 20 min followed by de-staining in 10% (v/v) acetic acid solution overnight.

2.1.6.4 Cultivation and Expression in 96-well Plates

For expression of the generated variants, each master plate was thawed, replicated into a new 96-well MTP (flat-bottomed, polystyrene plates; Greiner Bio-One GmbH, Frickenhausen, Germany) containing LB_{Amp} medium (150 µL per well) using a replicator (EnzyScreen BV, Leiden, Netherlands), then covered with a plastic lid and cultivated for 24 h (37 °C, 70% humidity, 900 rpm). Afterwards, the pre-cultures were replicated into new 96-well MTPs (V-bottomed, polystyrene plates; Greiner Bio-One GmbH, Frickenhausen, Germany) containing a modified auto-induction medium (Studier, 2005) (2.1.2) supplemented with ampicillin (150 µL per well). The expression was stopped after 16 h (900 rpm, 37 °C,

70% humidity) and the OD_{600} values were measured. BSLA is released into the culture supernatant when the vector pET22b(+) is employed in *E. coli* BL21-Gold (DE3) due to the pelB-signal sequence of the vector which enables the translocation of the lipase in the periplasma as previously described (Funke *et al.*, 2005; Reetz *et al.*, 2007; Yedavalli and Rao, 2013). The supernatant was therefore, harvested by centrifugation (Eppendorf 5804R/5810R, 4 °C, 4000 rpm, 20 min) and used for activity measurements.

2.1.7 Biochemical Methods

2.1.7.1 Selection of Substrates for BSLA in MTP Based Screening System

Although BSLA is described to exhibit maximum activity towards substrates with C8 chain length, the C16 substrate *p*-nitrophenyl palmitate (*p*NPP) is widely used for lipase activity detection. In addition to *p*NPP, the C8 substrate *p*-nitrophenyl caprylate (*p*NPC) and the C4 *p*-nitrophenyl butyrate (*p*NPB) were selected to be characterized. Detection of lipase activity for the wild type clones was performed with a modified protocol of Winkler and Stuckmann (Winkler and Stuckmann, 1979) using a spectrophotometric assay. The substrate solution (8 mM, in isopropanol) was mixed (in ratio 1:18) with Sørensen phosphate buffer with pH 8 (2.1.2), supplemented with 5 mM NaDOC (sodium desoxycholic acid) and 1 mg/mL gum arabic to 0.4 mM final concentration. Afterwards, 180 µL of the substrate emulsion were added to 20 µL of the supernatant. Absorbance at 410 nm was recorded in an endpoint measurement using a MTP reader (Tecan infinite® M200Pro Austria GmbH, Männedorf, Switzerland) after 10 min incubation at room temperature on a Bench top shaker Certomat (Sartorius, Göttingen, Germany).

2.1.7.2 Selection of Buffer

Different buffers (2.1.2) were used to investigate the stability of BSLA in presence of co-solvents. Kp_i (50 mM; pH 7.2), MOPS (50 mM, pH 7.2), Tris-HCl (50 mM, pH 7.5), Tris-HCl (50 mM, pH 8.0), HEPES (50 mM, pH 7.1), and TEA (50 mM, pH 7.4). Reactions were carried out in glass test tubes (Sigma Aldrich, Steinheim, Germany) by incubating 500 µL co-solvent (50% v/v in the incubation mixture before adding the substrate solution) with 400 µL buffer and 100 µL supernatant. DMSO (dimethyl sulfoxide), TFE (2,2,2,-trifluoroethanol) and [BMIM][Cl] (1-Butyl-3-methylimidazolium chloride) were the co-solvents used in this part. The mixtures were incubated (2 h, 1000 rpm, RT) in glass test tubes on a Bench top shaker

Certomat (Sartorius, Göttingen, Germany). Afterwards, 1000 µL of the selected substrate was added to the incubation mixture and pH shifts were measured.

2.1.7.3 *p*NPB Assay in MTP for BSLA Activity Measurement

Microtiter plates (96-well) were used for the screening of lipase activity based on the degradation of esters of chromogenic phenols. The activity is most generally determined spectrophotometrically using a *p*-nitrophenyl ester as substrate. This method is based on the quantification of the level of released *p*-nitrophenolate (λmax 400 to 410 nm) followed by hydrolysis of the substrate by lipase (Figure 2-3). In this work, *p*-nitrophenyl butyrate (*p*NPB) was selected as substrate to detect the activity in the generated BSLA libraries (Shirai *et al.*, 1982). The standard deviation of the assay (2.1.7.4) was determined by the addition of 90 µL of assay buffer or co-solvent to 10 µL of supernatant and 100 µL of the freshly prepared substrate solution (0.5 mM *p*NPB final concentration instead of 0.4 mM used for all three substrates in (2.1.7.1)), 10% (v/v) acetonitrile and 90% (v/v) TEA buffer (pKa 7.76, pH 7.4, 50 mM)). Afterwards, the release of *p*-nitrophenol was recorded (410 nm, RT, 8 min) each 20 sec on a MTP reader (Tecan infinite® M200Pro Austria GmbH, Männedorf, Switzerland).

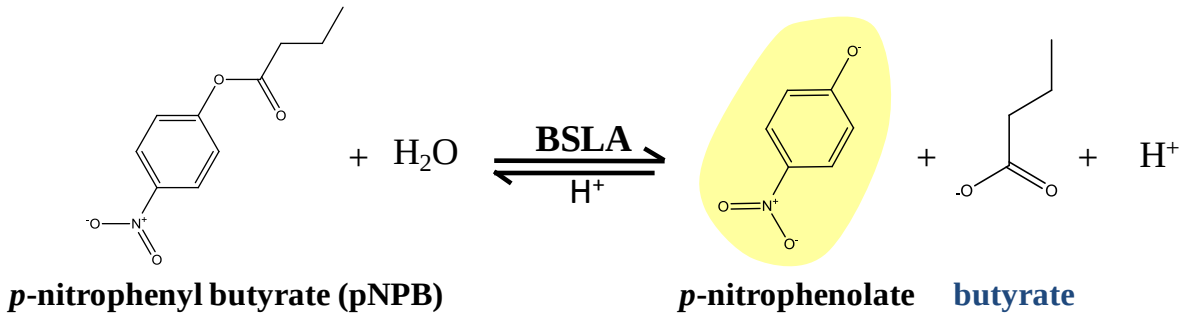


Figure 2-3: Schematic overview of an assay based lipase hydrolysis reaction. *p*-Nitrophenyl butyrate (*p*NPB) was used as substrate. The formation of *p*-nitrophenolate is detected by the intensity of the yellow colorat 410 nm.

To determine the *p*-nitrophenol production by BSLA during the assay and the linear range of the assay, a calibration curve was prepared using different concentrations (0-4 mM) of a *p*-nitrophenol standard solution.

2.1.7.4 Standard Deviation of pNPB Assay in Buffer

After optimization of all factors (expression medium, substrate and buffer) necessary for screening of SSM-BSLA libraries, the standard deviation of *p*NPB assay was determined. Therefore, a 96-well MTP containing only BSLA WT and another 96-well MTP containing

only EV were cultured and assayed as mentioned above (2.1.6.4) and 90 µL assay buffer was added to each 10 µL supernatant followed by activity measurement (2.1.7.3).

2.1.7.5 Selection of Non-conventional Media

For an ideal investigation of enzymatic reactions in OS, several factors should be investigated such as cofactor independence, availability and cheap enzymes, non-reaction of water as well as solubility of substrates in the used OS (Zaks and Klibanov, 1985). BSLA meets all this requirements. The aim therefore, in this part was to select non-conventional media for the investigation of BSLA WT solvent resistance. An important factor for the selection of the non-conventional media for BSLA screening was the water-miscibility of the solvent since the reaction system of the assay is a hydrolysis. Three OS with different polarities and four different ionic liquids (ILs) should be selected. The experiments were carried out in 96-well MTPs. In the first steps of the experiment, two OS and eight ILs (Table 2-2) were analyzed. Each 10 µL supernatant (BSLA WT or EV) was incubated (2 h, 1000 rpm, RT) on a shaker (Edmund Bühler Microtiter shaker, Hechingen, Germany) with 50% (v/v) co-solvent (or buffer as control). The freshly prepared substrate solution (100 µL/well) was added to the incubation mixture and the absorbance was measured (410 nm, 8 min, RT) in a MTP reader (Tecan infinite® M200Pro Austria GmbH, Männedorf, Switzerland).

Table 2-2: List of first selected three OS and eight ILs.

OS	Ionic liquids
DMSO: dimethyl sulfoxide	[BMIM][Cl] : 1-butyl-3-methylimidazolium chloride
1,4-dioxane	[BMIM][TfO]: 1-butyl-3-methylimidazolium trifluoromethanesulfonate
TFE: 2,2,2-trifluoroethanol	[BMPy][TFA]: 1-methylpyrrolidinium trifluoroacetat
	[EMIM][$MMPO_4$]: 1-ethyl-3-methylimidazolium dimethyl phosphate
	[EMIM][TFA]: 1-ethyl-3-methylimidazolium trifluoroacetat
	[EMIM][$MeSO_3$]: 1-ethyl-3-methylimidazolium methanesulphate
	[EMIM][$MeSO_4$]: 1-bthyl-3-methylimidazolium methylsulphate
	[MMIM][MMPO4]: 1,3 dimethylimidazolium dimethyl dimethyl phosphate

[BMIM][Cl] obtained as solid were dissolved by adding water while stirring (2-3 days) until homogenous IL solutions were obtained. In order to determine the concentration for screening, each non-conventional media was supplemented with the assay buffer to obtain OS or IL solutions with different concentrations. Concentrations leading to a residual activity of the BSLA WT between 30% and 40% were chosen for screening. The screening assay with ILs was performed in polystyrene MTPs (flat-bottomed, polystyrene plates, Greiner Bio-One

GmbH, Frickenhausen, Germany) while screening with OS was performed in polypropylene MTPs (flat-bottomed, polystyrene plates, Greiner Bio-One GmbH, Frickenhausen, Germany), which are resistant to the aggressivity of some OS as 1,4-dioxane.

2.2 Results and Discussion

2.2.1 PCR Amplification by SSM and SDM

Site saturation mutagenesis (SSM) was performed at every amino acid position of BSLA to elucidate the contribution of each amino acid substitution at each position of BSLA IL resistance and OS resistance. Obtaining the expected full diversity coverage remains an ideal goal of each SSM experiment. To achieve this goal, the choice of the appropriate degenerate codon plays an important role. NNS codons were selected to reduce the amount of possible inactive variants resulting from introduction of stop codons. NNS and NNK degenerated primers encode all 20 canonical amino acids with the advantage of creating 32 codons and only one stop codon instead of 64 codons and three stop codons as observed with NNN codons. In total, 181 SSMs and 341 SDMs were performed (after sequencing of SSM libraries, missing substitutions were generated by SDMs to complete the libraries). The success of the SSM and SDM amplifications was verified on 1% agarose gel (Figure 2-4). The amplified products with expected size of 6019 bp were digested with *DpnI* as described before (section 2.1.5.3) and used for electro transformation in *E. coli* BL21-Gold (DE3) (section 2.1.5.4) or chemical transformation in *E. coli* DH5α (section 2.1.6.1).

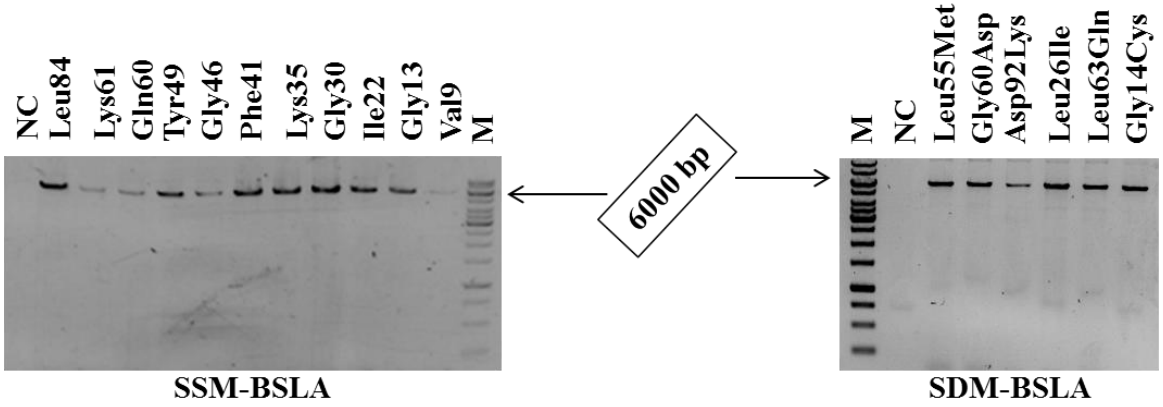


Figure 2-4: Exemplary agarose gel for SSM and SDM (QuikChange) at selected BSLA amino acid positions. 3 µL of each sample were mixed with 0.5 µL 6X loading dye and loaded. M (DNA marker, GeneRuler 1 kb DNA Ladder, Fermentas, St. Leon-Rot, Germany) is used for estimation of obtained DNA fragment sizes; NC= negative control. The expected size for each product was 6019 bp.

2.2.2 Sequencing of Generated Libraries

In total, 91 SSM-BLSA libraries were generated in RWTH Aachen (this work) and 90 SSM-BLSA libraries (Fulton, 2015) were generated in Research Center Juelich (Alexander Fulton in a BioNoCo project cooperation, FZJ). The quality control of the library generation was performed by sending 10 clones per library for sequencing. Our strategy to ensure full diversity coverage in the created libraries was to regenerate libraries displaying more than 20% wild type. In total, 16652 clones were picked in microtiter plates (MTPs) from the 181 SSM libraries (92 clones per amino acid-position x 181 amino acid-positions). The 16652 clones were fully sequenced and 3279 different BSLA variants were obtained (represent 90.6% completeness). In order to achieve 100% coverage, the missing 341 variants were generated by individual site directed mutagenesis (SDM), and each variant was confirmed by sequencing. Overall, 18547 clones corresponding to 3620 (181 BSLA amino acid positions x 20 naturally occurring amino acids) variants were generated and used for the screening in presence of ionic liquids and OS. Once all 181 Libraries were generated and confirmed by sequencing, they were replicated and exchanged between the cooperation partners to enable each PhD fellow to work on the complete BSLA libraries.

2.2.3 Selection of Optimal Medium for BSLA Expression Medium in pET22b(+)

This part focuses on the selection of the appropriate medium for BSLA expression. Protein expression (37 °C, 12 h, 250 rpm) in pET22b(+)-BSLA WT was successfully performed in all three auto-induction media (ZYP-5052, LS-5052 and MAIM) in flasks (2.1.6.2) followed by lipase activity detection by formation of *p*-nitrophenolate using *p*NPP as substrate. The highest lypolytic activity was measured in the MAIM media (Figure 2-5A), which was selected as expression media for the BSLA screening assay. Consequently, the BSLA expression level in MAIM as auto-induction medium was verified by SDS-PAGE (2.1.6.3) loading crude cell extracts on 12% SDS-gel (Figure 2-5B).

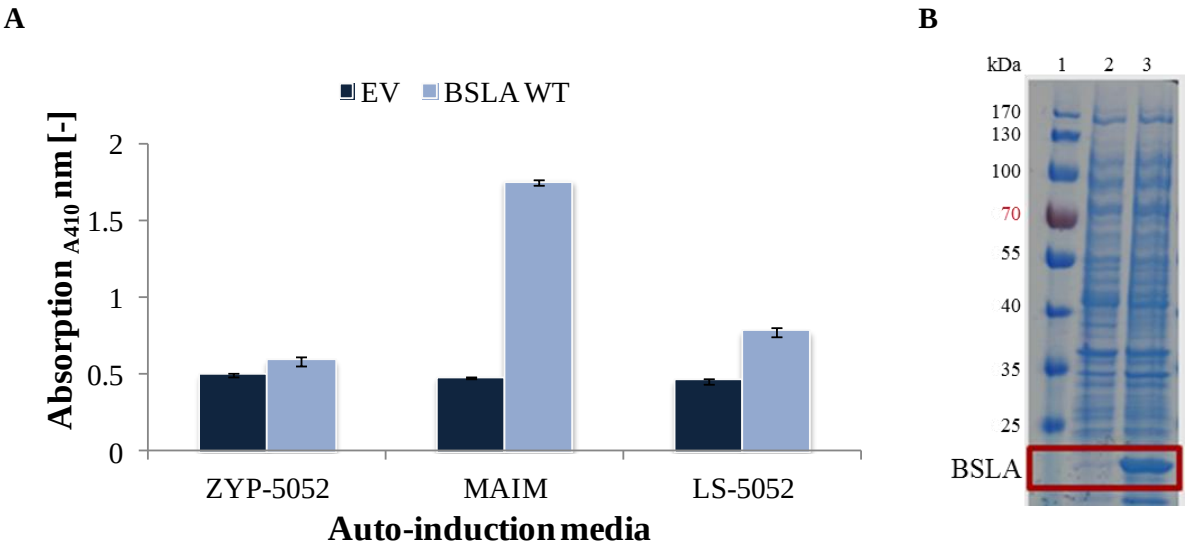


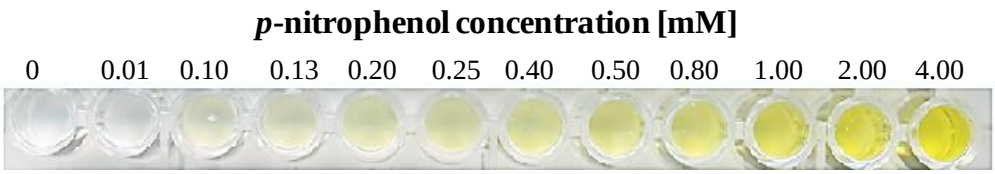
Figure 2-5: Comparison of BSLA WT activity in different auto-induction media (A) and BSLA protein expression in MAIM (B). A: ZYP-5052; MAIM and LS-5052 were used for expression followed by activity assay with *p*NPP as substrate. All measurements were performed in triplicate and errors were calculated and are represented as bar. B: Protein visualization of BSLA cultures on a 12% SDS-PAGE gel: 1) Molecular weight standard (PageRuler, Fermentas), 2) EV (empty vector), 3) BSLA WT. The red box marks the expected molecular weight of BSLA WT ≈ 19.3 kDa.

2.2.4 Optimization of Screening Conditions

2.2.4.1 Standard Curve of *p*-Nitrophenol

To monitor the release of *p*-nitrophenolate during the assay, a calibration curve (Figure 2-6B) was generated using *p*-nitrophenolate dissolved in Sørensen buffer (50 mM, pH 8.0). *p*-Nitrophenolate is released during the hydrolysis reaction, which is visible by a yellow coloration (Figure 2-6A) whose absorption was monitored to quantify the activity of BSLA. A linear detection range at least up to 0.5 mM *para*-nitrophenol could be detected.

A



B

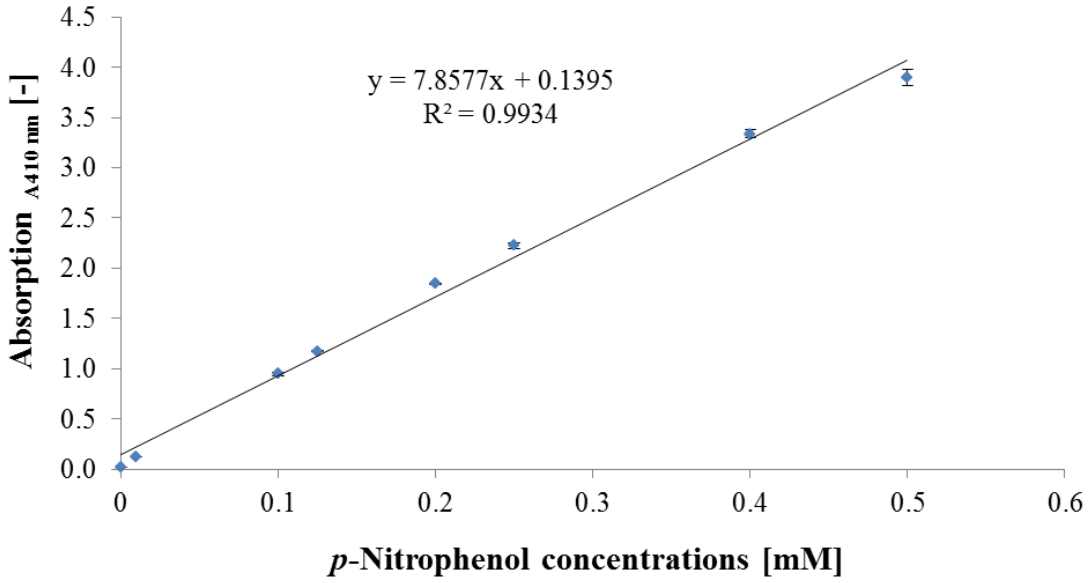


Figure 2-6: (A) Color development of *p*-nitrophenol relating to the concentration and (B) standard curve of *p*-nitrophenol. *p*-Nitrophenol was dissolved in Sörensen buffer (50 mM, pH 8.0) and different concentrations (0 to 4.0 mM) were obtained by dilution. Absorbance at A410 nm was measured in triplicate for each sample. Errors were calculated and are represented as bar.

2.2.4.2 Selection of Suitable BSLA Model Substrate

BSLA WT activity was measured with *p*-nitrophenyl palmitate (pNPP), *p*-nitrophenyl caprylate (pNPC) and *p*-nitrophenyl butyrate (pNPB) as substrates (2.1.7.1). Experiments showed that higher activity was obtained for BSLA WT as well as a higher substrate background for the empty vector using *p*NPP (Figure 2-7). Despite our attempts to increase *p*NPP solubility by addition of emulsifiers (NADOC, gum arabic) in the substrate solution, a considerably high background remained. This may also be the reason for the higher activity obtained in BSLA WT. The higher background observed in *p*NPP could negatively impact on activity determination (for example values outside the linear assay range) and should therefore be avoided. Since the intention was to establish an assay with very low background absorbance and avoid usage of emulsifiers, *p*NPB was selected for the screening of BSLA libraries. Minimal spontaneous hydrolysis and high solubility in water compared to other long chain substrates are properties which favor *p*NPB use as substrate for lipases (Yedavalli and Rao, 2013). Eggert and co-workers tested BSLA towards *p*-nitrophenylesters with different chain lengths (C4 to C18) and reported higher specific activities for substrates with C4 to C8

chain lengths while very low catalytic activities were observed for C18 substrates (Eggert *et al.*, 2000).

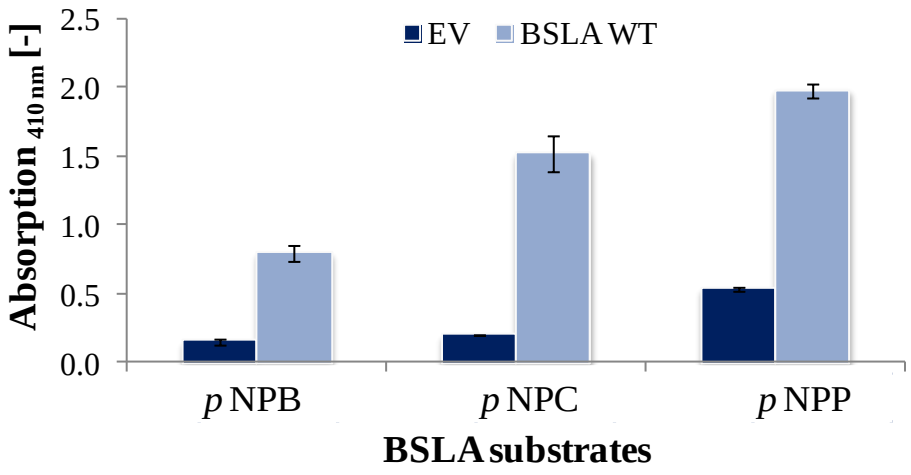


Figure 2-7: Comparison of BSLA WT activity using *p*NPB, *p*NPC, and *p*NPP. Endpoint measurement was performed at 410 nm (after 10 min incubation at RT) to determine BSLA WT activity with *p*NPB, *p*NPC and *p*NPP as substrates. All measurements were performed in sextuple and errors were calculated and are represented as bar.

2.2.4.3 Selection of Suitable Reaction Buffer

Six buffers (50 mM KP_i with pH 7.2, 50 mM MOPS with pH 7.2, 50 mM Tris-HCl with pH 7.5, 50 mM Tris-HCl with pH 8.0, 50 mM HEPES with pH 7.1, and 50 mM TEA with pH 7.4) were evaluated in preliminary experiments to identify the best one for screening of BSLA resistance in presence of 50% v/v non-conventional media (in the incubation mixture with the supernatant before adding the substrate solution) such as DMSO, TFE and [BMIM][Cl]. BSLA culture supernatant was incubated with each buffer (2.1.7.2) and pH values were measured. The pH shifts were calculated by subtracting the measured pH values from the expected ones. High pH shifts were particularly observed in DMSO for the following buffers: Kpi, HEPES, Tris-HCl (pH 8.0), and MOPS. [BMIM][Cl] in contrast, seemed to be more tolerant to most of the buffers except Tris-HCl (pH 7.5) and KP_i in which higher pH shifts were measured. Best buffer capacity meaning less pH shift was obtained with 50 mM TEA buffer (pH 7.4) (Figure 2-8). Therefore, TEA buffer was selected for the screening assay.

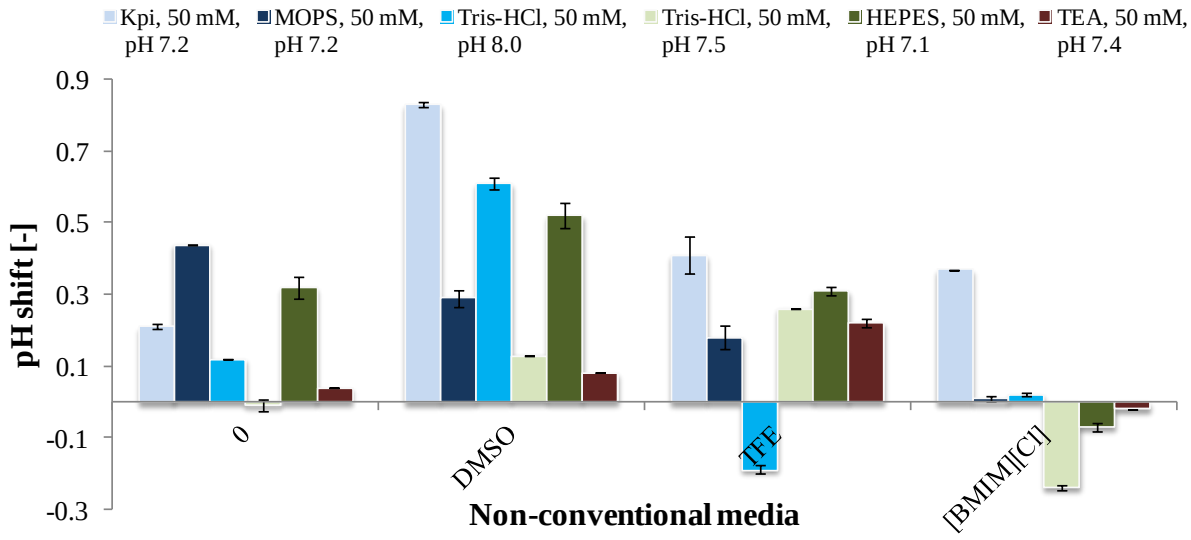


Figure 2-8: Comparison of buffer capacity for BSLA screening. The co-solvents (50% v/v) were incubated with BSLA culture supernatants (2h, RT, 1000 rpm) and substrate solution was added. Afterwards, pH values were measured and pH shifts were calculated (expected pH - measured pH). The measurements were performed in triplicate and errors were calculated and are represented as bar. DMSO: dimethyl sulfoxide; TFE: 2,2,2-trifluoroethanol; [BMIM][Cl]: 1-Butyl-3-methylimidazolium chloride.

2.2.4.4 Determination of the Standard Deviation of *p*NPB Assay in Buffer

To determine the standard deviation of the screening assay, the activity was measured in a 96-well MTP containing only BSLA WT and another 96-well MTP containing only EV. The apparent standard deviation was calculated without subtracting the background, while the true standard deviation was obtained after background subtraction. The true and apparent standard deviations of the assay in buffer were 11% and 9% (Figure 2-9), respectively. Screening systems with standard deviations up to 25 % have successfully been used in directed evolution experiments (Kardashliev *et al.*, 2014; Tee and Schwaneberg, 2007).

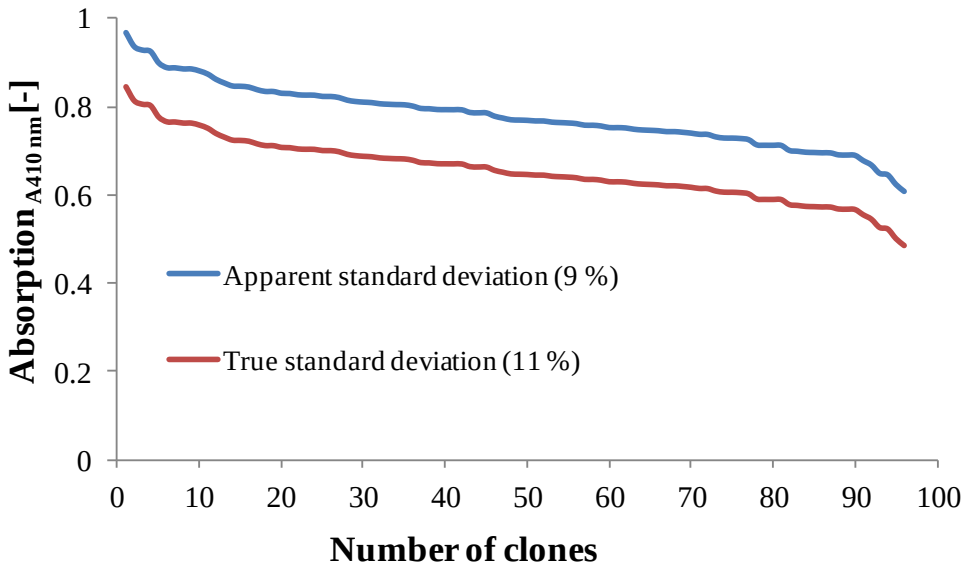


Figure 2-9: **Standard deviation of BSLA activity in buffer.** Activity values in decreasing order of the BSLA WT in a 96-well MTP using the optimized assay conditions. The apparent standard deviations were calculated without subtracting the background and the true standard deviations after background subtraction.

2.2.4.5 Selection of Non-conventional Media for the Screening of BSLA Mutant Libraries

In the first experiment, three OS and eight ILs were pre-selected. Each non-conventional media was incubated with BSLA/EV in 50% v/v concentration following by activity measurements at 410 nm (2.1.7.5). Residual activity was calculated using the formula below.

$$\text{Residual activity (R) [\%]} = \frac{\text{Slope (BSLA variant/WT - EV) in non-conventional media}}{\text{Slope (BSLA variant/WT - EV) in buffer}}$$

Results showed that DMSO, 1,4-dioxane, and TFE (Figure 2-10) were appropriate for BSLA screening assay. Since a residual activity of 30-40% for BSLA WT was required in order to enable an adequate range for improvements, the concentration of both OS needed to be adjusted. In a first experiment with 50% v/v co-solvent for each non-conventional media, few patterns were observed in presence of ILs. [BMIM][Cl] (1-Butyl-3-methylimidazolium Chloride, 100%) showed 4-fold higher residual activity than [BMIM][TfO] (1-butyl-3-methylimidazolium trifluoromethanesulfonate, 25%) although both ILs share the same cation. Likewise, [EMIM][$MMPO_4$] (1-ethyl-3-methylimidazolium dimethyl phosphate, 53%) and [EMIM][TFA] (1-ethyl-3-methylimidazolium trifluoroacetat, 42%) displayed 42-fold and 53-fold higher residual activity than [EMIM][$MeSO_3$] (1-ethyl-3-methylimidazolium methanesulphate, 0%) and [EMIM][$MeSO_4$] (1-ethyl-3-methylimidazolium methylsulphate,

0%), respectively despite sharing the same cation. [EMIM][$MeSO_3$] and [EMIM][$MeSO_4$] were experimentally found to be acidic and should reduce the pH of the assay solution inhibiting therefore, product detection. Indeed, the low pH values of both ILs did not allow the yellow color formation of the product (*p*-nitrophenolate, pKa 7.16), which is detectable in neutral to basic range at 410 nm. *p*-Nitrophenol was reported to have two states, with unique spectral characteristics in the 240 nm to 600 nm range: absorbance of phenol happens in acidic conditions in the lower range, while absorbance of phenolate occurs in basic conditions in the higher range (Max *et al.*, 2012). The results obtained suggested the foremost importance of the anion part of ILs over the cation part as reported in literatures (Anderson *et al.*, 2002; Huddleston *et al.*, 2001). Furthermore, residual activity of [EMIM][TFA] (53%) was only 1.3-fold increased than residual activity of [BMPy][TFA] (1-methylpyrrolidinium trifluoroacetat, 40%) although both ILs comprise the same anion. This confirmed our suggestions as well as the reports about the crucial role of IL-anion in the interactions with enzymes. Therefore, [BMIM][TfO] and [BMIM][Cl] were selected for further use. [BMIM]-based ILs are well characterized and numerous reports certified their broad application.

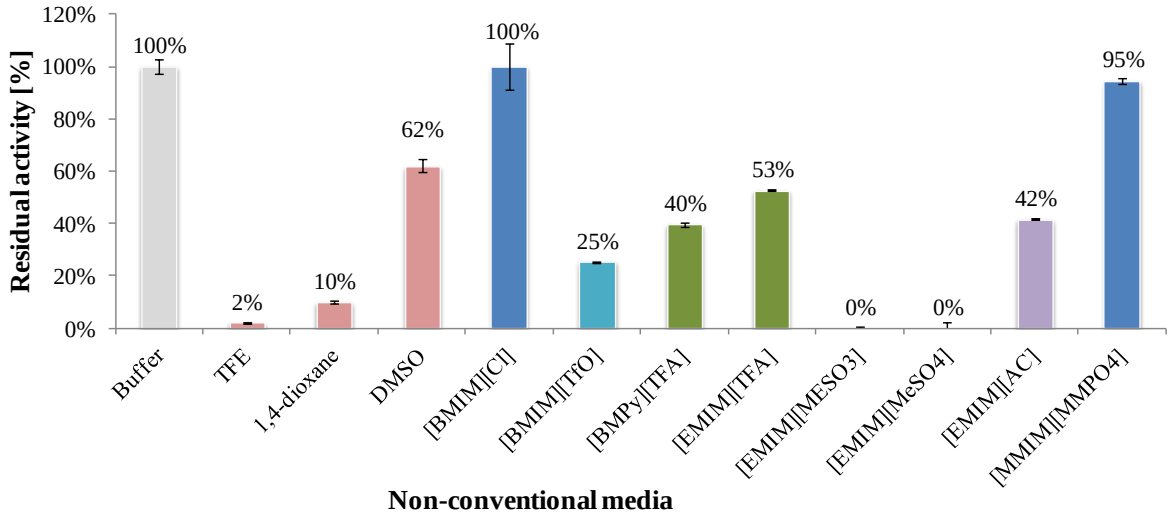


Figure 2-10: Selection of non-conventional media. EV and each BSLA sample were incubated with 50% v/v non-conventional media (2 h, RT, 1000 rpm) and the absorbance was recorded (410 nm, 8 min). The given co-solvent concentrations are related to the concentration in the 100 µL incubation mixture (10 µLsupernatant + 90 µL co-solvent solution). A buffer MTP was also measured as control to enable residual activity calculation. Each sample was measured in triplicate and errors were calculated and are represented as bar. OS: DMSO (dimethyl sulfoxide), 1,4-dioxane, and TFE (2,2,2-Trifluoroethanol). ILs: [BMIM]: 1-butyl-3-methylimidazolium; [Cl]: chloride; [TfO]: trifluoromethanesulfonate, [BMPy]: 1-methylpyrrolidinium, [TFA]: trifluoroacetat, [EMIM]: 1-ethyl-3-methylimidazolium, [$MeSO_3$]: methanesulphate, [$MeSO_4$]: methylsulphate; [MMIM]: 1,3-Dimethylimidazolium Dimethyl, [$MMPO_4$]: dimethyl phosphate, [AC]: acetate.

In conclusion, the three OS tested (DMSO, 1,4-dioxane, and TFE) as well as two ILs ([BMIM][TfO] and [BMIM][Cl]) were selected. BSLA had to be tested for resistance in presence of three OS and four ionic liquids therefore, one OS and two [BMIM]-based ILs with different anions had to be further tested. New batches of OS were tested to select the concentration leading to 30 to 40% BSLA WT residual activity in presence of each non-conventional media. Co-solvent concentrations applied in the incubation mixtures were between 0 to 70% v/v for ILs (Figure 2-11A) and between 0 to 80% v/v for OS (Figure 2-11B). From these results, [BMIM]]Cl] and [BMIM]]Br] should be used at concentration ≤30% v/v. [BMIM]]I] should be used at concentration ≥30% v/v and [BMIM]]TfO] should be used at concentration ≥15% v/v. Both ILs deactivated BSLA WT at relatively low concentrations. [BMIM][Br] appeared to have high deactivation effects on BSLA since only 10% residual activity was observed for a concentration range from 40 to 70% v/v. Among the OS, DMSO caused less deactivation effects and BSLA WT retained one quarter of its residual activity in DMSO. TFE and 1,4-dioxane in contrast, deactivated BSLA WT in low concentrations range. After preliminary tests (results not shown), [BMIM][I], [BMIM][Br] were selected to complete the number of solvents required for the screening (three OS and four ILs).

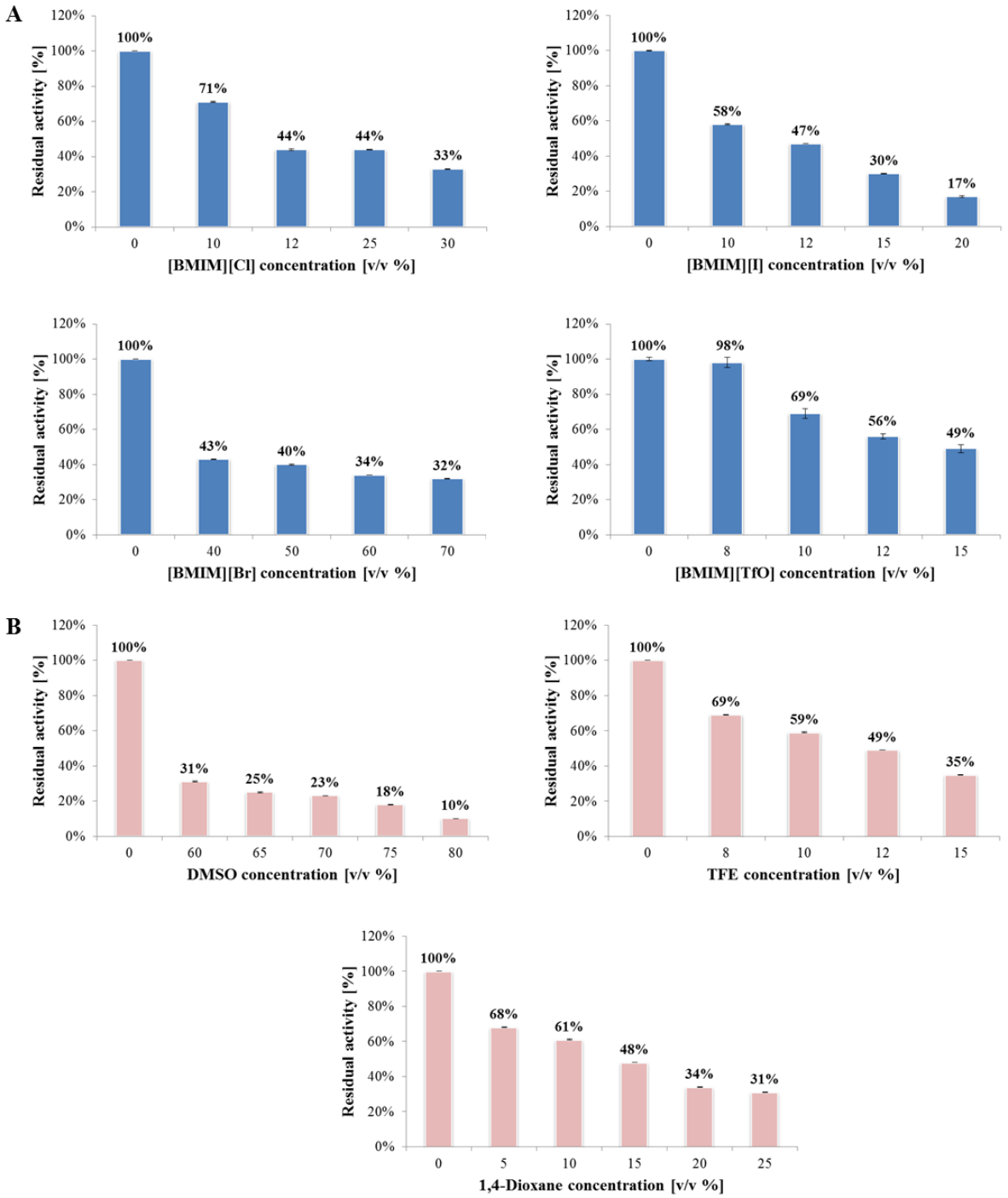


Figure 2-11: Adjustment of the concentrations on non-conventional media for BSLA screening. The aim was to adjust the concentration of each IL and OS to obtain 30 to 40% BSLA WT residual activity. EV and each BSLA sample were incubated with concentrations between 0 and 70% v/v OS or 0 to 80% v/v IL (2 h, RT, 1000 rpm) and the absorbance was recorded (410 nm, 8 min). The given co-solvent concentrations are related to the concentration in the 100 µL incubation mixture (10 µL supernatant + 90 µL co-solvent solution). A buffer MTP was also measured as control to enable residual activity calculation. Each sample was measured in triplicate and errors were calculated **A**: selected ILs (blue): [BMIM]: 1-butyl-3-methylimidazolium, [Cl]: chloride, [Br]: bromide, [I]: Iodide, [TfO]: trifluoromethanesulfonate, **B:** selected OS (pink): DMSO (dimethyl sulfoxide), TFE (2,2,2-Trifluoroethanol) and 1,4-dioxane. Each sample was measured in triplicate and errors were calculated and are represented as bar.

2.3 Conclusions

This chapter outlined experiments performed to establish and optimize all factors (expression medium, screening buffer, substrate for screening, and selection of four ILs and three OS) and conditions required for BSLA libraries generation as well as the screening of the generated libraries in presence of non-conventional media. Therefore, SSM on the whole BSLA sequence was performed (BSLA-SSM 1-91 in this work and SSM-BSLA 92-181 in Research Center Juelich by Alexander Fulton). After sequencing of the SSM libraries, the missing 341 variants were generated by SDM to complete the full diversity. In total, 18547 clones corresponding to 3620 BSLA variants (181*20) were generated. In parallel to libraries generation, the expression system and the screening system for BSLA were optimized. Hence, *p*NPB was selected as substrate, TEA buffer (pKA 7.76, pH7.4, 50 mM) was chosen as assay buffer and the standard deviation of the assay in buffer was determined (apparent standard deviation: 9% and true standard deviation: 11%). After testing three OS and ten ILs (results not all shown in this chapter), all three OS (DMSO, TFE, and 1,4-dioxane) and four ILs ([BMIM][Cl], [BMIM][Br], [BMIM][I], and [BMIM][TfO]) were selected to screen BSLA resistance. The concentration of OS and ILs leading to 30-40% residual activity of BSLA WT was adjusted and used to screen the SSM-BSLA libraries for the selected ILs (chapter 3) and OS (chapter 4) on BSLA resistance.

