RATIONAL PROTEIN DESIGN FOR ENHANCING THERMAL STABILITY OF INDUSTRIAL ENZYMES

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ABSTRACT

Enzymes possessing many excellent properties such as high selectivity, consuming less energy, and producing less side products or waste have been widely applied as biocatalysts in pharmaceutical production and many industries such as biofuel, biomaterials, biosensor, food, and environmental treatment. Although enzymes have shown its potential as biocatalysts for many industrial applications, natural enzymes were not originated for manufacturing process which requires harsh reaction conditions such as high temperature, alkaline pH, and organics solvents. It was reported that reduction of final conversion of several enzymatic reactions was declined at high temperature. Protein engineering to improve the enzymes' thermostability is crucial to extend the use of the industrial enzymes and maximize effectiveness of the enzyme-based procesess. Various industrial enzymes with improved thermostability were produced through rational protein engineering using different strategies. This review is not aimed to cover all successful rational protein engineering studies. The review focuses on some effective strategies which have widely used to increase the thermostability of several industrial enzymes through introduction of disulfide bonds and introduction of proline.

Keywords: Industrial enzymes; Introduction of disulfide bonds; Introduction of proline; Rational protein design; Thermostability.

1. Introduction

Enzymes as biocatalysts have been widely used for synthesis of molecules for pharmaceutical and industrial sector due to their excellent properties such as being highly selective, consuming less energy, and producing less side products or waste (Bornscheuer et al., 2012), (Bornscheuer & Pohl, 2001), (Lutz, 2010), (Woodley, 2006), (Akoh, Chang, Lee, & Shaw, 2007). In addition, many enzymes do not require toxic metal ions for their catalytic properties; thus, it would be beneficial for applying them in environmentally benign processing for production of industrial products (Turner, Mamo, & Karlsson, 2007). Enzymes were applied as biocatalysts for various industries such as biofuel, biomaterials, biosensor, food, and environmental treatment. Lipases were used for production of biodiesel (Ko¨se, Tu¨ter, & Aksoy, 2002), (Shimada et al., 1999), (Akoh, Chang, Lee, & Shaw, 2007) and polysaccharide-degrading enymes such as cellulases and xylanases were used for production of second-generation ethanol for biofuel from cellulosic biomass (Saha, 2003), (Wang et al., 2014), (Badieyan, Bevan, & Zhang, 2012). The applications of enzymes for production of various industrial products are seen in Table 1. The global market for industrial enzymes reached nearly $4.9 billion in 2015. Furthermore, the market is expected to grow 4.7% annually from 2016 through 2021 (Global Markets for Enzymes in Industrial Applications, 2017).

To apply enzymes for production of industrial products, thermal stability of the enzymes is one of the most desired characteristics because enzyme-catalyzed reactions increase exponentially with increasing reaction temperature up to enzyme denaturation temperature (Peterson ME, 2007). The Conversion of biodiesel (i.e., methyl esters) from cotton-seed oil and methanol catalyzed by immobilized *Candida antarctica* lipase B, for example, increased

with increasing reaction temperature in range of 30–50°C and reached maximum methyl ester yield of 91.5% at 50°C after 7h. In addition, the reactions at high temperature are essential for increasing the solubility and/or decreasing viscosity of reactants as well as decreasing the risk of bacterial contamination (Eijsink et al., 2004). Production of high fructose corn syrup (HFCS), for example, the liquefaction of starch is usually performed at high temperature to increase the solubility of starch which is insoluble at cold water (e.g., the liquefaction of starch into soluble, short-chain dextrin catalyzed by alpha-amylases is performed 95–105°C for 90 minutes (Maarel, Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002). Thermostable enzymes have many advantages in promoting better enzyme penetration and cell-wall disorganisation of the raw materials in biorefinery which produces chemicals, commodities and fuels from renewable raw materials such as agricultural crops (Turner, Mamo, & Karlsson, 2007). Thermostable enzymes also provide opportunities to perform the reaction at high temperature to reduce the risk of microbial contamination such as *Lactobacillus* in the ethanol production from non-sterilized cellulosic biomass (Badieyan, Bevan, & Zhang, 2012). Conversion of starch to oligomaltodextrin in production of high fructose corn syrup catalyzed by α-amylase, for instance, required the high thermalstable enzyme because the process concomitantly injected with steam (Ole Kirk, 2002).