Chapter 3

Towards Understanding of Directed Evolution: More Than Half of All Amino Acid Positions Contribute to Ionic Liquid Resistance of *Bacillus subtilis* Lipase A

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3 Towards Understanding of Directed Evolution: More Than Half of All Amino Acid Positions Contributed to Ionic Liquid Resistance of *Bacillus subtilis* Lipase A

3.1 Abstract

Ionic liquids (ILs) have been used as (co)solvents in biocatalytic reactions and are attractive for biocatalysis due to unique solvent properties. However, in solutions with high concentration of IL (>10%), enzymes usually show significantly decreased activity. No general principles have so far been identified to improve IL resistance of enzymes by protein engineering despite of a few successful directed evolution campaigns. In a systematic study to elucidate first general engineering principles, site saturation mutagenesis (SSM) on the complete sequence of *bsla* was performed and sequencing confirmed that each naturally possible single amino acid exchange was generated at each amino acid position of BLSA. Improvements in IL resistances that are obtainable by a single amino acid substitution were determined by screening in presence of four [BMIM]-based ILs. Surprisingly, two unexpected lessons on directed evolution were discovered: 1) resistance improvements were obtained at 50-69% of all amino acid positions of BLSA, which explained why improved enzyme variants can often be identified from small sized random mutant libraries (1000-2000 clones) and 2) often only few substitutions per amino acid position led to improved resistance (6-13% of all substitutions). Substitutions with chemically different amino acids (e.g. aromatic amino acids substituted by polar/aliphatic/charged amino acids) were predominantly preferred (66-95%) among the beneficial substitutions. The latter indicates that mutagenesis methods that introduce transversions should at least for lipases like BSLA be favored to generate variants with improved IL resistance.

Keyswords: BSLA, protein engineering, directed evolution, site saturation mutagenesis, ionic liquid (IL).

3.2 Highlights

Project objective:

- A systematic study to understand the contribution of single amino acid substitution, position and location to BSLA resistance towards [BMIM][Cl], [BMIM][Br], [BMIM][I], and [BMIM][TfO].

Main Findings:

- 6-13% of all amino acid substitutions at 50-69% of all amino acid positions led to improvement in IL resistance of BSLA.
- Polar or charged amino acid substitutions primarily lead to improvement of BSLA´s IL resistance.
- At solvent exposed positions: charged substitutions are more preferential for improving BSLA´s IL resistance towards [BMIM][Cl], [BMIM][Br], and [BMIM][I]. But opposite trend was observed for [BMIM][TfO].
- At buried positions: polar substitutions are more preferential for improving BSLA´s IL resistance.
- Substitutions with chemically different amino acids significantly improved BSLA resistance indicating that mutagenesis methods introducing transversions will be favorable for improving IL resistance of lipases at least for BSLA.

Novelty:

- The first systematic investigation of whole sequence SSM-BSLA library for improving the IL resistance.
- For the first time from an experimental point of view, it was explained why often the screening of small sized libraries (1000-2000 variants) is sufficient to find improved variants.

3.3 Introduction

The ionic nature of ionic liquids (ILs) is critical in affecting enzyme performance resulting in enzyme inactivation through unfolding and/or aggregation (Yang, 2009) as enzymes have been designed by nature to efficiently function in aqueous solutions. Enzyme activity in IL solutions is influenced by the physicochemical properties of ILs, which are governed by the proper combination of cation and anion. Numerous studies on the impact of ILs on enzymes performance revealed the dominant role of the IL-anion (Anderson *et al.*, 2002; Huddleston *et al.*, 2001) while cations held a rather secondary role (Micaêlo and Soares, 2008). This is mainly due to the usually small size of anions as they possess a smaller surface than cations, leading therefore to stronger Coulomb interactions with enzymes (Klähn *et al.*, 2011). H-bond basicity and nucleophilicity of IL-anions as well as the experimental Hofmeister series of salts (kosmotropic/chaotropic) are mostly applied to explain the impact of IL-anions on enzyme performance. Nucleophilic anions (as Cl^-, Br^-, OAc^-) were reported to coordinate more strongly to the positively charged amino acids in an enzyme, causing conformational changes (De Diego *et al.*, 2005; Kaar *et al.*, 2003; Lau *et al.*, 2004; Sheldon *et al.*, 2002). Hydrogen bonding of the IL-anions is an important factor influencing interactions between enzymes and ILs and therefore, affecting the enzyme activity (Anderson *et al.*, 2002; Huddleston *et al.*, 2001; Kaar *et al.*, 2003; Lou *et al.*, 2006; Yang and Pan, 2005). The IL-protein interactions may (Constantinescu *et al.*, 2007; Constatinescu *et al.*, 2010) or may not follow (Yang *et al.*, 2009; Zhao *et al.*, 2006) the Hofmeister series. A strong propensity for protein denaturation as well as dissociation of the hydrogen bonds maintaining the enzyme structure can be obtained in presence of IL anions with high H-bond basicity as halides, acetate, dicyanamide (Domínguez de María, 2012; Lau *et al.*, 2004). However, the influence of ILs on enzymes on a molecular level currently remains not fully understood since additional factors such as viscosity, hydrophobicity/hydrophilicity or ILs impurities can also influence.

Improved activity, stability and/or resistance of enzymes in ILs can be achieved by chemical modification of enzymes through PEGylation (Maruyama *et al.*, 2002; Nakashima *et al.*, 2006) and succinylation (Jia *et al.*, 2013; Nordwald and Kaar, 2013; Nordwald *et al.*, 2014b) as well as by immobilization (Madeira Lau *et al.*, 2000; Toral *et al.*, 2007). Additionally, protein engineering by directed evolution can be applied as an attractive alternative to improve enzymes properties through iterative cycles of diversity generation and screening (Lehmann *et al.*, 2014; Liu *et al.*, 2013). Enzyme classes such as oxidoreductases

(e.g. laccase (Liu *et al.*, 2013), formate dehydrogenase (Carter *et al.*, 2014)) and hydrolases (e.g. celullase (Chen *et al.*, 2013; Lehmann *et al.*, 2014; Pottkämper *et al.*, 2009) have been successfully reengineered by protein engineering for improved IL resistance/tolerance. However, up to now, only six reports on improving IL resistance of enzymes are described (Carter *et al*., 2014; Chen *et al*., 2013; Lehmann *et al.*, 2014; Liu *et al*., 2013; Nordwald *et al.*, 2014a; Pottkämper *et al.*, 2009). These reports mostly focused on identifying beneficial substitutions that govern IL resistance. Despite first success stories on improving IL resistance, there is still lack of basic knowledge indispensable to the understanding and improvement of enzyme towards ILs. General design principles to guide protein engineering campaigns have still not been discovered and are therefore needed.

Hydrolases and particularly lipases are an industrially important enzyme class (e.g. *Candida antarctica* lipase B (CALB), *Candida rugosa* lipase (CRL); and *Pseudomonas cepacia lipase* (PCL)). *Bacillus subtilis* lipase A (BSLA) has been investigated for its organic solvent resistance (Reetz *et al.*, 2010; Yedavalli and Rao, 2013), thermostability (Acharya *et al.*, 2004; Ahmad and Rao, 2009; Ahmad *et al.*, 2008; Kumar *et al.*, 2014) and enantioselectivity (Funke *et al.*, 2003). BLSA possesses a minimal α/β-hydrolase fold, a small size (181 amino acids and 19.3 kDa) and does not show interfacial activation (van Pouderoyen *et al.*, 2001).

In this chapter, a systematic study was carried out to understand to what extend a single amino acid substitution at each position of BSLA can contribute to improve the resistance of BSLA towards four ILs ([BMIM][Cl], [BMIM][Br], [BMIM][I], and [BMIM][TfO]). All the natural diversity was generated at each amino acid position of BSLA (181 positions; SSM + SDM) and screened after sequencing of all 18547 clones to confirm the ‘completeness’ of the libraries. In summary for the first time, a data set was generated enabling 1) identification of the amino acid positions in BSLA, which improve ILs resistance, 2) study of the influence of each amino acid substitution on the molecular level, and 3) performance of a comprehensive statistical data analysis (number of amino acid positions contributing to ILs resistance; trends in chemical composition of amino acid substitutions, and the influence of residues location (surface exposed and buried amino acids).

3.4 Experimental Section

All chemicals, reagents and methods used in this part were described in chapter 2.

3.4.1 Optimization of Screening Conditions in Presence of ILs

Four [BMIM] containing ILs with different anions were used in this chapter to screen the generated BSLA libraries: [BMIM][Cl], [BMIM][Br], [BMIM][I], and [BMIM][TfO] (Figure 3-1). BMIM][Cl] and [BMIM][Br] were obtained as solids and were dissolved by adding 8.6% (v/w) and 10% (v/w) water, respectively. IL solutions with different concentrations were prepared by supplementing ILs with TEA buffer (pKa 7.76, pH 7.4, 50 mM). These solutions were used to determine the concentration of the four ILs for the screening. All four ILs were miscible with water as described in the data sheets provided by SOLVIONIC company (en.solvionic.com, Toulouse, France). Besides, pH shifts less than 0.2 unit of pH were obtained in the presence of ILs. Concentrations leading to a residual activity between 30% and 40% of the BSLA WT (2.2.4.5) were chosen for the screening. Polystyrene MTPs (flat-bottomed, polystyrene plates, Greiner Bio-One GmbH, Frickenhausen, Germany) were used for the screening assay with ILs. In each well of a 96-well MTP, 10 µL supernatant were incubated with 90 µL of the IL solution (2 h, 1000 rpm, RT) on a MTP shaker (Edmund Bühler Microtiter shaker, Hechingen, Germany) at the adjusted IL concentration (Table 3-1). For each position, the plate for the reaction only in buffer was used as reference to enable the calculation of the residual activity given as percentage (2.2.4.5). The freshly prepared substrate solution (100 µL/well) was then added to the incubation mixture and the absorbance was measured (410 nm, 8 min, RT). For each well, the same amount of supernatant was incubated with or without ILs and the ratio of "activity in presence of IL divided by the activity in absence of IL" was used to evaluate the IL resistance of BSLA variants. No influence was observed even using two to three times more BSLA supernatant.

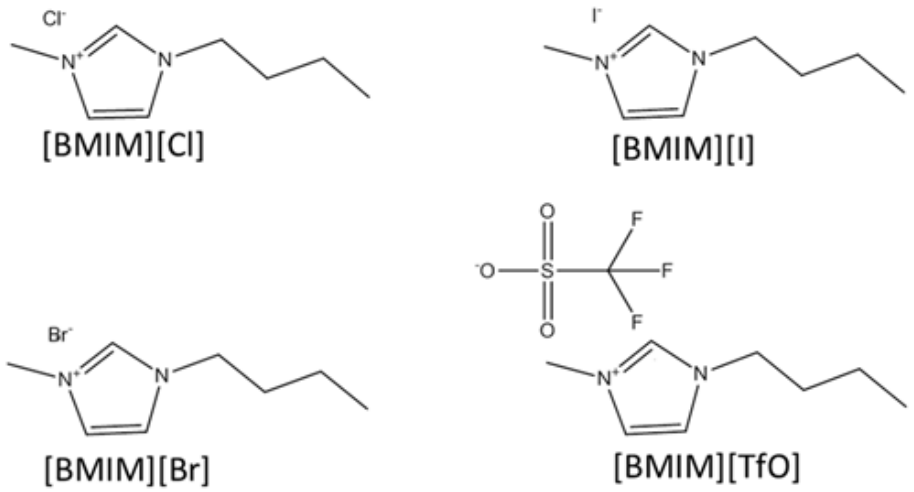

Figure 3-1: Chemical structures of the four selected ILs. [BMIM][Cl], [BMIM][Br], [BMIM][I], and [BMIM][TfO]. [BMIM] = 1-butyl-3-methylimidazolium [Cl] = chloride; [Br] = bromide, [I] =iodide, [TfO] = trifluoromethanesulfonate.

3.5 Results and Discussion

Can ILs resistance of hydrolases such as BSLA be efficiently improved by protein engineering? This question was elucidated in a systematic study by the screening of 181 BSLA libraries in presence of [BMIM][Cl], [BMIM][Br], [BMIM][I], and [BMIM][TfO]. The generated 181 BSLA libraries contained each of the 3620 naturally possible variants as confirmed by extensive sequencing. The results in this chapter were divided in eight sections. The first section summarizes the generation of the SSM libraries for the complete *lipA* gene from *B. subtilis*, the second section deals with the conditions used for the screening of SSM-BSLA libraries in presence of the four used ILs. The third section focuses on the analysis of the number of amino acids positions that influence IL resistance of BSLA (improved/decreased/unchanged IL resistance and inactivation). In the fourth section, all substitutions were analyzed to determine the number of single amino acid substitutions on BSLA resistance towards the four selected ILs. The amino acid substitutions were grouped into four types (aromatic, aliphatic, polar, and charged) and the influence of each type of amino acid on BSLA resistance was depicted in the fifth section. In the sixth section, the effect of substituting each type of amino acid with all the four types (aromatic, aliphatic, polar, and charged) was investigated. The seventh section discusses the effect of amino acid locations on IL resistance of BSLA. In the last section, the best variants contributing to the highest BSLA resistance in each IL were summarized.

The libraries containing all the variants generated with SSM and SDM were defined as "SSM-BSLA libraries" in this work. In all analysis, the SSM-BSLA variants were grouped as following: "**+**" representing variants with improved resistance (**R_V** $\geq$ R_{WT} + 3σ); "**=**" representing variants with unchanged resistance (**R_V** < R_{WT} + 3σ and **R_V** > R_{WT} - 3σ); "**-**" representing variants with decreased resistance (**R_V** $\leq$ R_{WT} - 3σ) and "**x**" representing inactive variants in buffer (**A_V** < **A_{EV}** + 3σ). **R**: Residual activity (2.2.4.5); **A**: activity; **V**: variant; **WT**: wild type; **EV**: empty vector; σ: standard deviation. Due to the different values obtained for BSLA WT in each solvent, comparison of the results using the residual activity values was almost impossible. Therefore, a ratio (R_V/R_{WT}) was applied to easily compare all results based on the same factor. This ratio ($R_V/R_{WT)}$ was obtained by dividing the residual activity of each BSLA variant (R_V) by the residual activity of BSLA WT (R_{WT}) at each position and in each IL.

3.5.1 Screening Conditions for BSLA Libraries in Presence of Four ILs

All 3620 BSLA variants were screened in the presence of four ILs containing the same cation $[BMIM]^+$ which allowed an analysis of the influence of IL-anion on BSLA resistance. IL concentrations were selected to obtain a residual BSLA WT activity of 30 to 40% (Table 3-1). True standard deviations of 11.3% to 14.5% were calculated for the screening system with the four ILs as co-solvents. Screening systems with standard deviations around 12% were generally applied in directed evolution experiments (Lehmann *et al.*, 2012; Tee and Schwaneberg, 2007).

Table 3-1: ILs and conditions used for the screening of BSLA libraries. Calculated standard deviations of the screening system are also showed. C: concentration, R_{WT}: residual activity of BSLA WT, stdev: standard deviation, App: apparent. [BMIM] = 1-butyl-3-methylimidazolium, [Cl] = chloride, [Br] = bromide, [I] =iodide, [TfO] = trifluoromethanesulfonate.

Selected ILs	C [M] (%v/v)*	R_{WT} [%]	App stdev [%]	True stdev [%]
[BMIM][Cl]	1.2 (≈ 18.3)	35.0	3.0	11.3
[BMIM][Br]	0.9 (≈ 13.2)	39.0	7.6	14.5
[BMIM][I]	0.6 (≈ 10.0)	37.0	7.6	12.3
[BMIM][TfO]	0.7 (≈ 15.0)	30.0	6.0	13.0

* Concentration used in the incubation steps.

The order of H-bond basicity of anions in aqueous solution is: $Cl^- > Br^- > I^- > TfO^-$ and the order in the Hofmeister series is: $Cl^- > Br^- > TfO^- > I^-$ (Naushad *et al.*, 2012). Deactivation effect of ILs on enzymes has been often reported to increase with higher H-bond basicity (Heller *et al.*, 2010; Lou *et al.*, 2006; Turner *et al.*, 2003). However, the results of the BSLA resistance screening do not correlate with the nucleophilicity/basicity of the anions. Among the four BMIM-based ILs, [BMIM][TfO] displayed the highest inactivation effect (Table 3-1). 0.7 M (15.0% v/v) of [BMIM][TfO] resulted in 70% activity loss which might be attributed to: 1) delocalized charge distribution of TfO^- which has a weaker solvation shell can therefore, easily form H-bonds with enzyme backbone amides, than the halides Cl^-, Br^- and I^-, 2) a single TfO^- might form multiple H-bonds with amino acids of BSLA and perturb the enzyme structure more dramatically than halides anions Cl^-, Br^- and I^- that can only form single H-bonds. In a MD simulations study, NO_3^-(with a similar structure to TfO^-) was described to form double the amount of H-bonds between the enzyme backbone amides and itself (Micaêlo and Soares, 2008). NMR was used in another study to determine the role of IL anion on protein destabilization: the binding of weakly hydrated anions to positively charged or polar residues destabilize protein structure as consequence of the partial dehydration of the protein backbone groups (Figueiredo *et al.*, 2013). This might justify why [BMIM][Cl]

displayed the least inactivation effect since among the four selected anions in this study, Cl^- is most hydrated and should therefore, be less available for interactions with BSLA.

Residual activity of a given variant (R_V) and the residual activity of the WT (R_{WT}) were calculated after the screening of all 181 BSLA libraries. The variants were classified into four categories as described above based on the Gaussian distribution curve (σ is the true coefficient of variation of the screening system, Table 3-1).

3.5.2 Influence of the Number of Amino Acid Positions on BSLA's Resistance Towards ILs

Figure 3-2 displays the number of amino acid positions of BSLA leading to improved/unchanged/decreased IL resistance as well as inactive BSLA variants after screening of all 181 BSLA libraries. [BMIM][Cl] (124 positions, 69% of all positions) yields 20 more positions than [BMIM][TfO] (104, 57%), 29 more than [BMIM][I] (95, 52%), and 33 more than [BMIM][Br] (91, 50%) for the IL resistance type "+". In case of the IL resistance type "=", at least one amino acid substitution was found that did not influence IL resistance of BSLA at each of the 181 amino acid positions. For the IL resistance type "-", it was found that [BMIM][Cl] (117, 65% of all positions) yields almost the same number of positions than [BMIM][I] (115; 64%) ~ [BMIM][TfO] (102; 56%) > [BMIM][Br] (89; 49%). For the resistance type "x", at least one amino acid substitution corresponding to the wild type was found at 99 (55%) amino acid positions which inactivated BSLA already in the buffer solution. Analysis of the number of substitution influencing BSLA´s IL resistance (improved/decreased/unchanged) as well as BSLA´s inactivation at each position of BSLA is detailed in Table 7-3 to Table 7-6.

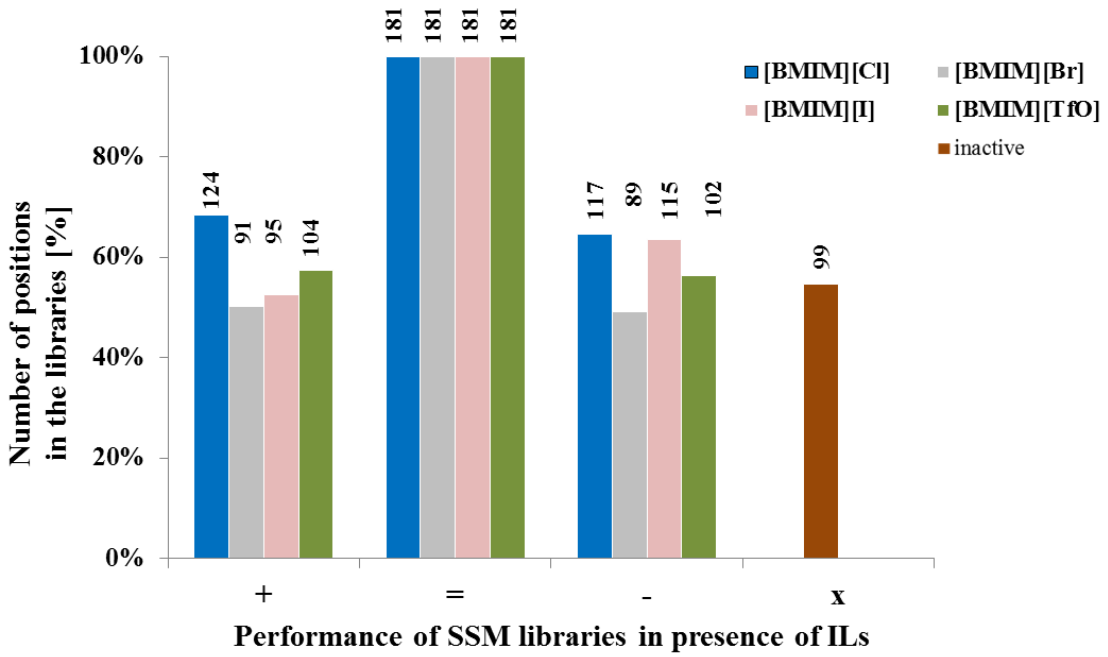

Figure 3-2: Number of amino acid positions, which lead to improved/unchanged/decreased IL resistance and/or inactivation of BSLA in buffer. All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

3.5.3 Influence of the Number of Single Amino Acid Substitutions on BSLA´s Resistance Towards ILs

Figure 3-3 depicts the number of variants with improved "+", unchanged "=", decreased "-" resistance and ''inactive'' variants "x" in presence of [BMIM][Cl], [BMIM][Br], [BMIM][I], and [BMIM][TfO]. [BMIM][Cl] (462 out of 3620; 13% of all substitutions) yields 170 substitutions more than [BMIM][TfO] (292, 8%), 199 more than [BMIM][I] (263, 7%) and 256 more than [BMIM][Br] (206, 6%) for the IL resistance type "+". Among the variants with increased resistance towards [BMIM][Cl], 1% displayed a resistance improvement higher than 2.5-fold. In the remaining three ILs, less than 0.3% of the BSLA variants were obtained with resistances higher than 2.5-fold. In general, a large majority 62-72% (2254-2591 out of 3620) of all substitutions does not affect BSLA resistance type "=" towards the four selected ILs. [BMIM][I] (544 out of 3620; 15% of all substitutions) yields 138 more substitutions than [BMIM][Cl] (406; 11%), 139 more substitutions than [BMIM][TfO] (405, 11%), and 219 more than [BMIM][Br] (325, 9%) for the IL resistance type "-". In case of IL resistance type "x", 498 (14%) substitutions resulted in inactivation of BSLA.

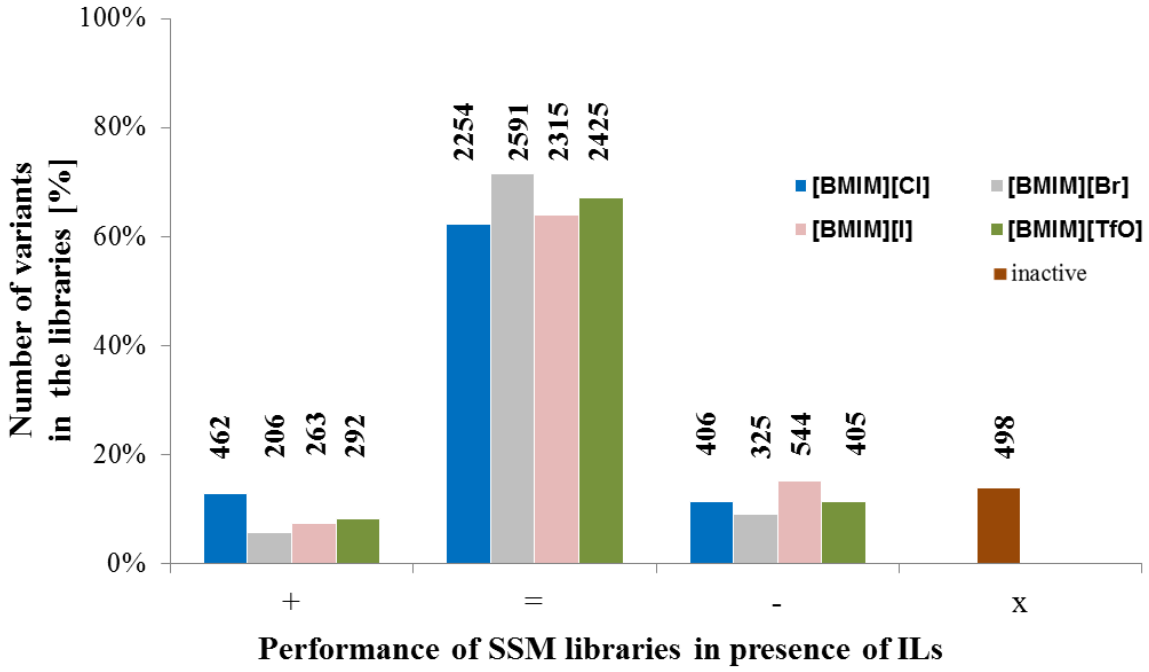

Figure 3-3: Number of SSM-BSLA variants, which lead to improved/unchanged/decreased IL resistance towards four ILs and/or inactivation of BSLA in buffer. All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Overall, 50% to 69% amino acid positions (Figure 3-2) contributed to the improved BSLA´s IL resistance towards [BMIM][Br] (50%), [BMIM][I] (52%), [BMIM][TfO] (57%), and [BMIM][Cl] (69%). A large fraction of the amino acid positions in BSLA can therefore contribute to its stabilization in presence of ILs. Among the 3620 BSLA variants that cover the naturally occurring diversity at each amino acid position, only 6-13% of all the amino acid exchanges improved IL resistance. In case of [BMIM][Cl] 1% and in case of [BMIM][Br], [BMIM][I] and [BMIM][TfO], 0.3% of the variants exhibit a significantly increased IL-resistance (>2.5-fold). Analysis of these screening results reveals an important new lesson in directed evolution and provides a first experimental explanation of why the screening of small libraries with a few thousand variants yields often improved variants in directed evolution experiments, especially for properties that have not been under selection pressure. In addition, the on average low number of amino acid substitutions per amino acid positions discloses why saturation mutagenesis experiments on amino acid positions that have been discovered by random mutagenesis yield only <50 % variants with better improvements.

3.5.4 Effect of Different Type of Amino Acid Substitutions (Aromatic, Aliphatic, Charged, Polar) on BSLA´s Resistance Towards ILs

The 20 amino acid substitutions were classified into four "types" in order to be consistent with previous reports (Verma *et al.*, 2014; Wong *et al.*, 2006b, 2007a): 15% aromatic (F, Y, and W), 25% aliphatic (A, V, L, I, and G), 25% charged (D, E, H, K, and R) and 35% polar (termed neutral in the cited papers; C, M, P, S, T, N, and Q) amino acids. Glycine (G), cysteine (C) and proline (P) could also be classified as special amino acids. Figure 3-4 depicts the effect of each type of amino acid substitution on BSLA resistance towards the four [BMIM]-based ILs. Inactive variants were defined as the variants which showed no activity in buffer. Supplementing of IL was not in single case able to recover BSLA activity. Overall, 183 out of 498 inactive BSLA variants ("x" type in buffer) harbored charged substitutions (37%), 152 polar (31%), 87 aromatic (17%), and 76 aliphatic (15%).

Figure 3-4A-D illustrates improvements for the IL resistance type "+" towards [BMIM][Cl] resistance in case of 148 out of 462 to polar amino acids (32%), 144 to charged (31%), 98 to aliphatic (21%), and 72 to aromatic (16%). For [BMIM][Br] resistance, 64 out of 206 to charged (31%), 62 to polar (30%), 48 to aliphatic (23%), and 32 to aromatic (16%). [BMIM][I] resulted in 87 out of 263 variants to charged (33%), 74 to polar (28%), 53 to aliphatic (20%), and 49 to aromatic (19%). [BMIM][TfO] led in 100 out of 292 to polar (34%), 87 to charged (30%), 54 to aliphatic (18%), and 51 to aromatic (17%). Taken together, the highest number of improved BSLA variants were achieved for [BMIM][Cl] (462) followed by [BMIM][TfO] (292), [BMIM][I] (263), and [BMIM][Br] (206) (Figure 3-4A-D). Overall, most substitutions improving BSLA resistance for all four ILs variants were achieved with polar or charged amino acids.

Table 3-2: Number of each type of amino acid substitution for each resistance type for all four [BMIM]-based ILs. All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer**.**

Resistance type	Aromatic [%]	Aliphatic [%]	Polar [%]	Charged [%]
+	17-19	18-21	28-34	30-33
=	14	28-29	35-36	22
-	14-19	19-24	36-41	21-27
x	17	15	31	37

A comparable amino acid substitution profile was obtained for all four ILs: 35-36% polar, 28-29% aliphatic, 22% charged, and 14% aromatic in the case of IL resistance type "=" (Table 3-2).

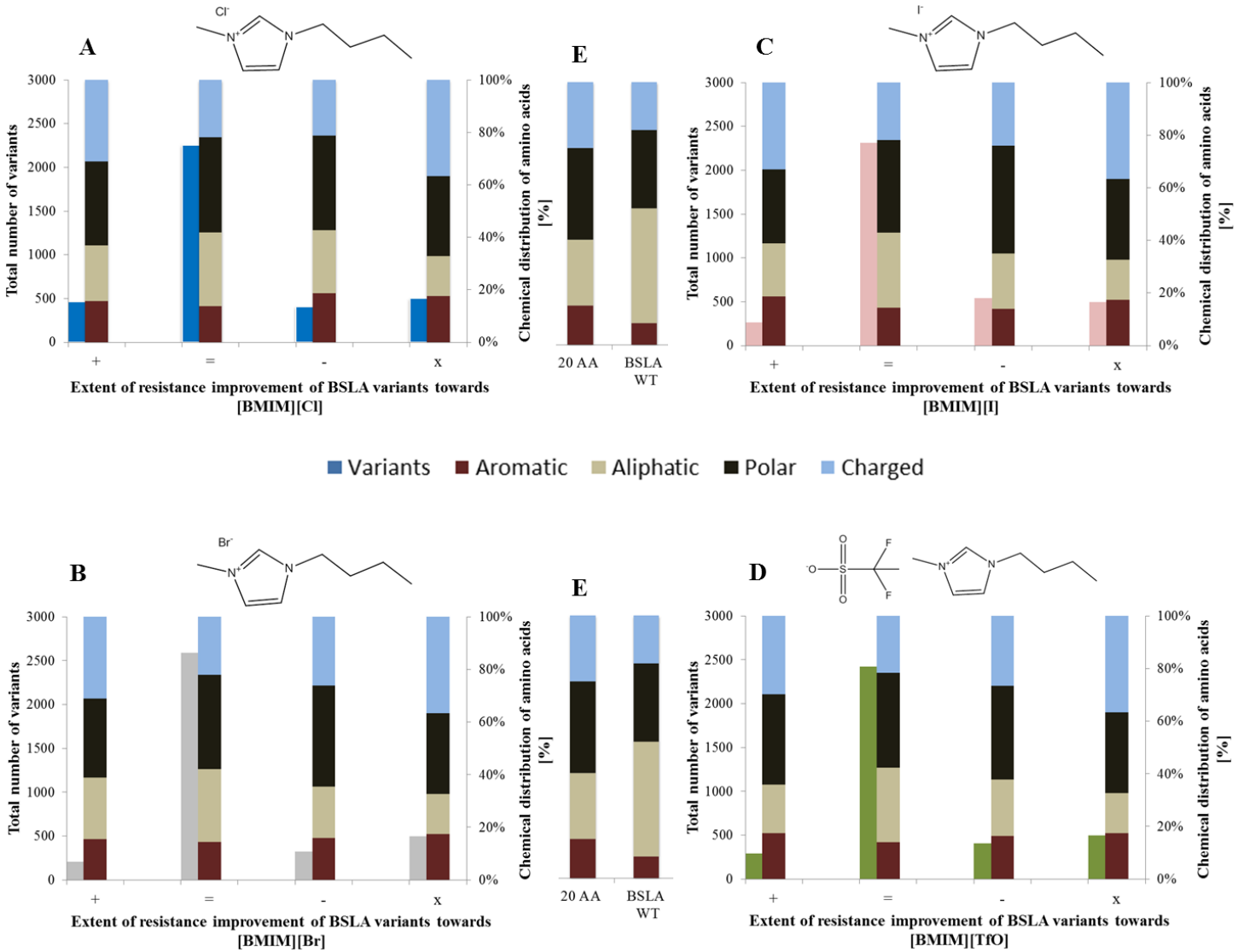

Figure 3-4: Distribution of amino acid substitutions of SSM-BSLA libraries with improved/unchanged /decreased IL resistance.
A: Resistance towards [BMIM][Cl], B: Resistance towards [BMIM][Br], C: Resistance towards [BMIM][I], D: Resistance towards [BMIM][TfO], **E:** Distribution of the 20 amino acids and amino acid composition of BSLA WT. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). The X-axis represents the extent of resistance improvement of BSLA variants toward different ILs in four categories ("+"; "="; "-", and "x"). The primary Y-axis (on the left) displays the total number of variants and positions, at which an effect on BSLA resistance is observed. The secondary Y-axis (on the right) represents the chemical distribution of the amino acids in categories. All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer. AA: amino acid.

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The IL resistance type "-" displayed for [BMIM][Cl], 147 out of 406 substitutions to polar amino acids (36%), 97 to aliphatic (24%), 86 to charged (21%), and 76 to aromatic (19%). For [BMIM][Br], 125 out of 325 to polar (38%), 85 to charged (26%), 63 to aliphatic (19%), 52 to aromatic (16%) were obtained. For [BMIM][I], 222 out of 544 to polar (41%), 131 to charged (24%), 115 to aliphatic (21%), 76 to aromatic (14%) were achieved. For [BMIM][TfO], 144 out of 405 to polar (36%), 108 to charged (27%), 87 to aliphatic (21%), and 66 to aromatic (16%) were found. Remarkably, BSLA variants with polar amino acid substitutions often led to a decreased resistance for all four ILs and significant discrepancy in the total number was observed (325 substitutions in [BMIM][Br] vs 544 in [BMIM][I]). The amino acid composition of BSLA (8.3% aromatic, 43.6% aliphatic, 29.8% polar, and 18.2% charged amino acids) is optimized for higher efficiency in aqueous solution with a common preference for aliphatic amino acids. Statistical analysis of chemical substitutions for all four ILs revealed improved variants by substitution to aromatic (17-19%), aliphatic (18-21%), polar (28-34%), and charged (30-33%) amino acids. As general trend, one can conclude that beneficial substitutions to polar and/or charged amino acid residues will lower the fraction of aliphatic amino acids in improved BSLA variants. Interestingly, the latter trend correlates roughly with natural distribution of amino acids: aromatic (15%), aliphatic (25%), polar (35%), and charged (25%).

For [BMIM][Cl] (Figure 3-4A) and [BMIM][Br] (Figure 3-4B), the number of beneficial substitutions followed this order: polar = charged > aliphatic > aromatic. The order for [BMIM][I] (Figure 3-4C) was however: charged > polar > aliphatic = aromatic and in [BMIM][TfO] (Figure 3-4D): polar > charged > aliphatic = aromatic. The following order: polar > aliphatic > charged > aromatic was observed for all four ILs for the variants with IL resistance type "=". The number of variants for IL resistance type "-" were ranked as follows: polar > charged > aliphatic > aromatic in presence of all ILs except [BMIM][Cl] (polar > aliphatic > charged >aromatic). To conclude, the main category of substitutions leading to improvements was either charged or polar for all four ILs. These findings were in agreement with reports of other groups on IL resistance of cellulases. Accordingly, three cellulase mutants with improved IL resistance (from SeSaM mutant libraries) were obtained with charged and polar substitutions (Pottkämper *et al.*, 2009). Introduction of charged amino acids at positions selected by using NMR were also reported to significantly improve IL tolerance of BSLA towards [BMIM][Cl] (Nordwald *et al.*, 2014a). Further, the stabilization of cellulase Cel5A Tma in [C2mim] solution by introducing two charged substitutions (distal from active sites) were described (Chen *et al.*, 2013).

3.5.5 Effect of Amino Acid Substitution Types on IL Resistance of BSLA: Comparison Between WT and Variants

Table 3-3 summarizes the effect of each type of amino acid substitution (to polar, aliphatic, charged and aromatic) on IL resistance of BSLA in a matrix of 16 substitution types. In all cases, improved IL resistance with significant differences in orders and trends was obtained with substitutions to chemically different amino acids as demonstrated in detail in the next four paragraphs. Table 3-3 provides general trends on amino acid substitution patterns and answers in contrast to Figure 3-4 the question what preferred types (aromatic, aliphatic, polar, charged) of amino acid exchanges exist. Table 7-7 displays the effect of charged acidic and charged basic amino acids. However, no trend equivalent to the one reported by Nordwald and co-workers was observed in this work (Nordwald and Kaar, 2013). They suggested stabilization and destabilization of chymotrypsin in [BMIM][Cl] by interactions between anions and cations, respectively. Indeed, chymotrypsin modified by anions was described to interact more with [BMIM] while chymotrypsin modified by cations was found to interact more with [Cl^-] and is destabilized (Nordwald and Kaar, 2013).

3.5.5.1 Substitution of Aromatic Amino Acids

The substitution of aromatic amino acids with chemically different amino acids (aliphatic, charged, and polar) led to significantly improved resistance "+" towards all four ILs. The number of improved variants were observed with the following order of substitution types: polar > aliphatic > charged > aromatic for [BMIM][Cl] and [BMIM][TfO]. In [BMIM][Br], the obtained order was: polar > aliphatic > aromatic > charged and in case of [BMIM][I] the order was: polar > charged > aromatic > aliphatic.

3.5.5.2 Substitution of Aliphatic Amino Acids

The substitution of aliphatic amino acids with chemically different amino acids (aromatic, charged, and polar) led to improved resistance "+" towards all four ILs. The number of improved variants was ranked in the following order of substitution types: polar ≥ charged > aliphatic ≥ aromatic for [BMIM][Cl], [BMIM][I], and [BMIM][TfO]. For [BMIM][Br], the order was: charged > polar > aliphatic > aromatic.

Table 3-3: Effect of each amino acid type substitution (polar, aliphatic, charged and aromatic) on IL resistance of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per IL are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Amino acid types in BSLA WT

[BMIM][Cl]

	BSLA: Aromatic (15; 8.3%)				BSLA: Aliphatic (79; 43.6%)				BSLA: Polar (54; 29.8%)				BSLA: Charged (33; 18.2%)			
	+ (42)	= (199)	- (32)	X (27)	+ (215)	= (852)	- (200)	x (313)	+ (121)	= (771)	- (111)	x (77)	+ (84)	= (432)	- (63)	x (81)
Aromatic	14%	19%	6%	0%	13%	13%	20%	19%	14%	14%	22%	19%	**25%**	13%	17%	15%
Aliphatic	26%	25%	28%	19%	20%	33%	18%	12%	21%	26%	32%	16%	**24%**	25%	25%	25%
Polar	**40%**	32%	41%	44%	**35%**	37%	39%	27%	28%	38%	28%	29%	**26%**	34%	41%	43%
Charged	19%	25%	25%	37%	33%	17%	24%	42%	**37%**	23%	18%	36%	**25%**	28%	16%	17%

[BMIM][Br]

	+ (25)	= (208)	- (40)	x (27)	+ (79)	= (1014)	- (174)	x (313)	+ (53)	= (869)	- (81)	x (77)	+ (49)	= (500)	- (30)	x (81)
Aromatic	20%	18%	8%	0%	14%	14%	17%	19%	13%	14%	17%	19%	18%	14%	20%	15%
Aliphatic	28%	25%	25%	19%	20%	31%	17%	12%	26%	26%	21%	16%	22%	25%	23%	25%
Polar	**36%**	34%	35%	44%	29%	37%	40%	27%	**32%**	36%	36%	29%	27%	34%	43%	43%
Charged	16%	23%	33%	37%	**37%**	19%	27%	42%	28%	24%	26%	36%	**33%**	26%	13%	17%

[BMIM][I]

	+ (18)	= (191)	- (64)	x (27)	+ (95)	= (854)	- (318)	x (313)	+ (88)	= (802)	- (113)	x (77)	+ (62)	= (468)	- (49)	x (81)
Aromatic	22%	20%	5%	0%	16%	14%	14%	19%	15%	14%	16%	19%	27%	13%	22%	15%
Aliphatic	17%	27%	23%	19%	19%	32%	20%	12%	22%	27%	22%	16%	21%	26%	24%	25%
Polar	**33%**	32%	41%	44%	**33%**	35%	43%	27%	28%	36%	35%	29%	19%	35%	39%	43%
Charged	28%	21%	31%	37%	**33%**	19%	23%	42%	**35%**	23%	27%	36%	**32%**	26%	14%	17%

BMIM][TfO]

	+ (21)	= (208)	- (44)	x (27)	+ (118)	= (925)	- (224)	x (313)	+ (82)	= (821)	- (100)	x (77)	+ (71)	= (471)	- (37)	x (81)
Aromatic	5%	20%	5%	0%	14%	13%	17%	19%	16%	14%	19%	19%	**28%**	13%	19%	15%
Aliphatic	33%	24%	30%	19%	15%	32%	19%	12%	18%	27%	21%	16%	20%	26%	27%	25%
Polar	**38%**	33%	39%	44%	**37%**	37%	38%	27%	**34%**	36%	29%	29%	**28%**	35%	32%	43%
Charged	24%	23%	27%	37%	33%	18%	25%	42%	32%	23%	31%	36%	24%	27%	22%	17%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6% aliphatic, 29.8% polar amino acids, 18.2% charged, and, 8.3% aromatic). Highest values among the resistance type "+" are highlighted in blue and dark red represents the values of substitutions preferring exchange with similar residues.

3.5.5.3 Substitution of Polar Amino Acids

The substitution of polar amino acids with chemically different amino acids (aromatic, aliphatic, and charged) led to improved resistance "+" towards all four ILs. For [BMIM][Cl] and [BMIM][I] the order was: charged > polar > aliphatic > aromatic. Interestingly, for [BMIM][Br] and [BMIM][TfO], polar amino acids were more frequently substituted by a chemically similar amino acid and the order was therefore: polar > charged > aliphatic > aromatic.

3.5.5.4 Substitution of Charged Amino Acids

The substitution of charged amino acids with chemically different amino acids (aromatic, aliphatic, and polar) led to improved resistance "+" towards all four ILs. The most common amino acid substitution type to substitute charged amino acids was different for the four ILs. For [BMIM][Cl], a comparable number of improved variants was observed for substitutions to aromatic, aliphatic, polar, and also charged residues. In case of [BMIM][Br], the observed order was: charged > polar > aliphatic > aromatic. In case of [BMIM][I], charged > aromatic > aliphatic > polar, and in case of [BMIM][TfO] polar = aromatic > charged > aliphatic. Overall, chemically different amino acids (e.g. aromatic to polar/aliphatic/charged amino acids) were preferred in 66-95% of all substitutions (Table 7-8).

This finding lectures one more lesson for directed enzyme evolution: the probability to find BSLA variants with improved IL resistance reducing thereby the screening effort increases by random mutagenesis methods generating chemically diverse amino acid substitution patterns (e.g. from aliphatic to charged or to polar ones). Chemically diverse mutant libraries are generated with a transversion bias or by subsequent mutations in a codon (Seng Wong *et al.*, 2004; Wong *et al.*, 2007a; Zhao *et al.*, 2014) and consequently, transversion enriched methods should be preferred (Mundhada *et al.*, 2011). Remarkably, frequently used epPCR random mutagenesis methods possess a strong transition bias preserving the chemical properties (Wong *et al.*, 2007c).