Although the enzymes have shown its potential as biocatalysts for many industrial applications, natural enzymes were not originated for manufacturing process which requires the harsh reaction condition such as high temperature, alkaline pH, and organics solvents. It has been found that the reduction of final conversion of several enzymatic reactions was declined at high temperature. For example, the conversion of glycerol carbonate and biodiesel synthesis which are catalyzed by *Candida antarctica* lipase B were significantly decreased around 60°C (Ko¨se, Tu¨ter, & Aksoy, 2002); (Kim, Kim, Lee, Yoon, & Song, 2007). Enhancing thermostability of the applied enzymes would be beneficial for extension and maximizing effectiveness of the enzyme-based processes.

Protein engineering has been increasingly considered as an excellent choice to fulfill the above requirement. Protein engineering, one part of genetic engineering, performs the modification of protein sequence through mutations (i.e., single or multiple mutation at certain amino acid residues) to change the properties of a protein. The protein engineering is classified into two groups: directed evolution and rational protein design. The directed evolution (also called molecular evolution) evolves the protein characteristics through random mutagenesis of gene encoding for target protein (e.g., error-prone PCR) or through combination of different fragments of DNA in molecular pool (Bornscheuer & Pohl, 2001), (Turner N. J., 2009). In this approach, the number of variants in library was so huge and it requires an efficient high-throughput screening to select the evolved variants. In contrast to the directed evolution, the rational protein design evolves the enzyme characteristics through site-directed mutagenesis at one or several certain amino acid residues which were predicted by analysis on sequence, structure, function of target enzymes (Bornscheuer & Pohl, 2001). In the rational protein design, the number of variants in experiments is relatively small and results depend highly on the prediction step. However, knowledge and information on sequence and/or structure of target enzyme is essential for the rational design. It has been reported that the thermal stability of enzymes was increased by various strategies such as introduction of additional disulfide

bonds (Jeong, Kim, Yun, Choi, & Cho, 2007), (Le, Joo, Yoo, & Kim, 2012), replacement of proline for some critical locations (Nakamura, Tanaka, Yada, & Nakai, 1997), (Tian et al., 2010), (Wang et al., 2014) or rigidifying the flexible residues (Kim, Le, & Kim, 2010), (Badieyan, Bevan, & Zhang, 2012), introduction of salt bridges (Chan, Yu, & Wong, 2011). In this review, some successful examples on rational protein design focusing on engineering disulfide bonds and introduction of proline to enhance thermostability of enzymes applying in industrial sector will be presented.

Table 1

Industrial applications of enzymes

Enzymes	Applications	References
Lipase B from *Candida antartica* (CalB) (Novozyme 435) and other lipses	Production of Biodiesel	(Ko¨se, Tu¨ter, & Aksoy, 2002); (Shimada et al., 1999); (Akoh, Chang, Lee, & Shaw, 2007).
Lipase B from *Candida antartica* (CalB) (Novozyme 435)	Production of Glycerol carbonate (a key multifunctional compound for chemical intermediate and cosmetic solvents)	(Kim, Kim, Lee, Yoon, & Song, 2007).
Lipase B from *Candida antarctica* (CalB)	Production of poly (D-lactide), a Biodegradable bioplastic	(Hans, Keul, & Moeller, 2009).
Lipase B from *Candida antartica* (CalB) (Novozyme 435)	Production of Gamma Butyrolactone Methacrylate (GBLMA) for production of phoresist resin	(Kim, Nam, Kim, & Song, 2010).
Xylanases	Degradation of hemicellosic biomass for biofuel synthesis and pulp industry	(Saha, 2003), (Wang et al., 2014).
Alpha-amylase family	Enzymatical hydrolysis of starch to product maltodextrin, modified starch or glucose and fructose syrup	(Maarel, Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002).
D-xylose isomerase from *Streptomyces diastaticus* No.7 (M1033)	Converting xylose into xylulose and catatalyze glucose into sweeter fructose. This is widely used in food industry for high fructose corn syrup (HFCS)	(Zhu et al., 1999).
Organophosphate hydrolase from *Pseudomonas diminuta*	Detoxification of harmful organophosphate pesticides	(Farnooshgholamreza, Khajeh, Latifi, & Aghamollaei, 2016).
Acetylcholinesterase from *Drosophila melanogaster*	Biosensor for detection of organophosphate and carbamate insecticides	(Siadat, Lougarre, Lamouroux, Ladurantie, & Fournier, 2006).
GH5 cellulases	Production of cellulosic ethanol from lignocellulose	(Badieyan, Bevan, & Zhang, 2012).

2. Rational protein engineering through introduction of proline

Introduction of proline has been successfully applied to increase the thermal stability of several enzyme such as neutral protease from *Bacillus stearothermophilus* (Nakamura, Tanaka, Yada, & Nakai, 1997), D-xylose isomerase from *Streptomyces diastaticus* No.7 (M1033) (Zhu et al., 1999), liquefing α-amylase from *Bacillus* sp. Strain KSM-1378 (Igarashi et al., 1999), methyl parathion hydrolase from *Ochrobactrum* sp. M231 (Ochr-MPH) (Tian et al., 2010), and xylanase from *Streptomyces* sp. strain S9 (Wang et al., 2014). Proline, which possesses a special cyclic structure of side chain, restricts backbone configuration and hence decrease backbone configurational entropy compared to other amino acids (Watanabe & Suzuki, 1998). General rule for engineering proline was proposed by Suzuki *et al.* based on their discovery that the increase in proline number in second positions of β-turns of *Bacillus* oligo-1,6-glucosidases with their thermostability (Suzuki, 1989), (Suzuki Y., 1999). The presence of proline at β-turns increase protein's thermostability mainly because of decrease in the backbone entropy of unfolding (Igarashi et al., 1999). In rational design, the selection of suitable residues for site-directed mutagenesis plays the central role. To engineer the enzyme belonging to a family with different thermal stability, multiple sequence alignment to identify the critical residues which conserved in higher thermal stability, but not in less thermal stability is a good strategy for engineering through introduction of proline. The combination of introduction of proline and multiple sequence alignment significantly increased 2.5-fold in half-life at 35°C for D-xylose isomerase from *Streptomyces diastaticus* No.7 (M1033) (Zhu et al., 1999) and 2.6-fold in half-life at 50°C for a liquefying α-amylase from *Bacillus* sp. Strain KSM-1378 (Igarashi et al., 1999) (Table 2). Protein engineering for target enzyme with limited available information, however, multiple sequence alignment does not work. Residual flexibility analysis based on Molecular Dynamics (MD) Simulations could be an excellent choice. Methyl parathion hydrolase from *Ochrobactrum* sp. M231 (Ochr-MPH) mutant exhibited a 3.3°C increase in melting temperature (Tm) and a 5°C increase in T_{50}^{10} (half-survial temperature at 50°C for 10 minutes) compared to the wild type (Tian et al., 2010). In addition, combination of flexibility analysis using MD simulations and multiple sequence alignment to increase >9-fold in half-life at 70°C and 7.0°C in T_m for xylanase from *Streptomyces* sp. strain S9 (Wang et al., 2014). The successful researches demonstrated the usefulness of introduction of proline on the thermostability of a given enzyme. However, the introduction of proline does not always get beneficial results. It was reported that introduction of proline at N-terminal of the helix region led to increase in enzyme's thermostability, while the introduction of proline in the middle of the helix region led to decrease in the enzyme's thermostability (Tian et al., 2010). The selection of suitable positions for proline subtitution is critical the success of the engineering.