3.5.6 Effect of the Location of Amino Acid Substitution on BSLA´s IL Resistance

Around 29% amino acid residues of BSLA are buried and 71% are surface exposed. The solvent accessible surface for each residue was determined using the Accelrys Discover

Studio software package employing a water probe with a radius of 1.5 Å and a cutoff of <5% solvent accessibility for buried residues (Chothia, 1976) based on the chain A of the BSLA X-ray crystal structure (PDB ID: 1I6W) (van Pouderoyen *et al.*, 2001). The effect of amino acid location on IL resistance of BSLA is illustrated in Table 3-4A. Exposed and buried amino acid positions led to improved IL resistance ("+") for all four types of substitutions. As reported for other enzymes (Chen *et al.*, 2013; Liu *et al.*, 2013; Nordwald and Kaar, 2013; Nordwald *et al.*, 2014a), beneficial substitutions were located on the surface and in buried regions of BSLA. Analysis of the amino acid substitution patterns of surface exposed and buried amino acid revealed discrepancies. At exposed positions, charged substitutions resulted in more improved variants than polar substitutions when exposed to [BMIM][Cl], [BMIM][Br], and [BMIM][I], which all contained a halide anion (Cl^-, Br^- or I^-). These results differ from the natural distribution in BSLA WT (Table 3-4B) showing more aliphatic amino acids exposed as well as buried. An opposite trend was however, observed for [BMIM][TfO], in which more polar and charged amino acids were found to be beneficial for the resistance at exposed surfaces. Furthermore, at buried positions, polar substitutions were always preferred for improved IL resistance (type "+") and yielded more improved variants than charged substitutions for all four ILs. Remarkably, the fraction of polar substitutions resulting in inactive BSLA variants (type "x") was significantly higher (26% buried positions and 38% exposed positions). It is noteworthy that charged substitutions at the buried positions resulted in a high fraction of inactive variants (type "x"; 41%) and substitutions to polar amino acids predominantly lowered activity (type "-"; 39-44% for all four ILs). Moreover, 11 polar and only 4 charged amino acid residues were represented among all 53 buried positions (Table 3-4B). Introduction of charged substitutions with stabilizing effects is difficult to achieve in buried positions and it is harder to find the counterpart amino acid to form a salt bridge than introduction of a polar substitution which is stabilized through H-bonds. Moreover, the α/β-hydrolase fold is an evolutionary very successful protein fold which can be found in many hydrolases. Therefore, the results achieved for BSLA can likely be generalized to other hydrolases and enzyme classes with an α/β-hydrolase fold.

Table 3-4: Effect of the amino acid locations on IL resistance of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per IL are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

A

Location of amino acid positions in BSLA

		Exposed positions (128; 71%)				Buried positions (53; 29%)			
		+ (343)	= (1781)	- (247)	x (189)	+ (119)	= (473)	- (159)	x (309)
[BMIM][Cl]	Aromatic	15%	14%	22%	15%	16%	12%	14%	19%
	Aliphatic	22%	27%	23%	17%	18%	34%	25%	14%
	Polar	30%	36%	34%	38%	**38%**	39%	40%	26%
	Charged	**32%**	24%	21%	30%	28%	15%	22%	41%
		+ (172)	= (2077)	- (122)	x (189)	+ (34)	= (514)	- (203)	x (309)
[BMIM][Br]	Aromatic	16%	15%	20%	15%	12%	13%	14%	19%
	Aliphatic	23%	26%	17%	17%	26%	33%	21%	14%
	Polar	**30%**	35%	38%	38%	**32%**	39%	39%	26%
	Charged	**31%**	24%	25%	30%	29%	14%	27%	41%
		+ (236)	= (1897)	- (238)	x (189)	+ (27)	= (418)	- (306)	x (309)
[BMIM][I]	Aromatic	19%	15%	15%	15%	15%	13%	13%	19%
	Aliphatic	19%	27%	22%	17%	26%	37%	20%	14%
	Polar	27%	35%	37%	38%	**37%**	35%	44%	26%
	Charged	**34%**	23%	26%	30%	22%	15%	22%	41%
		+ (248)	= (1962)	- (161)	x (189)	+ (44)	= (463)	- (244)	x (309)
BMIM][TfO]	Aromatic	19%	14%	17%	15%	11%	12%	16%	19%
	Aliphatic	19%	27%	22%	17%	14%	36%	21%	14%
	Polar	**33%**	35%	31%	38%	**43%**	38%	39%	26%
	Charged	29%	24%	29%	30%	32%	14%	25%	41%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6% aliphatic, 29.8% polar amino acids, 18.2 % charged, and 8.3% aromatic). Highest values among the resistance type "+" are highlighted in blue. Column "x" shows that the number of inactive variants resulted from amino acid substitutions. The activity was measured in a buffer plate which was used as reference (absence of IL) for comparison. Therefore, the number of inactive variants was the same for the four ILs.

B

Number of each type of amino acids exposed and buried in BSLA

	Aliphatic	Aromatic	Polar	Charged	Total
Exposed AA residues					
Number of AA	44 (34%)	12 (9%)	43 (34%)	29 (23%)	128 (100%)
Buried AA residues					
Number of AA	35 (66%)	3 (6%)	11 (21%)	4 (8%)	53 (100%)

AA: amino acid

Figure 3-5 to Figure 3-8 represent 3D models of BSLA for visualization of the beneficial positions found during screening with all four ILs. [BMIM[[Cl] showed few positions holding more than eight variants (colored in red) with improved IL resistance in the core of BSLA (Figure 3-5). In contrast, for [BMIM][Br], [BMIM][I], and [BMIM][TfO] in most cases only one variant per position (colored in blue) at buried positions (Figure 3-6 to Figure 3-8) was able to improve BSLA resistance. Besides, considering secondary structure

elements of BSLA, more than 85% of the beneficial amino acid positions for IL resistance of BSLA were localized on the helices and loops of BSLA (Table 7-10).

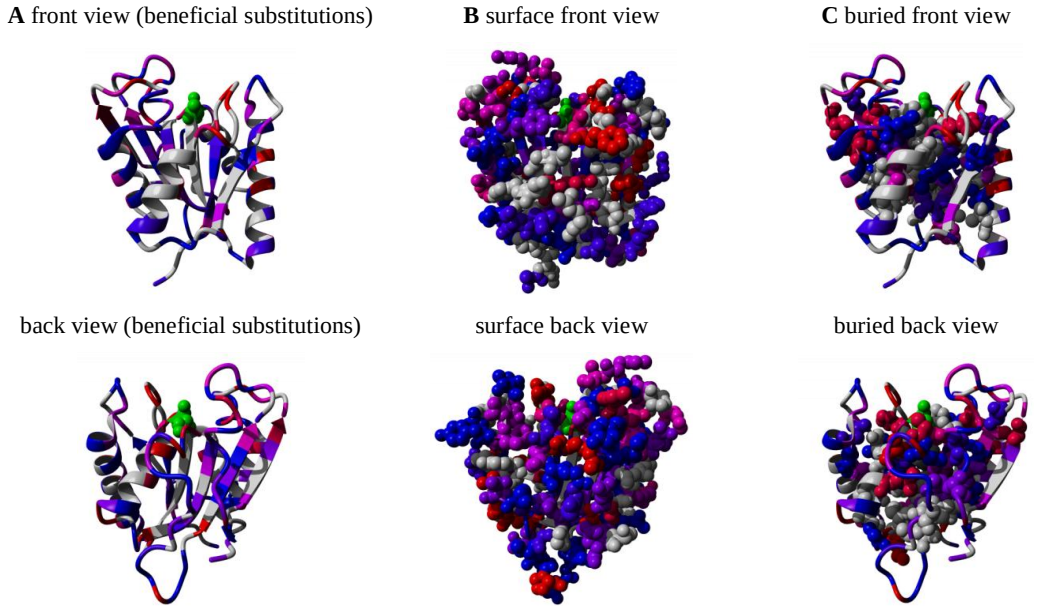


Figure 3-5: 3D model of BSLA (Protein Database code: 1I6W) displaying regions which contribute to the resistance of BSLA towards [BMIM][Cl] with a color scale. Positions at which no substitution led to improvements are colored grey. Positions with one substitution leading to improvements are colored blue. Positions with two to seven substitutions leading to improvements are colored purple (two positions) to magenta (seven positions). Red represents positions with eight or more substitutions leading to improvements. The active site residue Ser 77 is colored green. A) Cartoon representation of BSLA, B) Van der Waals representation of surface residues of BSLA, C) Van der Waals representation of buried residues of BSLA. The two N-terminal amino acids (Ala1 and Glu2) are also included. Yasara was used for visualization.

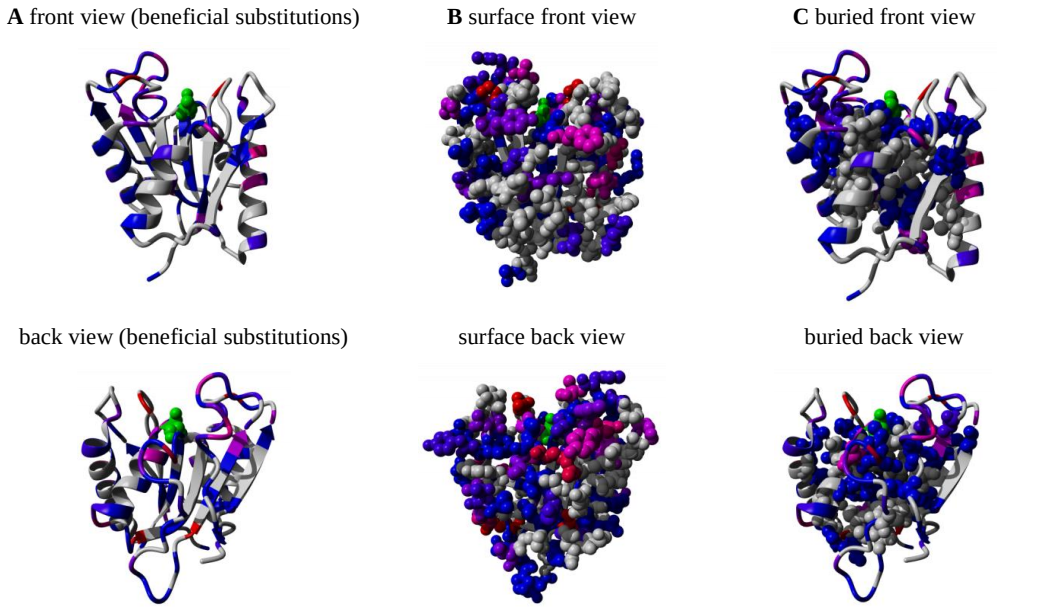


Figure 3-6: 3D model of BSLA (Protein Database code: 1I6W) displaying regions which favor BSLA resistance in presence of [BMIM][Br] in a color scale. Positions at which no substitution led to improvements are colored grey. Positions with one substitution leading to improvements are colored blue. Positions with two to seven substitutions leading to improvements are colored purple (two positions) to magenta (seven positions). Red represents positions with eight or more substitutions leading to improvements. The active site residue Ser 77 is colored green. A) Cartoon representation of BSLA, B) Van der Waals representation of surface residues of BSLA, C) Van der Waals representation of buried residues of BSLA. The two N-terminal amino acids (Ala1 and Glu2) are also included. Yasara was used for visualization.

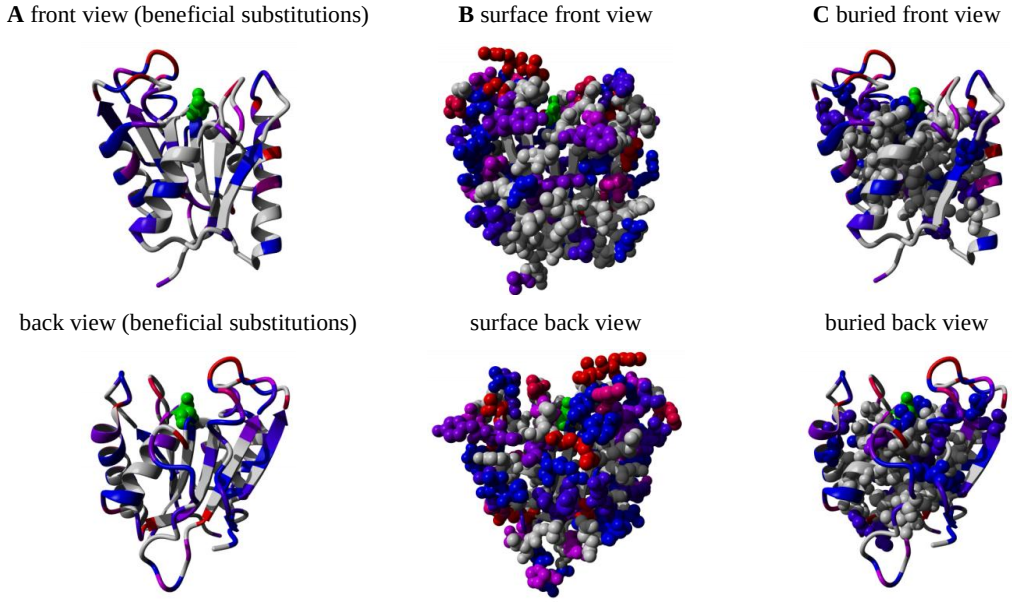


Figure 3-7: 3D model of BSLA (Protein Database code: 1I6W) displaying regions which favor BSLA resistance in presence of [BMIM][I] in a color scale. Positions at which no substitution led to improvements are colored grey. Positions with one substitution leading to improvements are colored blue. Positions with two to seven substitutions leading to improvements are colored purple (two positions) to magenta (seven positions). Red represents positions with eight or more substitutions leading to improvements. The active site residue Ser 77 is colored green. A) Cartoon representation of BSLA, B) Van der Waals representation of surface residues of BSLA, C) Van der Waals representation of buried residues of BSLA. The two N-terminal amino acids (Ala1 and Glu2) are also included. Yasara was used for visualization.

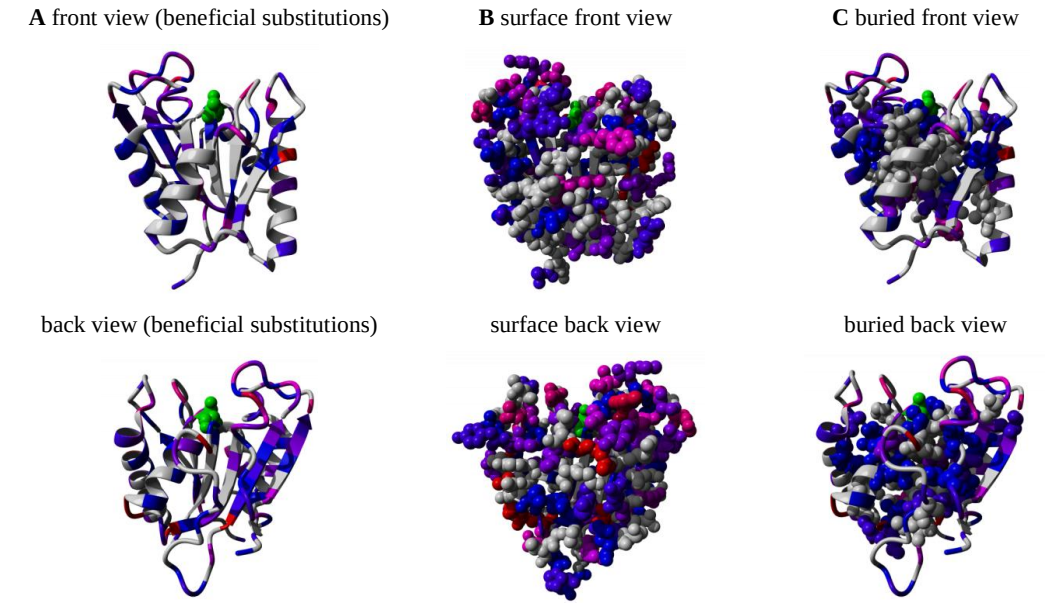


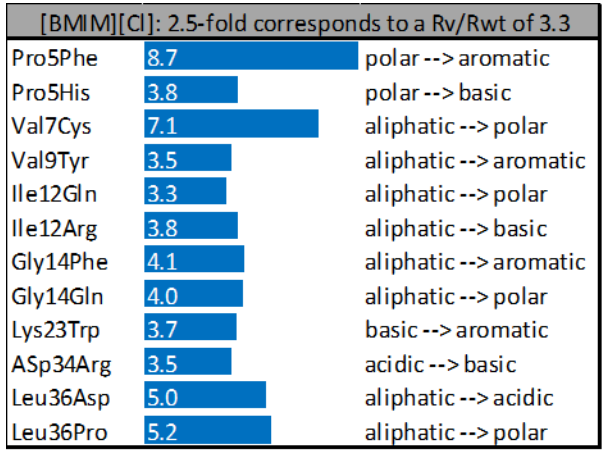
Figure 3-8: 3D model of BSLA (Protein Database code: 1I6W) displaying regions which favor BSLA resistance in presence of [BMIM][TfO] in a color scale. Positions at which no substitution led to improvements are colored grey. Positions with one substitution leading to improvements are colored blue. Positions with two to seven substitutions leading to improvements are colored purple (two positions) to magenta (seven positions). Red represents positions with eight or more substitutions leading to improvements. The active site residue Ser 77 is colored green. A) Cartoon representation of BSLA, B) Van der Waals representation of surface residues of BSLA, C) Van der Waals representation of buried residues of BSLA. The two N-terminal amino acids (Ala1 and Glu2) are also included. Yasara was used for visualization.

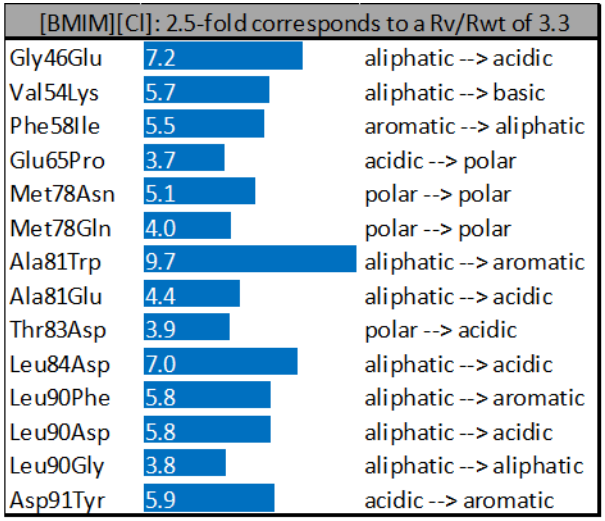
3.5.7 The SSM-BSLAVariants Showing Improved IL Resistance >2.5-fold

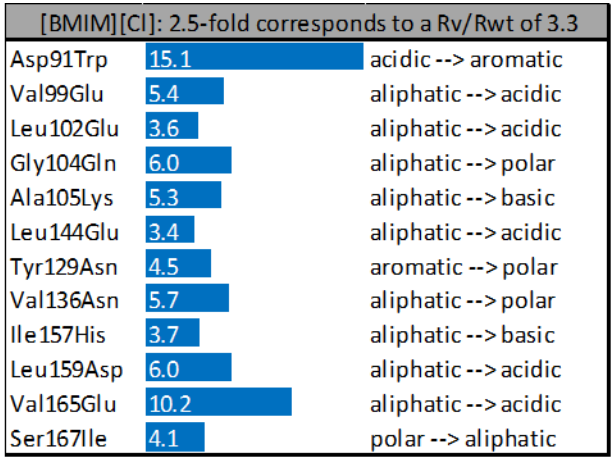
The results presented above (Figure 3-2 to Figure 3-8 and Table 3-2 to Table 3-4) are summarizing the overall general trends. At present section, the focus is on the best variants leading to an improvement of BSLA´s IL resistance >2.5-fold. As already mentioned, highest number of positions and substitutions for improved IL resistance were achieved for [BMIM][Cl]. Considering the single amino acid substitutions, 38 (1%) variants with resistance improvement >2.5-fold were found for [BMIM][Cl] which represented 28 (0.3%), 31 (0.2%) and 32 (0.2%) variants for [BMIM][TfO], [BMIM][I], and [BMIM][Br] (Table 3-5), respectively. Highest R_V/R_{WT} values (section 3.5) achieved for the ILs were 15.1, 12.5, 7.1, and 5.0 for [BMIM][Cl], [BMIM][Br], [BMIM][TfO], and [BMIM][I], respectively. R_V/R_{WT} was obtained dividing the residual activity of each BSLA variant (R_V) by the residual activity of BSLA WT (R_{WT}) at each position and in each IL. Positions Met78 (helix, exposed) and Asp91 (loop, exposed) are the only two positions at which highest improvements were obtained for all four ILs.

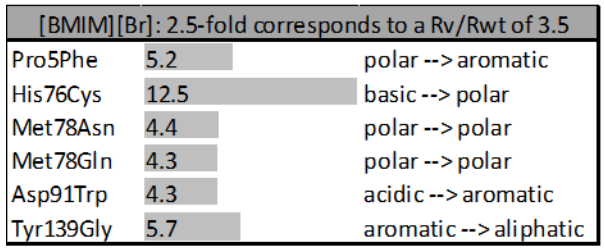
Comparing all types of amino acid exchanges (e.g aromatic exchanged by aromatic, aliphatic, polar, charged acidic, or charged basic), replacement of aliphatic residues in BSLA WT with charged acidic amino acids resulted in highest number of variants with improvement >2.5-fold for all four ILs followed by replacement of polar residues in BSLA WT (Figure 3-9). Interestingly, replacement of charged acidic residues with aromatic amino acids also resulted in many significantly more resistant variants. These results are in accordance with our previous findings on the general trend when considering all improved variants: substitutions of BSLA WT residues with polar and charged amino acids predominantly led to more improved variants. Furthermore, similar findings were obtained with variants displaying higher improvements (>2.5 fold) (Table 3-5). Met78 (helix, exposed) and Asp91 (loop, exposed) are both the only residues showing higher improvements for all four ILs. Chen *et al.* also found surface located residues contributing to increased specific activity for C2mim][OAc] pretreated substrate. They suggested that the presence of mutations on the surface of protein probably induced changes in the interaction mechanisms between protein and solvent system (Chen *et al.*, 2013).

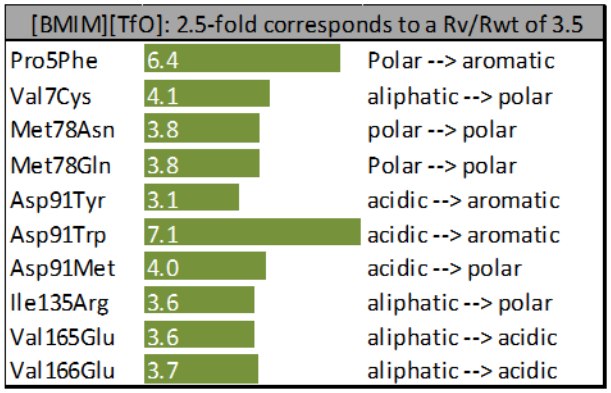
Table 3-5: Variants showing >2.5-fold improved IL resistance for all four ILs. The numbers represents improvement of each variant. All variants listed have a ratio >2.5-fold. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged acidic (D, E), charged basic (H, K, and R), and polar (C, M, P, S, T, N, and Q).

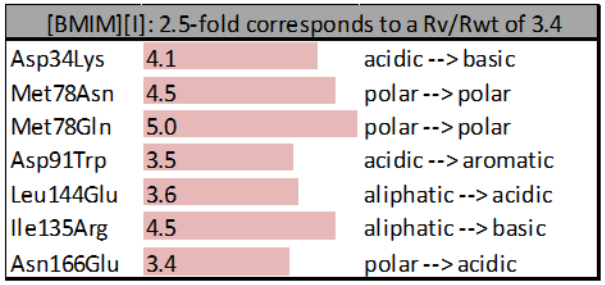












R_V/R_{WT} values are represented in blue bars for [BMIM][Cl], in grey bars for [BMIM][Br], in green bars for [BMIM][TfO], and in pink bars for [BMIM][I].

Although polar and charged substitutions led to higher number of improved variants for all four ILs when looking at all variants, highest improvements were however found by substitutions from charged acidic to aromatic amino acid (Asp91Trp) for [BMIM][Cl] (R_v/R_{WT} = 15.1) and [BMIM][Tfo] (R_v/R_{WT} = 7.1). This finding is supported by reports speculating that replacement of noncritical charged surface residues by uncharged amino acids can stabilize a protein for use in non-aqueous solvents (Arnold, 1988, 1990). This might be a result of the decreased hydrophobilicity of the protein surface, which is less dependent on solvation by water for proper folding (Arnold, 1988, 1990). In addition, Ala, Asn, and Leu were found to improve the stability of subtilisin E in presence of 80% v/v DMF (Martinez *et al.*, 1992).

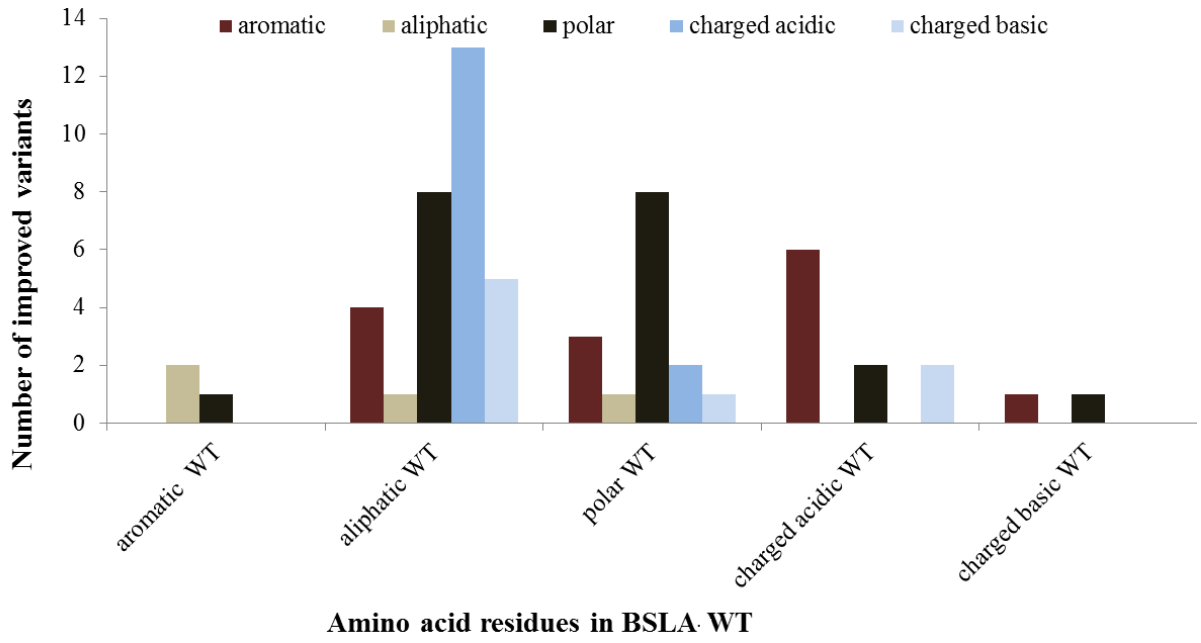


Figure 3-9: Number of improved variants with >2.5-fold improved IL resistance according to the amino acid substitution of BSLA WT for all four ILs. Variants with a value >2.5-fold are represented here. The nature of the substitution for each variant is also illustrated. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged acidic (D, E), charged basic (H, K, and R), and polar (C, M, P, S, T, N, and Q).

Substitutions from polar to polar led to the variants with highest improvements (3.6-fold) for [BMIM][I], and substitutions from charged basic to polar led to variants with highest improvements (8.7-fold) for [BMIM][Br]. Substitution of aliphatic residues in BSLA WT with charged acidic residues were found to lead to the highest number of improved variants (42%, 13 variants out of 31) with improvements >2.5-fold (figure 3-9). This correlates with a report speculating that addition of negative surface residues may repulse the binding of anions to the enzymes via electrostatic effects since the clustering of acidic amino acids on the surface of halophilic proteins were found to attract water molecules and therefore, increase

salt bridges, protein interaction and reduce aggregation. This might prevent salting out and stabilize the protein (Nordwald and Kaar, 2013).

3.6 Conclusions

This chapter attempted to elucidate the influence of each amino acid position, substitution, as well as the effect of their location on the resistance of *Bacillus subtilis* Lipase A (BSLA) in presence of four ILs. To achieve this goal, single site saturation mutagenesis at all the 181 amino acids of BSLA was performed and the obtained libraries were screened in the presence of four commonly used [BMIM]-based ILs ([BMIM][Cl], [BMIM][Br], [BMIM][I], and [BMIM][TfO]). Screening of the 181 generated BSLA libraries, revealed that >50% of amino acid positions contributed to improvements in four ILs resistance of BSLA. The latter demonstrates that directed evolution can very effectively improve IL resistance of BSLA and most likely other hydrolases. The high number of positions (50-69%) that improve IL resistance explains for the first time from an experimental point of view why often the screening of small sized libraries (1000-2000 variants) is sufficient to find improved variants. Additionally, screening efforts can be minimized by mutagenesis methods that introduce chemically different amino acids substitutions through transversions and consecutive mutations.

Chapter 4

Nature of Amino acid Residues Contributing to Organic Solvent Resistance of *Bacillus subtilis* Lipase A

4 Nature of Amino Acid Residues Contributing to Organic Solvent Resistance of *Bacillus subtilis* Lipase A

4.1 Abstract

Native enzymes have not been designed to work in non-aqueous medium such as organic solvents (OS) which usually cause destabilization. Therefore, numerous attempts have been invested to enhance OS resistance of enzymes by strategies as protein engineering. In this chapter, the nature and location of amino acids substitutions as well as the number of positions influencing enzyme resistance towards three polar OS was investigated. The complete *lipA* gene from *B. subtilis* was subjected to site saturation mutagenesis (SSM), followed by screening in presence of dimethyl sulfoxide (DMSO), 1,4-dioxane, and 2,2,2-trifluoroethanol (TFE). Increased OS resistance was obtained at 41-59% of BSLA positions but only with 4-10% of all possible BSLA single amino acid substitution variants. Additionally, improved OS resistance was mostly achieved with charged amino acids for 1,4-dioxane and TFE and rather with polar amino acids for DMSO. Furthermore, two patterns of amino acid substitutions were obtained: polar substitutions primarily led to improved BSLA resistance towards DMSO while charged amino acids resulted in highest number of improved BSLA variants for 1,4-dioxane and for TFE. Furthmore, on the surface of BSLA charged substitutions primarily led to improved resistance while in the buried region of BSLA polar ones were more preferred. This emphasizes the importance of engineering surface residues for improving OS resistance of enzymes. Among the beneficial substitutions, 58-93% of improved substitutions were achieved with chemically different amino acids (e.g. aromatic amino acids substituted by polar/aliphatic/charged amino acids). Therefore, mutagenesis methods introducing transversions which will encode chemically different amino acids will be beneficial for generating variants with improved OS resistance at least for lipases like BSLA.

Keyswords: BSLA, directed evolution, protein engineering, site saturation mutagenesis, Organic Solvents (OS).

4.2 Highlights

Project objective:

- A systematic study to understand the contribution of single amino acid substitution, position and location to BSLA resistance towards DMSO, 1,4-dioxane, and TFE.

Main Findings:

- 4-10% of all amino acid substitutions at 41-59% of all amino acid positions contributed to improved OS resistance of BSLA.
- Polar or charged amino acid substitutions primarily lead to improvement of BSLA´s OS resistance for DMSO and for 1,4-dioxane/TFE, respectively.
- At exposed positions, charged substitutions mainly improved BSLA´s OS resistance towards all three OS.
- At buried positions, polar substitutions were mainly favored improved BSLA´s OS resistance.
- Substitutions with chemically different amino acids predominantly improved OS resistance of BSLA indicating that mutagenesis methods introducing transversions will be favorable for improving organic solvent resistance of lipases at least for BSLA.

Novelty:

- The first systematic investigation of whole sequence SSM-BSLA library for improving the OS resistance.
- For the first time, from an experimental point of view explained why often the screening of small sized libraries (1000-2000 variants) is sufficient to find improved variants.

4.3 Introduction

Organic Solvent (OS) are commonly used in industrial enzymatic reactions because of the numerous advantages they offer: increased solubility of substrates and products, easy product recovery, suppression of unwanted side reactions (Zaks and Klibanov 1985), and suppression of microbial contamination (Arnold, 1990; Castro and Knubovets, 2003). However, enzymes usually display optimal function in aqueous solutions where they fold with a define pattern according to the properties of the amino acids. Therefore, most of the enzymes dramatically lose their activity in presence of OS only with a few exceptions, e.g. three lipases (porcine pancreatic, *Candida cylindracea*; and *Mucor sp.*) maintained a robust catalytic activity in 99.98% 2-pentanone by a lipase-catalyzed transesterification reaction between tributyrin and heptanol (Zaks and Klibanov, 1985). The low activity of most enzymes in the presence of OS considerably limits their industrial applicability.

Enzymes are usually designed by nature to mostly have ionizable amino acid residues forming a hydrophilic area on the surface which is in contact with the aqueous environment, while hydrophobic ones are sequestered inside the enzyme to form the hydrophobic core and neutral ones occupy intermediate positions (Oobatake and Ooi, 1993). The structural conformation of enzymes is maintained through intramolecular interactions among the amino acid residues (Ogino and Ishikawa, 2001). Besides, the aqueous phase surrounding enzymes also interacts with enzymes through electrostatic and hydrophobic interactions (Arnold, 1990; Doukyu and Ogino, 2010).

Effects of OS on enzymatic properties differ according to their polarity. Hydrophilic OS with similar polarity to water can form a homogeneous co-solvent system (Park *et al.*, 2012), in which organic molecules can strip or replace water molecules inside and around an enzyme causing deformation or denaturation (Ogino and Ishikawa, 2001; Yang *et al.*, 2004). Hydrophobic OS are in contrast unable to remove substantial amounts of water from enzyme (Gorman and Dordick, 1992) and are therefore more friendly to enzymes. Besides, it was found that OS molecules can compete with the substrate molecules to enter the substrate tunnel and coordinate the haem iron leading to inhibition of P450 BM3 (Kuper *et al.*, 2012; Seng Wong *et al.*, 2004). Almost total inactivation effect of organic cosolvent on enzyme can be expected at concentrations above at 60-70% (v/v) (Stepankova *et al.*, 2013).

In the last years, numerous studies have focused on investigating the effects of OS on enzymes properties such as stability (Kawata and Ogino, 2009, 2010; Koudelakova *et al.*,

2013; Liu *et al.*, 2009; Martinez and Arnold, 1991; Stepankova *et al.*, 2013; Timasheff, 1993), which plays a crucial role in the industrial applicability of enzymes. Isolation of novel enzymes, protein engineering, and medium engineering are strategies developed to achieve increased enzymes stability/resistance in presence of OS (Carrea and Riva, 2000; Illanes *et al.*, 2012; Stepankova *et al.*, 2013).

Protein engineering has been successfully used to improve catalytic properties of numerous enzymes under reaction conditions including also OS tolerance (Carrea and Riva, 2000; Klibanov, 2001; Stepankova *et al.*, 2013). Due to the lack of understanding of the complex interactions between enzyme and solvent molecules, improvement of OS stability of enzymes has been mostly obtained by means of directed evolution (Chen and Arnold, 1991, 1993; Dror *et al.*, 2014; Kawata and Ogino, 2009; Kumar *et al.*, 2006; Seng Wong *et al.*, 2004; Song and Rhee, 2001; Takahashi *et al.*, 2005; Zumárraga *et al.*, 2007a, 2007b). Only few cases for enhanced OS stability by rational design have been reported (Dror *et al.*, 2014; Kawata and Ogino, 2010; Martinez *et al.*, 1992; Park *et al.*, 2012, 2013; Takagi *et al.*, 2000) and semi-rational design yielded in few cases OS tolerant variants (Reetz *et al.*, 2006, 2010; Yedavalli and Rao, 2013).

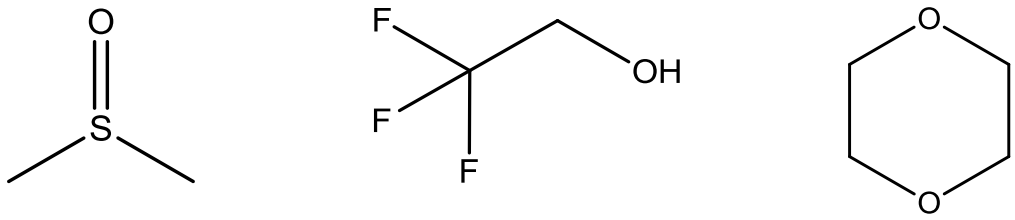
The goal of this study was to investigate the contribution of single amino acid substitutions at each amino acid position of BSLA to improve BSLA resistance towards the polar OS dimethyl sulfoxide (DMSO), 1,4-dioxane, and 2,2,2-trifluoroethanol (TFE). BSLA belongs to the lipase subfamily I-4, contains 181 amino acid residues in its mature form and shares 74-80% sequence identity with other members of this subfamily (Arpigny and Jaeger, 1999; Bustos-Jaimes *et al.*, 2010). Here for the first time, a comprehensive statistical data analysis was performed to determine the number of amino acid positions and substitutions contributing to BSLA´s OS resistance. Thus, all amino acid positions and substitutions displaying improved OS resistance of BSLA were identified. Further, trends in chemical composition of beneficial amino acid substitutions as well as the importance of residues location (surface exposed and buried amino acids) were elucidated.

4.4 Experimental Section

All chemicals, reagents and methods used in this part were described in chapter 2.

4.4.1 Optimization of Screening Conditions in OS

In this chapter, the generated 181 SSM-BSLA libraries were screened in the presence of DMSO, 1,4-dioxane and TFE (Figure 4-1). In order to determine the concentration of the three OS for SSM libraries screening, the solvents were supplemented with TEA buffer (pKa 7.76, pH 7.4, 50 mM) to obtain OS with different concentrations. All three OS were water-miscible as described in the safety sheets provided by the suppliers Sigma Aldrich (Steinheim, Germany) and Carl Roth (Karlsruhe, Germany). Besides, the shifts in pH of the buffer solutions were less than 0.22 units of pH in the presence of OS. Concentrations leading to a residual activity between 30% and 40% of the BSLA WT were chosen to screen all libraries. The screening assay with OS was performed in polypropylen MTP (flat-bottomed, polypropylen MTP plate, Greiner Bio-One GmbH, Frickenhausen, Germany), which are resistant to OS as 1,4-dioxane. In each well of a 96-well MTP, 10 µL supernatant were incubated with 90 µL of the OS solution (2 h, 1000 rpm, RT) on a shaker (Edmund Bühler Microtiter shaker, Hechingen, Germany) at the adjusted OS concentration (Table 4-1). An additional plate only with buffer as reference was measured for each position to enable the calculation of the residual activity given as percentage (2.2.4.5). The freshly prepared substrate solution (100 µL/well) was added to the incubation mixture and the change in absorbance was monitored (410 nm, 8 min, RT). For each well, the same amount of supernatant was incubated with or without OS and the ratio of 'activity in presence of OS divided by the activity in absence of OS' was used to evaluate the organic solvent resistance of BSLA variants. This ratio was not influenced even when using 2-3 times more BSLA supernatant.



DMSO TFE 1,4-Dioxane

Figure 4-1: Chemical structure of the three selected OS: DMSO = dimethyl sulfoxide, **TFE** = 2,2,2-trifluoroethanol.

4.5 Results and Discussion

Many different enzyme classes (e.g. hydrolases, oxidoreductases, transferases, etc.) have been successfully tailored for improved OS stability by using protein engineering (Carrea and Riva, 2000; Stepankova *et al.*, 2013). These studies also provided initial clues to

reengineer focused regions of enzymes (e.g. substrate access tunnel and substrate binding pocket) and to strengthen the interactions between amino acids (e.g. introducing disulfide bridges) for improved OS resistance stability or tolerance. However, in most cases, protein engineering strategies for stabilization in OS are usually based on empirical observations rather than guided by detailed molecular understanding. In a systematic study through screening of a complete saturation mutagenesis library of BSLA (181 SSM-BSLA libraries) in the presence of three OS, the following questions were addressed: 1) how many single positions can contribute to improved OS resistance of BSLA, 2) what types of amino acid substitutions influence the OS resistance of BSLA, and 3) how does the location of amino acids (surface exposed or buried) affect the OS resistance of BSLA.

The SSM-BSLA libraries covered all 181 amino acid positions of BSLA and each of the 3620 naturally possible variants were generated and confirmed by extensive sequencing. The results in this chapter were analyzed in eight sections as described in chapter 3. The first section describes the generation of the SSM libraries for the complete gene encoding the BSLA amino sequence. The second section focuses on the conditions used for the screening of SSM-BSLA libraries in the presence of the three used OS. The third section deals with the analysis of the number of amino acids positions which influence OS resistance of BSLA (improved/decreased/unchanged OS resistance and inactivation). In the fourth section, all substitutions were analyzed to determine the number of single amino acid substitutions on BSLA resistance towards the three OS. The amino acid substitutions were categorized into four types (aromatic, aliphatic, polar, and charged) and the influence of each type of amino acid on BSLA resistance was elucidated in the fifth section. The sixth section mainly focuses on the effect of substituting each type of amino acid with all four types. The seventh section discusses the effect of amino acid locations on OS resistance of BSLA. In the last section, best variants contributing to highest BSLA resistance in each OS were investigated. The results for all variants and positions were categorized as described in chapter 3 to allow efficient comparison.

4.5.1 Screening Conditions for BSLA Libraries in the Presence of Three OS

All 3620 BSLA variants were screened in the presence of three OS (DMSO, 1,4 dioxane and TFE). Organic solvent concentrations were selected to obtain a residual BSLA WT activity of 30% to 40% (Table 4-1). True standard deviations of 9.6% to 12.0% were obtained for the screening system with the three OS as co-solvents. Screening systems with

true standard deviations around 12% were commonly used in directed evolution experiments (Wong *et al.*, 2007c).

Table 4-1: OS used for the screening of BSLA libraries. Calculated standard deviations of the screening system are also showed. C: concentration, RA: residual activity, stdev: standard deviation, App: apparent. DMSO = Dimethyl sulfoxide, TFE = 2,2,2-trifluoroethanol.

Selected OS	C [M](%v/v)	R_{WT} [%]	App stdev [%]	True stdev [%]
DMSO	8.4 (□□60.0)	29.0	8.0	10.5
1,4-dioxane	2.6 (□ 22.0)	31.0	6.5	9.6
TFE	2.9 (□ 12.0)	30.0	7.6	12.0

* Concentration used in the incubation steps.

Table 4-1 shows that BSLA is more resistant to DMSO than to TFE and 1,4-dioxane. Indeed, maybe because of its strong acceptor capacity, DMSO (8.4 M) resulted in around 70% deactivation effect as well as the weak acceptor 1,4-dioxane (2.6 M) and the weak donor/strong acceptor TFE (2.9 M). The dependence of enzyme stability/resistance to the hydrophobicity/hydrophilicity of OS has already been established (Carrea and Riva, 2000; Klibanov, 1997; Serdakowski and Dordick, 2008). It is well known that the hydrophobic OS are less harmful than hydrophilic ones (Zaks and Klibanov, 1988) because of their weak ability to remove water from the enzymes. LogP value (defined as the octanol water partition of OS and also used as a measure of hydrophobicity) is commonly used to describe the hydrophobicity/hydrophilicity of OS (Laane *et al.*, 1987). It is worth mentioning that hydrophobicity of OS increases with increased logP and hydrophilicity increased with decreased logP. However, the obtained results (Table 4-1) did not correlate with hydrophobicity/hydrophilicity of OS (as quantified by logP) (Degn and Zimmermann, 2001; Lee *et al.*, 2004) and showed that more improved BSLA variants were obtained for DMSO, the most hydrophilic of the three OS. The hydrophilicity order of the selected OS is as following: DMSO (-1.12) > 1,4-dioxane (0.17) > TFE (0.31) (Chakravorty *et al.*, 2012; Wu *et al.*, 2004). Molecular docking studies predicted that hydrophilic OS (logP < 2, e.g ethanol, DMSO, 1,4-dioxane) inactivated the lipase BSLP1 by disrupting the interactions between the active sites with water molecules (Chakravorty *et al.*, 2012). Therefore, the inactivation of enzymes by OS could be due to multiple reasons. Improved DMSO resistance has been also successfully described in other studies: directed evolution of subtilisin E (Chen and Arnold, 1993), P450 monooxygenase (Wong *et al.*, 2004b), a lipase from *Pseudomonas aeruginosa* (Kawata and Ogino, 2009), and BSLA (Reetz *et al.*, 2010; Yedavalli and Rao, 2013).

After screening of all 181 SSM-BSLA libraries in buffer and in the presence of each of the three OS, residual activity of variant (R_V) and the residual activity of WT (R_{WT}) were

calculated. Based on the Gaussian distribution curve (σ is the true coefficient of variation of the screening system see Table 4-1), all the variants were grouped into four categories as described previously in chapter 3.

4.5.2 Influence of the Number of Amino Acid Positions on BSLA's OS Resistance

Figure 3-2 depicts the number of amino acid positions of BSLA that led to improved/unchanged/decreased OS resistance as well as inactivation of BSLA. For the OS resistance type "+", it was found that DMSO (107; 59% of all positions) yields 32 to 33 more positions than 1,4-dioxane (75; 41%) and TFE (74; 41%) , respectively. For the OS resistance type "=", at each of the 181 amino acid positions at least one amino acid substitution was found that did not influence OS resistance of BSLA. For the OS resistance type "-", it was found that 1,4-dioxane (136, 75% of all positions) yields more positions than TFE (120; 66%) ~ DMSO (118; 65%). For the resistance type "x", at least one amino acid substitution was found at 99 (55%) amino acid positions which inactivated BSLA already in the buffer solution. Detailed analysis of the number of substitutions at each amino acid position of BSLA leading to improved/decreased/unchanged OS resistance and inactivation are given in Table 7-11 to Table 7-13.

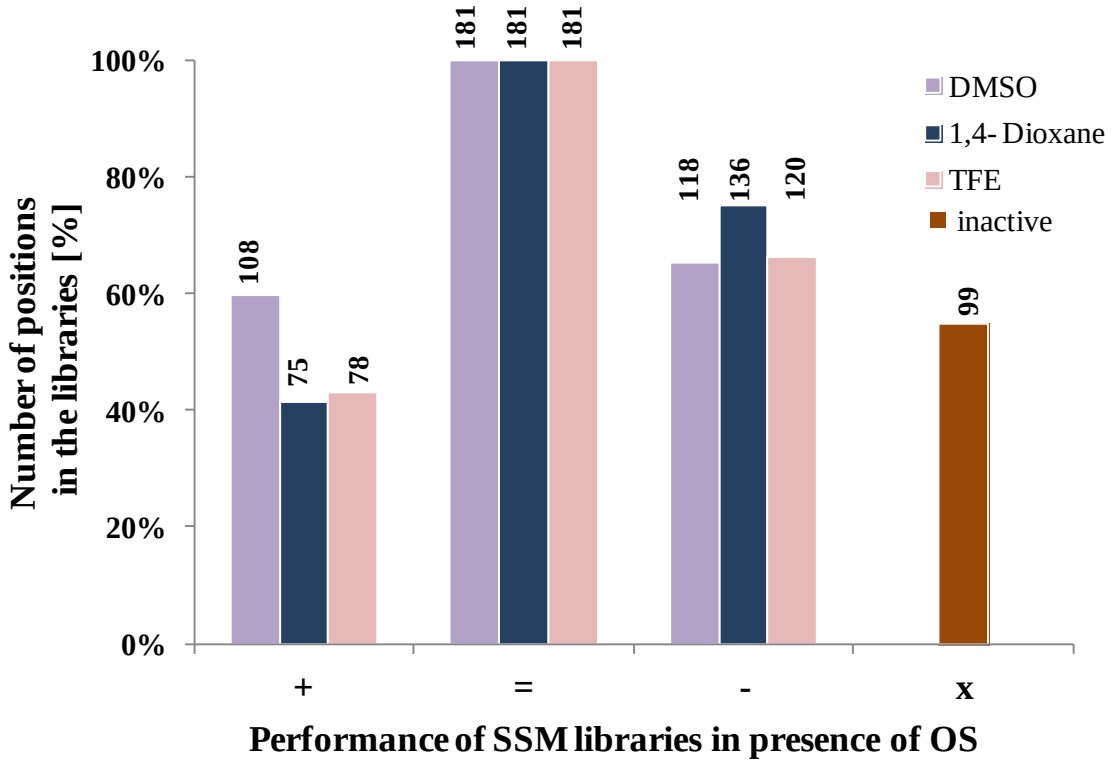


Figure 4-2: Number of amino acid positions which lead to improved/unchanged/decreased OS and/or inactivation of BSLA in buffer. All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

4.5.3 Influence of the Number of Single Amino Acid Substitutions on BSLA´s OS Resistance

The number of variants with improved "+", unchanged "=", decreased "-" resistance in presence of DMSO, 1,4-dioxane, and TFE as well as ''inactive'' variants "x" is depicted in Figure 4-3. DMSO (371 out of 3620, 10% of all substitutions) yields 190 substitutions more than TFE (181, 5%) and 212 more than 1,4-dioxane (159, 4%) for the OS resistance type "+". Among the variants with increased resistance towards TFE, 0.17% showed a resistance improvement higher than 2.5-fold, which is 6-fold higher than the values obtained for DMSO and 1,4-dioxane (0.03%). Overall, a large majority 61-63% (2212-2288 out of 3620) of all substitutions did not influence BSLA resistance type "=" towards the three selected OS. Similar results were achieved for the OS resistance type "-" in 1,4-dioxane (675 out of 3620, 19% of all substitutions) and TFE (664, 18%) in which 136 and 125 more substitutions than DMSO (539, 15%) were identified, respectively. BSLA inactivation type "x", was obtained for 498 (14%) substitutions.

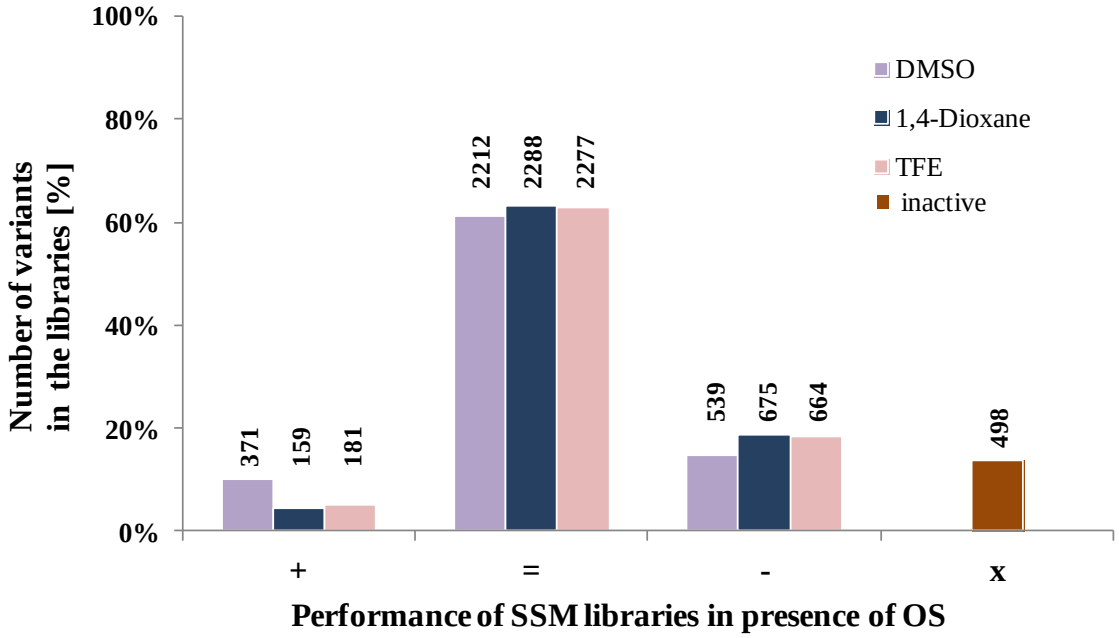


Figure 4-3: Number of SSM-BSLA variants leading to improved/unchanged/decreased OS resistance and inactivation of BSLA. All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Interestingly, a similar number of positions and substitutions/variants contributing to improved BSLA resistance towards 1,4-dioxane and TFE were observed. As strong acceptor, DMSO however, displayed around 1.4-fold more positions (59%) for improved resistance than 1,4-dioxane (41%) and TFE (41%) (Figure 4-2). Around 2-fold more variants with improved OS resistance were obtained for DMSO (10%) than for 1,4-dioxane (4%) and TFE (5%) (Figure 4-3). Therefore, there is a higher chance to identify improved variants for DMSO resistance than for 1,4-dioxane and TFE from a random mutagenesis library with single substitutions. Furthermore, 6-fold more variants with >2.5-fold improved resistance were obtained for TFE (0.17%) than for DMSO (0.03%) and for 1,4-dioxane (0.03%), which are very difficult to be identified from a random mutagenesis library. This also explains why it is feasible to use strategy of accumulating single mutations with modest improvements to obtain high improvements by screening iterative rounds of random mutagenesis library.

4.5.4 Effect of Amino Acid Substitution "Types" (Aromatic, Aliphatic, Charged, Polar) on BSLA´s OS Resistance

The 20 amino acid substitutions were grouped into four "types" in order to be consistent with previous reports (Verma *et al.*, 2014; Wong *et al.*, 2006b, 2007a) as mentioned before: 15% aromatic (F, Y, and W), 25% aliphatic (A, V, L, I, and G), 25%

charged (D, E, H, K, and R) and 35% polar (termed neutral in the cited paper; C, M, P, S, T, N, and Q) amino acids. Proline (P), cysteine (C), and glycine (G) are classified elsewhere as special amino acids. Figure 4-4 provides information about the effect of each type of amino acid substitution on BSLA resistance towards the three OS. Inactive variants were defined as the variants which showed no activity in buffer. Supplementing of OS did not in single case lead to regain of BSLA activity. Among the 498 inactive BSLA variants ("x" type in buffer), charged substitutions harbored 183 (37%), polar 152 (31%), aromatic 87 (17%), and aliphatic 76 (15%).

Figure 4-4 A to C provides an overview of improvements for the OS resistance type "+" towards DMSO resistance in case of 126 out of 371 to polar amino acids (34%), 117 to charged (33%), 88 to aliphatic (24%), and 40 to aromatic (11%). For 1,4-dioxane resistance, 62 out of 159 to charged (39%), 47 to polar (30%), 26 to aliphatic (16%), and 24 to aromatic (15%) were obtained. TFE resulted in 62 out of 181 variants to charged (34%), 57 to polar (31%), 34 to aromatic (18%), and 28 to aliphatic (15%). The highest number of improved BSLA variants were obtained for DMSO (126) followed by 1,4-dioxane and TFE with each 62 variants (Figure 4-4 A-C). In summary, BSLA variants with predominantly charged amino acid substitutions represented the majority of substitutions that improved resistance in TFE and 1,4-dioxane. However, polar amino acids were preferential for improved DMSO resistance of BSLA.

A similar amino acid substitution pattern was observed for all three OS for the organic solvent resistance type "=": 36% polar, 28-29% aliphatic, 20-22% charged, and 14-15% aromatic (Table 4-2).

Table 4-2: Number of each type of amino acid substitution for each resistance type for all three OS. All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer**.**

Resistance type	Aromatic [%]	Aliphatic [%]	Polar [%]	Charged [%]
+	11-19	15-28	30-34	32-39
=	14	28-29	36	20-22
-	15-19	21-23	35-37	24-28
x	17	15	31	37

The organic solvent resistance type "-" showed 197 polar amino acids (37%), 127 charged (24%), 111 aliphatic (21%), and 104 aromatic (19%) out of 539 substitutions for DMSO. For 1,4-dioxane, 242 polar (36%), 191 charged (28%), 132 aliphatic (20%), and 110

(16%) aromatic out of 675 substitutions were obtained. In case of TFE, 230 out of 664 to polar (41%), 181 to charged (27%), 153 to aliphatic (23%), and 100 to aromatic (15%) were achieved. BSLA variants with polar amino acid substitutions largely led to decreased resistance for the three OS and significant difference in the total number was observed (539 substitutions for DMSO vs 675 in 1,4-dioxane). A similar trend was also observed for the four ILs as described in chapter 3. The amino acid composition of BSLA (8.3% aromatic, 43.6% aliphatic, 29.8% polar, and 18.2% charged amino acids) was optimized by nature for its performance in aqueous solution with a preference for aliphatic amino acids. The investigation of individual effect of each type of substitution when replacing each type of amino acid in BSLA WT showed that improved variants can be obtained by substitutions with aromatic (11-19%), aliphatic (15-28%), polar (30-34%), and charged (32-39%) amino acids.

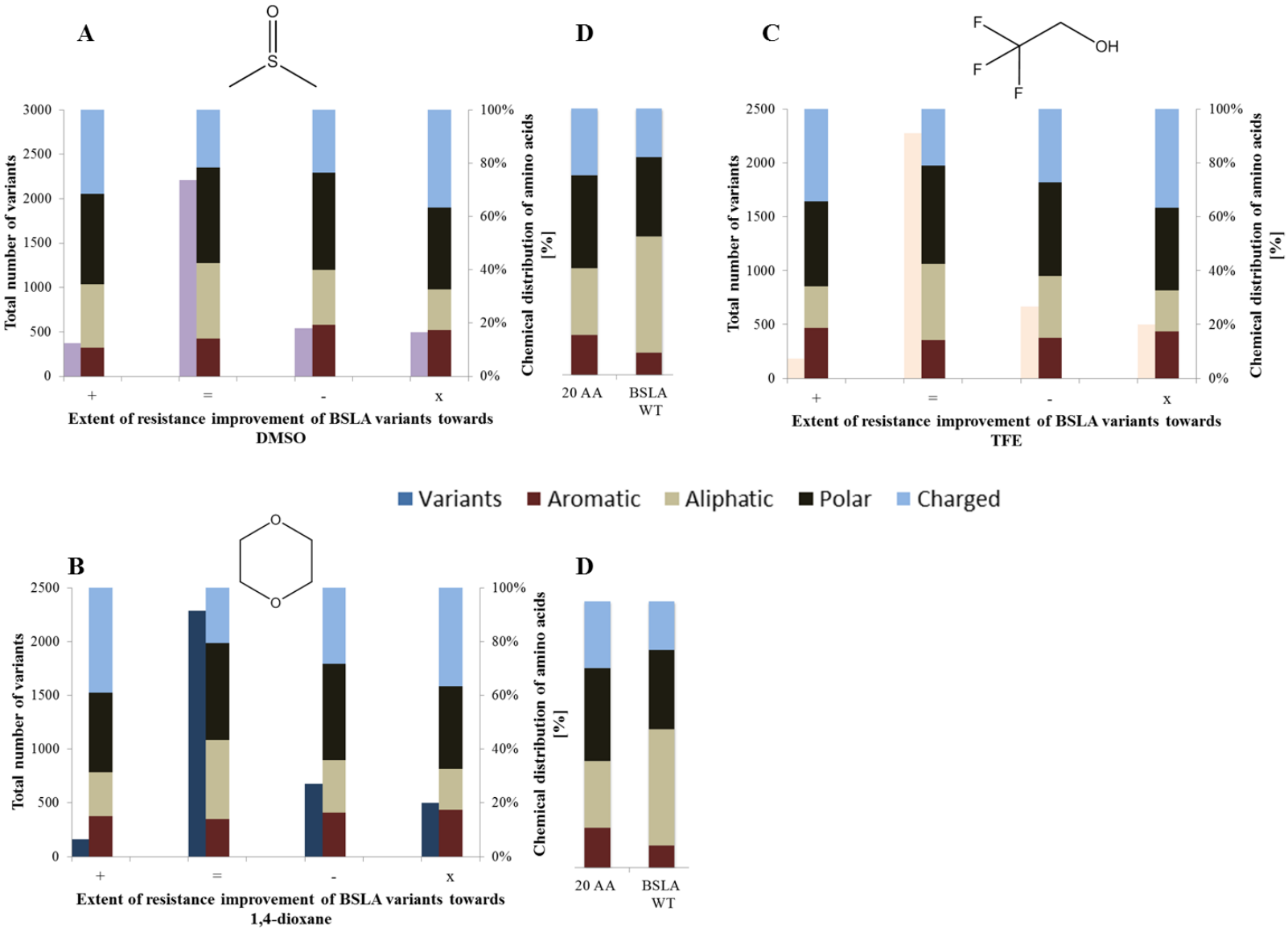


Figure 4-4: Distribution of amino acid substitutions of SSM-BSLA libraries with improved/unchanged /decreased OS resistance.
A: Resistance towards DMSO,
B: Resistance towards 1,4-dioxane,
C: Resistance towards TFE,
D: Distribution of the 20 amino acids, and amino acid composition of BSLA WT. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). The X-axis represents the extent of resistance improvement of BSLA variants toward different OS in four categories ("+"; "="; "-", and "x"). The primary Y-axis (on the left) displays the total number of variants and positions, at which an effect on BSLA resistance is observed. The secondary Y-axis (on the right) represents the chemical distribution of the amino acids in categories. All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer. AA: amino acid.

The number of beneficial substitutions for DMSO (Figure 4-4A) was obtained in the order: polar > charged > aliphatic > aromatic. The order for 1,4-dioxane (Figure 4-4B) was rather: charged > polar > aliphatic ≥ aromatic and for TFE (Figure 4-4C): charged > polar > aromatic > aliphatic. The following order was observed for the variants with OS resistance type "=": polar > aliphatic > charged > aromatic for all three OS. For organic solvent resistance type "-", the number of variants followed this order: polar > charged > aliphatic > aromatic in presence of all three OS. Overall, substitutions to charged amino acids mainly led to improvements in 1,4-dioxane and TFE. In contrast, substitutions to polar residues mostly improved BSLA for DMSO resistance (Table 4-2). Both charged and polar residues have been reported to contribute to improved enzymes properties in OS (Chen *et al.*, 1991; Park *et al.*, 2012, 2013; Song and Rhee, 2001; Takahashi *et al.*, 2005; Zumárraga *et al.*, 2007a). It is likely that charged and polar residues could enable the formation of hydrogen bonds between amino acid residues or between surface residues and water molecules which help maintaining the enzyme structure intact (Park *et al.*, 2012).

4.5.5 Effect of Amino Acid Substitution Types on OS Resistance of BSLA: Comparison Between WT and SSM-BSLA Variants

The effect of each type of amino acid substitution (polar, aliphatic, charged and aromatic) on organic solvent resistance of BSLA is presented in Table 4-3 in a matrix of 16 substitution types. In all cases, substitutions to chemically different amino acids led to improved OS resistance with significant differences in orders and trends which are reported in detail in the following four paragraphs. Table 4-2 provides general trends on amino acid substitution patterns and answers in contrast to Figure 4-4 the question what kind of preferred types (aromatic, aliphatic, polar and charged) of amino acid exchanges exist. Table 7-14 shows the effect of charged acidic and charged basic amino acids.

Table 4-3: Effect of each amino acid type substitution (polar, aliphatic, charged and aromatic) on OS resistance of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per OS are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Amino acid types in BSLA WT

		BSLA: Aromatic (15; 8.3%)				BSLA: Aliphatic (79; 43.6%)				BSLA: Polar (54; 29.8%)				BSLA: Charged (33; 18.2%)			
		+ (44)	= (193)	- (36)	x (27)	+ (155)	= (843)	- (269)	x (313)	+ (108)	= 759)	- (136)	x 77)	+ (64)	= (417)	- 98)	x 81)
DMSO	Aromatic	7%	20%	8%	0%	9%	14%	17%	19%	8%	14%	24%	19%	**22%**	12%	21%	15%
	Aliphatic	32%	26%	17%	19%	21%	32%	20%	12%	23%	28%	16%	16%	**25%**	24%	30%	25%
	Polar	27%	36%	33%	44%	**41%**	36%	39%	27%	32%	36%	34%	29%	**25%**	35%	36%	43%
	Charged	**34%**	18%	42%	37%	29%	18%	24%	42%	**36%**	22%	26%	36%	**28%**	29%	13%	17%
		+ (19)	= 195)	- (59)	x (27)	+ (60)	= (827)	- (380)	x (313)	+ (56)	= (780)	- 167)	x (77)	+ (24)	= (486)	- (69)	x (81)
1,4-dioxane	Aromatic	5%	22%	3%	0%	17%	13%	15%	19%	16%	13%	22%	19%	17%	14%	19%	15%
	Aliphatic	26%	26%	25%	19%	13%	34%	18%	12%	16%	28%	19%	16%	17%	26%	22%	25%
	Polar	21%	35%	34%	44%	32%	37%	39%	27%	32%	37%	32%	29%	25%	35%	32%	43%
	Charged	**47%**	17%	37%	37%	**38%**	16%	28%	42%	**36%**	23%	27%	36%	**42%**	25%	28%	17%
		+ (12)	= (211)	- (50)	x (27)	+ (69)	= 836)	- 362)	x 313)	+ (62)	= (751)	- (190)	x 77)	+ (38)	= (479)	- (62)	x (81)
TFE	Aromatic	8%	19%	6%	0%	23%	12%	17%	19%	18%	15%	14%	19%	16%	15%	16%	15%
	Aliphatic	0%	27%	28%	19%	14%	33%	20%	12%	19%	26%	28%	16%	16%	26%	23%	25%
	Polar	33%	34%	34%	44%	29%	38%	37%	27%	26%	38%	31%	29%	**45%**	33%	32%	43%
	Charged	**58%**	20%	32%	37%	**33%**	18%	26%	42%	**37%**	22%	28%	36%	24%	26%	29%	17%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6% aliphatic, 29.8% polar amino acids, 18.2% charged, and, 8.3% aromatic). Highest values among the resistance type "+" are highlighted in blue and dark red represents the values of substitutions preferring exchange with similar residues.

4.5.5.1 Substitution of Aromatic Amino Acids

The substitution of aromatic amino acids with chemically different amino acids (aliphatic, charged, and polar) led to significantly improved resistance "+" towards all three OS. The number of improved variants for DMSO and 1,4-dioxane were obtained in the following order of substitution types: charged > aliphatic > polar > aromatic. The order obtained in TFE was: charged > polar > aromatic > aliphatic.

4.5.5.2 Substitution of Aliphatic Amino Acids

The substitution of aliphatic amino acids with chemically different amino acids (polar and charged) led to more variants with improved resistance type "+" towards all three OS. The number of improved variants achieved in DMSO was with the substitution types: polar > charged > aliphatic > aromatic. The order obtained in 1,4-dioxane and TFE was: charged > polar > aromatic > aliphatic.

4.5.5.3 Substitution of Polar Amino Acids

The substitution of polar amino acids with chemically different amino acids (charged) led to more variants with the resistance type "+" than its own type towards all three OS. The order obtained in DMSO was: charged > polar > aliphatic > aromatic. However, in 1,4-dioxane and TFE, the order was rather: charged > polar > aliphatic = aromatic.

4.5.5.4 Substitution of Charged Amino Acids

The substitution of charged amino acids with chemically different amino acids (aromatic, aliphatic, and polar) did not yield more variants with resistance type "+" towards any of the three OS. For DMSO and 1,4-dioxane, more resitant variants were in many cases the results of an exchange from a charged amino acid to a chemically similar amino acid (charged amino acid). Accordingly, the obtained order was: charged > polar = aliphatic > aromatic for DMSO and charged > polar > aliphatic = aromatic for 1,4-dioxane. TFE was the only OS with a preference for substitution with chemically different amino acids: polar > charged > aliphatic = aromatic.

Overall, substitution of aromatic, polar, charged and aliphatic amino acid residues with chemically different ones yielded more improved variants (68-95%) than with its own type for all three OS (Table 7-15). Similar trends were also reported for BSLA resistance towards ionic liquids (Frauenkron-Machedjou *et al.*, 2015). Chemically different amino acids

substitutions were preferred to achieve improved BSLA resistance for four ionic liquids by replacement of aromatic, polar and aliphatic amino acids. As described here, charged residues were also found to mostly favor replacements with chemically similar residues (Frauenkron-Machedjou *et al.*, 2015).

4.5.6 Effect of the Location of Amino Acid Substitution on BSLA´s OS Resistance

About 71% amino acid residues of BSLA are surface exposed and 29% are buried, solvent accessible surface for each residue was calculated as described in chapter 3 based on the chain A of the BSLA X-ray crystal structure (PDB ID: 1I6W)(van Pouderoyen *et al.*, 2001). Table 4-4 and Table 7-16 illustrate the effect of amino acid location on OS resistance of BSLA.

Table 4-4 Effect of amino acid location on organic OS of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per OS are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

A

Location of amino acid positions in BSLA

		Exposed positions (128; 71%)				Buried positions (53; 29%)			
		+ (275)	= (1777)	- (319)	x (189)	+ (96)	= (435)	- (220)	x (309)
DMSO	Aromatic	10%	15%	22%	15%	14%	12%	16%	19%
	Aliphatic	25%	27%	21%	17%	21%	36%	20%	14%
	Polar	31%	36%	33%	38%	**44%**	36%	41%	26%
	Charged	**35%**	23%	24%	30%	22%	15%	23%	41%
		+ (138)	= (1903)	- (330)	x (189)	+ (21)	= (385)	- (345)	x (309)
1,4-dioxane	Aromatic	16%	15%	17%	15%	10%	12%	15%	19%
	Aliphatic	17%	27%	19%	17%	14%	39%	20%	14%
	Polar	26%	35%	35%	38%	**52%**	40%	37%	26%
	Charged	**41%**	23%	28%	30%	24%	9%	28%	41%
		+ (152)	= (1883)	- (336)	x (189)	+ (29)	= (394)	- (328)	x (309)
TFE	Aromatic	19%	15%	15%	15%	17%	12%	15%	19%
	Aliphatic	16%	27%	24%	17%	10%	37%	22%	14%
	Polar	30%	36%	32%	38%	**38%**	40%	38%	26%
	Charged	**34%**	23%	29%	30%	**34%**	11%	26%	41%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6% aliphatic, 29.8% polar amino acids, 18.2% charged, and 8.3% aromatic). Highest values among the resistance type "+" are highlighted in blue. Column "x" shows that the number of inactive variants resulted from amino acid substitutions. The activity was measured in a buffer plate which was used as reference (absence of OS) for comparison. Therefore, the number of inactive variants was the same for three OS.

B

Number of each type of amino acids exposed and buried in BSLA

	Aliphatic	Aromatic	Polar	Charged	Total
Exposed AA residues					
Number of AA	44 (34%)	12 (9%)	43 (34%)	29 (23%)	128 (100%)
Buried AA residues					
Number of AA	35 (66%)	3 (6%)	11 (21%)	4 (8%)	53 (100%)

AA: amino acid

The substitutions with charged amino acids on the surface of BSLA yielded to more improved variants than the other three type of substitutions for all three OS: DMSO (35%), 1,4-dioxane (41%), and TFE (34%). In contrast, substitutions with polar amino acids in the buried part of BSLA yielded more improved variants than the other kinds of substitutions for all three OS: DMSO (44%), 1,4-dioxane (52%), and TFE (38%) (Table 4-4). Substitutions of surface located residues were found in numerous cases to contribute to the O resistance of enzymes (Kawata and Ogino, 2009; Martinez *et al.*, 1992; Park *et al.*, 2013, 2012). For instance, amino acid substitutions located on the surface of a *Pseudonomas aeruginosa*

LST03 lipase were identified by directed evolution to play an important role in improving its organic solvent-stability. Ogino *et al.* speculated that these substitutions on the surface increased enzyme stability in OS because they maybe efficient to prevent the penetration of OS inside of the enzyme (Kawata and Ogino, 2009; Ogino *et al.*, 2007). As previously reported (Frauenkron-Machedjou *et al.*, 2015), the fraction of polar substitutions resulting in inactive BSLA variants (type "x") was significantly enhanced from 26% (buried positions) to 38% (exposed positions). Remarkably, charged substitutions at the buried positions rose the fraction of inactive variants (type "x"; 41%) and substitutions to polar amino acids predominantly lowered activity (type "-"; 32-41% for all three OS). Moreover, substitutions that improved OS resistance in this study were mostly located on loops (44% for DMSO, 47% for 1,4-dioxane, and 43% for TFE and on helices (34 % for DMSO, 36% for 1,4-dioxane, and 43% for TFE) as described in Table 7-17. Yedavalli and Rao performed site saturation mutagenesis (SSM) of the loop regions of *Bacillus subtilis* lipase A (BSLA) to find DMSO resistant BSLA variants. They identified six mutations that led to 8-fold in higher catalytic turnover in 60% DMSO after one round of selection. Additionally, three of the six mutations increased the surface polarity suggesting that substitution of more polar amino acids may be helpful to improve the stability of a protein in a water-miscible OS (Yedavalli and Rao, 2013). Since BSLA shows high sequence identity with the other members of the lipase subfamily I-4, the trend observed here can likely be applicable for other members and possibly in all other enzymes with an α/β hydrolase fold.

Figure 4-5 to Figure 4-7 illustrates 3D models of BSLA for visualization of the beneficial positions (front and back view of surface/buried positions) found during screening with the three OS. Most positions harboring more than eight substitutions (colored in red) with improved OS resistance were observed for DMSO. Those beneficial positions were gathered located on the back site of the protein surface (Figure 4-5). Interestingly, positions harboring more than eight beneficial substitutions in the core were observed only for DMSO (Figure 4-5 to Figure 4-7). Positions in the core with only one improved variant (colored blue) or no improved variants (colored grey) can be observed for 1,4-dioxane and TFE in Figure 4-6 and Figure 4-7, respectively implying that the wild type amino acids located in BSLA´s core could be weak residues for BSLA resistance. Besides, considering BSLA secondary structure elements, 43 to 47% of the beneficial amino acid positions for OS resistance of BSLA were localized on the loops of BSLA (Table 7-17).

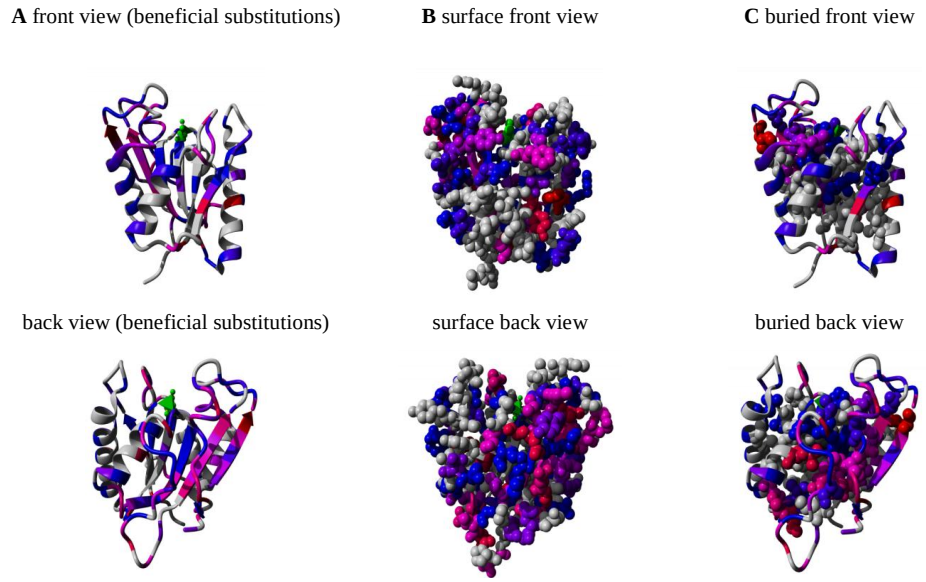


Figure 4-5: 3D model of BSLA (Protein Database code: 1I6W) displaying regions which favor BSLA resistance in presence of DMSO in a color scale. Positions at which no substitution led to improvements are colored grey. Positions with one substitution leading to improvements are colored blue. Positions with two to seven substitutions leading to improvements are colored purple (two positions) to magenta (seven positions). Red represents positions with eight or more substitutions leading to improvements. The active site residue Ser 77 is colored green. A) Cartoon representation of BSLA, B) Van der Waals representation of surface residues of BSLA, C) Van der Waals representation of buried residues of BSLA. The two N-terminal amino acids (Ala1 and Glu2) are also included. Yasara was used for visualization.

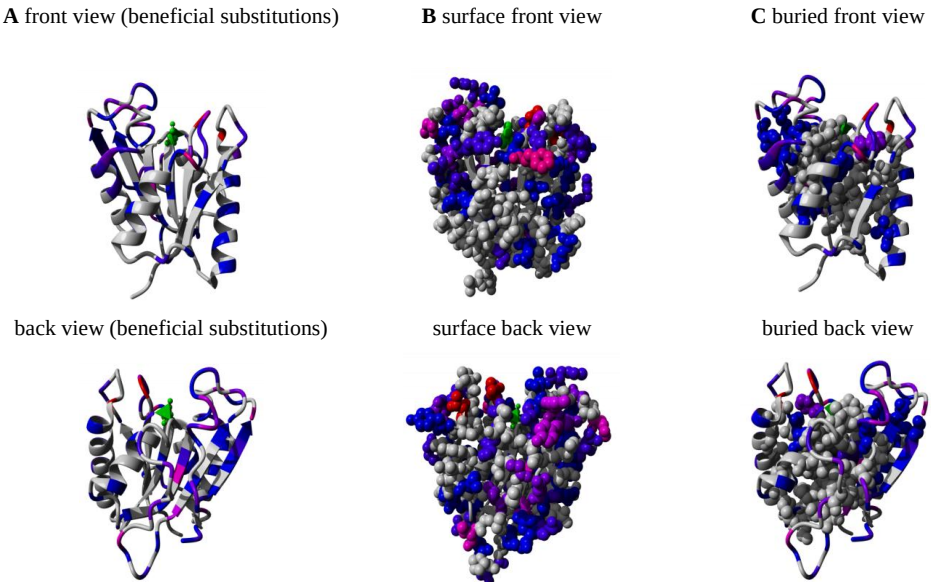


Figure 4-6: 3D model auf BSLA (Protein Database code: 1I6W) displaying regions which favor BSLA resistance in presence of 1,4-dioxane in a color scale. Positions at which no substitution led to improvements are colored grey. Positions with one substitution leading to improvements are colored blue. Positions with two to seven substitutions leading to improvements are colored purple (two positions) to magenta (seven positions). Red represents positions with eight or more substitutions leading to improvements. The active site residue Ser 77 is colored green. A) Cartoon representation of BSLA, B) Van der Waals representation of surface residues of BSLA, C) Van der Waals representation of buried residues of BSLA. The two N-terminal amino acids (Ala1 and Glu2) are also included. Yasara was used for visualization.

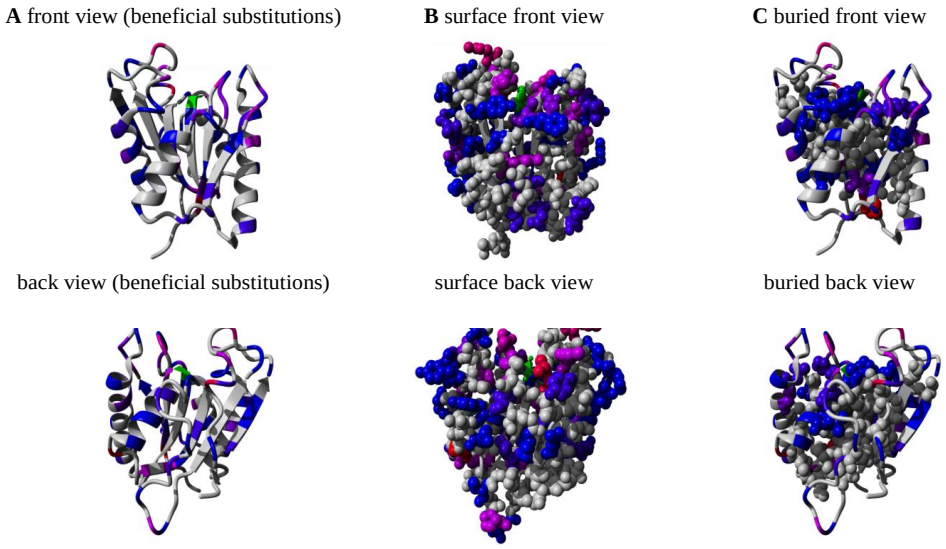


Figure 4-7: 3D model auf BSLA (Protein Database code: 1I6W) displaying regions which favor BSLA resistance in presence of TFE in a color scale. Positions at which no substitution led to improvements are colored grey. Positions with one substitution leading to improvements are colored blue. Positions with two to seven substitutions leading to improvements are colored purple (two positions) to magenta (seven positions). Red represents positions with eight or more substitutions leading to improvements. The active site residue Ser 77 is colored green. A) Cartoon representation of BSLA, B) Van der Waals representation of surface residues of BSLA, C) Van der Waals representation of buried residues of BSLA. The two N-terminal amino acids (Ala1 and Glu2) are also included. Yasara was used for visualization.

4.5.7 The SSM-BSLAVariants Showing Improved OS Resistance >2.5-fold

In contrast to the results presented above 4.5.6, the focus in this section is on the best variants with improved BSLA´s organic solvent resistance >2.5-fold. As previously observed, highest number of beneficial positions (59%) and substitutions (10%) for improved OS resistance were achieved for DMSO whereas a lower number (4%) of variants with improvements were obtained in the presence of 1,4-dioxane and TFE. Considering the single amino acid substitutions, 6 (0.17%) variants with higher resistance (>2.5-fold improvements) were found for DMSO, which represented 5 (0.03%) variants more than for TFE and 1,4-dioxane (Table 4-5). The highest R_V/R_{WT} values (see definition in section 3.5) achieved in the OS were 9.0, 7.9, and 3.5 for DMSO, TFE, and 1,4-dioxane, respectively. Variants with the highest improvements were obtained at positions Gly21 (helix, exposed) for DMSO, Pro5 (loop, buried) and Asp91 (loop, exposed) for TFE and at position Val62 (helix, buried) for 1,4-dioxane. Fewer variants (eight variants) with highest improvements in different organic solvents were found than in ionic liquids (61 variants).

Table 4-5: Best variants in all three OS. The numbers represents the ratio of residual activity of each variant to the residual activity of the wild type. All variants listed have a ratio >2.5-fold. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged acidic (D, E), charged basic (H, K, and R), and polar (C, M, P, S, T, N, and Q).

DMSO: 2.5-fold corresponds to a Rv/Rwt of 3.3		
Gly21Gln	9.0	polar --> polar

1,4-dioxane: 2.5-fold corresponds to a Rv/Rwt of 3.3		
Val62Glu	3.5	aliphatic --> acidic

TFE: 2.5-fold corresponds to a Rv/Rwt of 3.4		
Pro5Phe	6.7	Polar --> aromatic
Pro5Asn	7.9	Polar --> polar
Val7Cys	3.6	aliphatic --> polar
Ala81Trp	3.7	aliphatic --> aromatic
Asp91Trp	7.8	acidic --> aromatic
Ile157His	3.7	aliphatic --> acidic

R_V/R_{WT} values are represented in purple bars for DMSO, in dark blue bars for 1,4-dioxane, and in pink bars TFE.

The nature of the substitutions that led to the improvement >2.5-fold for all three OS were different among the three OS (Table 4-5). Replacement of charged acidic residues with aromatic amino acids and polar residues with polar residues resulted in variants with highest resistance improvements >2.5-fold for TFE (Figure 4-8). Replacement of polar residues in BSLA WT to chemically equivalent residues (polar ones) led to the best variants for DMSO resistance. For 1,4-dioxane, replacement of aliphatic residues with charged acidic amino acids also led to the best improved variants. These observations implied that polar substitutions gave higher improvement while charged substitutions led to higher number of improved variants.

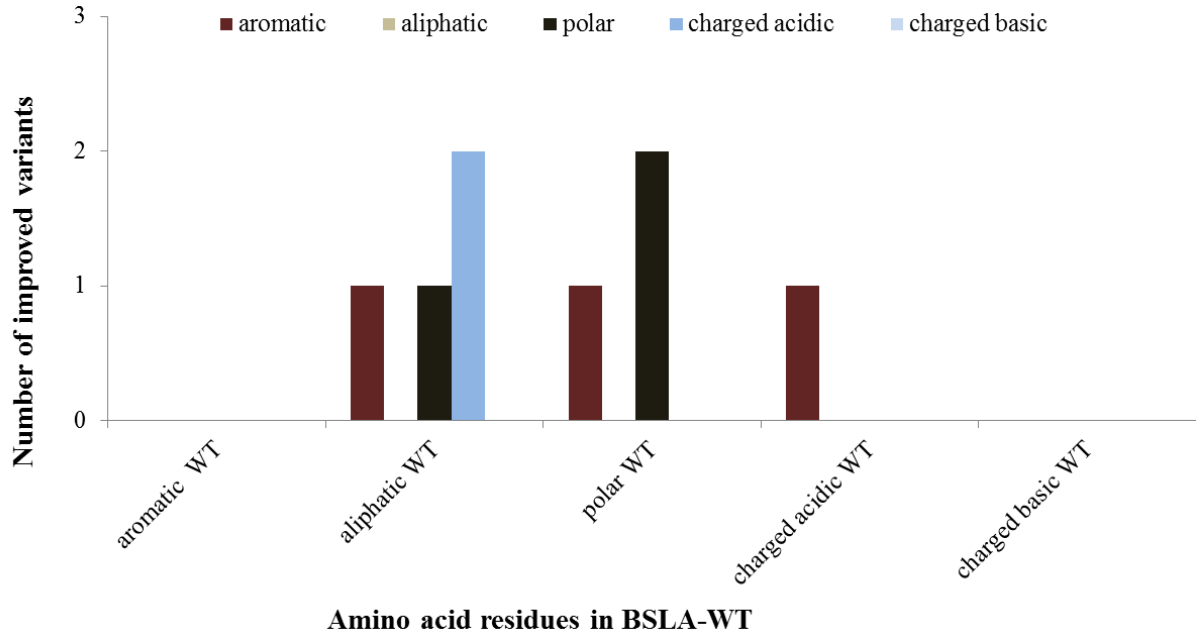


Figure 4-8: Number of improved variants according to the amino acid substitution of BSLA WT in all three OS. Variants with a value ≥2.5-fold are represented here. The nature of the substitution for each variant is also illustrated. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged acidic (D, E), charged basic (H, K, and R), and polar (C, M, P, S, T, N, and Q).

4.6 Conclusion

In this chapter, all the 181 amino acid positions of the model enzyme BSLA were saturated to study the resistance towards three hydrophilic OS. The effect of each position and each type of amino acid substitution (charged, polar, aliphatic, and aromatic) on BSLA resistance towards the three selected OS was investigated. For the first time, the evolutionary potential by single substitution of all amino acid positions of an enzyme to improve its OS resistance was shown. Less improved positions and substitutions were found to contribute to BSLA OS resistance (41-59%) than ionic liquid resistance (50-69%). In addition, the number of substitutions which improved BSLA resistance for OS (4-10%) was slightly lower than for ILs (6-13%). Both findings imply that engineering of BSLA for improving IL resistance likely is easier than for OS resistance by single amino acid exchanges. For both types of solvents, polar and charged amino acids substitutions are more beneficial than aliphatic and aromatic substitutions. In contrast to ILs, a clear trend was observed for OS: charged residues are more favorable for improved BSLA resistance at the surface and polar substitutions are mostly favorable at buried positions. For both kinds of solvents, substitutions of amino acids residues with chemically different ones led to significantly more BSLA variants with improved solvent resistance than the chemically similar ones. These results explained again as reported in chapter 3 why screening of small libraries is often sufficient to find improved

variants and confirmed that substitutions with chemically different residues can minimize screening effort. Athough chemically different amino acid substitutions preferentially dominantly led to improved variants, the variant with highest improvement for DMSO showed a substitution from polar amino acid to a polar amino acid (Gly21Gln with 6.9-fold improvements). In order to understand this, MD simulation has to be performed in the future.

Chapter 5

Final Summary and Conclusions

5 Final Summary and Conclusions

Enzymes have been designed by nature to function in aqueous environment. Very often, dramatically loss of enzymatic activity was observed in presence of non-conventional media (e.g. ionic liquids (ILs) and organic solvents (OS)) which are often needed in industrial applications. Protein engineering has been used to improve the resistance of enzymes towards ILs and OS. Despite of the success, geneneral principles for protein engineering are still missing. The aim of this PhD project was to conclude some general principles for improving the resistance of enzymes toward ILs and OS. *Bacillus subtilis* lipase A (BSLA) was used as a model hydrolase in this work. BSLA is a lipase that does not require interfacial activation and belongs to the lipase subfamily I-4 (comprised solely of monomeric enzymes), contains 181 amino acid residues in their mature forms, have molecular masses close to 19.5 kDa and share 74-80% sequence identity. The latter characteristic allows drawing more general conclusions from the results obtained for BSLA to other members of the subfamily and all hydrolases in general. Site saturation mutagenesis (SSM) libraries targeting the complete *bsla* were generated to systematically analyze how much single substitutions can influence the IL/OS resistance and what types of amino acid substitution can contribute to the improvement. A colorimetric screening assay was optimized based on the detection of *p*-nitrophenolate formation in 96-well MTPs (second chapter). In order to cover the full diversity, 18547 clones representing 3620 unique variants were generated and fully sequenced (second chapter). The BSLA-SSM libraries were screened and systematically analyzed in the presence of four ILs (third chapter) and three OS (fourth chapter).

The systematic study of all SSM-BSLA variants elucidated first general engineering principles for improving IL resistance of BSLA. 6-13% of all substitutions at 50-69% of all amino acid positions contributed to the improved BSLA resistance. More than half of the amino acid positions contributed to the improved IL resistance. It explained why improved enzyme variants can often be identified from small sized random mutant libraries (1000-2000 clones). Highest number of improved variants were obtained for [BMIM][Cl] among the four selected ILs. Moreover, replacement of BSLA WT residues with charged and/or polar substitutions led to more improved variants for all four ILs. However, the substitutions for the best variants do not show a preference to specific type of amino acid substitution. Among the beneficial substitutions, 66-95% of the improved variants were achieved with chemically different amino acids (e.g. aromatic amino acids substituted by polar/aliphatic/charged amino

acids). The latter indicates that mutagenesis methods that introduce transversions should at least for lipases like BSLA be favored to generate variants with improved IL resistance.

The systematic study of the performance of all SSM-BSLA variants in the presence of three OS (fourth chapter) showed that 4-10% substitutions at 41-59% of BSLA positions displayed an increased OS resistance. Resistance pattern for TFE and 1,4-dioxane was almost similar but different from DMSO resistance pattern of BSLA. In contrast to ILs for which the charged and polar amino acid substitution led to improvements, predominantly beneficial substitutions for improved OS resistance were charged ones. Lower number of improved variants and lower number of variants with higher improvement (>2.5-fold) was obtained for OS than that for ILs. In total 61 variants with improvements >2.5-fold were observed for the four ILs while only eight variants were obtained for OS. This implies that improving BSLA's OS resistance is more difficult than IL resistance. It was also observed that more improved variants were obtained on the surface than in buried region, showing the importance of surface residues on improving enzyme stability. Further, analysis of the location of the beneficial substitutions on the secondary structure elements indicated that most improvements for IL and OS were achieved with substitutions located on the loops. Similar to ILs, high number (58-93%) of improved variants were obtained by substitution with chemically different amino acids (e.g. aromatic amino acids substituted by polar/aliphatic/charged amino acids).

In summary, an exhaustive set of experimental data to elucidate the general principles governing the resistance of BSLA in the presence of four ILs and three OS was used. Screening of 181 SSM libraries of BSLA, revealed that ≥50% of amino acid positions contributed to improve in four ILs the resistance of BSLA while ≥41% of the positions contributed to improved BSLA resistance in OS. The high number of positions which improve IL resistance (≥50%) and OS resistance (≥41%) explains for the first time from an experimental point of view why often the screening of small sized libraries (1000-2000 variants) is sufficient to find improved variants. Additionally, screening efforts can be minimized by mutagenesis methods, which introduce chemical different amino acids substitutions through transversions and consecutive mutations.

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Appendix

7 Appendix

7.1 List of Abbreviations

AA	Amino Acid
amp	Ampicillin (100 µg/µL Finale Concentration)
AmpR	Ampicillin Resistance
APS	Ammonium Persulphate
Br^-	Bromide
BMIM	1-Butyl-3-Methylimidazolium
bp	Base Pair
BSL	Lipase from *Bacillus Subtilis*
BSLA	Lipase A from *Bacillus Subtilis*
BSLB	Lipase B from *Bacillus Subtilis*
Cl^-	Chloride
CMC	Critical Micellar Concentration
Da	Dalton
ddH_2O	double-distillated water
epPCR	Error-Prone Polymerase Chain Reaction
EV	Empty Vector
g	Gram
GRAS	Generally Recognized As Safe
H	Hour
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
H6	6xhistag
HTS	High-Throughput Screening
I^-	Iodide
IL	Ionic Liquid
k	Kilo
kb	Kilobases
kDa	Kilodalton
Kpi	Potassium Phosphate Inorganic
L	Liter
LB	Lysogeny Broth
LipA	*Bacillus Subtilis* Lipase A
M	Molarity (Mol/L)
m	Mili
MAIM	Modified Auto-Induction Medium
min	Minutes
MTP	Microtiter Plate
MOPS	(N-Morpholino)Propansulfonic Acid Buffer
OD	Optical Density
OS	Organic Solvent

PAGE	Polyacrylamid Gel Electrophoresis
PCR	Polymerase Chain Reaction
pNPB	p-Nitrophenyl Butyrate
pNPC	*p*-Nitrophenyl Caprylate
pNPP	*p*-Nitrophenyl Palmitate
rpm	Rounds per Minute
RT	Room Temperature
SDM	Side Directed Mutagenesis
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOC	Super Optimal Broth with Catabolite Repression
SS	Signal Sequence
SSM	Site Saturation Mutagenesis
TEA	Triethanolamine
TEMED	Tetramethylethylenediamine
TfO	Trifluoromethane Sulfonate
Tris/HCl$^-$	Tris(Hydroxymethyl)Aminomethane Buffer
U	Units
V	Volt
v/v	Volume per Volume
w/v	Weight per Volume
WT	Wild Type

°C	Degree Celsius
µ	Micro
nm	Nanometer
3D	Three-dimensional

7.2 Nomeclature

NaOH	Sodium Hydroxyde
HCl	Hydrogen Chloride
[BMIM][Br]	1-Butyl-3-Methylimidazolium Bromide
[BMIM][Cl]	1-Butyl-3-Methylimidazolium Chloride
[BMIM][I]	1-Butyl-3-Methylimidazolium Iodide
[BMIM][Tfo]	1-Butyl-3-Methylimidazolium Trifluoromethanesulfonate
[BMPy][TFA]	1-Methylpyrrolidinium Trifluoroacetate
[EMIM][TFA]	1-Ethyl-3-Methylimidazolium Trifluoroacetate
[EMIM][MESO3]	1-Ethyl-3-Methylimidazolium Methanesulphate
[EMIM][MeSO4]	1-Ethyl-3-Methylimidazolium Methylsulphate
[EMIM][AC]	1-Ethyl-3-Methylimidazolium Acetate
[MMIM][MMPO4]	1,3-Dimethylimidazolium Dimethyl Phosphate
DMSO	Dimethyl Sulfoxide
TFE	2,2,2-Trifluoroethanol

7.3 List of Tables

7.4 List of Figures

7.5 Vector Maps

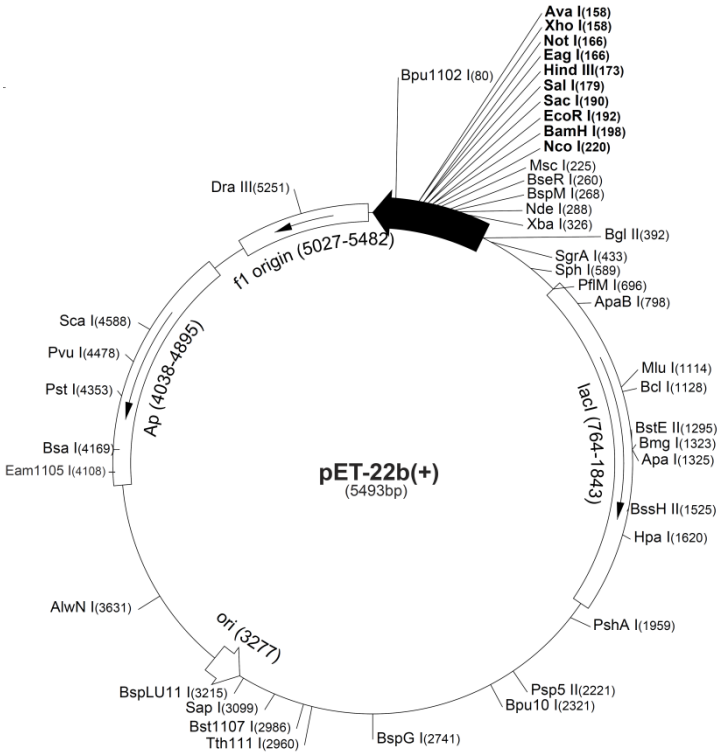

Figure 7-1: Plasmid map of of the expression vector pET22b(+) from Novagen (Cat. No. 69744-3). Ap: ampicillin resistance. The black arrow represents the multiple cloning site (MCS).

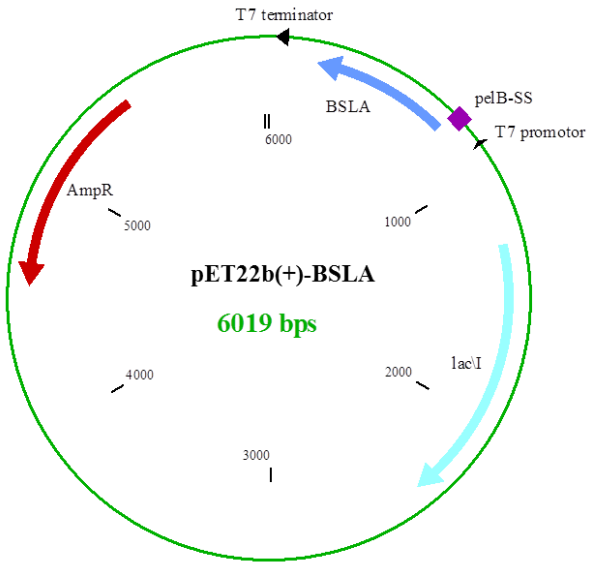

Figure 7-2: Plasmid map of pET22b(+) BSLA. The expression vector pET22b(+) contains the gene *bsla* encoding for lipase A from *Bacillus subtilis*. SS: signal sequence, pelB-SS is the sequence responsible for periplasmid localization. Amp^R: ampicillin resistance. LacI: gene encoding for the lactose repressor.

7.6 List of 20 Canonical Amino Acids

Types of amino acids	One-letter code	Three-letter code	Full name	Polarity
Aromatic	F	Phe	Phenylalanine	hydrophobic
	Y	Tyr	Tyrosine	hydrophilic
	W	Trp	Tryptophan	hydrophilic
Aliphatic	A	Ala	Alanine	hydrophobic
	V	Val	Valine	
	L	Leu	Leucine	
	I	Ile	Isoleucine	
	G	Gly	Glycine	
Polar	C	Cys	Cysteine	hydrophilic
	M	Met	Methionine	hydrophilic
	P	Pro	Proline	hydrophobic
	S	Ser	Serine	hydrophilic
	T	Thr	Threonine	hydrophilic
	N	Asn	Asparagine	hydrophilic
	Q	Gln	Glutamine	hydrophilic
Charged acidic	D	Asp	Aspartic Acid	hydrophilic
	E	Glu	Glutamic Acid	
Charged basic	H	His	Histidine	
	K	Lys	Lysine	
	R	Arg	Arginine	

7.7 List of Primers

Protein Sequence of BSLA (181 amino acids)

AGHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNYNNGPVL
SRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANVVTLGGANRLTT
GKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNVQIHGVGHIGLLYSSQVNSL
IKEGLNGGGQNTN

Table 7-1: List of all primers used for the site saturation mutagenesis (SSM).

Primer name	Forward primer	Reverse primer
Ala1-**NNS**	GATGGCCATGGATATC**NNS**GAACACAATCCAG	CTGGATTGTGTTC**SNN**GATATCCATGGCCATC
Glu2-**NNS**	CCATGGATATCGCT**NNS**CACAATCCAGTCG	CGACTGGATTGTG**SNN**AGCGATATCCATGG
His3-**NNS**	CATGGATATCGCTGAA**NNS**AATCCAGTCGTTATG	CATAACGACTGGATT**SNN**TTCAGCGATATCCATG
Asn4-**NNS**	GATATCGCTGAACAC**NNS**CCAGTCGTTATGGTT	AACCATAACGACTGG**SNN**GTGTTCAGCGATATC
Pro5-**NNS**	GATATCGCTGAACACAAT**NNS**GTCGTTATGGTTCAC	GTGAACCATAACGAC**SNN**ATTGTGTTCAGCGATATC
Val6-**NNS**	GCTGAACACAATCCA**NNS**GTTATGGTTCACGGT	ACCGTGAACCATAAC**SNN**TGGATTGTGTTCAGC
Val7-**NNS**	GAACACAATCCAGTC**NNS**ATGGTTCACGGTATT	AATACCGTGAACCAT**SNN**GACTGGATTGTGTTC
Met8-**NNS**	CACAATCCAGTCGTT**NNS**GTTCACGGTATTGGA	TCCAATACCGTGAAC**SNN**AACGACTGGATTGTG
Val9-**NNS**	CAATCCAGTCGTTATG**NNSC**ACGGTATTGGAGGG	CCCTCCAATACCGTG**SNN**CATAACGACTGGATTG

Table 7-1: List of all primers used for the site saturation mutagenesis (SSM).

Primer name	Forward primer	Reverse primer
His10-**NNS**	CCAGTCGTTATGGTT**NNS**GGTATTGGAGGGGCA	TGCCCCTCCAATACC**SNN**AACCATAACGACTGG
Gly11-**NNS**	GTCGTTATGGTTCAC**NNS**ATTGGAGGGGCATCA	TGATGCCCCTCCAAT**SNN**GTGAACCATAACGAC
Ile12-**NNS**	GTTATGGTTCACGGT**NNS**GGAGGGGCATCATTC	GAATGATGCCCCTCC**SNN**ACCGTGAACCATAAC
Gly13-**NNS**	GTTATGGTTCACGGTATT**NNS**GGGGCATCATTC	GAATGATGCCCC**SNN**AATACCGTGAACCATAAC
Gly14-**NNS**	GTTCACGGTATTGGA**NNS**GCATCATTCAATTTT	AAAATTGAATGATGC**SNN**TCCAATACCGTGAAC
Ala15-**NNS**	CACGGTATTGGAGGG**NNS**TCATTCAATTTTGCG	CGCAAAATTGAATGA**SNN**CCCTCCAATACCGTG
Ser16-**NNS**	GGTATTGGAGGGGCA**NNS**TTCAATTTTGCGGGA	TCCCGCAAAATTGAA**SNN**TGCCCCTCCAATACC
Phe17-**NNS**	GTATTGGAGGGGCATCA**NNS**AATTTTGCGGGAATT	AATTCCCGCAAAATT**SNN**TGATGCCCCTCCAATAC
Asn18-**NNS**	GGAGGGGCATCATTC**NNS**TTTGCGGGAATTAAG	CTTAATTCCCGCAAA**SNN**GAATGATGCCCCTCC
Phe19**NNS**	GGGGCATCATTCAAT**NNS**GCGGGAATTAAGAGC	GCTCTTAATTCCCGC**SNN**ATTGAATGATGCCCC
Ala20-**NNS**	GCATCATTCAATTTT**NNS**GGAATTAAGAGC	GCTCTTAATTCC**SNN**AAAATTGAATGATGC
Gly21-**NNS**	GCATCATTCAATTTTGCG**NNS**ATTAAGAGCTAT	ATAGCTCTTAAT**SNN**CGCAAAATTGAATGATGC
Ile22-**NNS**	CAATTTTGCGGGA**NNS**AAGAGCTATCTCGTA	TACGAGATAGCTCTT**SNN**TCCCGCAAAATTG
Lys23-**NNS**	CAATTTTGCGGGAATT**NNS**AGCTATCTCGTATCT	AGATACGAGATAGCT**SNN**AATTCCCGCAAAATTG
Ser24-**NNS**	GCGGGAATTAAG**NNS**TATCTCGTATCTCAG	CTGAGATACGAGATA**SNN**CTTAATTCCCGC
Tyr25-**NNS**	GCGGGAATTAAGAGC**NNS**CTCGTATCTCAGGGC	GCCCTGAGATACGAG**SNN**GCTCTTAATTCCCGC
Leu26-**NNS**	GGAATTAAGAGCTAT**NNS**GTATCTCAGGGCTGG	CCAGCCCTGAGATAC**SNN**ATAGCTCTTAATTCC
Val27-**NNS**	GAATTAAGAGCTATCTC**NNS**TCTCAGGGCTGGTCG	CGACCAGCCCTGAGA**SNN**GAGATAGCTCTTAATTC
Ser28-**NNS**	GAGCTATCTCGTA**NNS**CAGGGCTGGTCGCGG	CCGCGACCAGCCCTG**SNN**TACGAGATAGCTC
Gln29-**NNS**	AGCTATCTCGTATCT**NNS**GGCTGGTCGCGGGAC	GTCCCGCGACCAGCC**SNN**AGATACGAGATAGCT
Gly30-**NNS**	CTATCTCGTATCTCAG**NNS**TGGTCGCGGGACAAG	CTTGTCCCGCGACCA**SNN**CTGAGATACGAGATAG
Trp31-**NNS**	CTCGTATCTCAGGGC**NNS**TCGCGGGACAAGCTG	CAGCTTGTCCCGCGA**SNN**GCCCTGAGATACGAG
Ser32-**NNS**	GTATCTCAGGGCTGG**NNS**CGGGACAAGCTGTAT	ATACAGCTTGTCCCG**SNN**CCAGCCCTGAGATAC
Leu36-**NNS**	CTGGTCGCGGGACAAG**NNS**TATGCAGTTGATTTT	AAAATCAACTGCATA**SNN**CTTGTCCCGCGACCAG
Tyr37-**NNS**	GTCGCGGGACAAGCTG**NNS**GCAGTTGATTTTTGG	CCAAAAATCAACTGC**SNN**CAGCTTGTCCCGCGAC
Ala38-**NNS**	CGGGACAAGCTGTAT**NNS**GTTGATTTTTGGGAC	GTCCCAAAAATCAAC**SNN**ATACAGCTTGTCCCG
Val39-**NNS**	GACAAGCTGTATGCA**NNS**GATTTTTGGGACAAG	CTTGTCCCAAAAATC**SNN**TGCATACAGCTTGTC
Asp40-**NNS**	AAGCTGTATGCAGTT**NNS**TTTTGGGACAAGACA	TGTCTTGTCCCAAAA**SNN**AACTGCATACAGCTT
Phe41-**NNS**	CTGTATGCAGTTGAT**NNST**GGGACAAGACAGGC	GCCTGTCTTGTCCCA**SNN**ATCAACTGCATACAG
Trp42-**NNS**	TATGCAGTTGATTTT**NNS**GACAAGACAGGCACA	TGTGCCTGTCTTGTC**SNN**AAAATCAACTGCATA
Asp43-**NNS**	GCAGTTGATTTTTGG**NNS**AAGACAGGCACAAAT	ATTTGTGCCTGTCTT**SNN**CCAAAAATCAACTGC
Lys44-**NNS**	GTTGATTTTTGGGAC**NNS**ACAGGCACAAATTAT	ATAATTTGTGCCTGT**SNN**GTCCCAAAAATCAAC
Thr45-**NNS**	GATTTTTGGGACAAG**NNS**GGCACAAATTATAAC	GTTATAATTTGTGCC**SNN**CTTGTCCCAAAAATC
Gly46-**NNS**	GATTTTTGGGACAAGACA**NNS**ACAAATTATAACAAT	ATTGTTATAATTTGT**SNN**TGTCTTGTCCCAAAAATC
Thr47-**NNS**	GATTTTTGGGACAAGACAGGC**NNS**AATTATAACAATG GA	TCCATTGTTATAATT**SNN**GCCTGTCTTGTCCCAAAAAT C
Asn48-**NNS**	GACAAGACAGGCACA**NNS**TATAACAATGGACCG	CGGTCCATTGTTATA**SNN**TGTGCCTGTCTTGTC
Tyr49-**NNS**	AAGACAGGCACAAAT**NNS**AACAATGGACCGGTA	TACCGGTCCATTGTT**SNN**ATTTGTGCCTGTCTT
Asn50-**NNS**	ACAGGCACAAATTAT**NNS**AATGGACCGGTATTA	TAATACCGGTCCATT**SNN**ATAATTTGTGCCTGT
Asn51-**NNS**	GGCACAAATTATAAC**NNS**GGACCGGTATTATCA	TGATAATACCGGTCC**SNN**GTTATAATTTGTGCC
Gly52-**NNS**	CACAAATTATAACAAT**NNS**CCGGTATTATCACGA	TCGTGATAATACCGG**SNN**ATTGTTATAATTTGTG
Pro53-**NNS**	AATTATAACAATGGA**NNS**GTATTATCACGATTT	AAATCGTGATAATAC**SNN**TCCATTGTTATAATT
Val54-**NNS**	TATAACAATGGACCG**NNS**TTATCACGATTTGTG	CACAAATCGTGATAA**SNN**CGGTCCATTGTTATA
Leu55-**NNS**	CAATGGACCGGTA**NNS**TCACGATTTGTGCAA	TTGCACAAATCGTGA**SNN**TACCGGTCCATTG
Ser56-**NNS**	CAATGGACCGGTATTA**NNS**CGATTTGTGCAAAAG	CTTTTGCACAAATCG**SNN**TAATACCGGTCCATTG
Arg57-**NNS**	GGACCGGTATTATCA**NNS**TTTGTGCAAAAGGTT	AACCTTTTGCACAAA**SNN**TGATAATACCGGTCC
Phe58-**NNS**	CCGGTATTATCACGA**NNS**GTGCAAAAGGTTTTA	TAAAACCTTTTGCAC**SNN**TCGTGATAATACCGG
Val59-**NNS**	GTATTATCACGATTT**NNS**CAAAAGGTTTTAGAT	ATCTAAAACCTTTTG**SNN**AAATCGTGATAATAC
Gln60-**NNS**	TTATC**AC**GATTTGTG**NNS**AAGGTTTTAGATGAA	TTCATCTAAAACCTT**SNN**CACAAATCGTGATAA

Table 7-1: List of all primers used for the site saturation mutagenesis (SSM).

Primer name	Forward primer	Reverse primer
Lys61-**NNS**	CACGATTTGTGCAA**NNS**GTTTTAGATG**A**AACG	CGTTTCATCTAAAAC**SNN**TTGCACAAATCGTG
Val62-**NNS**	CGATTTGTGCAAAAG**NNS**TTAGATGAAACGGGT	ACCCGTTTCATCTAA**SNN**CTTTTGCACAAATCG
Leu63-**NNS**	GATTTGTGCAAAAGGTT**NNS**GATGAAACGGGTGCG	CGCACCCGTTTCATC**SNN**AACCTTTTGCACAAATC
Asp64-**NNS**	GTGCAAAAGGTTTTA**NNS**GAAACGGGTGCGAAA	TTTCGCACCCGTTTC**SNN**TAAAACCTTTTGCAC
Glu65-**NNS**	CAAAAGGTTTTAGAT**NNS**ACGGGTGCGAAAAAA	TTTTTTCGCACCCGT**SNN**ATCTAAAACCTTTTG
Thr66-**NNS**	GGTTTTAGATGAA**NNS**GGTGCGAAAAAAGTG	CACTTTTTTCGCACC**SNN**TTCATCTAAAACC
Gly67-**NNS**	GTTTTAGATGAAACG**NNS**GCGAAAAAAGTGGAT	ATCCACTTTTTTCGC**SNN**CGTTTCATCTAAAAC
Lys69-**NNS**	GATGAAACGGGTGCG**NNS**AAAGTGGATATTGTC	GACAATATCCACTTT**SNN**CGCACCCGTTTCATC
Lys70-**NNS**	GAAACGGGTGCGAAA**NNS**GTGGATATTGTCGCT	AGCGACAATATCCAC**SNN**TTTCGCACCCGTTTC
Asp72-**NNS**	GGTGCGAAAAAAGTG**NNS**ATTGTCGCTCACAGC	GCTGTGAGCGACAAT**SNN**CACTTTTTTCGCACC
Ile73-**NNS**	GCGAAAAAAGTGGAT**NNS**GTCGCTCACAGCATG	CATGCTGTGAGCGAC**SNN**ATCCACTTTTTTCGC
Val74-**NNS**	GAAAAAAGTGGATATT**NNS**GCTCACAGCATGGGG	CCCCATGCTGTGAGC**SNN**AATATCCACTTTTTTC
Ala75-**NNS**	GAAAAAAGTGGATATTGTC**NNS**CACAGCATGGGGGGC	GCCCCCCATGCTGTG**SNN**GACAATATCCACTTTTTTC
His76-**NNS**	GTGGATATTGTCGCT**NNS**AGCATGGGGGGCGCG	CGCGCCCCCCATGCT**SNN**AGCGACAATATCCAC
Ser77-**NNS**	GATATTGTCGCTCAC**NNS**ATGGGGGGCGCGAAC	GTTCGCGCCCCCCAT**SNN**GTGAGCGACAATATC
Met78-**NNS**	GATATTGTCGCTCACAGC**NNS**GGGGGCGCGAACACA	TGTGTTCGCGCCCCC**SNN**GCTGTGAGCGACAATATC
Gly79-**NNS**	GTCGCTCACAGCATG**NNS**GGCGCGAACACACTT	AAGTGTGTTCGCGCC**SNN**CATGCTGTGAGCGAC
Gly80-**NNS**	GCTCACAGCATGGGG**NNS**GCGAACACACTTTAC	GTAAAGTGTGTTCGC**SNN**CCCCATGCTGTGAGC
Ala81-**NNS**	CACAGCATGGGGGGC**NNS**AACACACTTTACTAC	GTAGTAAAGTGTGTT**SNN**GCCCCCCATGCTGTG
Asn82-**NNS**	CAGCATGGGGGGCGCG**NNS**ACACTTTACTACATA	TATGTAGTAAAGTGT**SNN**CGCGCCCCCCATGCTG
Thr83-**NNS**	CATGGGGGGCGCGAAC**NNS**CTTTACTACATAAAA	TTTTATGTAGTAAAG**SNN**GTTCGCGCCCCCCATG
Leu84-**NNS**	GGGGGCGCGAACACA**NNS**TACTACATAAAAAAT	ATTTTTTATGTAGTA**SNN**TGTGTTCGCGCCCCC
Tyr85-**NNS**	GGCGCGAACACACTT**NNS**TACATAAAAAATCTG	CAGATTTTTTATGTA**SNN**AAGTGTGTTCGCGCC
Tyr86-**NNS**	GCGAACACACTTTAC**NNS**ATAAAAAATCTGGAC	GTCCAGATTTTTTAT**SNN**GTAAAGTGTGTTCGC
Ile87-**NNS**	GAACACACTTTACTAC**NNS**AAAAATCTGGACGGC	GCCGTCCAGATTTTT**SNN**GTAGTAAAGTGTGTTC
Lys88-**NNS**	CACACTTTACTACATA**NNS**AATCTGGACGGCGGA	TCCGCCGTCCAGATT**SNN**TATGTAGTAAAGTGTG
Asn89-**NNS**	CTTTACTACATAAAA**NNS**CTGGACGGCGGAAAT	ATTTCCGCCGTCCAG**SNN**TTTTATGTAGTAAAG
Leu90-**NNS**	CTTTACTACATAAAAAAT**NNS**GACGGCGGAAATAAA	TTTATTTCCGCCGTC**SNN**ATTTTTTATGTAGTAAAG
Asp91-**NNS**	CTACATAAAAAATCTG**NNS**GGCGGAAATAAAGTT	AACTTTATTTCCGCC**SNN**CAGATTTTTTATGTAG
Gly92-**NNS**	TGCAACTTTATTTCC**SNN**GTCCAGATTTTTTATG	CATAAAAAATCTGGAC**NNS**GGAAATAAAGTTGCA
Gly93-**NNS**	GTTTGCAACTTTATT**SNN**GCCGTCCAGATTTTTTATG	CATAAAAAATCTGGACGGC**NNS**AATAAAGTTGCAAAC
Asn94-**NNS**	GACGTTTGCAACTTT**SNN**TCCGCCGTCCAGATTTTTTA TG	TAAAAAATCTGGACGGCGGA**NNS**AAAGTTGCAAACGT C
Lys95-**NNS**	CACGACGTTTGCAAC**SNN**ATTTCCGCCGTCCAG	CTGGACGGCGGAAAT**NNS**GTTGCAAACGTCGTG
Val96-**NNS**	CGTCACGACGTTTGC**SNN**TTTATTTCCGCCGTC	GACGGCGGAAATAAA**NNS**GCAAACGTCGTGACG
Ala97-**NNS**	AAGCGTCACGACGTT**SNN**AACTTTATTCCTGCC	GGCAGGAATAAAGTT**NNS**AACGTCGTGACGCTT
Asn98-**NNS**	GCCAAGCGTCACGAC**SNN**TGCAACTTTATTTCC	GGAAATAAAGTTGCA**NNS**GTCGTGACGCTTGGC
Val99-**NNS**	GCCGCCAAGCGTCAC**SNN**GTTTGCAACTTTATTTC	GAAATAAAGTTGCAAAC**NNS**GTGACGCTTGGCGGC
Val100-**NNS**	CGCGCCGCCAAGCGT**SNN**GACGTTTGCAAC	GTTGCAAACGTC**NNS**ACGCTTGGCGGCGCG
Thr101-**NNS**	GTTCGCGCCGCCAAG**SNN**CACGACGTTTGCAAC	GTTGCAAACGTCGTG**NNS**CTTGGCGGCGCGAAC
Leu102-**NNS**	ACGGTTCGCGCCGCC**SNN**CGTCACGACGTTTGC	GCAAACGTCGTGACG**NNS**GGCGGCGCGAACCGT
Gly103-**NNS**	TAAACGGTTCGCGCC**SNN**AAGCGTCACGACGTT	AACGTCGTGACGCTT**NNS**GGCGCGAACCGTTTA
Gly104-**NNS**	CGTTAAACGGTTCGC**SNN**GCCAAGCGTCACGAC	GTCGTGACGCTTGGC**NNS**GCGAACCGTTTAACG
Ala105-**NNS**	TGTCGTTAAACGGTT**SNN**GCCGCCAAGCGTCAC	GTGACGCTTGGCGGC**NNS**AACCGTTTAACGACA
Asn106-**NNS**	GGCGGCGCG**NNS**CGTTTGACGACAGGC	CAAACG**SNN**CGCGCCGCCAAGCGTCACGAC
Arg107-**NNS**	CTTGGCGGCGCGAAC**NNS**TTGACGACAGGCAAG	CTTGCCTGTCGTCAA**SNN**GTTCGCGCCGCCAAG
Leu108-**NNS**	CGCCTTGCCTGTCGT**SNN**ACGGTTCGCGCCGCC	GGCGGCGCGAACCGT**NNS**ACGACAGGCAAGGCG
Thr109-**NNS**	AAGCGCCTTGCCTGT**SNN**CAAACGGTTCGCGCC	GGCGCGAACCGTTTG**NNS**ACAGGCAAGGCGCTT
Thr110-**NNS**	GCGAACCGTTTGACG**NNS**GGCAAGGCGCTTCCG	CGGAAGCGCCTTGCC**SNN**CGTCAAACGGTTCGC

Table 7-1: List of all primers used for the site saturation mutagenesis (SSM).

Primer name	Forward primer	Reverse primer
Gly111-**NNS**	GAACCGTTTGACGACA**NNS**AAGGCGCTTCCGGG	CCCGGAAGCGCCTT**SNN**TGTCGTCAAACGGTTC
Lys112-**NNS**	TGTTCCCCGAAGCGC**SNN**GCCTGTCGTCAAACG	CGTTTGACGACAGGC**NNS**GCGCTTCGGGGAACA
Ala113-**NNS**	ATCTGTTCCCGGAAG**SNN**CTTGCCTGTCGTTAAAC	GTTTAACGACAGGCAAG**NNS**CTTCCGGGAACAGAT
Leu114-**NNS**	TGGATCTGTTCCCGG**SNN**CGCCTTGCCTGTCG	CGACAGGCAAGGCG**NNS**CCGGGAACAGATCCA
Pro115-**NNS**	ATTTGGATCTGTTCC**SNN**AAGCGCCTTGCCTGTC	GACAGGCAAGGCGCTT**NNS**GGAACAGATCCAAAT
Gly116-**NNS**	TTGATTTGGATCTGT**SNN**CGGAAGCGCCTTGCC	GGCAAGGCGCTTCCG**NNS**ACAGATCCAAATCAA
Thr117-**NNS**	CTTTTGATTTGGATC**SNN**TCCCGGAAGCGCCTTG	CAAGGCGCTTCCGGGA**NNS**GATCCAAATCAAAAG
Asp118-**NNS**	AATCTTTTGATTTGG**SNN**TGTTCCCGGAAGCGC	GCGCTTCCGGGAACA**NNS**CCAAATCAAAAGATT
Pro119-**NNS**	TAAAATCTTTTGATT**SNN**ATCTGTTCCCGGAAG	CTTCCGGGAACAGAT**NNS**AATCAAAAGATTTTA
Asn120-**NNS**	CCGGGAACAGATCCA**NNS**CAAAAGATTTTATAC	GTATAAAATCTTTTG**SNN**TGGATCTGTTCCCGG
Gln121-**NNS**	TGTGTATAAAATCTT**SNN**ATTTGGATCTGTTCC	GGAACAGATCCAAAT**NNS**AAGATTTTATACACA
Lys122-**NNS**	GGATGTGTATAAAAT**SNN**TTGATTTGGATCTG	CAGATCCAAATCAA**NNS**ATTTTATACACATCC
Ile123-**NNS**	AATGGATGTGTATAA**SNN**CTTTTGATTTGGATC	GATCCAAATCAAAAG**NNS**TTATACACATCCATT
Leu124-**NNS**	CCAAATCAAAAGATT**NNS**TACACATCCATTTAC	GTAAATGGATGTGTA**SNN**AATCTTTTGATTTGG
Tyr125-**NNS**	GCTGTAAATGGATGT**SNN**TAAAATCTTTTGATTTG	CAAATCAAAAGATTTTA**NNS**ACATCCATTTACAGC
Thr126-**NNS**	ACTGCTGTAAATGGA**SNN**GTATAAAATCTTTTG	CAAAAGATTTTATAC**NNS**TCCATTTACAGCAGT
Ser127-**NNS**	GGCACTGCTGTAAAT**SNN**TGTGTATAAAATCTT	AAGATTTTATACACA**NNS**ATTTACAGCAGTGCC
Ile128-**NNS**	ATCGGCACTGCTGTA**SNN**GGATGTGTATAAAATC	GATTTTATACACATCC**NNS**TACAGCAGTGCCGAT
Tyr129-**NNS**	CATATCGGCACTGCT**SNN**AATGGATGTGTATAAAATC	GATTTTATACACATCCATT**NNS**AGCAGTGCCGATATG
Ser130-**NNS**	AATCATATCGGCACT**SNN**GTAAATGGATGTG	CACATCCATTTAC**NNS**AGTGCCGATATGATT
Ser131-**NNS**	ACATCCATTTACAGC**NNS**GCCGATATGATTGTC	GACAATCATATCGGC**SNN**GCTGTAAATGGATGT
Ala132-**NNS**	CATGACAATCATATC**SNN**ACTGCTGTAAATGG	CCATTTACAGCAGT**NNS**GATATGATTGTCATG
Asp133-**NNS**	ATTCATGACAATCAT**SNN**GGCACTGCTGTAAATG	CATTTACAGCAGTGCC**NNS**ATGATTGTCATGAAT
Met134-**NNS**	GTAATTCATGACAAT**SNN**ATCGGCACTGCTGTAAATG	CATTTACAGCAGTGCCGAT**NNS**ATTGTCATGAATTAC
Ile135-**NNS**	TAAGTAATTCATGAC**SNN**CATATCGGCACTGCTG	CAGCAGTGCCGATATG**NNS**GTCATGAATTACTTA
Val136-**NNS**	TGATAAGTAATTCAT**SNN**AATCATATCGGCACTG	CAGTGCCGATATGATT**NNS**ATGAATTACTTATCA
Met137-**NNS**	TCTTGATAAGTAATT**SNN**GACAATCATATCGGC	GCCGATATGATTGTC**NNS**AATTACTTATCAAGA
Asn138-**NNS**	TAATCTTGATAAGTA**SNN**CATGACAATCATATC	GATATGATTGTCATG**NNS**TACTTATCAAGATTA
Tyr139-**NNS**	ATCTAATCTTGATAA**SNN**ATTCATGACAATCATATCGGC	GCCGATATGATTGTCATGAAT**NNS**TTATCAAGATTAGAT
Leu140-**NNS**	ACCATCTAATCTTGA**SNN**GTAATTCATGACAATC	GATTGTCATGAATTAC**NNS**TCAAGATTAGATGGT
Ser141-**NNS**	AGCACCATCTAATCT**SNN**TAAGTAATTCATGAC	GTCATGAATTACTTA**NNS**AGATTAGATGGTGCT
Arg142-**NNS**	TCTAGCACCATCTAA**SNN**TGATAAGTAATTCATG	CATGAATTACTTATCA**NNS**TTAGATGGTGCTAGA
Leu143-**NNS**	GTTTCTAGCACCATC**SNN**TCTTGATAAGTAATTC	GAATTACTTATCAAGA**NNS**GATGGTGCTAGAAAC
Asp144-**NNS**	AACGTTTCTAGCACC**SNN**TAATCTTGATAAGTAATT	AATTACTTATCAAGATTA**NNS**GGTGCTAGAAACGTT
Gly145-**NNS**	TTGAACGTTTCTAGC**SNN**ATCTAATCTTGATAAG	CTTATCAAGATTAGAT**NNS**GCTAGAAACGTTCAA
Ala146-**NNS**	TCAAGATTAGATGGT**NNS**AGAAACGTTCAAATC	GATTTGAACGTTTCT**SNN**ACCATCTAATCTTGA
Arg147-**NNS**	ATGGATTTGAACGTT**SNN**AGCACCATCTAATCTTG	CAAGATTAGATGGTGCT**NNS**AACGTTCAAATCCAT
Asn148-**NNS**	GCCATGGATTTGAAC**SNN**TCTAGCACCATCTAATC	GATTAGATGGTGCTAGA**NNS**GTTCAAATCCATGGC
Val149-**NNS**	AACGCCATCGATTTG**SNN**GTTTCTAGCACCATC	GATGGTGCTAGAAAC**NNS**CAAATCGATGGCGTT
Gln150-**NNS**	TCCAACGCCATGGAT**SNN**AACGTTTCTAGCACC	GGTGCTAGAAACGTT**NNS**ATCCATGGCGTTGGA
Ile151-**NNS**	GTGTCCAACGCCATG**SNN**TTGAACGTTTCTAGC	GCTAGAAACGTTCAA**NNS**CATGGCGTTGGACAC
His152-**NNS**	GATGTGTCCAACGCC**SNN**GATTTGAACGTTTCTAG	CTAGAAACGTTCAAATC**NNS**GGCGTTGGACACATC
Gly153-**NNS**	GCCGATGTGTCCAAC**SNN**ATGGATTTGAACGTTTC	GAAACGTTCAAATCCAT**NNS**GTTGGACACATCGGC
Val154-**NNS**	AAGGCCGATGTGTCC**SNN**GCCATGGATTTGAAC	GTTCAAATCCATGGC**NNS**GGACACATCGGCCTT
Gly155-**NNS**	CAGAAGGCCGATGTG**SNN**AACGCCATGGATTTG	CAAATCCATGGCGTT**NNS**CACATCGGCCTTCTG
His156-**NNS**	GTACAGAAGGCCGAT**SNN**TCCAACGCCATGGATTTG	CAAATCCATGGCGTTGGA**NNS**ATCGGCCTTCTGTAC
Ile157-**NNS**	GCTGTACAGAAGGCC**SNN**GTGTCCAACGCCATG	CATGGCGTTGGACAC**NNS**GGCCTTCTGTACAGC
Gly158-**NNS**	GCTGCTGTACAGAAG**SNN**GATGTGTCCAACGCC	GGCGTTGGACACATC**NNS**CTTCTGTACAGCAGC

Table 7-1: List of all primers used for the site saturation mutagenesis (SSM).

Primer name	Forward primer	Reverse primer
Leu159-**NNS**	TTGGCTGCTGTACAG**SNN**GCCGATGTGTCCAAC	GTTGGACACATCGGC**NNS**CTGTACAGCAGCCAA
Leu160-**NNS**	GACTTGGCTGCTGTA**SNN**AAGGCCGATGTGTCC	GGACACATCGGCCTT**NNS**TACAGCAGCCAAGTC
Tyr161-**NNS**	GTTGACTTGGCTGCT**SNN**CAGAAGGCCGATGTG	CACATCGGCCTTCTG**NNS**AGCAGCCAAGTCAAC
Ser162-**NNS**	GCTGTTGACTTGGCT**SNN**GTACAGAAGGCCGATG	CATCGGCCTTCTGTAC**NNS**AGCCAAGTCAACAGC
Ser163-**NNS**	CAGGCTGTTGACTTG**SNN**GCTGTACAGAAGGCC	GGCCTTCTGTACAGC**NNS**CAAGTCAACAGCCTG
Gln164-**NNS**	AATCAGGCTGTTGAC**SNN**GCTGCTGTACAGAAG	CTTCTGTACAGCAGC**NNS**GTCAACAGCCTGATT
Val165-**NNS**	TTTAATCAGGCTGTT**SNN**TTGGCTGCTGTACAG	CTGTACAGCAGCCAA**NNS**AACAGCCTGATTAAA
Asn166-**NNS**	TTCTTTAATCAGGCT**SNN**GACTTGGCTGCTGTAC	GTACAGCAGCCAAGTC**NNS**AGCCTGATTAAAGAA
Ser167-**NNS**	CCCTCCTTTAATCAG**SNN**GTTGACTTGGCTGCTG	CAGCAGCCAAGTCAAC**NNS**CTGATTAAAGGAGGG
Leu168-**NNS**	CAGCCCTTCTTTAAT**SNN**GCTGTTGACTTGGCTGCTG	CAGCAGCCAAGTCAACAGC**NNS**ATTAAAGAAGGGCTG
Ile169-**NNS**	GTTCAGCCCTTCTTT**SNN**CAGGCTGTTGACTTG	CAAGTCAACAGCCTG**NNS**AAAGAAGGGCTGAAC
Lys170-**NNS**	GCCGTTCAGCCCTTC**SNN**AATCAGGCTGTTGAC	GTCAACAGCCTGATT**NNS**GAAGGGCTGAACGGC
Glu171-**NNS**	CCCGCCGTTCAGCCC**SNN**TTTAATCAGGCTGTT	AACAGCCTGATTAAA**NNS**GGGCTGAACGGCGGG
Gly172-**NNS**	AGCCTGATTAAAGAA**NNS**CTGAACGGCGGGGGC	GCCCCCGCCGTTCAG**SNN**TTCTTTAATCAGGCT
Leu173-**NNS**	CTGATTAAAGAAGGG**NNS**AACGGCGGGGGCCAG	CTGGCCCCCGCCGTT**SNN**CCCTTCTTTAATCAG
Asn174-**NNS**	AATCAGGCTGTTGAC**SNN**GCTGCTGTACAGAAG	GAAGGGCTG**NNS**GGCGGGGGCGAGAATACGAAT
Gly175-**NNS**	AAAGAAGGGCTGAAC**NNS**GGGGGCCAGAATACG	CGTATTCTGGCCCCC**SNN**GTTCAGCCCTTCTTT
Gly176-**NNS**	GAAGGGCTGAACGGC**NNS**GGCCAGAATACGAAT	ATTCGTATTCTGGCC**SNN**GCCGTTCAGCCCTTC
Gly177-**NNS**	GGGCTGAACGGCGGG**NNS**CAGAATACGAATTAA	TTAATTCGTATTCTG**SNN**CCCGCCGTTCAGCCC
Gln178-**NNS**	GCTTGTCGACGGAGCTCTCATTAATTCGTATT**SNN**GCC	GGC**NNS**AATACGAATTAATGAGAGCTCCGTCGACAAG C
Asn179-**NNS**	GCTTGTCGACGGAGCTCTCATTAATTCGT**SNN**CTC	GAG**NNS**ACGAATTAATGAGAGCTCCGTCGACAAGC
Thr180-**NNS**	GGCGGGGGCCAGAAT**NNS**AATTAATGAGAGCTC	GAGCTCTCATTAATT**SNN**ATTCTGGCCCCCGCC
Asn181-**NNS**	GCTTGTCGACGGAGCTCTCATTA**SNN**CGT	ACG**NNS**TAATGAGAGCTCCGTCGACAAGC

Table 7-2: List of all primers used for the site directed mutagenesis (SDM).

Primer name	Forward primer	Reverse primer
Ala1phe	GATGGCCATGGATATCTTTGAACACAATCCAG	CTGGATTGTGTTCAAAGATATCCATGGCCATC
Ala1Asp	GATGGCCATGGATATCGATGAACACAATCCAG	CTGGATTGTGTTCATCGATATCCATGGCCATC
Glu2Lys	CCATGGATATCGCTAAACACAATCCAGTCG	CGACTGGATTGTGTTTAGCGATATCCATGG
Gly2Tyr	CCATGGATATCGCTTATCACAATCCAGTCG	CGACTGGATTGTGATAAGCGATATCCATGG
Glu2Gln	CCATGGATATCGCTCAGCACAATCCAGTCG	CGACTGGATTGTGCTGAGCGATATCCATGG
Glu2Cys	CCATGGATATCGCTTGCCACAATCCAGTCG	CGACTGGATTGTGGCAAGCGATATCCATGG
His3Lys	CATGGATATCGCTGAAAAGAATCCAGTCGTTATG	CATAACGACTGGATTCTTTTCAGCGATATCCATG
His3Met	CATGGATATCGCTGAAATGAATCCAGTCGTTATG	CATAACGACTGGATTCATTTCAGCGATATCCATG
His3Gln	CATGGATATCGCTGAACAGAATCCAGTCGTTATG	CATAACGACTGGATTCTGTTCAGCGATATCCATG
His3Cys	CATGGATATCGCTGAATGCAATCCAGTCGTTATG	CATAACGACTGGATTGCATTCAGCGATATCCATG
Asn4Phe	GATATCGCTGAACACTTTCCAGTCGTTATGGTT	AACCATAACGACTGGAAAGTGTTCAGCGATATC
Val6Lys	GCTGAACACAATCCAAAAGTTATGGTTCACGGT	ACCGTGAACCATAACTTTTGGATTGTGTTCAGC
Val6lle	GCTGAACACAATCCAATTGTTATGGTTCACGGT	ACCGTGAACCATAACAATTGGATTGTGTTCAGC
Met8Lys	CACAATCCAGTCGTTAAGGTTCACGGTATTGGA	TCCAATACCGTGAACCTTAACGACTGGATTGTG
Met8Phe	CACAATCCAGTCGTTTTTGTTCACGGTATTGGA	TCCAATACCGTGAACAAAAACGACTGGATTGTG
Val9Glu	CAATCCAGTCGTTATGGAACACGGTATTGGAGGG	CCCTCCAATACCGTGTTCCATAACGACTGGATTG
Val9His	CAATCCAGTCGTTATGCACCACGGTATTGGAGGG	CCCTCCAATACCGTGGTGCATAACGACTGGATTG
Val9Met	CAATCCAGTCGTTATGATGCACGGTATTGGAGGG	CCCTCCAATACCGTGCATCATAACGACTGGATTG
Val9Trp	CAATCCAGTCGTTATGTGGCACGGTATTGGAGGG	CCCTCCAATACCGTGCCACATAACGACTGGATTG

Table 7-2: List of all primers used for the site directed mutagenesis (SDM).

Primer name	Forward primer	Reverse primer
Gly11Asp	GTCGTTATGGTTCACGATATTGGAGGGGCATCA	TGATGCCCCTCCAATATCGTGAACCATAACGAC
lle12Glu	GTTATGGTTCACGGTGAAGGAGGGGCATCATTC	GAATGATGCCCCTCCTTCACCGTGAACCATAAC
lle12His	GTTATGGTTCACGGTCACGGAGGGGCATCATTC	GAATGATGCCCCTCCGTGACCGTGAACCATAAC
Gly14lle	GTTCACGGTATTGGAATTGCATCATTCAATTTT	AAAATTGAATGATGCAATTCCAATACCGTGAAC
Gly14Tyr	GTTCACGGTATTGGATATGCATCATTCAATTTT	AAAATTGAATGATGCATATCCAATACCGTGAAC
Gly14Ser	GTTCACGGTATTGGAAGCGCATCATTCAATTTT	AAAATTGAATGATGCGCTTCCAATACCGTGAAC
Gly14Cys	GTTCACGGTATTGGATGCGCATCATTCAATTTT	AAAATTGAATGATGCGCATCCAATACCGTGAAC
Ala15lle	CACGGTATTGGAGGGATTTCATTCAATTTTGCG	CGCAAAATTGAATGAAATCCCTCCAATACCGTG
Ser16His	GGTATTGGAGGGGCACACTTCAATTTTGCGGGA	TCCCGCAAAATTGAAGTGTGCCCCTCCAATACC
Ser16lle	GGTATTGGAGGGGCAATTTTCAATTTTGCGGGA	TCCCGCAAAATTGAAAATTGCCCCTCCAATACC
Ser16Phe	GGTATTGGAGGGGCATTTTTCAATTTTGCGGGA	TCCCGCAAAATTGAAAAATGCCCCTCCAATACC
Asn18Trp	GGAGGGGCATCATTCTGGTTTGCGGGAATTAAG	CTTAATTCCCGCAAACCAGAATGATGCCCCTCC
Phe19Lys	GGGGCATCATTCAATAAGGCGGGAATTAAGAGC	GCTCTTAATTCCCGCCTTATTGAATGATGCCCC
Phe19lle	GGGGCATCATTCAATATTGCGGGAATTAAGAGC	GCTCTTAATTCCCGCAATATTGAATGATGCCCC
Phe19Gln	GGGGCATCATTCAATCAGGCGGGAATTAAGAGC	GCTCTTAATTCCCGCCTGATTGAATGATGCCCC
Ala20-Met	GCATCATTCAATTTTATGGGAATTAAGAGC	GCTCTTAATTCCCATAAAATTGAATGATGC
Ala20-Phe	GCATCATTCAATTTTTTTGGAATTAAGAGC	GCTCTTAATTCCAAAAAAATTGAATGATGC
Gly21Asp	GCATCATTCAATTTTGCGGATATTAAGAGCTAT	ATAGCTCTTAATATCCGCAAAATTGAATGATGC
Gly21Phe	GCATCATTCAATTTTGCGTTTATTAAGAGCTAT	ATAGCTCTTAATAAACGCAAAATTGAATGATGC
Gly21Gln	GCATCATTCAATTTTGCGCAGATTAAGAGCTAT	ATAGCTCTTAATCTGCGCAAAATTGAATGATGC
Lys23-Asp	CAATTTTGCGGGAATTGATAGCTATCTCGTATCT	AGATACGAGATAGCTATCAATTCCCGCAAAATTG
Ser24-Met	GCGGGAATTAAGATGTATCTCGTATCTCAG	CTGAGATACGAGATACATCTTAATTCCCGC
Ser24lle	GCGGGAATTAAGATTTATCTCGTATCTCAG	CTGAGATACGAGATAAATCTTAATTCCCGC
Ser24Phe	GCGGGAATTAAGTTTTATCTCGTATCTCAG	CTGAGATACGAGATAAAACTTAATTCCCGC
Ser24Cys	GCGGGAATTAAGTGCTATCTCGTATCTCAG	CTGAGATACGAGATAGCACTTAATTCCCGC
Tyr25Asp	GCGGGAATTAAGAGCGATCTCGTATCTCAGGGC	GCCCTGAGATACGAGATCGCTCTTAATTCCCGC
Leu26lle	GGAATTAAGAGCTATATTGTATCTCAGGGCTGG	CCAGCCCTGAGATACAATATAGCTCTTAATTCC
Leu26Tyr	GGAATTAAGAGCTATTATGTATCTCAGGGCTGG	CCAGCCCTGAGATACATAATAGCTCTTAATTCC
Leu26Trp	GGAATTAAGAGCTATTGGGTATCTCAGGGCTGG	CCAGCCCTGAGATACCCAATAGCTCTTAATTCC
Val27Asp	GAATTAAGAGCTATCTCGATTCTCAGGGCTGGTCG	CGACCAGCCCTGAGAATCGAGATAGCTCTTAATTC
Val27lle	GAATTAAGAGCTATCTCATTTCTCAGGGCTGGTCG	CGACCAGCCCTGAGAAATGAGATAGCTCTTAATTC
Val27Tyr	GAATTAAGAGCTATCTCTATTCTCAGGGCTGGTCG	CGACCAGCCCTGAGAATAGAGATAGCTCTTAATTC
Ser28Asp	GAGCTATCTCGTAGATCAGGGCTGGTCGCGG	CCGCGACCAGCCCTGATCTACGAGATAGCTC
Ser28His	GAGCTATCTCGTACACCAGGGCTGGTCGCGG	CCGCGACCAGCCCTGGTGTACGAGATAGCTC
Ser28Trp	GAGCTATCTCGTATGGCAGGGCTGGTCGCGG	CCGCGACCAGCCCTGCCATACGAGATAGCTC
Gly29His	AGCTATCTCGTATCTCACGGCTGGTCGCGGGAC	GTCCCGCGACCAGCCGTGAGATACGAGATAGCT
Gly29Phe	AGCTATCTCGTATCTTTTGGCTGGTCGCGGGAC	GTCCCGCGACCAGCCAAAAGATACGAGATAGCT
Gly30His	CTATCTCGTATCTCAGCACTGGTCGCGGGACAAG	CTTGTCCCGCGACCAGTGCTGAGATACGAGATAG
Trp31Tyr	CTCGTATCTCAGGGCTATTCGCGGGACAAGCTG	CAGCTTGTCCCGCGAATAGCCCTGAGATACGAG
Trp31-Ile	CTCGTATCTCAGGGCATTTCGCGGGACAAGCTG	CAGCTTGTCCCGCGAAATGCCCTGAGATACGAG
Trp31-His	CTCGTATCTCAGGGCCACTCGCGGGACAAGCTG	CAGCTTGTCCCGCGAGTGGCCCTGAGATACGAG
Trp31-Cys	CTCGTATCTCAGGGCTGCTCGCGGGACAAGCTG	CAGCTTGTCCCGCGAGCAGCCCTGAGATACGAG
Trp31-Pro	CTCGTATCTCAGGGCCCGTCGCGGGACAAGCTG	CAGCTTGTCCCGCGACGGGCCCTGAGATACGAG
Ser32Trp	GTATCTCAGGGCTGGTGGCGGGACAAGCTGTAT	ATACAGCTTGTCCCGCCACCAGCCCTGAGATAC
Ser32Gln	GTATCTCAGGGCTGGCAGCGGGACAAGCTGTAT	ATACAGCTTGTCCCGCTGCCAGCCCTGAGATAC
Leu36His	CTGGTCGCGGGACAAGCACTATGCAGTTGATTTT	AAAATCAACTGCATAGTGCTTGTCCCGCGACCAG
Leu36Met	CTGGTCGCGGGACAAGATGTATGCAGTTGATTTT	AAAATCAACTGCATACATCTTGTCCCGCGACCAG
Tyr37His	GTCGCGGGACAAGCTGCACGCAGTTGATTTTTGG	CCAAAAATCAACTGCGTGCAGCTTGTCCCGCGAC
Tyr37Met	GTCGCGGGACAAGCTGATGGCAGTTGATTTTTGG	CCAAAAATCAACTGCCATCAGCTTGTCCCGCGAC
Tyr37Gln	GTCGCGGGACAAGCTGCAGGCAGTTGATTTTTGG	CCAAAAATCAACTGCCTGCAGCTTGTCCCGCGAC
Ala38Asp	CGGGACAAGCTGTATGATGTTGATTTTTGGGAC	GTCCCAAAAATCAACATCATACAGCTTGTCCCG

Table 7-2: List of all primers used for the site directed mutagenesis (SDM).

Primer name	Forward primer	Reverse primer
Ala38Arg	CGGGACAAGCTGTATCGGGTTGATTTTTGGGAC	GTCCCAAAAATCAACCCGATACAGCTTGTCCCG
Val39Cys	GACAAGCTGTATGCATGCGATTTTTGGGACAAG	CTTGTCCCAAAAATCGCATGCATACAGCTTGTC
Asp40Tyr	AAGCTGTATGCAGTTTATTTTTGGGACAAGACA	TGTCTTGTCCCAAAAATAAACTGCATACAGCTT
Asp40Ile	AAGCTGTATGCAGTTATTTTTTGGGACAAGACA	TGTCTTGTCCCAAAAAATAACTGCATACAGCTT
Phy41Val	CTGTATGCAGTTGATGTTTGGGACAAGACAGGC	GCCTGTCTTGTCCCAAACATCAACTGCATACAG
Trp42Asp	TATGCAGTTGATTTTGATGACAAGACAGGCACA	TGTGCCTGTCTTGTCATCAAAATCAACTGCATA
Trp42Glu	TATGCAGTTGATTTTGAAGACAAGACAGGCACA	TGTGCCTGTCTTGTCTTCAAAATCAACTGCATA
Trp42Ala	TATGCAGTTGATTTTGCGGACAAGACAGGCACA	TGTGCCTGTCTTGTCCGCAAAATCAACTGCATA
Trp42Gln	TATGCAGTTGATTTTCAGGACAAGACAGGCACA	TGTGCCTGTCTTGTCCTGAAAATCAACTGCATA
Asp43-Gln	GCAGTTGATTTTTGGCAGAAGACAGGCACAAAT	ATTTGTGCCTGTCTTCTGCCAAAAATCAACTGC
Lys44-Asp	GTTGATTTTTGGGACGATACAGGCACAAATTAT	ATAATTTGTGCCTGTATCGTCCCAAAAATCAAC
Lys44-Glu	GTTGATTTTTGGGACGAAACAGGCACAAATTAT	ATAATTTGTGCCTGTTTCGTCCCAAAAATCAAC
Lys44-Ile	GTTGATTTTTGGGACATTACAGGCACAAATTAT	ATAATTTGTGCCTGTAATGTCCCAAAAATCAAC
Lys44-Tyr	GTTGATTTTTGGGACTATACAGGCACAAATTAT	ATAATTTGTGCCTGTATAGTCCCAAAAATCAAC
Thr45Ile	GATTTTTGGGACAAGATTGGCACAAATTATAAC	GTTATAATTTGTGCCAATCTTGTCCCAAAAATC
Thr47Asp	GATTTTTGGGACAAGACAGGCGATAATTATAACAATGGA	TCCATTGTTATAATTATCGCCTGTCTTGTCCCAAAAATC
Thr47Ala	GATTTTTGGGACAAGACAGGCGCGAATTATAACAATGGA	TCCATTGTTATAATTCGCGCCTGTCTTGTCCCAAAAATC
Thr47Gln	GATTTTTGGGACAAGACAGGCCAGAATTATAACAATGGA	TCCATTGTTATAATTCTGGCCTGTCTTGTCCCAAAAATC
Thr47-Ile	GATTTTTGGGACAAGACAGGCATTAATTATAACAATGGA	TCCATTGTTATAATTAATGCCTGTCTTGTCCCAAAAATC
Asn50Met	ACAGGCACAAATTATATGAATGGACCGGTATTA	TAATACCGGTCCATTCATATAATTTGTGCCTGT
Asn51Asp	GGCACAAATTATAACGATGGACCGGTATTATCA	TGATAATACCGGTCCATCGTTATAATTTGTGCC
Asn51Cys	GGCACAAATTATAACTGCGGACCGGTATTATCA	TGATAATACCGGTCCGCAGTTATAATTTGTGCC
Gly52Met	CACAAATTATAACAATATGCCGGTATTATCACGA	TCGTGATAATACCGGCATATTGTTATAATTTGTG
Gly52Asn	CACAAATTATAACAATAATCCGGTATTATCACGA	TCGTGATAATACCGGATTATTGTTATAATTTGTG
Gly52Cys	CACAAATTATAACAATTGCCCGGTATTATCACGA	TCGTGATAATACCGGGCAATTGTTATAATTTGTG
Pro53Ile	ACAAATTATAACAATGGAATTGTATTATCACGATTTGTG	CACAAATCGTGATAATACAATTCCATTGTTATAATTTGT
Val54Ile	TATAACAATGGACCGATTTTATCACGATTTGTG	CACAAATCGTGATAAAATCGGTCCATTGTTATA
Leu55Asp	CAATGGACCGGTAGATTCACGATTTGTGCAA	TTGCACAAATCGTGAATCTACCGGTCCATTG
Leu55Ile	CAATGGACCGGTAATTTCACGATTTGTGCAA	TTGCACAAATCGTGAAATTACCGGTCCATTG
Leu55Met	CAATGGACCGGTAATGTCACGATTTGTGCAA	TTGCACAAATCGTGACATTACCGGTCCATTG
Arg57-Met	GGACCGGTATTATCAATGTTTGTGCAAAAGGTT	AACCTTTTGCACAAACATTGATAATACCGGTCC
Arg57-Trp	GGACCGGTATTATCATGGTTTGTGCAAAAGGTT	AACCTTTTGCACAAACCATGATAATACCGGTCC
Phe58-Asp	CCGGTATTATCACGAGATGTGCAAAAGGTTTTA	TAAAACCTTTTGCACATCTCGTGATAATACCGG
Phe58-Ala	CCGGTATTATCACGAGCGGTGCAAAAGGTTTTA	TAAAACCTTTTGCACCGCTCGTGATAATACCGG
Phe58-Gln	CCGGTATTATCACGACAGGTGCAAAAGGTTTTA	TAAAACCTTTTGCACCTGTCGTGATAATACCGG
Val59Asp	GTATTATCACGATTTGATCAAAAGGTTTTAGAT	ATCTAAAACCTTTTGATCAAATCGTGATAATAC
Val59Tyr	GTATTATCACGATTTTATCAAAAGGTTTTAGAT	ATCTAAAACCTTTTGATAAAATCGTGATAATAC
Gly60Asp	TTATCACGATTTGTGGATAAGGTTTTAGATGAA	TTCATCTAAAACCTTATCCACAAATCGTGATAA
Lys61Ile	CACGATTTGTGCAAATTGTTTTAGATGAAACG	CGTTTCATCTAAAACAATTTGCACAAATCGTG
Val62Asp	CGATTTGTGCAAAAGGATTTAGATGAAACGGGT	ACCCGTTTCATCTAAATCCTTTTGCACAAATCG
Val62Trp	CGATTTGTGCAAAAGTGGTTAGATGAAACGGGT	ACCCGTTTCATCTAACCACTTTTGCACAAATCG
Val62Cys	CGATTTGTGCAAAAGTGCTTAGATGAAACGGGT	ACCCGTTTCATCTAAGCACTTTTGCACAAATCG
Leu63Ile	GATTTGTGCAAAAGGTTATTGATGAAACGGGTGCG	CGCACCCGTTTCATCAATAACCTTTTGCACAAATC
Leu63Gln	GATTTGTGCAAAAGGTTCAGGATGAAACGGGTGCG	CGCACCCGTTTCATCCTGAACCTTTTGCACAAATC
Asp64Ile	GTGCAAAAGGTTTTAATTGAAACGGGTGCGAAA	TTTCGCACCCGTTTCAATTAAAACCTTTTGCAC
Glu65Asp	CAAAAGGTTTTAGATGATACGGGTGCGAAAAAA	TTTTTTCGCACCCGTATCATCTAAAACCTTTTG
Glu65Tyr	CAAAAGGTTTTAGATTATACGGGTGCGAAAAAA	TTTTTTCGCACCCGTATAATCTAAAACCTTTTG
Thr66Asp	GGTTTTAGATGAAGATGGTGCGAAAAAAGTG	CACTTTTTTCGCACCATCTTCATCTAAAACC
Thr66Cys	GGTTTTAGATGAATGCGGTGCGAAAAAAGTG	CACTTTTTTCGCACCGCATTCATCTAAAACC
Gly67Asp	GTTTTAGATGAAACGGATGCGAAAAAAGTGGAT	ATCCACTTTTTTCGCATCCGTTTCATCTAAAAC
Gly67Gln	GTTTTAGATGAAACGCAGGCGAAAAAAGTGGAT	ATCCACTTTTTTCGCCTGCGTTTCATCTAAAAC

Table 7-2: List of all primers used for the site directed mutagenesis (SDM).

Primer name	Forward primer	Reverse primer
Ala68-Lys	AGATGAAACGGGTAAGAAAAAAGTGGATATTGTCGC	GCGACAATATCCACTTTTTTCTTACCCGTTTCATCT
Ala68-Met	AGATGAAACGGGTATGAAAAAAGTGGATATTGTCGC	GCGACAATATCCACTTTTTTCATACCCGTTTCATCT
Lys69Trp	GATGAAACGGGTGCGTGGAAAGTGGATATTGTC	GACAATATCCACTTTCCACGCACCCGTTTCATC
Lys69Cys	GATGAAACGGGTGCGTGCAAAGTGGATATTGTC	GACAATATCCACTTTGCACGCACCCGTTTCATC
Lys70Asp	GAAACGGGTGCGAAAGATGTGGATATTGTCGCT	AGCGACAATATCCACATCTTTCGCACCCGTTTC
Lys70Ile	GAAACGGGTGCGAAAATTGTGGATATTGTCGCT	AGCGACAATATCCACAATTTTCGCACCCGTTTC
Lys70Met	GAAACGGGTGCGAAAATGGTGGATATTGTCGCT	AGCGACAATATCCACCATTTTCGCACCCGTTTC
Val71Asp	CGGGTGCGAAAAAAGATGATATTGTCGCTCACAG	CTGTGAGCGACAATATCATCTTTTTTCGCACCCG
His76Gln	GTGGATATTGTCGCTCAGAGCATGGGGGGCGCG	CGCGCCCCCCATGCTCTGAGCGACAATATCCAC
Ser77His	GATATTGTCGCTCACCACATGGGGGGCGCGAAC	GTTCGCGCCCCCCATGTGGTGAGCGACAATATC
Ser77Tyr	GATATTGTCGCTCACTATATGGGGGGCGCGAAC	GTTCGCGCCCCCCATATAGTGAGCGACAATATC
Ser77Cys	GATATTGTCGCTCACTGCATGGGGGGCGCGAAC	GTTCGCGCCCCCCATGCAGTGAGCGACAATATC
Met78Trp	GATATTGTCGCTCACAGCTGGGGGGGCGCGAACACA	TGTGTTCGCGCCCCCCCAGCTGTGAGCGACAATATC
Met78Cys	GATATTGTCGCTCACAGCTGCGGGGGCGCGAACACA	TGTGTTCGCGCCCCCGCAGCTGTGAGCGACAATATC
Gly79Asp	GTCGCTCACAGCATGGATGGCGCGAACACACTT	AAGTGTGTTCGCGCCATCCATGCTGTGAGCGAC
Gly79His	GTCGCTCACAGCATGCACGGCGCGAACACACTT	AAGTGTGTTCGCGCCGTGCATGCTGTGAGCGAC
Gly79lle	GTCGCTCACAGCATGATTGGCGCGAACACACTT	AAGTGTGTTCGCGCCAATCATGCTGTGAGCGAC
Gly79Trp	GTCGCTCACAGCATGTGGGGCGCGAACACACTT	AAGTGTGTTCGCGCCCCACATGCTGTGAGCGAC
Gly80Glu	GCTCACAGCATGGGGGAAGCGAACACACTTTAC	GTAAAGTGTGTTCGCTTCCCCCATGCTGTGAGC
Gly80lle	GCTCACAGCATGGGGATTGCGAACACACTTTAC	GTAAAGTGTGTTCGCAATCCCCATGCTGTGAGC
Gly80Met	GCTCACAGCATGGGGATGGCGAACACACTTTAC	GTAAAGTGTGTTCGCCATCCCCATGCTGTGAGC
Ala81Asp	CACAGCATGGGGGGCGATAACACACTTTACTAC	GTAGTAAAGTGTGTTATCGCCCCCCATGCTGTG
Ala81Gln	CACAGCATGGGGGGCCAGAACACACTTTACTAC	GTAGTAAAGTGTGTTCTGGCCCCCCATGCTGTG
Ala81cys	CACAGCATGGGGGGCTGCAACACACTTTACTAC	GTAGTAAAGTGTGTTGCAGCCCCCCATGCTGTG
Ala81-Phe	CACAGCATGGGGGGCTTTAACACACTTTACTAC	GTAGTAAAGTGTGTTAAAGCCCCCCATGCTGTG
Thr83Ala	CATGGGGGGCGCGAACGCGCTTTACTACATAAAA	TTTTATGTAGTAAAGCGCGTTCGCGCCCCCCATG
Thr83lle	CATGGGGGGCGCGAACATTCTTTACTACATAAAA	TTTTATGTAGTAAAGAATGTTCGCGCCCCCCATG
Thr83Ser	CATGGGGGGCGCGAACAGCCTTTACTACATAAAA	TTTTATGTAGTAAAGGCTGTTCGCGCCCCCCATG
Thr83Gln	CATGGGGGGCGCGAACCAGCTTTACTACATAAAA	TTTTATGTAGTAAAGCTGGTTCGCGCCCCCCATG
Thr83Cys	CATGGGGGGCGCGAACTGCCTTTACTACATAAAA	TTTTATGTAGTAAAGGCAGTTCGCGCCCCCCATG
Tyr85Met	GGCGCGAACACACTTATGTACATAAAAAATCTG	CAGATTTTTTATGTACATAAGTGTGTTCGCGCC
Tyr85Gln	GGCGCGAACACACTTCAGTACATAAAAAATCTG	CAGATTTTTTATGTACTGAAGTGTGTTCGCGCC
Tyr85Glu	GGCGCGAACACACTTGAATACATAAAAAATCTG	CAGATTTTTTATGTATTCAAGTGTGTTCGCGCC
Tyr85Ile	GGCGCGAACACACTTATTTACATAAAAAATCTG	CAGATTTTTTATGTAAATAAGTGTGTTCGCGCC
Tyr86Phe	GCGAACACACTTTACTTTATAAAAAATCTGGAC	GTCCAGATTTTTTATAAAGTAAAGTGTGTTCGC
Tyr86Gln	GCGAACACACTTTACCAGATAAAAAATCTGGAC	GTCCAGATTTTTTATCTGGTAAAGTGTGTTCGC
Ile87Gln	GAACACACTTTACTACCAGAAAAATCTGGACGGC	GCCGTCCAGATTTTTCTGGTAGTAAAGTGTGTTC
Lys88Trp	CACACTTTACTACATATGGAATCTGGACGGCGGA	TCCGCCGTCCAGATTCCATATGTAGTAAAGTGTG
Asn89Cys	CTTTACTACATAAAATGCCTGGACGGCGGAAAT	ATTTCCGCCGTCCAGGCATTTTATGTAGTAAAG
Asp91Lys	CTACATAAAAAATCTGAAGGGCGGAAATAAAGTT	AACTTTATTTCCGCCCTTCAGATTTTTTATGTAG
Gly92Gln	AAAAATCTGGACCAGGGAAATAAAGTT	AACTTTATTTCCCTGGTCCAGATTTTT
Gly92Cys	AAAAATCTGGACTGCGGAAATAAAGTT	AACTTTATTTCCGCAGTCCAGATTTTT
Gly93Asp	AATCTGGACGGCGATAATAAAGTTGCA	TGCAACTTTATTATCGCCGTCCAGATT
Gly93Glu	AATCTGGACGGCGAAAATAAAGTTGCA	TGCAACTTTATTTTCGCCGTCCAGATT
Gly93Lys	AATCTGGACGGCAAAAATAAAGTTGCA	TGCAACTTTATTTTTGCCGTCCAGATT
Gly93Ile	AATCTGGACGGCATTAATAAAGTTGCA	TGCAACTTTATTAATGCCGTCCAGATT
Gly93Gln	AATCTGGACGGCCAGAATAAAGTTGCA	TGCAACTTTATTCTGGCCGTCCAGATT
Gly93Cys	AATCTGGACGGCTGCAATAAAGTTGCA	TGCAACTTTATTGCAGCCGTCCAGATT
Asn94Cys	CTGGACGGCGGATGCAAAGTTGCAAAC	GTTTGCAACTTTGCATCCGCCGTCCAG
Asn94Glu	CTGGACGGCGGAGAAAAAGTTGCAAAC	GTTTGCAACTTTTTCTCCGCCGTCCAG
Asn94Lys	CTGGACGGCGGAAAAAAAGTTGCAAAC	GTTTGCAACTTTTTTTCCGCCGTCCAG

Table 7-2: List of all primers used for the site directed mutagenesis (SDM).

Primer name	Forward primer	Reverse primer
Asn94Trp	CTGGACGGCGGATGGAAAGTTGCAAAC	GTTTGCAACTTTCCATCCGCCGTCCAG
Asn94Thr	GGACGGCGGAACCAAAGTTGCAA	TTGCAACTTTGGTTCCGCCGTCC
Lys95His	GACGGCGGAAATCATGTTGCAAACGTC	GACGTTTGCAACATGATTTCCGCCGTC
Lys95Ile	GACGGCGGAAATATTGTTGCAAACGTC	GACGTTTGCAACAATATTTCCGCCGTC
Lys95Gln	GACGGCGGAAATCAGGTTGCAAACGTC	GACGTTTGCAACCTGATTTCCGCCGTC
Val96Asp	GGCGGAAATAAAGATGCAAACGTCGTG	CACGACGTTTGCATCTTTATTTCCGCC
Val96Lys	GGCGGAAATAAAAAAGCAAACGTCGTG	CACGACGTTTGCTTTTTTATTTCCGCC
Val96Gln	GGCGGAAATAAACAGGCAAACGTCGTG	CACGACGTTTGCCTGTTTATTTCCGCC
Ala97Asp	GGAAATAAAGTTGATAACGTCGTGACG	CGTCACGACGTTATCAACTTTATTTCC
Ala97His	GGAAATAAAGTTCATAACGTCGTGACG	CGTCACGACGTTATGAACTTTATTTCC
Ala97Asn	GGAAATAAAGTTAACAACGTCGTGACG	CGTCACGACGTTGTTAACTTTATTTCC
Asn98Lys	AATAAAGTTGCAAAAGTCGTGACGCTT	AAGCGTCACGACTTTTGCAACTTTATT
Asn98Thr	AATAAAGTTGCAACCGTCGTGACGCTT	AAGCGTCACGACGGTTGCAACTTTATT
Asn98Gln	AATAAAGTTGCACAGGTCGTGACGCTT	AAGCGTCACGACCTGTGCAACTTTATT
Val100His	GTTGCAAACGTCCATACGCTTGGCGGC	GCCGCCAAGCGTATGGACGTTTGCAAC
Val100Ala	GTTGCAAACGTCGCCACGCTTGGCGGC	GCCGCCAAGCGTGGCGACGTTTGCAAC
Val100Ile	GTTGCAAACGTCATTACGCTTGGCGGC	GCCGCCAAGCGTAATGACGTTTGCAAC
Thr101His	GCAAACGTCGTGCATCTTGGCGGCGCG	CGCGCCGCCAAGATGCACGACGTTTGC
Leu102Lys	CGTCGTGACGAAAGGCGGCGCGA	TCGCGCCGCCTTTCGTCACGACG
Ala105Cys	ACGCTTGGCGGCTGCAACCGTTTGACG	CGTCAAACGGTTGCAGCCGCCAAGCGT
Thr109Glu	GCGAACCGTTTGGAAACAGGCAAGGCG	CGCCTTGCCTGTTTCCAAACGGTTCGC
Thr109Lys	GCGAACCGTTTGAAAACAGGCAAGGCG	CGCCTTGCCTGTTTTCAAACGGTTCGC
Thr109Ile	GCGAACCGTTTGATTACAGGCAAGGCG	CGCCTTGCCTGTAATCAAACGGTTCGC
Thr109Asn	GAACCGTTTGAACACAGGCAAGG	CCTTGCCTGTGTTCAAACGGTTC
Lys112Asp	TTGACGACAGGCGATGCGCTTCCGGGA	TCCCGGAAGCGCATCGCCTGTCGTCAA
Lys112Glu	TTGACGACAGGCGAAGCGCTTCCGGGA	TCCCGGAAGCGCTTCGCCTGTCGTCAA
Ala113Cys	ACGACAGGCAAGTGCCTTCCGGGAACA	TGTTCCCGGAAGGCACTTGCCTGTCGT
Ala113His	ACGACAGGCAAGCATCTTCCGGGAACA	TGTTCCCGGAAGATGCTTGCCTGTCGT
Ala113Asn	ACGACAGGCAAGAACCTTCCGGGAACA	TGTTCCCGGAAGGTTCTTGCCTGTCGT
Ala113Gln	ACGACAGGCAAGCAGCTTCCGGGAACA	TGTTCCCGGAAGCTGCTTGCCTGTCGT
Leu114Glu	ACAGGCAAGGCGGAACCGGGAACAGAT	ATCTGTTCCCGGTTCCGCCTTGCCTGT
Leu114Trp	ACAGGCAAGGCGTGGCCGGGAACAGAT	ATCTGTTCCCGGCCACGCCTTGCCTGT
Pro115Trp	GGCAAGGCGCTTTGGGGAACAGATCCA	TGGATCTGTTCCCCAAAGCGCCTTGCC
Gly116Met	AAGGCGCTTCCGATGACAGATCCAAAT	ATTTGGATCTGTCATCGGAAGCGCCTT
Thr117Tyr	GCGCTTCCGGGATATGATCCAAATCAA	TTGATTTGGATCATATCCCGGAAGCGC
Asp118Ile	CTTCCGGGAACAATTCCAAATCAAAAG	CTTTTGATTTGGAATTGTTCCCGGAAG
Asp118Tyr	CTTCCGGGAACATATCCAAATCAAAAG	CTTTTGATTTGGATATGTTCCCGGAAG
Pro119Cys	CCGGGAACAGATTGCAATCAAAAGATT	AATCTTTTGATTGCAATCTGTTCCCGG
Asn120Glu	GGAACAGATCCAGAACAAAAGATTTTA	TAAAATCTTTTGTTCTGGATCTGTTCC
Asn120Arg	GGAACAGATCCACGCCAAAAGATTTTA	TAAAATCTTTTGGCGTGGATCTGTTCC
Gln121Ala	ACAGATCCAAATGCCAAGATTTTATAC	GTATAAAATCTTGGCATTTGGATCTGT
Gln121Met	ACAGATCCAAATATGAAGATTTTATAC	GTATAAAATCTTCATATTTGGATCTGT
Lys122Tyr	GATCCAAATCAATATATTTTATACACA	TGTGTATAAAATATATTGATTTGGATC
Lys122Cys	GATCCAAATCAATGCATTTTATACACA	TGTGTATAAAATGCATTGATTTGGATC
Lys122Asp	GATCCAAATCAAGATATTTTATACACA	TGTGTATAAAATATCTTGATTTGGATC
Lys122Ile	GATCCAAATCAAATTATTTTATACACA	TGTGTATAAAATAATTTGATTTGGATC
Lys122Phe	GATCCAAATCAATTTATTTTATACACA	TGTGTATAAAATAAATTGATTTGGATC
Ile123Tyr	CCAAATCAAAAGTATTTATACACATCC	GGATGTGTATAAATACTTTTGATTTGG
Leu124Trp	AATCAAAAGATTTGGTACACATCCATT	AATGGATGTGTACCAAATCTTTTGATT
Leu124Gln	AATCAAAAGATTCAGTACACATCCATT	AATGGATGTGTACTGAATCTTTTGATT
Leu124Glu	AATCAAAAGATTGAATACACATCCATT	AATGGATGTGTATTCAATCTTTTGATT
Leu124Val	AATCAAAAGATTGTGTACACATCCATT	AATGGATGTGTACACAATCTTTTGATT

Table 7-2: List of all primers used for the site directed mutagenesis (SDM).

Primer name	Forward primer	Reverse primer
Leu124Tyr	AATCAAAAGATTTATTACACATCCATT	AATGGATGTGTAATAAATCTTTTGATT
Tyr125Ile	CAAAAGATTTTAATTACATCCATTTAC	GTAAATGGATGTAATTAAAATCTTTTG
Tyr125Met	CAAAAGATTTTAATGACATCCATTTAC	GTAAATGGATGTCATTAAAATCTTTTG
Tyr125Gln	CAAAAGATTTTACAGACATCCATTTAC	GTAAATGGATGTCTGTAAAATCTTTTG
Thr126Asp	AAGATTTTATACGATTCCATTTACAGC	GCTGTAAATGGAATCGTATAAAATCTT
Thr126Met	AAGATTTTATACATGTCCATTTACAGC	GCTGTAAATGGACATGTATAAAATCTT
Thr126Phe	AAGATTTTATACTTTTCCATTTACAGC	GCTGTAAATGGAAAAGTATAAAATCTT
Thr126Lys	GATTTTATACAAATCCATTTACA	TGTAAATGGATTTGTATAAAATC
Thr126Cys	GATTTTATACTGCTCCATTTACA	TGTAAATGGAGCAGTATAAAATC
Ser127Gln	ATTTTATACACACAGATTTACAGCAGT	ACTGCTGTAAATCTGTGTGTATAAAAT
Ser127Glu	ATTTTATACACAGAAATTTACAGCAGT	ACTGCTGTAAATTTCTGTGTATAAAAT
Ser127Met	ATTTTATACACAATGATTTACAGCAGT	ACTGCTGTAAATCATTGTGTATAAAAT
Ser127Trp	ATTTTATACACATGGATTTACAGCAGT	ACTGCTGTAAATCCATGTGTATAAAAT
Ile128Asp	TTATACACATCCGATTACAGCAGTGCC	GGCACTGCTGTAATCGGATGTGTATAA
Ile128Cys	TTATACACATCCTGCTACAGCAGTGCC	GGCACTGCTGTAGCAGGATGTGTATAA
Tyr129His	TACACATCCATTCATAGCAGTGCCGAT	ATCGGCACTGCTATGAATGGATGTGTA
Tyr129Asn	TACACATCCATTAACAGCAGTGCCGAT	ATCGGCACTGCTGTTAATGGATGTGTA
Tyr129Cys	TACACATCCATTTGCAGCAGTGCCGAT	ATCGGCACTGCTGCAAATGGATGTGTA
Ser130Asp	ACATCCATTTACGATAGTGCCGATATG	CATATCGGCACTATCGTAAATGGATGT
Ser130Trp	ACATCCATTTACTGGAGTGCCGATATG	CATATCGGCACTCCAGTAAATGGATGT
Ser131Gln	TCCATTTACAGCCAGGCCGATATGATT	AATCATATCGGCCTGGCTGTAAATGGA
Ser131Ala	CATTTACAGCGCCGCCGATATGA	TCATATCGGCGGCGCTGTAAATG
Ala132Gln	ATTTACAGCAGTCAGGATATGATTGTC	GACAATCATATCCTGACTGCTGTAAAT
Ala132Lys	ATTTACAGCAGTAAAGATATGATTGTC	GACAATCATATCTTTACTGCTGTAAAT
Ala132Ile	ATTTACAGCAGTATTGATATGATTGTC	GACAATCATATCAATACTGCTGTAAAT
Ala132Met	ATTTACAGCAGTATGGATATGATTGTC	GACAATCATATCCATACTGCTGTAAAT
Met134Phe	AGCAGTGCCGATTTTATTGTCATGAAT	ATTCATGACAATAAAATCGGCACTGCT
Met134Tyr	AGCAGTGCCGATTATATTGTCATGAAT	ATTCATGACAATATAATCGGCACTGCT
Ile135Gln	AGTGCCGATATGCAGGTCATGAATTAC	GTAATTCATGACCTGCATATCGGCACT
Val136Trp	GCCGATATGATTTGGATGAATTACTTA	TAAGTAATTCATCCAAATCATATCGGC
Val136Cys	GCCGATATGATTTGCATGAATTACTTA	TAAGTAATTCATGCAAATCATATCGGC
Val136His	CGATATGATTCATATGAATTACT	AGTAATTCATATGAATCATATCG
Met137Asn	GATATGATTGTCAACAATTACTTATCA	TGATAAGTAATTGTTGACAATCATATC
Met137Cys	GATATGATTGTCTGCAATTACTTATCA	TGATAAGTAATTGCAGACAATCATATC
Met137Pro	GATATGATTGTCCCGAATTACTTATCA	TGATAAGTAATTCGGGACAATCATATC
Met137Asp	GATATGATTGTCGATAATTACTTATCA	TGATAAGTAATTATCGACAATCATATC
Met137His	GATATGATTGTCCATAATTACTTATCA	TGATAAGTAATTATGGACAATCATATC
Met137Tyr	GATATGATTGTCTATAATTACTTATCA	TGATAAGTAATTATAGACAATCATATC
Asn138Cys	ATGATTGTCATGTGCTACTTATCAAGA	TCTTGATAAGTAGCACATGACAATCAT
Tyr139Phe	ATTGTCATGAATTTTTTATCAAGATTA	TAATCTTGATAAAAAATTCATGACAAT
Tyr139Gln	ATTGTCATGAATCAGTTATCAAGATTA	TAATCTTGATAACTGATTCATGACAAT
Tyr139Pro	TGTCATGAATCCGTTATCAAGAT	ATCTTGATAACGGATTCATGACA
Tyr139Ile	TGTCATGAATATTTTATCAAGAT	ATCTTGATAAAATATTCATGACA
Tyr139Met	TGTCATGAATATGTTATCAAGAT	ATCTTGATAACATATTCATGACA
Leu140Gln	GTCATGAATTACCAGTCAAGATTAGAT	ATCTAATCTTGACTGGTAATTCATGAC
Arg142Phe	AATTACTTATCATTTTTAGATGGTGCT	AGCACCATCTAAAAATGATAAGTAATT
Leu143Asp	TACTTATCAAGAGATGATGGTGCTAGA	TCTAGCACCATCATCTCTTGATAAGTA
Asp144Phe	TTATCAAGATTATTTGGTGCTAGAAAC	GTTTCTAGCACCAAATAATCTTGATAA
Gly145Phe	TCAAGATTAGATTTTGCTAGAAACGTT	AACGTTTCTAGCAAAATCTAATCTTGA
Gly145Gln	TCAAGATTAGATCAGGCTAGAAACGTT	AACGTTTCTAGCCTGATCTAATCTTGA
Arg147Trp	TTAGATGGTGCTTGGAACGTTCAAATC	GATTTGAACGTTCCAAGCACCATCTAA
Arg147Glu	TTAGATGGTGCTGAAAACGTTCAAATC	GATTTGAACGTTTTCAGCACCATCTAA

Table 7-2: List of all primers used for the site directed mutagenesis (SDM).

Primer name	Forward primer	Reverse primer
Arg147Lys	TTAGATGGTGCTAAAAACGTTCAAATC	GATTTGAACGTTTTTAGCACCATCTAA
Arg147Met	TTAGATGGTGCTATGAACGTTCAAATC	GATTTGAACGTTCATAGCACCATCTAA
Asn148Trp	GATGGTGCTAGATGGGTTCAAATCCAT	ATGGATTTGAACCCATCTAGCACCATC
Asn148Val	TGGTGCTAGAGTGGTTCAAATCC	GGATTTGAACCACTCTAGCACCA
Val149Gln	GGTGCTAGAAACCAGCAAATCCATGGC	GCCATGGATTTGCTGGTTTCTAGCACC
Gln150Asn	GCTAGAAACGTTAACATCCATGGCGTT	AACGCCATGGATGTTAACGTTTCTAGC
Ile151Glu	AGAAACGTTCAAGAACATGGCGTTGGA	TCCAACGCCATGTTCTTGAACGTTTCT
Ile151Arg	AGAAACGTTCAACGCCATGGCGTTGGA	TCCAACGCCATGGCGTTGAACGTTTCT
Ile151Cys	AGAAACGTTCAATGCCATGGCGTTGGA	TCCAACGCCATGGCATTGAACGTTTCT
His152Asp	AACGTTCAAATCGATGGCGTTGGACAC	GTGTCCAACGCCATCGATTTGAACGTT
His152Cys	AACGTTCAAATCTGCGGCGTTGGACAC	GTGTCCAACGCCGCAGATTTGAACGTT
Val154His	AATCCATGGCCATGGACACATCG	CGATGTGTCCATGGCCATGGATT
His156Met	CATGGCGTTGGAATGATCGGCCTTCTG	CAGAAGGCCGATCATTCCAACGCCATG
His156Cys	CATGGCGTTGGATGCATCGGCCTTCTG	CAGAAGGCCGATGCATCCAACGCCATG
Ile157Gln	GGCGTTGGACACCAGGGCCTTCTGTAC	GTACAGAAGGCCCTGGTGTCCAACGCC
Ile157Asp	GGCGTTGGACACGATGGCCTTCTGTAC	GTACAGAAGGCCATCGTGTCCAACGCC
Ile157Glu	GGCGTTGGACACGAAGGCCTTCTGTAC	GTACAGAAGGCCTTCGTGTCCAACGCC
Ile157Met	GGCGTTGGACACATGGGCCTTCTGTAC	GTACAGAAGGCCCATGTGTCCAACGCC
Gly158Met	GTTGGACACATCATGCTTCTGTACAGC	GCTGTACAGAAGCATGATGTGTCCAAC
Gly158Phe	GTTGGACACATCTTTCTTCTGTACAGC	GCTGTACAGAAGAAAGATGTGTCCAAC
Gly158Asn	GTTGGACACATCAACCTTCTGTACAGC	GCTGTACAGAAGGTTGATGTGTCCAAC
Leu159Ile	GGACACATCGGCATTCTGTACAGCAGC	GCTGCTGTACAGAATGCCGATGTGTCC
Leu160Asp	CACATCGGCCTTGATTACAGCAGCCAA	TTGGCTGCTGTAATCAAGGCCGATGTG
Tyr161Asp	ATCGGCCTTCTGGATAGCAGCCAAGTC	GACTTGGCTGCTATCCAGAAGGCCGAT
Tyr161Val	ATCGGCCTTCTGGTGAGCAGCCAAGTC	GACTTGGCTGCTCACCAGAAGGCCGAT
Ser163Phe	CTTCTGTACAGCTTTCAAGTCAACAGC	GCTGTTGACTTGAAAGCTGTACAGAAG
Ser163Gln	CTTCTGTACAGCCAGCAAGTCAACAGC	GCTGTTGACTTGCTGGCTGTACAGAAG
Ser163Cys	CTTCTGTACAGCTGCCAAGTCAACAGC	GCTGTTGACTTGGCAGCTGTACAGAAG
Gln164Asp	CTGTACAGCAGCGATGTCAACAGCCTG	CAGGCTGTTGACATCGCTGCTGTACAG
Gln164Ile	CTGTACAGCAGCATTGTCAACAGCCTG	CAGGCTGTTGACAATGCTGCTGTACAG
Gln164Trp	CTGTACAGCAGCTGGGTCAACAGCCTG	CAGGCTGTTGACCCAGCTGCTGTACAG
Val165Gln	TACAGCAGCCAACAGAACAGCCTGATT	AATCAGGCTGTTCTGTTGGCTGCTGTA
Val165Cys	TACAGCAGCCAATGCAACAGCCTGATT	AATCAGGCTGTTGCATTGGCTGCTGTA
Asn166Glu	AGCAGCCAAGTCGAAAGCCTGATTAAA	TTTAATCAGGCTTTCGACTTGGCTGCT
Asn166Met	AGCAGCCAAGTCATGAGCCTGATTAAA	TTTAATCAGGCTCATGACTTGGCTGCT
Asn166Tyr	AGCAGCCAAGTCTATAGCCTGATTAAA	TTTAATCAGGCTATAGACTTGGCTGCT
Ser167Val	AGCCAAGTCAACGTGCTGATTAAAGAA	TTCTTTAATCAGCACGTTGACTTGGCT
Ser167Trp	AGCCAAGTCAACTGGCTGATTAAAGAA	TTCTTTAATCAGCCAGTTGACTTGGCT
Ser167Gln	AGCCAAGTCAACCAGCTGATTAAAGAA	TTCTTTAATCAGCTGGTTGACTTGGCT
Ser167Met	CCAAGTCAACATGCTGATTAAAG	CTTTAATCAGCATGTTGACTTGG
Leu168Thr	CAAGTCAACAGCACCATTAAAGAAGGG	CCCTTCTTTAATGGTGCTGTTGACTTG
Leu168Asn	CAAGTCAACAGCAACATTAAAGAAGGG	CCCTTCTTTAATGTTGCTGTTGACTTG
Ile169Glu	GTCAACAGCCTGGAAAAAGAAGGGCTG	CAGCCCTTCTTTTTCCAGGCTGTTGAC
Lys170Cys	AACAGCCTGATTTGCGAAGGGCTGAAC	GTTCAGCCCTTCGCAAATCAGGCTGTT
Lys170Gly	AACAGCCTGATTGGCGAAGGGCTGAAC	GTTCAGCCCTTCGCCAATCAGGCTGTT
Glu171Trp	AGCCTGATTAAATGGGGGCTGAACGGC	GCCGTTCAGCCCCCATTTAATCAGGCT
Glu171Gln	AGCCTGATTAAACAGGGGCTGAACGGC	GCCGTTCAGCCCCTGTTTAATCAGGCT
Gly172Phe	CTGATTAAAGAATTTCTGAACGGCGGG	CCCGCCGTTCAGAAATTCTTTAATCAG
Leu173Asp	ATTAAAGAAGGGGATAACGGCGGGGGC	GCCCCCGCCGTTATCCCCTTCTTTAAT
Leu173Lys	ATTAAAGAAGGGAAAAACGGCGGGGGC	GCCCCCGCCGTTTTTCCCTTCTTTAAT
Gly175Lys	GAAGGGCTGAACAAAGGGGGCCAGAAT	ATTCTGGCCCCCTTTGTTCAGCCCTTC
Gly175Asn	GAAGGGCTGAACAACGGGGGCCAGAAT	ATTCTGGCCCCCGTTGTTCAGCCCTTC

Table 7-2: List of all primers used for the site directed mutagenesis (SDM).

Primer name	Forward primer	Reverse primer
Gly176Lys	GGGCTGAACGGCAAAGGCCAGAATACG	CGTATTCTGGCCTTTGCCGTTCAGCCC
Gly176Cys	GGGCTGAACGGCTGCGGCCAGAATACG	CGTATTCTGGCCGCAGCCGTTCAGCCC
Gly177Asp	CTGAACGGCGGGGATCAGAATACGAAT	ATTCGTATTCTGATCCCCGCCGTTCAG
Gly177Gln	CTGAACGGCGGGCAGCAGAATACGAAT	ATTCGTATTCTGCTGCCCGCCGTTCAG
Gly177Cys	CTGAACGGCGGGTGCCAGAATACGAAT	ATTCGTATTCTGGCACCCGCCGTTCAG
Gly177His	GAACGGCGGGCATCAGAATACGA	TCGTATTCTGATGCCCGCCGTTC
Gln178His	AACGGCGGGGGCCATAATACGAATTAA	TTAATTCGTATTATGGCCCCCGCCGTT
Asn179Ala	GGCGGGGGCCAGGCCACGAATTAATGA	TCATTAATTCGTGGCCTGGCCCCCGCC
Asn179Met	GGCGGGGGCCAGATGACGAATTAATGA	TCATTAATTCGTCATCTGGCCCCCGCC
Thr180Gln	GGGGGCCAGAATCAGAATTAATGAGAG	CTCTCATTAATTCTGATTCTGGCCCCC
Thr180Gly	GGGGGCCAGAATGGCAATTAATGAGAG	CTCTCATTAATTGCCATTCTGGCCCCC
Asn181Glu	GGCCAGAATACGGAATAATGAGAGCTC	GAGCTCTCATTATTCCGTATTCTGGCC
Asn181Met	GGCCAGAATACGATGTAATGAGAGCTC	GAGCTCTCATTACATCGTATTCTGGCC

Table 7-3: Number of amino acid substitutions in BSLA which lead to increased/unchanged/decreased resistance towards [BMIM][Cl] and inactivation of BSLA. The secondary structure characteristic (loop, helix and β-sheet) of every position is shown. Loops are labelled in black, sheets in white and helix in light grey. The results in the table are given with a color scale from dark grey (highest values) to light grey (lowest values.). "**+**": improved resistance; "**=**": unchanged resistance**;** "**-**": decreased resistance and "**x**" inactivated.

[BMIM][Cl]

BSLA secondary structure	BSLA amino acid positions	Number of amino acid substitutions with +	=	-	x
loop	Ala1	2	18	0	0
loop	Glu2	0	19	1	0
loop	HIS3	0	19	1	0
loop	ASN4	1	18	1	0
loop	PRO5	5	8	5	2
Sheet	VAL6	2	11	2	5
Sheet	VAL7	3	2	0	15
Sheet	MET8	1	8	7	4
Sheet	VAL9	2	9	4	5
loop	HIS10	7	5	8	0
loop	GLY11	0	1	1	18
loop	ILE12	10	5	1	4
loop	GLY13	0	8	12	0
loop	GLY14	8	5	4	3
loop	ALA15	0	17	2	1
Helix H1	SER16	0	19	1	0
Helix H1	PHE17	15	5	0	0
Helix H1	ASN18	6	11	2	1
Helix H1	PHE19	0	8	11	1
Helix H1	ALA20	0	15	5	0
Helix H1	GLY21	0	17	3	0
Helix H1	ILE22	5	13	0	2
Helix H1	LYS23	7	9	3	1
Helix H1	SER24	0	17	3	0
Helix H1	TYR25	0	18	1	1
Helix H1	LEU26	0	7	3	10
Helix H1	VAL27	2	16	1	1
Helix H1	SER28	2	14	4	0
loop	GLN29	1	18	0	1
loop	GLY30	0	3	15	2
loop	TRP31	1	16	1	2
loop	SER32	1	18	1	0
Helix H3	ARG33	0	20	0	0
Helix H3	ASP34	2	7	10	1
Helix H3	LYS35	0	16	1	3
Sheet	LEU36	4	12	2	2
Sheet	TYR37	0	19	1	0
Sheet	ALA38	0	18	2	0
loop	VAL39	1	19	0	0
loop	ASP40	0	18	2	0
loop	PHE41	4	4	10	2
loop	TRP42	0	19	1	0
loop	ASP43	0	20	0	0
loop	LYS44	1	19	0	0
loop	THR45	1	19	0	0
loop	GLY46	4	12	4	0
loop	THR47	4	16	0	0
Helix H4	ASN48	4	16	0	0
Helix H4	TYR49	1	19	0	0
Helix H4	ASN50	1	19	0	0
Helix H4	ASN51	2	17	1	0
loop	GLY52	1	3	16	0
Helix H4	PRO53	0	20	0	0
Helix H4	VAL54	13	6	0	1
Helix H4	LEU55	4	7	0	9
Helix H4	SER56	0	18	2	0
Helix H4	ARG57	2	10	8	0
Helix H4	PHE58	12	7	0	1
Helix H4	VAL59	1	3	4	12
Helix H4	GLN60	0	8	12	0
Helix H4	LYS61	2	15	2	1
Helix H4	VAL62	0	8	10	2
Helix H4	LEU63	1	15	3	1
Helix H4	ASP64	6	11	2	1
Helix H4	GLU65	2	14	4	0
Helix H4	THR66	3	15	1	1
loop	GLY67	0	15	4	1
loop	ALA68	1	14	5	0
loop	LYS69	0	20	0	0
loop	LYS70	9	6	4	1
Sheet	VAL71	0	1	8	11
Sheet	ASP72	0	8	6	6
Sheet	ILE73	0	2	2	16
Sheet	VAL74	0	6	1	13
Sheet	ALA75	0	8	2	10
Sheet	HIS76	0	1	1	18
loop	SER77	0	1	0	19
Helix H5	MET78	6	6	0	8
Helix H5	GLY79	0	2	1	17
Helix H5	GLY80	2	3	1	14
Helix H5	ALA81	9	8	2	1
Helix H5	ASN82	0	9	9	2
Helix H5	THR83	1	14	2	3
Helix H5	LEU84	7	11	0	2
Helix H5	TYR85	0	18	1	1
Helix H5	TYR86	0	16	0	4
Helix H5	ILE87	2	9	1	8
Helix H5	LYS88	1	15	3	1
loop	ASN89	3	9	4	4
loop	LEU90	14	3	3	0
loop	ASP91	3	10	5	2

[BMIM][Cl]

BSLA secondary structure	BSLA amino acid positions	Number of amino acid substitutions with +	=	-	x
Helix H6	GLY92	0	7	13	0
Helix H6	GLY93	1	19	0	0
Helix H6	ASN94	3	17	0	0
loop	LYS95	9	10	1	0
Sheet	VAL96	0	10	2	8
Sheet	ALA97	2	16	1	1
Sheet	ASN98	3	15	2	0
Sheet	VAL99	3	12	1	4
Sheet	VAL100	0	10	2	8
Sheet	THR101	4	14	1	1
Sheet	LEU102	2	8	1	9
loop	GLY103	0	2	3	15
loop	GLY104	6	7	1	6
loop	ALA105	7	10	1	2
Helix H7	ASN106	1	14	4	1
Helix H7	ARG107	8	9	0	3
Helix H7	LEU108	2	15	2	1
loop	THR109	4	13	3	0
loop	THR110	1	17	2	0
loop	GLY111	1	13	6	0
loop	LYS112	2	17	1	0
loop	ALA113	0	19	1	0
loop	LEU114	3	17	0	0
loop	PRO115	0	20	0	0
loop	GLY116	2	18	0	0
loop	THR117	1	12	4	3
loop	ASP118	1	19	0	0
loop	PRO119	12	7	1	0
loop	ASN120	1	19	0	0
loop	GLN121	1	19	0	0
loop	LYS122	1	18	0	1
loop	ILE123	0	11	5	4
Sheet	LEU124	12	8	0	0
Sheet	TYR125	0	11	2	7
Sheet	THR126	1	12	5	2
Sheet	SER127	3	12	1	4
Sheet	ILE128	1	11	1	7
Sheet	TYR129	5	10	2	3
Sheet	SER130	2	12	4	2
loop	SER131	0	20	0	0
loop	ALA132	4	16	0	0
loop	ASP133	1	1	0	18
loop	MET134	4	16	0	0
loop	ILE135	5	14	1	0
loop	VAL136	1	11	2	6
loop	MET137	7	13	0	0
Helix H8	ASN138	6	13	0	1
Helix H8	TYR139	1	13	2	4
Helix H8	LEU140	4	16	0	0
Helix H8	SER141	2	4	5	9
loop	ARG142	1	19	0	0
loop	LEU143	3	4	6	7
loop	ASP144	3	17	0	0
loop	GLY145	7	13	0	0
loop	ALA146	0	17	2	1
Sheet	ARG147	4	15	0	1
Sheet	ASN148	0	14	5	1
Sheet	VAL149	0	20	0	0
Sheet	GLN150	3	17	0	0
Sheet	ILE151	7	11	2	0
loop	HIS152	5	14	0	1
loop	GLY153	0	19	1	0
loop	VAL154	5	10	5	0
loop	GLY155	17	3	0	0
loop	HIS156	0	1	0	19
loop	ILE157	2	11	3	4
Helix H9	GLY158	1	19	0	0
Helix H9	LEU159	7	12	0	1
Helix H9	LEU160	1	10	3	6
Helix H9	TYR161	3	16	0	1
loop	SER162	1	18	1	0
Helix H10	SER163	1	19	0	0
Helix H10	GLN164	3	14	3	0
Helix H10	VAL165	7	10	0	3
Helix H10	ASN166	1	2	11	6
Helix H10	SER167	5	12	2	1
Helix H10	LEU168	3	4	3	10
Helix H10	ILE169	0	1	1	18
Helix H10	LYS170	1	18	0	1
Helix H10	GLU171	6	12	0	2
Helix H10	GLY172	1	19	0	0
Helix H10	LEU173	0	8	6	6
loop	ASN174	2	18	0	0
loop	GLY175	0	20	0	0
loop	GLY176	0	19	1	0
loop	GLY177	0	20	0	0
loop	GLN178	1	17	2	0
loop	ASN179	3	16	0	1
loop	THR180	0	20	0	0
loop	ASN181	3	17	0	0
	Total	124	181	117	99

Table 7-4: Number of amino acid substitutions in BSLA which lead to increased/unchanged/decreased resistance towards [BMIM][Br] and inactivation of BSLA. The secondary structure characteristic (loop, helix and β-sheet) of every position is shown. Loops are labelled in black, sheets in white and helix in light grey. The results in the table are given with a color scale from dark grey (highest values) to light grey (lowest values.). "**+**": improved resistance; "**=**": unchanged resistance**;** "**-**": decreased resistance and "**x**" inactivated.

[BMIM][Br]

BSLA secondary structure	BSLA amino acid positions	Number of amino acid substitutions with +	=	-	x
loop	Ala1	1	19	0	0
loop	Glu2	0	20	0	0
loop	HIS3	0	20	0	0
loop	ASN4	0	20	0	0
loop	PRO5	4	10	4	2
Sheet	VAL6	1	11	3	5
Sheet	VAL7	1	4	0	15
Sheet	MET8	0	8	8	4
Sheet	VAL9	0	5	10	5
loop	HIS10	1	8	11	0
loop	GLY11	0	1	1	18
loop	ILE12	8	8	0	4
loop	GLY13	0	18	2	0
loop	GLY14	0	11	6	3
loop	ALA15	0	18	1	1
Helix H1	SER16	0	20	0	0
Helix H1	PHE17	5	15	0	0
Helix H1	ASN18	1	17	1	1
Helix H1	PHE19	0	7	12	1
Helix H1	ALA20	0	20	0	0
Helix H1	GLY21	2	18	0	0
Helix H1	ILE22	0	14	4	2
Helix H1	LYS23	3	16	0	1
Helix H1	SER24	0	20	0	0
Helix H1	TYR25	0	19	0	1
Helix H1	LEU26	0	10	0	10
Helix H1	VAL27	0	19	0	1
Helix H1	SER28	1	18	1	0
loop	GLN29	1	18	0	1
loop	GLY30	0	17	1	2
loop	TRP31	0	18	0	2
loop	SER32	0	20	0	0
Helix H3	ARG33	0	20	0	0
Helix H3	ASP34	2	11	6	1
Helix H3	LYS35	0	17	0	3
Sheet	LEU36	0	17	1	2
Sheet	TYR37	0	20	0	0
Sheet	ALA38	0	19	1	0
loop	VAL39	1	19	0	0
loop	ASP40	0	20	0	0
loop	PHE41	1	5	12	2
loop	TRP42	0	20	0	0
loop	ASP43	0	20	0	0
loop	LYS44	0	20	0	0
loop	THR45	0	20	0	0
loop	GLY46	3	16	1	0
loop	THR47	3	17	0	0
Helix H4	ASN48	1	19	0	0
Helix H4	TYR49	2	18	0	0
Helix H4	ASN50	1	19	0	0
Helix H4	ASN51	1	19	0	0
loop	GLY52	0	10	10	0
Helix H4	PRO53	0	20	0	0
Helix H4	VAL54	6	13	0	1
Helix H4	LEU55	1	10	0	9
Helix H4	SER56	0	18	2	0
Helix H4	ARG57	1	15	4	0
Helix H4	PHE58	5	14	0	1
Helix H4	VAL59	0	5	3	12
Helix H4	GLN60	0	15	5	0
Helix H4	LYS61	0	18	1	1
Helix H4	VAL62	0	4	14	2
Helix H4	LEU63	0	15	4	1
Helix H4	ASP64	1	18	0	1
Helix H4	GLU65	2	18	0	0
Helix H4	THR66	0	18	1	1
loop	GLY67	0	18	1	1
loop	ALA68	0	20	0	0
loop	LYS69	0	20	0	0
loop	LYS70	1	18	0	1
Sheet	VAL71	0	1	8	11
Sheet	ASP72	0	9	5	6
Sheet	ILE73	1	1	2	16
Sheet	VAL74	1	5	1	13
Sheet	ALA75	0	6	4	10
Sheet	HIS76	1	1	0	18
loop	SER77	0	1	0	19
Helix H5	MET78	4	8	0	8
Helix H5	GLY79	0	2	1	17
Helix H5	GLY80	1	5	0	14
Helix H5	ALA81	5	13	1	1
Helix H5	ASN82	0	8	10	2
Helix H5	THR83	0	13	4	3
Helix H5	LEU84	1	11	6	2
Helix H5	TYR85	0	19	0	1
Helix H5	TYR86	0	10	6	4
Helix H5	ILE87	0	10	2	8
Helix H5	LYS88	1	18	0	1
loop	ASN89	2	14	0	4
loop	LEU90	3	15	2	0
loop	ASP91	6	11	1	2

[BMIM][Br]

BSLA secondary structure	BSLA amino acid positions	Number of amino acid substitutions with +	=	-	x
Helix H6	GLY92	0	4	16	0
Helix H6	GLY93	1	19	0	0
Helix H6	ASN94	1	19	0	0
loop	LYS95	9	11	0	0
Sheet	VAL96	0	11	1	8
Sheet	ALA97	1	18	0	1
Sheet	ASN98	1	19	0	0
Sheet	VAL99	0	9	7	4
Sheet	VAL100	0	9	3	8
Sheet	THR101	0	18	1	1
Sheet	LEU102	0	10	1	9
loop	GLY103	0	3	2	15
loop	GLY104	0	8	6	6
loop	ALA105	1	16	1	2
Helix H7	ASN106	1	15	3	1
Helix H7	ARG107	7	10	0	3
Helix H7	LEU108	1	17	1	1
loop	THR109	3	16	1	0
loop	THR110	1	18	1	0
loop	GLY111	0	20	0	0
loop	LYS112	2	18	0	0
loop	ALA113	0	19	1	0
loop	LEU114	1	19	0	0
loop	PRO115	0	20	0	0
loop	GLY116	0	20	0	0
loop	THR117	3	14	0	3
loop	ASP118	1	19	0	0
loop	PRO119	1	16	3	0
loop	ASN120	1	19	0	0
loop	GLN121	0	19	1	0
loop	LYS122	1	18	0	1
loop	ILE123	0	10	6	4
Sheet	LEU124	8	11	1	0
Sheet	TYR125	0	10	3	7
Sheet	THR126	1	14	3	2
Sheet	SER127	0	15	1	4
Sheet	ILE128	1	6	6	7
Sheet	TYR129	4	7	6	3
Sheet	SER130	2	9	7	2
loop	SER131	0	20	0	0
loop	ALA132	2	18	0	0
loop	ASP133	1	1	0	18
loop	MET134	2	18	0	0
loop	ILE135	5	15	0	0
loop	VAL136	1	10	3	6
loop	MET137	3	17	0	0
Helix H8	ASN138	6	6	7	1
Helix H8	TYR139	5	10	1	4
Helix H8	LEU140	2	18	0	0
Helix H8	SER141	1	5	5	9
loop	ARG142	0	18	2	0
loop	LEU143	1	7	5	7
loop	ASP144	0	20	0	0
loop	GLY145	1	18	1	0
loop	ALA146	0	18	1	1
Sheet	ARG147	1	18	0	1
Sheet	ASN148	0	15	4	1
Sheet	VAL149	0	20	0	0
Sheet	GLN150	0	20	0	0
Sheet	ILE151	1	17	2	0
loop	HIS152	4	15	0	1
loop	GLY153	0	19	1	0
loop	VAL154	1	17	2	0
loop	GLY155	9	11	0	0
loop	HIS156	0	1	0	19
loop	ILE157	0	14	2	4
Helix H9	GLY158	2	18	0	0
Helix H9	LEU159	2	17	0	1
Helix H9	LEU160	0	12	2	6
Helix H9	TYR161	3	16	0	1
loop	SER162	0	20	0	0
Helix H10	SER163	1	19	0	0
Helix H10	GLN164	0	19	1	0
Helix H10	VAL165	1	15	1	3
Helix H10	ASN166	1	7	6	6
Helix H10	SER167	2	17	0	1
Helix H10	LEU168	0	9	1	10
Helix H10	ILE169	0	1	1	18
Helix H10	LYS170	1	18	0	1
Helix H10	GLU171	3	15	0	2
Helix H10	GLY172	1	17	2	0
Helix H10	LEU173	0	6	8	6
loop	ASN174	0	20	0	0
loop	GLY175	0	20	0	0
loop	GLY176	0	20	0	0
loop	GLY177	0	20	0	0
loop	GLN178	1	18	1	0
loop	ASN179	2	17	0	1
loop	THR180	0	20	0	0
loop	ASN181	0	20	0	0
	Total	91	181	89	99

Table 7-5: Number of amino acid substitutions in BSLA which lead to increased/unchanged/decreased resistance towards [BMIM][I] and inactivation of BSLA. The secondary structure characteristic (loop, helix and β-sheet) of every position is shown. Loops are labelled in black, sheets in white and helix in light grey. The results in the table are given with a color scale from dark grey (highest values) to light grey (lowest values.). "**+**": improved resistance; "**=**": unchanged resistance**;** "**-**": decreased resistance and "**x**" inactivated.

[BMIM][I]

BSLA secondary structure	BSLA amino acid positions	Number of amino acid substitutions with +	=	-	x
loop	Ala1	3	17	0	0
loop	Glu2	0	19	1	0
loop	HIS3	0	20	0	0
loop	ASN4	0	20	0	0
loop	PRO5	2	13	3	2
Sheet	VAL6	0	8	7	5
Sheet	VAL7	1	4	0	15
Sheet	MET8	0	4	12	4
Sheet	VAL9	0	4	11	5
loop	HIS10	0	1	19	0
loop	GLY11	0	1	1	18
loop	ILE12	7	7	2	4
loop	GLY13	0	8	12	0
loop	GLY14	0	5	12	3
loop	ALA15	4	14	1	1
Helix H1	SER16	0	19	1	0
Helix H1	PHE17	3	16	1	0
Helix H1	ASN18	0	15	4	1
Helix H1	PHE19	0	5	14	1
Helix H1	ALA20	0	20	0	0
Helix H1	GLY21	0	19	1	0
Helix H1	ILE22	0	12	6	2
Helix H1	LYS23	3	16	0	1
Helix H1	SER24	1	17	2	0
Helix H1	TYR25	0	18	1	1
Helix H1	LEU26	0	3	7	10
Helix H1	VAL27	0	18	1	1
Helix H1	SER28	2	16	2	0
loop	GLN29	2	16	1	1
loop	GLY30	0	7	11	2
loop	TRP31	0	10	8	2
loop	SER32	0	20	0	0
Helix H3	ARG33	0	20	0	0
Helix H3	ASP34	2	10	7	1
Helix H3	LYS35	0	16	1	3
Sheet	LEU36	0	8	10	2
Sheet	TYR37	0	18	2	0
Sheet	ALA38	1	16	3	0
loop	VAL39	1	19	0	0
loop	ASP40	0	17	3	0
loop	PHE41	0	5	13	2
loop	TRP42	0	20	0	0
loop	ASP43	0	20	0	0
loop	LYS44	2	18	0	0
loop	THR45	1	19	0	0
loop	GLY46	2	15	3	0
loop	THR47	9	11	0	0
Helix H4	ASN48	3	17	0	0
Helix H4	TYR49	3	17	0	0
Helix H4	ASN50	4	16	0	0
Helix H4	ASN51	2	16	2	0
loop	GLY52	1	5	14	0
Helix H4	PRO53	1	19	0	0
Helix H4	VAL54	10	9	0	1
Helix H4	LEU55	0	7	4	9
Helix H4	SER56	0	18	2	0
Helix H4	ARG57	1	15	4	0
Helix H4	PHE58	5	14	0	1
Helix H4	VAL59	2	5	1	12
Helix H4	GLN60	0	8	12	0
Helix H4	LYS61	0	18	1	1
Helix H4	VAL62	0	4	14	2
Helix H4	LEU63	0	13	6	1
Helix H4	ASP64	0	19	0	1
Helix H4	GLU65	1	19	0	0
Helix H4	THR66	0	17	2	1
loop	GLY67	0	15	4	1
loop	ALA68	0	18	2	0
loop	LYS69	0	20	0	0
loop	LYS70	2	15	2	1
Sheet	VAL71	0	1	8	11
Sheet	ASP72	0	6	8	6
Sheet	ILE73	0	2	2	16
Sheet	VAL74	0	5	2	13
Sheet	ALA75	0	4	6	10
Sheet	HIS76	1	1	0	18
loop	SER77	0	1	0	19
Helix H5	MET78	4	7	1	8
Helix H5	GLY79	0	2	1	17
Helix H5	GLY80	0	3	3	14
Helix H5	ALA81	3	13	3	1
Helix H5	ASN82	0	12	6	2
Helix H5	THR83	0	11	6	3
Helix H5	LEU84	0	12	6	2
Helix H5	TYR85	0	18	1	1
Helix H5	TYR86	0	7	9	4
Helix H5	ILE87	0	9	3	8
Helix H5	LYS88	1	18	0	1
loop	ASN89	1	15	0	4
loop	LEU90	2	16	2	0
loop	ASP91	6	10	2	2
Helix H6	GLY92	0	3	17	0
Helix H6	GLY93	2	15	3	0
Helix H6	ASN94	4	16	0	0
loop	LYS95	9	10	1	0
Sheet	VAL96	0	8	4	8
Sheet	ALA97	3	16	0	1
Sheet	ASN98	3	16	1	0
Sheet	VAL99	0	7	9	4
Sheet	VAL100	0	9	3	8
Sheet	THR101	2	13	4	1
Sheet	LEU102	0	5	6	9
loop	GLY103	0	2	3	15
loop	GLY104	0	3	11	6
loop	ALA105	1	10	7	2
Helix H7	ASN106	0	11	8	1
Helix H7	ARG107	10	7	0	3
Helix H7	LEU108	0	10	9	1
loop	THR109	3	16	1	0
loop	THR110	1	18	1	0
loop	GLY111	1	19	0	0
loop	LYS112	2	18	0	0
loop	ALA113	0	11	9	0
loop	LEU114	2	18	0	0
loop	PRO115	0	20	0	0
loop	GLY116	0	16	4	0
loop	THR117	0	14	3	3
loop	ASP118	1	19	0	0
loop	PRO119	4	13	3	0
loop	ASN120	1	19	0	0
loop	GLN121	0	19	1	0
loop	LYS122	4	15	0	1
loop	ILE123	0	8	8	4
Sheet	LEU124	10	10	0	0
Sheet	TYR125	0	8	5	7
Sheet	THR126	1	13	4	2
Sheet	SER127	0	13	3	4
Sheet	ILE128	0	6	7	7
Sheet	TYR129	3	6	8	3
Sheet	SER130	1	10	7	2
loop	SER131	2	17	1	0
loop	ALA132	4	16	0	0
loop	ASP133	1	1	0	18
loop	MET134	8	12	0	0
loop	ILE135	8	12	0	0
loop	VAL136	1	9	4	6
loop	MET137	6	14	0	0
Helix H8	ASN138	3	16	0	1
Helix H8	TYR139	1	13	2	4
Helix H8	LEU140	1	19	0	0
Helix H8	SER141	2	3	6	9
loop	ARG142	0	20	0	0
loop	LEU143	1	6	6	7
loop	ASP144	2	18	0	0
loop	GLY145	2	14	4	0
loop	ALA146	0	17	2	1
Sheet	ARG147	2	17	0	1
Sheet	ASN148	0	14	5	1
Sheet	VAL149	1	19	0	0
Sheet	GLN150	2	18	0	0
Sheet	ILE151	2	16	2	0
loop	HIS152	7	12	0	1
loop	GLY153	0	19	1	0
loop	VAL154	1	17	2	0
loop	GLY155	9	11	0	0
loop	HIS156	0	1	0	19
loop	ILE157	0	13	3	4
Helix H9	GLY158	3	17	0	0
Helix H9	LEU159	2	15	2	1
Helix H9	LEU160	0	6	8	6
Helix H9	TYR161	3	16	0	1
loop	SER162	1	19	0	0
Helix H10	SER163	1	19	0	0
Helix H10	GLN164	2	17	1	0
Helix H10	VAL165	0	16	1	3
Helix H10	ASN166	4	6	4	6
Helix H10	SER167	1	17	1	1
Helix H10	LEU168	1	8	1	10
Helix H10	ILE169	0	1	1	18
Helix H10	LYS170	1	18	0	1
Helix H10	GLU171	4	14	0	2
Helix H10	GLY172	1	16	3	0
Helix H10	LEU173	0	5	9	6
loop	ASN174	1	19	0	0
loop	GLY175	0	20	0	0
loop	GLY176	0	20	0	0
loop	GLY177	2	18	0	0
loop	GLN178	1	16	3	0
loop	ASN179	1	18	0	1
loop	THR180	1	19	0	0
loop	ASN181	0	20	0	0
	Total	95	181	115	99

Table 7-6: Number of amino acid substitutions in BSLA which lead to increased/unchanged/decreased resistance towards [BMIM][TfO] and inactivation of BSLA. The secondary structure characteristic (loop, helix and β-sheet) of every position is shown. Loops are labelled in black, sheets in white and helix in light grey. The results in the table are given with a color scale from dark grey (highest values) to light grey (lowest values.). "+": improved resistance; "**=**": unchanged resistance**;** "**-**": decreased resistance and "**x**" inactivated.

[BMIM][TfO]

BSLA secondary structure	BSLA amino acid positions	Number of amino acid substitutions with +	=	-	x
loop	Ala1	2	18	0	0
loop	Glu2	0	19	1	0
loop	HIS3	0	20	0	0
loop	ASN4	0	20	0	0
loop	PRO5	5	9	4	2
Sheet	VAL6	2	11	2	5
Sheet	VAL7	1	4	0	15
Sheet	MET8	0	15	1	4
Sheet	VAL9	0	5	10	5
loop	HIS10	0	6	14	0
loop	GLY11	0	1	1	18
loop	ILE12	6	10	0	4
loop	GLY13	0	17	3	0
loop	GLY14	0	12	5	3
loop	ALA15	1	17	1	1
Helix H1	SER16	0	20	0	0
Helix H1	PHE17	5	15	0	0
Helix H1	ASN18	3	15	1	1
Helix H1	PHE19	0	7	12	1
Helix H1	ALA20	0	18	2	0
Helix H1	GLY21	0	20	0	0
Helix H1	ILE22	0	13	5	2
Helix H1	LYS23	5	13	1	1
Helix H1	SER24	0	20	0	0
Helix H1	TYR25	0	18	1	1
Helix H1	LEU26	3	7	0	10
Helix H1	VAL27	1	18	0	1
Helix H1	SER28	0	20	0	0
loop	GLN29	2	17	0	1
loop	GLY30	0	15	3	2
loop	TRP31	0	15	3	2
loop	SER32	3	17	0	0
Helix H3	ARG33	0	20	0	0
Helix H3	ASP34	3	15	1	1
Helix H3	LYS35	0	17	0	3
Sheet	LEU36	2	15	1	2
Sheet	TYR37	0	20	0	0
Sheet	ALA38	0	18	2	0
loop	VAL39	1	19	0	0
loop	ASP40	0	19	1	0
loop	PHE41	1	9	8	2
loop	TRP42	0	20	0	0
loop	ASP43	0	20	0	0
loop	LYS44	2	18	0	0
loop	THR45	0	20	0	0
loop	GLY46	4	13	3	0
loop	THR47	6	14	0	0
Helix H4	ASN48	3	17	0	0
Helix H4	TYR49	2	18	0	0
Helix H4	ASN50	1	19	0	0
Helix H4	ASN51	2	17	1	0
loop	GLY52	1	6	13	0
Helix H4	PRO53	0	20	0	0
Helix H4	VAL54	9	10	0	1
Helix H4	LEU55	1	10	0	9
Helix H4	SER56	0	18	2	0
Helix H4	ARG57	3	17	0	0
Helix H4	PHE58	3	16	0	1
Helix H4	VAL59	0	5	3	12
Helix H4	GLN60	0	17	3	0
Helix H4	LYS61	0	18	1	1
Helix H4	VAL62	0	11	7	2
Helix H4	LEU63	0	15	4	1
Helix H4	ASP64	3	15	1	1
Helix H4	GLU65	2	18	0	0
Helix H4	THR66	0	18	1	1
loop	GLY67	2	17	0	1
loop	ALA68	0	20	0	0
loop	LYS69	0	20	0	0
loop	LYS70	2	15	2	1
Sheet	VAL71	0	1	8	11
Sheet	ASP72	0	6	8	6
Sheet	ILE73	0	3	1	16
Sheet	VAL74	0	5	2	13
Sheet	ALA75	0	7	3	10
Sheet	HIS76	1	1	0	18
loop	SER77	0	1	0	19
Helix H5	MET78	3	9	0	8
Helix H5	GLY79	0	2	1	17
Helix H5	GLY80	1	5	0	14
Helix H5	ALA81	3	15	1	1
Helix H5	ASN82	1	11	6	2
Helix H5	THR83	1	12	4	3
Helix H5	LEU84	1	16	1	2
Helix H5	TYR85	0	19	0	1
Helix H5	TYR86	0	8	8	4
Helix H5	ILE87	1	9	2	8
Helix H5	LYS88	1	18	0	1
loop	ASN89	0	14	2	4
loop	LEU90	10	8	2	0
loop	ASP91	10	7	1	2

[BMIM][TfO]

BSLA secondary structure	BSLA amino acid positions	Number of amino acid substitutions with +	=	-	x
Helix H6	GLY92	0	1	19	0
Helix H6	GLY93	2	17	1	0
Helix H6	ASN94	5	15	0	0
loop	LYS95	10	5	5	0
Sheet	VAL96	1	10	1	8
Sheet	ALA97	3	16	0	1
Sheet	ASN98	3	16	1	0
Sheet	VAL99	0	8	8	4
Sheet	VAL100	0	9	3	8
Sheet	THR101	3	11	5	1
Sheet	LEU102	0	7	4	9
loop	GLY103	0	2	3	15
loop	GLY104	0	4	10	6
loop	ALA105	1	13	4	2
Helix H7	ASN106	0	11	8	1
Helix H7	ARG107	8	9	0	3
Helix H7	LEU108	0	10	9	1
loop	THR109	3	12	5	0
loop	THR110	0	16	4	0
loop	GLY111	0	20	0	0
loop	LYS112	2	18	0	0
loop	ALA113	0	11	9	0
loop	LEU114	1	19	0	0
loop	PRO115	0	20	0	0
loop	GLY116	0	12	8	0
loop	THR117	0	16	1	3
loop	ASP118	1	19	0	0
loop	PRO119	2	17	1	0
loop	ASN120	1	19	0	0
loop	GLN121	0	20	0	0
loop	LYS122	4	15	0	1
loop	ILE123	0	9	7	4
Sheet	LEU124	13	6	1	0
Sheet	TYR125	2	6	5	7
Sheet	THR126	1	9	8	2
Sheet	SER127	2	10	4	4
Sheet	ILE128	1	5	7	7
Sheet	TYR129	3	11	3	3
Sheet	SER130	0	6	12	2
loop	SER131	3	17	0	0
loop	ALA132	5	15	0	0
loop	ASP133	1	1	0	18
loop	MET134	3	17	0	0
loop	ILE135	5	14	1	0
loop	VAL136	1	10	3	6
loop	MET137	7	13	0	0
Helix H8	ASN138	5	14	0	1
Helix H8	TYR139	3	9	4	4
Helix H8	LEU140	4	16	0	0
Helix H8	SER141	1	5	5	9
loop	ARG142	0	20	0	0
loop	LEU143	1	10	2	7
loop	ASP144	2	18	0	0
loop	GLY145	3	16	1	0
loop	ALA146	0	17	2	1
Sheet	ARG147	4	15	0	1
Sheet	ASN148	0	12	7	1
Sheet	VAL149	1	18	1	0
Sheet	GLN150	3	17	0	0
Sheet	ILE151	2	15	3	0
loop	HIS152	6	13	0	1
loop	GLY153	0	19	1	0
loop	VAL154	1	17	2	0
loop	GLY155	4	16	0	0
loop	HIS156	0	1	0	19
loop	ILE157	2	10	4	4
Helix H9	GLY158	3	17	0	0
Helix H9	LEU159	2	16	1	1
Helix H9	LEU160	0	11	3	6
Helix H9	TYR161	2	17	0	1
loop	SER162	0	20	0	0
Helix H10	SER163	1	19	0	0
Helix H10	GLN164	2	17	1	0
Helix H10	VAL165	1	14	2	3
Helix H10	ASN166	1	3	10	6
Helix H10	SER167	2	17	0	1
Helix H10	LEU168	3	5	2	10
Helix H10	ILE169	0	1	1	18
Helix H10	LYS170	1	17	1	1
Helix H10	GLU171	0	18	0	2
Helix H10	GLY172	1	13	6	0
Helix H10	LEU173	0	5	9	6
loop	ASN174	1	19	0	0
loop	GLY175	0	20	0	0
loop	GLY176	1	19	0	0
loop	GLY177	4	16	0	0
loop	GLN178	0	17	3	0
loop	ASN179	2	17	0	1
loop	THR180	0	20	0	0
loop	ASN181	1	19	0	0
	Total	104	181	102	99

Table 7-7: Effect of each amino acid type substitution (polar, aliphatic, charged and aromatic) on IL resistance of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per IL are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Amino acid types in BSLA WT

		BSLA: Aromatic (15; 8.3%)				BSLA: Aliphatic (79; 43.6%)				BSLA: Polar (54; 29.8%)				BSLA: Charged (33; 18.2%)			
		+ (42)	= (199)	- (32)	X (27)	+ (215)	= (852)	- (200)	x (313)	+ (121)	= (771)	- (111)	x (77)	+ (84)	= (432)	- (63)	x (81)
[BMIM][Cl]	Aromatic	14%	19%	6%	0%	13%	13%	20%	19%	14%	14%	22%	19%	**25%**	13%	17%	15%
	Aliphatic	26%	25%	28%	19%	20%	33%	18%	12%	21%	26%	32%	16%	**24%**	25%	25%	25%
	Polar	**40%**	32%	41%	44%	**35%**	37%	39%	27%	28%	38%	28%	29%	**26%**	34%	41%	43%
	Charged	19%	25%	25%	37%	33%	17%	24%	42%	**37%**	23%	18%	36%	**25%**	28%	16%	17%
	acidic	7%	10%	13%	15%	14%	6%	14%	15%	13%	10%	6%	10%	12%	10%	10%	6%
	basic	12%	15%	13%	22%	19%	11%	11%	27%	24%	13%	12%	26%	13%	17%	6%	11%
		+ (25)	= (208)	- (40)	x (27)	+ (79)	= (1014)	- (174)	x (313)	+ (53)	= (869)	- (81)	x (77)	+ (49)	= (500)	- (30)	x (81)
[BMIM][Br]	Aromatic	20%	18%	8%	0%	14%	14%	17%	19%	13%	14%	17%	19%	18%	14%	20%	15%
	Aliphatic	28%	25%	25%	19%	20%	31%	17%	12%	26%	26%	21%	16%	22%	25%	23%	25%
	Polar	**36%**	34%	35%	44%	29%	37%	40%	27%	**32%**	36%	36%	29%	27%	34%	43%	43%
	Charged	16%	23%	33%	37%	**37%**	19%	27%	42%	28%	24%	26%	36%	**33%**	26%	13%	17%
	acidic	8%	9%	15%	15%	15%	7%	16%	15%	9%	10%	12%	10%	16%	10%	7%	6%
	basic	8%	14%	18%	22%	22%	12%	11%	27%	19%	14%	14%	26%	16%	16%	7%	11%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6% aliphatic, 29.8% polar amino acids, 18.2% charged, and, 8.3% aromatic). Highest values among the resistance type "+" are highlighted in blue and dark red represents the values of substitutions preferring exchange with similar residues.

Table 7-7: Effect of each amino acid type substitution (polar, aliphatic, charged and aromatic) on IL resistance of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per IL are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Amino acid types in BSLA WT

[BMIM][I]

	BSLA: Aromatic (15; 8.3%)				BSLA: Aliphatic (79; 43.6%)				BSLA: Polar (54; 29.8%)				BSLA: Charged (33; 18.2%)			
	+ (18)	= (191)	- (64)	x (27)	+ (95)	= (854)	- (318)	x (313)	+ (88)	= (802)	- (113)	x (77)	+ (62)	= (468)	- (49)	x (81)
Aromatic	22%	20%	5%	0%	16%	14%	14%	19%	15%	14%	16%	19%	27%	13%	22%	15%
Aliphatic	17%	27%	23%	19%	19%	32%	20%	12%	22%	27%	22%	16%	21%	26%	24%	25%
Polar	**33%**	32%	41%	44%	**33%**	35%	43%	27%	28%	36%	35%	29%	19%	35%	39%	43%
Charged	28%	21%	31%	37%	**33%**	19%	23%	42%	**35%**	23%	27%	36%	**32%**	26%	14%	17%
acidic	17%	6%	17%	15%	15%	6%	13%	15%	14%	9%	12%	10%	15%	10%	6%	6%
basic	11%	15%	14%	22%	18%	12%	10%	27%	22%	13%	14%	26%	18%	16%	8%	11%

BMIM][TfO]

	+ (21)	= (208)	- (44)	x (27)	+ (118)	= (925)	- (224)	x (313)	+ (82)	= (821)	- (100)	x (77)	+ (71)	= (471)	- (37)	x (81)
Aromatic	5%	20%	5%	0%	14%	13%	17%	19%	16%	14%	19%	19%	**28%**	13%	19%	15%
Aliphatic	33%	24%	30%	19%	15%	32%	19%	12%	18%	27%	21%	16%	20%	26%	27%	25%
Polar	**38%**	33%	39%	44%	**37%**	37%	38%	27%	**34%**	36%	29%	29%	**28%**	35%	32%	43%
Charged	24%	23%	27%	37%	33%	18%	25%	42%	32%	23%	31%	36%	24%	27%	22%	17%
acidic	14%	9%	11%	15%	14%	7%	13%	15%	16%	9%	12%	10%	11%	10%	19%	6%
basic	10%	14%	16%	22%	19%	11%	12%	27%	16%	13%	19%	26%	13%	17%	3%	11%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6% aliphatic, 29.8% polar amino acids, 18.2% charged, and, 8.3% aromatic). Highest values among the resistance type "+" are highlighted in blue and dark red represents the values of substitutions preferring exchange with similar residues.

Table 7-8: Chemical diversity for improved variants in all four ILs. The percentage values were calculated by addition of the values for the chemically different amino acids in each case. In case of [BMIM][Cl] for example, 86% (addition of values from aliphatic, polar, and charged) of the aromatic WT residues preferred substitutions with chemically different amino acids**. Lowest and highest values are in bold.**

	Amino acid types in BSLA WT			
	Aromatic [%]	Aliphatic [%]	Polar [%]	Charged [%]
[BMIM][Cl]	86	80	72	75
[BMIM][Br]	80	80	68	67
[BMIM][I]	78	81	72	68
[BMIM][TfO]	**95**	85	**66**	76

Table 7-9. Effect of amino acid location (exposed, buried) on IL resistance of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per IL are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged acidic (D and E), charged basic (H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Location of amino acid positions in BSLA

[BMIM][Cl]

	Exposed positions (128; 71%)				Buried positions (53; 29%)			
	+ (343)	= (1781)	- (247)	x (189)	+ (119)	= (473)	- (159)	x (309)
Aromatic	15%	14%	22%	15%	16%	12%	14%	19%
Aliphatic	22%	27%	23%	17%	18%	34%	25%	14%
Polar	30%	36%	34%	38%	**38%**	39%	40%	26%
Charged	**32%**	24%	21%	30%	28%	15%	22%	41%
acidic	13%	9%	11%	11%	13%	6%	11%	14%
basic	20%	14%	10%	19%	15%	9%	11%	27%

[BMIM][Br]

	+ (172)	= (2077)	- (122)	x (189)	+ (34)	= (514)	- (203)	x (309)
Aromatic	16%	15%	20%	15%	12%	13%	14%	19%
Aliphatic	23%	26%	17%	17%	26%	33%	21%	14%
Polar	**30%**	35%	38%	38%	**32%**	39%	39%	26%
Charged	**31%**	24%	25%	30%	29%	14%	27%	41%
acidic	12%	10%	14%	11%	18%	5%	14%	14%
basic	19%	14%	11%	19%	12%	9%	12%	27%

[BMIM][I]

	+ (236)	= (1897)	- (238)	x (189)	+ (27)	= (418)	- (306)	x (309)
Aromatic	19%	15%	15%	15%	15%	13%	13%	19%
Aliphatic	19%	27%	22%	17%	26%	37%	20%	14%
Polar	27%	35%	37%	38%	**37%**	35%	44%	26%
Charged	**34%**	23%	26%	30%	22%	15%	22%	41%
acidic	14%	9%	14%	11%	15%	5%	12%	14%
basic	20%	14%	12%	19%	7%	10%	10%	27%

BMIM][TfO]

	+ (248)	= (1962)	- (161)	x (189)	+ (44)	= (463)	- (244)	x (309)
Aromatic	19%	14%	17%	15%	11%	12%	16%	19%
Aliphatic	19%	27%	22%	17%	14%	36%	21%	14%
Polar	**33%**	35%	31%	38%	**43%**	38%	39%	26%
Charged	29%	24%	29%	30%	32%	14%	25%	41%
acidic	14%	9%	14%	11%	14%	5%	13%	14%
basic	15%	15%	16%	19%	18%	8%	12%	27%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6% aliphatic, 29.8% polar amino acids, 18.2% charged, and, 8.3% aromatic). Highest values among the resistance type "+" are highlighted in blue. Column "x" shows that the number of inactive variants resulted from amino acid substitutions. The activity was measured in a buffer plate which was used as reference (absence of IL) for comparison. Therefore, the number of inactive variants was the same for three OS.

Table 7-10: **Overall number of amino acid substitutions on different secondary elements of BSLA (loop, helix and β-sheet)** contributing to increased/unchanged/decreased resistance towards **all four ILs** and inactivation of BSLA. "**+**": improved resistance; "**=**": unchanged resistance**;** "**-**": decreased resistance and "**x**" inactivated.

[BMIM][Cl]

	+ (124)	**= (181)**	**- (117)**	**x (99)**
Loops	**44%**	44%	39%	28%
Sheets	15%	18%	23%	25%
Helices	40%	39%	38%	46%
Total	100%	100%	100%	100%

[BMIM][Br]

	+ (91)	**= (181)**	**- (89)**	**x (99)**
Loops	41%	44%	35%	28%
Sheets	15%	18%	27%	25%
Helices	**44%**	39%	38%	46%
Total	100%	100%	100%	100%

[BMIM][I]

	+ (95)	**= (181)**	**- (115)**	**x (99)**
Loops	**47%**	44%	36%	28%
Sheets	15%	18%	22%	25%
Helices	38%	39%	43%	46%
Total	100%	100%	100%	100%

[BMIM][TfO]

	+ (104)	**= (181)**	**- (112)**	**x (99)**
Loops	**42%**	44%	36%	28%
Sheets	17%	18%	25%	25%
Helices	40%	39%	38%	46%
Total	100%	100%	100%	100%

Highest values among the resistance type "+" are highlighted in blue.

Table 7-11: **Number of amino acid substitutions in BSLA which lead to increased/unchanged/decreased resistance towards DMSO and inactivation of BSLA.** The secondary structure characteristic (loop, helix and β-sheet) of every position is shown. Loops are labelled in black, sheets in white and helix in light grey. The results in the table are given with a color scale from dark grey (highest values) to light grey (lowest values.). "**+**": improved resistance; "**=**": unchanged resistance**;** "**-**": decreased resistance and "**x**" inactivated.

		DMSO			
		Number of amino acid substitutions with			
BSLA secondary structure	BSLA amino acid positions	+	=	-	x
loop	Ala1	0	20	0	0
loop	Glu2	0	15	5	0
loop	HIS3	0	20	0	0
loop	ASN4	9	9	2	0
loop	PRO5	0	3	15	2
Sheet	VAL6	0	9	6	5
Sheet	VAL7	0	3	2	15
Sheet	MET8	1	4	11	4
Sheet	VAL9	0	8	7	5
loop	HIS10	0	4	16	0
loop	GLY11	0	1	1	18
loop	ILE12	6	7	3	4
loop	GLY13	0	14	6	0
loop	GLY14	2	6	9	3
loop	ALA15	4	13	2	1
Helix H1	SER16	0	14	6	0
Helix H1	PHE17	5	14	1	0
Helix H1	ASN18	0	16	3	1
Helix H1	PHE19	0	11	8	1
Helix H1	ALA20	1	18	1	0
Helix H1	GLY21	2	15	3	0
Helix H1	ILE22	0	11	7	2
Helix H1	LYS23	0	13	6	1
Helix H1	SER24	0	17	3	0
Helix H1	TYR25	0	19	0	1
Helix H1	LEU26	0	7	3	10
Helix H1	VAL27	0	19	0	1
Helix H1	SER28	2	16	2	0
loop	GLN29	1	15	3	1
loop	GLY30	0	10	8	2
loop	TRP31	0	6	12	2
loop	SER32	5	13	2	0
Helix H3	ARG33	0	16	4	0
Helix H3	ASP34	0	9	10	1
Helix H3	LYS35	0	7	10	3
Sheet	LEU36	0	9	9	2
Sheet	TYR37	7	11	2	0
Sheet	ALA38	3	17	0	0
loop	VAL39	1	17	2	0
loop	ASP40	0	19	1	0
loop	PHE41	2	12	4	2
loop	TRP42	2	18	0	0
loop	ASP43	0	15	5	0
loop	LYS44	0	11	9	0
loop	THR45	0	13	7	0
loop	GLY46	1	13	6	0
loop	THR47	0	20	0	0
Helix H4	ASN48	0	17	3	0
Helix H4	TYR49	0	17	3	0
Helix H4	ASN50	1	15	4	0
Helix H4	ASN51	1	13	6	0
loop	GLY52	0	16	4	0
Helix H4	PRO53	1	18	1	0
Helix H4	VAL54	3	16	0	1
Helix H4	LEU55	0	6	5	9
Helix H4	SER56	0	19	1	0
Helix H4	ARG57	1	11	8	0
Helix H4	PHE58	9	10	0	1
Helix H4	VAL59	0	4	4	12
Helix H4	GLN60	0	11	9	0
Helix H4	LYS61	0	18	1	1
Helix H4	VAL62	0	4	14	2
Helix H4	LEU63	0	9	10	1
Helix H4	ASP64	1	18	0	1
Helix H4	GLU65	2	15	3	0
Helix H4	THR66	1	18	0	1
loop	GLY67	0	16	3	1
loop	ALA68	1	17	2	0
loop	LYS69	1	18	1	0
loop	LYS70	7	12	0	1
Sheet	VAL71	0	1	8	11
Sheet	ASP72	0	8	6	6
Sheet	ILE73	0	3	1	16
Sheet	VAL74	3	3	1	13
Sheet	ALA75	0	7	3	10
Sheet	HIS76	1	1	0	18
loop	SER77	0	1	0	19
Helix H5	MET78	1	6	5	8
Helix H5	GLY79	0	2	1	17
Helix H5	GLY80	0	3	3	14
Helix H5	ALA81	1	14	4	1
Helix H5	ASN82	0	6	12	2
Helix H5	THR83	0	14	3	3
Helix H5	LEU84	7	8	3	2
Helix H5	TYR85	0	19	0	1
Helix H5	TYR86	0	12	4	4
Helix H5	ILE87	0	12	0	8
Helix H5	LYS88	1	16	2	1
loop	ASN89	3	10	3	4
loop	LEU90	0	4	16	0
loop	ASP91	1	12	5	2
Helix H6	GLY92	5	15	0	0
Helix H6	GLY93	6	13	1	0
Helix H6	ASN94	6	14	0	0
loop	LYS95	14	6	0	0
Sheet	VAL96	2	7	3	8
Sheet	ALA97	1	18	0	1
Sheet	ASN98	5	15	0	0
Sheet	VAL99	5	11	0	4
Sheet	VAL100	1	8	3	8
Sheet	THR101	1	14	4	1
Sheet	LEU102	1	7	3	9
loop	GLY103	0	3	2	15
loop	GLY104	0	2	12	6
loop	ALA105	2	7	9	2
Helix H7	ASN106	1	13	5	1
Helix H7	ARG107	7	9	1	3
Helix H7	LEU108	0	4	15	1
loop	THR109	5	11	4	0
loop	THR110	1	13	6	0
loop	GLY111	1	18	1	0
loop	LYS112	1	19	0	0
loop	ALA113	2	9	9	0
loop	LEU114	4	16	0	0
loop	PRO115	0	20	0	0
loop	GLY116	1	8	11	0
loop	THR117	5	12	0	3
loop	ASP118	7	13	0	0
loop	PRO119	0	18	2	0
loop	ASN120	5	15	0	0
loop	GLN121	0	20	0	0
loop	LYS122	3	16	0	1
loop	ILE123	0	14	2	4
Sheet	LEU124	10	9	1	0
Sheet	TYR125	6	7	0	7
Sheet	THR126	4	14	0	2
Sheet	SER127	5	8	3	4
Sheet	ILE128	4	9	0	7
Sheet	TYR129	6	11	0	3
Sheet	SER130	2	14	2	2
loop	SER131	0	20	0	0
loop	ALA132	2	18	0	0
loop	ASP133	1	1	0	18
loop	MET134	0	19	1	0
loop	ILE135	0	15	5	0
loop	VAL136	2	9	3	6
loop	MET137	5	15	0	0
Helix H8	ASN138	7	11	1	1
Helix H8	TYR139	3	11	2	4
Helix H8	LEU140	5	15	0	0
Helix H8	SER141	2	8	1	9
loop	ARG142	1	14	5	0
loop	LEU143	5	5	3	7
loop	ASP144	6	14	0	0
loop	GLY145	7	12	1	0
loop	ALA146	0	17	2	1
Sheet	ARG147	3	16	0	1
Sheet	ASN148	4	13	2	1
Sheet	VAL149	2	18	0	0
Sheet	GLN150	5	15	0	0
Sheet	ILE151	9	11	0	0
loop	HIS152	5	14	0	1
loop	GLY153	1	18	1	0
loop	VAL154	1	15	4	0
loop	GLY155	4	16	0	0
loop	HIS156	0	1	0	19
loop	ILE157	0	7	9	4
Helix H9	GLY158	2	16	2	0
Helix H9	LEU159	3	13	3	1
Helix H9	LEU160	4	10	0	6
Helix H9	TYR161	4	15	0	1
loop	SER162	3	17	0	0
Helix H10	SER163	2	18	0	0
Helix H10	GLN164	0	18	2	0
Helix H10	VAL165	0	17	0	3
Helix H10	ASN166	1	12	1	6
Helix H10	SER167	2	17	0	1
Helix H10	LEU168	4	5	1	10
Helix H10	ILE169	0	1	1	18
Helix H10	LYS170	1	18	0	1
Helix H10	GLU171	0	18	0	2
Helix H10	GLY172	10	10	0	0
Helix H10	LEU173	0	9	5	6
loop	ASN174	1	18	1	0
loop	GLY175	5	15	0	0
loop	GLY176	0	20	0	0
loop	GLY177	9	11	0	0
loop	GLN178	1	19	0	0
loop	ASN179	6	13	0	1
loop	THR180	0	20	0	0
loop	ASN181	3	17	0	0
	Total	107	181	118	99

Table 7-12: Number of amino acid substitutions in BSLA which lead to increased/unchanged/decreased resistance towards 1,4-dioxane and inactivation of BSLA. The secondary structure characteristic (loop, helix and β-sheet) of every position is shown. Loops are labelled in black, sheets in white and helix in light grey. The results in the table are given with a color scale from dark grey (highest values) to light grey (lowest values.). "**+**": improved resistance; "**=**": unchanged resistance**;** "**-**": decreased resistance and "**x**" inactivated.

1,4-dioxane

Number of amino acid substitutions with

BSLA secondary structure	BSLA amino acid positions	+	=	-	x
loop	Ala1	0	20	0	0
loop	Glu2	0	20	0	0
loop	HIS3	0	16	4	0
loop	ASN4	0	18	2	0
loop	PRO5	0	10	8	2
Sheet	VAL6	0	6	9	5
Sheet	VAL7	0	5	0	15
Sheet	MET8	0	9	7	4
Sheet	VAL9	0	7	8	5
loop	HIS10	3	3	14	0
loop	GLY11	0	1	1	18
loop	ILE12	8	7	1	4
loop	GLY13	4	14	2	0
loop	GLY14	2	11	4	3
loop	ALA15	2	17	0	1
Helix H1	SER16	0	18	2	0
Helix H1	PHE17	6	14	0	0
Helix H1	ASN18	1	12	6	1
Helix H1	PHE19	1	12	6	1
Helix H1	ALA20	0	20	0	0
Helix H1	GLY21	0	19	1	0
Helix H1	ILE22	0	8	10	2
Helix H1	LYS23	0	19	0	1
Helix H1	SER24	0	18	2	0
Helix H1	TYR25	1	17	1	1
Helix H1	LEU26	1	5	4	10
Helix H1	VAL27	0	18	1	1
Helix H1	SER28	0	17	3	0
loop	GLN29	0	19	0	1
loop	GLY30	0	4	14	2
loop	TRP31	0	10	8	2
loop	SER32	2	17	1	0
Helix H3	ARG33	0	20	0	0
Helix H3	ASP34	1	14	4	1
Helix H3	LYS35	0	16	1	3
Sheet	LEU36	0	9	9	2
Sheet	TYR37	0	15	5	0
Sheet	ALA38	1	18	1	0
loop	VAL39	0	18	2	0
loop	ASP40	1	19	0	0
loop	PHE41	0	15	3	2
loop	TRP42	2	16	2	0
loop	ASP43	2	18	0	0
loop	LYS44	0	10	10	0
loop	THR45	0	19	1	0
loop	GLY46	0	15	5	0
loop	THR47	8	12	0	0
Helix H4	ASN48	0	18	2	0
Helix H4	TYR49	1	19	0	0
Helix H4	ASN50	3	17	0	0
Helix H4	ASN51	0	15	5	0
loop	GLY52	0	9	11	0
Helix H4	PRO53	0	19	1	0
Helix H4	VAL54	1	18	0	1
Helix H4	LEU55	0	4	7	9
Helix H4	SER56	0	19	1	0
Helix H4	ARG57	2	13	5	0
Helix H4	PHE58	1	16	2	1
Helix H4	VAL59	0	5	3	12
Helix H4	GLN60	0	17	3	0
Helix H4	LYS61	0	14	5	1
Helix H4	VAL62	1	3	14	2
Helix H4	LEU63	0	11	8	1
Helix H4	ASP64	0	19	0	1
Helix H4	GLU65	1	18	1	0
Helix H4	THR66	0	17	2	1
loop	GLY67	0	16	3	1
loop	ALA68	0	16	4	0
loop	LYS69	0	19	1	0
loop	LYS70	0	16	3	1
Sheet	VAL71	0	3	6	11
Sheet	ASP72	0	8	6	6
Sheet	ILE73	0	3	1	16
Sheet	VAL74	1	4	2	13
Sheet	ALA75	0	5	5	10
Sheet	HIS76	0	1	1	18
loop	SER77	0	1	0	19
Helix H5	MET78	2	5	5	8
Helix H5	GLY79	0	1	2	17
Helix H5	GLY80	0	4	2	14
Helix H5	ALA81	0	11	8	1
Helix H5	ASN82	1	12	5	2
Helix H5	THR83	0	10	7	3
Helix H5	LEU84	0	9	9	2
Helix H5	TYR85	0	15	4	1
Helix H5	TYR86	0	5	11	4
Helix H5	ILE87	0	4	8	8
Helix H5	LYS88	1	15	3	1
loop	ASN89	0	16	0	4
loop	LEU90	0	17	3	0
loop	ASP91	1	16	1	2

1,4-dioxane

Number of amino acid substitutions with

BSLA secondary structure	BSLA amino acid positions	+	=	-	x
Helix H6	GLY92	0	15	5	0
Helix H6	GLY93	0	14	6	0
Helix H6	ASN94	2	18	0	0
loop	LYS95	1	15	4	0
Sheet	VAL96	0	10	2	8
Sheet	ALA97	0	19	0	1
Sheet	ASN98	1	14	5	0
Sheet	VAL99	0	11	5	4
Sheet	VAL100	5	5	2	8
Sheet	THR101	2	11	6	1
Sheet	LEU102	0	6	5	9
loop	GLY103	0	1	4	15
loop	GLY104	0	2	12	6
loop	ALA105	0	8	10	2
Helix H7	ASN106	0	7	12	1
Helix H7	ARG107	0	15	2	3
Helix H7	LEU108	0	7	12	1
loop	THR109	2	8	10	0
loop	THR110	0	8	12	0
loop	GLY111	3	14	3	0
loop	LYS112	0	19	1	0
loop	ALA113	0	8	12	0
loop	LEU114	3	17	0	0
loop	PRO115	0	20	0	0
loop	GLY116	0	3	17	0
loop	THR117	5	12	0	3
loop	ASP118	1	19	0	0
loop	PRO119	0	17	3	0
loop	ASN120	2	18	0	0
loop	GLN121	0	18	2	0
loop	LYS122	1	18	0	1
loop	ILE123	0	6	10	4
Sheet	LEU124	4	15	1	0
Sheet	TYR125	0	5	8	7
Sheet	THR126	1	9	8	2
Sheet	SER127	0	7	9	4
Sheet	ILE128	0	4	9	7
Sheet	TYR129	1	11	5	3
Sheet	SER130	1	4	13	2
loop	SER131	0	20	0	0
loop	ALA132	3	16	1	0
loop	ASP133	0	2	0	18
loop	MET134	2	18	0	0
loop	ILE135	1	14	5	0
loop	VAL136	1	7	6	6
loop	MET137	4	16	0	0
Helix H8	ASN138	3	15	1	1
Helix H8	TYR139	4	8	4	4
Helix H8	LEU140	2	17	1	0
Helix H8	SER141	1	5	5	9
loop	ARG142	0	17	3	0
loop	LEU143	0	7	6	7
loop	ASP144	3	17	0	0
loop	GLY145	1	11	8	0
loop	ALA146	2	13	4	1
Sheet	ARG147	1	18	0	1
Sheet	ASN148	0	10	9	1
Sheet	VAL149	1	17	2	0
Sheet	GLN150	2	18	0	0
Sheet	ILE151	1	11	8	0
loop	HIS152	5	14	0	1
loop	GLY153	0	19	1	0
loop	VAL154	1	15	4	0
loop	GLY155	4	15	1	0
loop	HIS156	0	1	0	19
loop	ILE157	0	6	10	4
Helix H9	GLY158	0	18	2	0
Helix H9	LEU159	1	9	9	1
Helix H9	LEU160	0	6	8	6
Helix H9	TYR161	2	17	0	1
loop	SER162	3	16	1	0
Helix H10	SER163	2	18	0	0
Helix H10	GLN164	0	19	1	0
Helix H10	VAL165	1	12	4	3
Helix H10	ASN166	2	6	6	6
Helix H10	SER167	0	18	1	1
Helix H10	LEU168	1	7	2	10
Helix H10	ILE169	0	1	1	18
Helix H10	LYS170	0	19	0	1
Helix H10	GLU171	0	18	0	2
Helix H10	GLY172	0	13	7	0
Helix H10	LEU173	0	9	5	6
loop	ASN174	0	20	0	0
loop	GLY175	0	20	0	0
loop	GLY176	0	19	1	0
loop	GLY177	4	15	1	0
loop	GLN178	0	20	0	0
loop	ASN179	1	18	0	1
loop	THR180	2	18	0	0
loop	ASN181	1	19	0	0
	Total	75	181	136	99

Table 7-13: **Number of amino acid substitutions in BSLA which lead to increased/unchanged/decreased resistance towards TFE and inactivation of BSLA**. The secondary structure characteristic (loop, helix and β-sheet) of every position is shown. Loops are labelled in black, sheets in white and helix in light grey. The results in the table are given with a color scale from dark grey (highest values) to light grey (lowest values.). "**+**": improved resistance; "**=**": unchanged resistance**;** "**-**": decreased resistance and "**x**" inactivated.

		TFE			
		Number of amino acid substitutions with			
BSLA secondary structure	**BSLA amino acid positions**	**+**	**=**	**-**	**x**
loop	Ala1	0	20	0	0
loop	Glu2	0	20	0	0
loop	HIS3	0	20	0	0
loop	ASN4	0	20	0	0
loop	PRO5	8	9	1	2
Sheet	VAL6	3	12	0	5
Sheet	VAL7	1	4	0	15
Sheet	MET8	0	15	1	4
Sheet	VAL9	0	6	9	5
loop	HIS10	2	7	11	0
loop	GLY11	0	1	1	18
loop	ILE12	5	11	0	4
loop	GLY13	1	16	3	0
loop	GLY14	3	11	3	3
loop	ALA15	3	16	0	1
Helix H1	SER16	0	20	0	0
Helix H1	PHE17	1	18	1	0
Helix H1	ASN18	3	13	3	1
Helix H1	PHE19	0	9	10	1
Helix H1	ALA20	0	19	1	0
Helix H1	GLY21	1	17	2	0
Helix H1	ILE22	0	8	10	2
Helix H1	LYS23	4	15	0	1
Helix H1	SER24	0	17	3	0
Helix H1	TYR25	0	16	3	1
Helix H1	LEU26	1	9	0	10
Helix H1	VAL27	0	19	0	1
Helix H1	SER28	0	20	0	0
loop	GLN29	1	18	0	1
loop	GLY30	0	17	1	2
loop	TRP31	0	18	0	2
loop	SER32	2	18	0	0
Helix H3	ARG33	0	20	0	0
Helix H3	ASP34	2	15	2	1
Helix H3	LYS35	0	17	0	3
Sheet	LEU36	2	13	3	2
Sheet	TYR37	0	20	0	0
Sheet	ALA38	0	19	1	0
loop	VAL39	1	18	1	0
loop	ASP40	0	20	0	0
loop	PHE41	4	12	2	2
loop	TRP42	2	18	0	0
loop	ASP43	3	17	0	0
loop	LYS44	0	19	1	0
loop	THR45	1	19	0	0
loop	GLY46	0	17	3	0
loop	THR47	10	10	0	0
Helix H4	ASN48	0	20	0	0
Helix H4	TYR49	1	19	0	0
Helix H4	ASN50	1	19	0	0
Helix H4	ASN51	2	17	1	0
loop	GLY52	3	7	10	0
Helix H4	PRO53	0	20	0	0
Helix H4	VAL54	4	15	0	1
Helix H4	LEU55	0	11	0	9
Helix H4	SER56	0	20	0	0
Helix H4	ARG57	1	13	6	0
Helix H4	PHE58	0	15	4	1
Helix H4	VAL59	0	5	3	12
Helix H4	GLN60	0	20	0	0
Helix H4	LYS61	0	17	2	1
Helix H4	VAL62	0	16	2	2
Helix H4	LEU63	0	13	6	1
Helix H4	ASP64	2	16	1	1
Helix H4	GLU65	2	18	0	0
Helix H4	THR66	0	19	0	1
loop	GLY67	0	18	1	1
loop	ALA68	0	20	0	0
loop	LYS69	0	20	0	0
loop	LYS70	0	14	5	1
Sheet	VAL71	0	6	3	11
Sheet	ASP72	0	4	10	6
Sheet	ILE73	0	2	2	16
Sheet	VAL74	0	5	2	13
Sheet	ALA75	0	8	2	10
Sheet	HIS76	1	1	0	18
loop	SER77	0	1	0	19
Helix H5	MET78	4	7	1	8
Helix H5	GLY79	0	2	1	17
Helix H5	GLY80	0	4	2	14
Helix H5	ALA81	2	11	6	1
Helix H5	ASN82	0	9	9	2
Helix H5	THR83	1	11	5	3
Helix H5	LEU84	1	6	11	2
Helix H5	TYR85	1	18	0	1
Helix H5	TYR86	0	13	3	4
Helix H5	ILE87	0	6	6	8
Helix H5	LYS88	1	18	0	1
loop	ASN89	0	16	0	4
loop	LEU90	10	9	1	0
loop	ASP91	9	7	2	2
Helix H6	GLY92	0	5	15	0
Helix H6	GLY93	0	9	11	0
Helix H6	ASN94	0	19	1	0
loop	LYS95	4	14	2	0
Sheet	VAL96	0	12	0	8
Sheet	ALA97	4	15	0	1
Sheet	ASN98	1	15	4	0
Sheet	VAL99	0	11	5	4
Sheet	VAL100	2	9	1	8
Sheet	THR101	0	10	9	1
Sheet	LEU102	0	6	5	9
loop	GLY103	0	3	2	15
loop	GLY104	0	3	11	6
loop	ALA105	1	13	4	2
Helix H7	ASN106	0	4	15	1
Helix H7	ARG107	0	7	10	3
Helix H7	LEU108	0	5	14	1
loop	THR109	2	2	16	0
loop	THR110	0	2	18	0
loop	GLY111	0	19	1	0
loop	LYS112	1	18	1	0
loop	ALA113	0	4	16	0
loop	LEU114	2	15	3	0
loop	PRO115	0	19	1	0
loop	GLY116	0	1	19	0
loop	THR117	1	16	0	3
loop	ASP118	0	17	3	0
loop	PRO119	4	15	1	0
loop	ASN120	1	19	0	0
loop	GLN121	0	18	2	0
loop	LYS122	0	17	2	1
loop	ILE123	0	4	12	4
Sheet	LEU124	0	15	5	0
Sheet	TYR125	0	2	11	7
Sheet	THR126	0	10	8	2
Sheet	SER127	0	4	12	4
Sheet	ILE128	0	4	9	7
Sheet	TYR129	0	4	13	3
Sheet	SER130	0	3	15	2
loop	SER131	1	17	2	0
loop	ALA132	1	17	2	0
loop	ASP133	0	1	1	18
loop	MET134	6	14	0	0
loop	ILE135	0	17	3	0
loop	VAL136	0	10	4	6
loop	MET137	4	16	0	0
Helix H8	ASN138	0	13	6	1
Helix H8	TYR139	2	11	3	4
Helix H8	LEU140	7	12	1	0
Helix H8	SER141	0	1	10	9
loop	ARG142	0	20	0	0
loop	LEU143	0	3	10	7
loop	ASP144	1	19	0	0
loop	GLY145	0	2	18	0
loop	ALA146	0	14	5	1
Sheet	ARG147	2	15	2	1
Sheet	ASN148	0	1	18	1
Sheet	VAL149	1	17	2	0
Sheet	GLN150	1	19	0	0
Sheet	ILE151	0	7	13	0
loop	HIS152	1	18	0	1
loop	GLY153	0	18	2	0
loop	VAL154	0	9	11	0
loop	GLY155	0	20	0	0
loop	HIS156	0	1	0	19
loop	ILE157	4	10	2	4
Helix H9	GLY158	0	17	3	0
Helix H9	LEU159	1	7	11	1
Helix H9	LEU160	1	3	10	6
Helix H9	TYR161	1	18	0	1
loop	SER162	0	12	8	0
Helix H10	SER163	2	18	0	0
Helix H10	GLN164	2	18	0	0
Helix H10	VAL165	1	13	3	3
Helix H10	ASN166	1	3	10	6
Helix H10	SER167	3	15	1	1
Helix H10	LEU168	3	7	0	10
Helix H10	ILE169	0	1	1	18
Helix H10	LYS170	1	18	0	1
Helix H10	GLU171	1	16	1	2
Helix H10	GLY172	0	10	10	0
Helix H10	LEU173	0	7	7	6
loop	ASN174	0	19	1	0
loop	GLY175	0	20	0	0
loop	GLY176	0	19	1	0
loop	GLY177	0	6	14	0
loop	GLN178	0	17	3	0
loop	ASN179	0	14	5	1
loop	THR180	0	20	0	0
loop	ASN181	0	20	0	0
	Total	74	181	120	99

Table 7-14: Effect of each type of amino acid substitution (polar, aliphatic, charged and aromatic) on OS resistance of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per OS are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Amino acid types in BSLA WT

		BSLA: Aromatic (15; 8.3%)				BSLA: Aliphatic (79; 43.6%)				BSLA: Polar (54; 29.8%)				BSLA: Charged (33; 18.2%)			
		+ (44)	= (193)	- (36)	x (27)	+ (155)	= (843)	- (269)	x (313)	+ (108)	= 759)	- (136)	x 77)	+ (64)	= (417)	- 98)	x 81)
DMSO	Aromatic	7%	20%	8%	0%	9%	14%	17%	19%	8%	14%	24%	19%	**22%**	12%	21%	15%
	Aliphatic	32%	26%	17%	19%	21%	32%	20%	12%	23%	28%	16%	16%	**25%**	24%	30%	25%
	Polar	27%	36%	33%	44%	**41%**	36%	39%	27%	32%	36%	34%	29%	**25%**	35%	36%	43%
	Charged	**34%**	18%	42%	37%	29%	18%	24%	42%	**36%**	22%	26%	36%	**28%**	29%	13%	17%
	acidic	11%	6%	25%	15%	8%	8%	10%	15%	11%	9%	16%	10%	11%	12%	6%	6%
	basic	23%	12%	17%	22%	21%	10%	13%	27%	25%	13%	10%	26%	17%	17%	7%	11%
		+ (19)	= 195)	- (59)	x (27)	+ (60)	= (827)	- (380)	x (313)	+ (56)	= (780)	- 167)	x (77)	+ (24)	= (486)	- (69)	x (81)
1,4-dioxane	Aromatic	5%	22%	3%	0%	17%	13%	15%	19%	16%	13%	22%	19%	17%	14%	19%	15%
	Aliphatic	26%	26%	25%	19%	13%	34%	18%	12%	16%	28%	19%	16%	17%	26%	22%	25%
	Polar	21%	35%	34%	44%	32%	37%	39%	27%	32%	37%	32%	29%	25%	35%	32%	43%
	Charged	**47%**	17%	37%	37%	**38%**	16%	28%	42%	**36%**	23%	27%	36%	**42%**	25%	28%	17%
	acidic	11%	5%	24%	15%	8%	6%	15%	15%	4%	9%	15%	10%	4%	9%	23%	6%
	basic	37%	12%	14%	22%	30%	10%	13%	27%	32%	13%	12%	26%	38%	16%	4%	11%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6% aliphatic, 29.8% polar amino acids, 18.2% charged, and, 8.3% aromatic). Highest values among the resistance type "+" are highlighted in blue and dark red represents the values of substitutions preferring exchange with similar residues.

Table 7-14: Effect of each type of amino acid substitution (polar, aliphatic, charged and aromatic) on OS resistance of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per OS are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Amino acid types in BSLA WT

		BSLA: Aromatic (15; 8.3%)				BSLA: Aliphatic (79; 43.6%)				BSLA: Polar (54; 29.8%)				BSLA: Charged (33; 18.2%)			
		+ (12)	= (211)	- (50)	x (27)	+ (69)	= 836)	- 362)	x 313)	+ (62)	= (751)	- (190)	x 77)	+ (38)	= (479)	- (62)	x (81)
TFE	Aromatic	8%	19%	6%	0%	23%	12%	17%	19%	18%	15%	14%	19%	16%	15%	16%	15%
	Aliphatic	0%	27%	28%	19%	14%	33%	20%	12%	19%	26%	28%	16%	16%	26%	23%	25%
	Polar	33%	34%	34%	44%	29%	38%	37%	27%	26%	38%	31%	29%	**45%**	33%	32%	43%
	Charged	**58%**	20%	32%	37%	**33%**	18%	26%	42%	**37%**	22%	28%	36%	24%	26%	29%	17%
	acidic	8%	8%	16%	15%	14%	6%	14%	15%	16%	9%	12%	10%	5%	10%	21%	6%
	basic	50%	12%	16%	22%	19%	11%	12%	27%	21%	13%	16%	26%	18%	16%	8%	11%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6% aliphatic, 29.8% polar amino acids, 18.2% charged, and, 8.3% aromatic). Highest values among the resistance type "+" are highlighted in blue and dark red represents the values of substitutions preferring exchange with similar residues.

Table 7-15: Chemical diversity for improved variants in all three OS. The percentage values were calculated by addition of the values for the chemically different amino acids in each case. In case of DMSO for example, 93% (addition of values from aliphatic, polar, and charged) of the aromatic WT residues preferred substitutions with chemically different amino acid**. Lowest and highest values are in bold.**

	Amino acid types in BSLA WT			
	Aromatic [%]	Aliphatic [%]	Polar [%]	Charged [%]
DMSO	93	79	68	72
1,4-dioxane	**95**	87	68	**58**
TFE	92	86	74	76

Table 7-16: Effect of amino acid location (buried, exposed) on OS resistance of BSLA when replacing each type of amino acid in BSLA WT. 16 patterns per OS are shown. The absolute number of BSLA variants per type is shown in bracket. Amino acids are grouped into: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). All SSM-BSLA variants were grouped as described in the text: "+" representing variants with improved resistance, "=" representing variants with unchanged resistance, "-" representing variants with decreased resistance and "x" representing inactive variants in buffer.

Location of amino acid positions in BSLA

		Exposed positions (128; 71%)				Buried positions (53; 29%)			
		+ (275)	= (1777)	- (319)	x (189)	+ (96)	= (435)	- (220)	x (309)
DMSO	Aromatic	10%	15%	22%	15%	14%	12%	16%	19%
	Aliphatic	25%	27%	21%	17%	21%	36%	20%	14%
	Polar	31%	36%	33%	38%	**44%**	36%	41%	26%
	Charged	**35%**	23%	24%	30%	22%	15%	23%	41%
	acidic	9%	9%	13%	11%	10%	6%	11%	9%
	basic	26%	14%	11%	19%	11%	9%	12%	25%
		+ (138)	= (1903)	- (330)	x (189)	+ (21)	= (385)	- (345)	x (309)
1,4-dioxane	Aromatic	16%	15%	17%	15%	10%	12%	15%	19%
	Aliphatic	17%	27%	19%	17%	14%	39%	20%	14%
	Polar	26%	35%	35%	38%	**52%**	40%	37%	26%
	Charged	**41%**	23%	28%	30%	24%	9%	28%	41%
	acidic	6%	9%	19%	11%	10%	3%	14%	14%
	basic	36%	14%	10%	19%	14%	6%	14%	27%
		+ (152)	= (1883)	- (336)	x (189)	+ (29)	= (394)	- (328)	x (309)
TFE	Aromatic	19%	15%	15%	15%	17%	12%	15%	19%
	Aliphatic	16%	27%	24%	17%	10%	37%	22%	14%
	Polar	30%	36%	32%	38%	**38%**	40%	38%	26%
	Charged	**34%**	23%	29%	30%	**34%**	11%	26%	41%
	acidic	11%	9%	15%	11%	21%	3%	13%	14%
	basic	23%	14%	14%	19%	14%	8%	13%	27%

The percentage values were given here for a better comparison among columns since the amino acid composition of BSLA is not equally distributed (43.6 % aliphatic, 29.8 % polar amino acids, 18.2 % charged, and, 8.3 % aromatic). Column "x" shows that the number of inactive variants resulted from amino acid substitutions. The activity was measured in a buffer plate which was used as reference (absence of OS) for comparison. Therefore, the number of inactive variants was the same for the three OS.

Table 7-17: Overall number of amino acid substitutions on different secondary elements of BSLA (loop, helix and β-sheet) contributing to increased/unchanged/decreased resistance towards **all three OS** and inactivation of BSLA. "**+**": improved resistance; "**=**": unchanged resistance**;** "**-**": decreased resistance and "**x**" inactivated.

DMSO

	+ (107)	= (181)	- (118)	x (99)
Loops	**44%**	44%	41%	28%
Sheets	22%	18%	16%	25%
Helices	34%	39%	43%	46%
Total	100%	100%	100%	100%

1,4-Dioxane

	+ (75)	= (181)	- (136)	x (99)
Loops	**47%**	44%	38%	28%
Sheets	17%	18%	21%	25%
Helices	36%	39%	42%	46%
Total	100%	100%	100%	100%

TFE

	+ (74)	= (181)	- (120)	x (99)
Loops	**43%**	44%	41%	28%
Sheets	14%	18%	21%	25%
Helices	**43%**	39%	38%	46%
Total	100%	100%	100%	100%

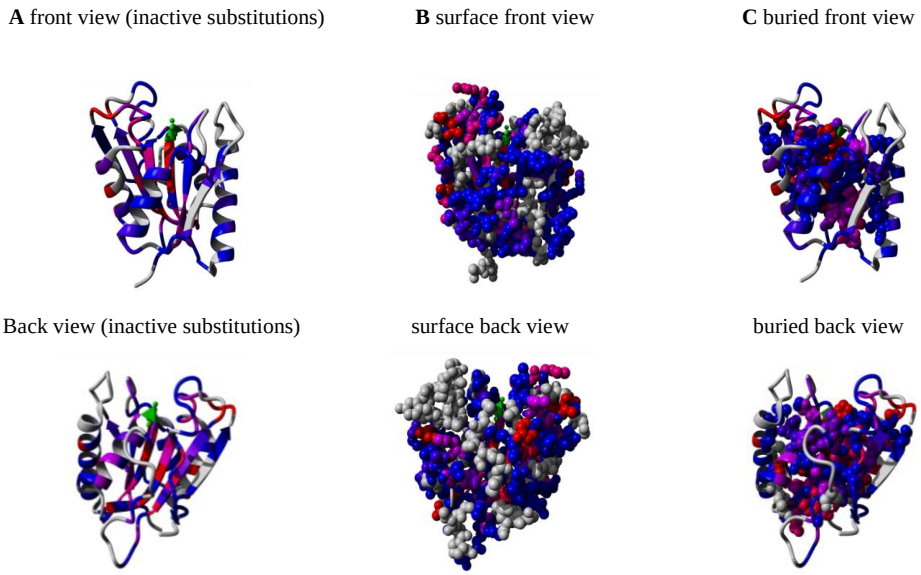

Figure 7-3: 3D model auf BSLA (Protein Database code: 1I6W) displaying regions which inactivated BSLA. These numbers are identical **in all OS and ILs** since inactivation resulted from the mutations and did not depend on the non-conventional media used. Positions which did not habor inactive substitutions are colored grey. Positions with one substitution leading to inactivation are colored blue. Positions with two to seven substitutions leading to inactivation are colored purple (two positions) to magenta (seven positions). Red represents positions with eight or more substitutions leading to inactivation. The active site residue Ser 77 is colored green. A) Cartoon representation of BSLA, B) Van der Waals representation of surface residues of BSLA, C) Van der Waals representation of buried residues of BSLA. The two N-terminal amino acids (Ala1 and Glu2) are also included. Yasara was used for visualization

8 Scientific Contributions

8.1 Oral Presentations

Frauenkron-Machedjou, V. J., Fulton, A., Zhu, L., Jaeger, KE, Schwaneberg, U., 2011. Deciphering rationales determining the stability of a lipase in non-conventional media. **12th BioNoCo workshop**, Schleiden-Gemünd, Germany.

Frauenkron-Machedjou, V. J., Fulton, A., Zhu, L., Jaeger, KE, Schwaneberg, U., 2011. Deciphering rationales determining the stability of a lipase in non-conventional media. **13th BioNoCo workshop**, Kleve, Germany.

Frauenkron-Machedjou, V. J., Fulton, A., Zhu, L., Jaeger, KE, Schwaneberg, U., 2012. Deciphering rationales determining the resistance of a lipase in non-conventional media. **14th BioNoCo workshop**, Monschau, Germany.

Frauenkron-Machedjou, V. J., Fulton, A., Zhu, L., Jaeger, KE, Schwaneberg, U., 2012. Deciphering rationales determining the resistance of a lipase in non-conventional media. **15th BioNoCo workshop**, Monschau, Germany.

Fulton, A., Frauenkron-Machedjou, V. J., Schwaneberg, U., Jaeger, K.-E. (2012). Systematic study of a model lipase towards stability in non-conventional media. **Zing Biocatalysis Conference** 2012, Xcaret, Mexico.

Frauenkron Machedjou, V. J., Fulton, A., Zhu, L., Jaeger, KE, Schwaneberg, U., 2013. Deciphering rationales determining the resistance of a lipase in non-conventional media. **16th BioNoCo workshop**, Monschau, Germany.

Frauenkron-Machedjou, V. J., Fulton, A., Zhu, L., Jaeger, KE, Schwaneberg, U.,2013. Systematic study of a model biocatalyst towards resistance in non-conventional “solvents”. BioNoCo Summer School, RWTH Aachen, Germany.

Nov, Y., Fulton, A., Frauenkron-Machedjou V. J., Schwaneberg U., Jaeger, K.-E. (2014) ANNealing bias in site saturation mutagenesis. **Biocat 2014**, Hamburg, Germany.

Fulton, A., Frauenkron-Machedjou, V.J., Skoczinski, P., Wilhelm, S., Schwaneberg, U., Jaeger, K.-E. (2014). Full protein stability landscape: Bacillus subtilis lipase lipA as a model for detergent resistance. **VAAM 2014**, Dresden, Germany.

8.2 Poster Presentations

Frauenkron-Machedjou V. J., Fulton A., Zhu L., Jaeger KE, Schwaneberg U., 2011. Deciphering rationales determining the stability of a lipase in non-conventional media. **Biotrans 2011**, Sicily, Italy.

Fulton, A., Frauenkron-Machedjou V. J., Schwaneberg, U., Wilhelm, S., Jaeger, K.-E. (2011). Systematic approach to decipher the rationales behind detergent and thermostability of a model lipase. **Biotrans 2011**, Sicily, Italy.

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9 Curriculum Vitae

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