3. Rational protein engineering of disulfide bonds in proteins

The engineering of disulfide bonds in protein by introducing additional disulfide bond(s) into proteins is another promising strategy for improving the thermostability of industrial enzymes. Disulfide bonds, which are additional covalent linkages in polypeptide sequence and occur naturally in secreted proteins, contribute significantly on protein's stability (Wells & Powers, 1986), (Petsko & Ringe, 2004), (Siadat, Lougarre, Lamouroux, Ladurantie, & Fournier, 2006). The disulfide bonds contribute on protein's

stability caused mainly by decreasing entropy of the unfolded form of protein (Anfinsen & Scheraga, 1975) or decreasing unfolding rate of irreversibly denatured protein (Clarke J, 1993), (Plaza, Ibarra-Molero, & Sanchez-Ruiz, 2000). Introducing disulfide bonds into protein demonstrated its usefulness to increase the thermal stability of several enzymes such as ribonuclease H from *Escherichia coli* (Kanaya et al., 1991), xylanase from *Bacillus stearothermophilus* No. 236 (Jeong, Kim, Yun, Choi, & Cho, 2007), Acetylcholinesterase from *Drosophila melanogaster* (Siadat, Lougarre, Lamouroux, Ladurantie, & Fournier, 2006), and lipase B from *Candida antarctica* (Le, Joo, Yoo, & Kim, 2012).

The engineering of protein's thermostability by introducing additional disulfide bonds include two important steps. The first step is to predict residue pairs having possibility to form disulfide bonds in protein. The second step is to select promising residue pairs among the predicted residue pairs for site-directed mutagenesis into cysteines having high potential to increase protein's thermostability. In the first step, the residue pairs having possibility to form disulfide bonds were usually identified by several automated prediction programs such as modeling of disulfide bonds in proteins (MODIP) (Dani, Ramakrishnan, & Varadarajan, 2003), (Sowdhamini et al., 1989) or Disulfide by Design (DbD) (Dombkowski, 2003). Different programs have different criteria to predict the residue pairs having possibility to form disulfide bonds. MODIP evaluates geometrical parameters (dihedral angles and S–S distance) of model disulfide bonds from each residue pairs and classified it into grade A or B or C depending on their geometrically satisfactory (Dani, Ramakrishnan, & Varadarajan, 2003), (Sowdhamini et al., 1989). On the while, DbD evaluates the formation energy of model disulfide bonds with fixed geometrical parameters (C_β–S_γ, Sγ–Sγ bond lengths and C_β–Sγ–Sγ bond angles are 1.81 Å, 2.04 Å and 104.15°, respectively). A residue pair which generated a lower energy value for a putative disulfide bond has a higher possibility to form a disulfide bond (Dombkowski, 2003). The thermal stability of several enzymes were significantly increased by applying MODIP and/or DbD such as indoleglycerol phosphate synthase by MODIP (Ivens et al., 2002) and xylanase from *Bacillus stearothermophilus* No. 236 by DbD (Jeong, Kim, Yun, Choi, & Cho, 2007) and lipase B from *Candida antarctica* by both MODIP and DbD in our previous study (Le, Joo, Yoo, & Kim, 2012). The successful researches that have applied MODIP and DbD to increase the thermal stability of enzymes demonstrated the usefulness of those computational tools to predict the possible disulfide bonds in proteins. However, both MODIP and DbD, which are focus only on identifying residue pairs having possibility to form disulfide bonds, do not have any tool to evaluate potential to increase thermostability of genetically engineered protein bearing the newly disulfide bond(s). Additionally, number of the residue pairs which could form new disulfide bonds which suggested by MODIP and/or DbD is relalively large for site-direted mutagenesis (Le, Joo, Yoo, & Kim, 2012). Therefore, efficient selection of promising residue pairs for the site-directed mutagenesis is crucial for time-saving and cost-effective engineering of disulfide bonds in improving the thermostability of target enzymes. Different selection strategies to select the promising residue pairs for site-directed mutagenesis were introduced and got success for improving the thermostability of several enzymes. The MODIP-predicted residue pairs for disulfide bond formation was further filtered for experimental mutagenesis by simply selecting the residue pairs which are localized nearby active site of enzyme and/or

may lead to short-range disulfide bond formation of indoleglycerol phosphate synthase (Ivens et al., 2002). Multiple sequence alignment having ability to identify the conserved residues and critical variants from the protein family has shown its usefulness for selection of the promising residue pairs in protein engineering of disulfide bonds of enzymes belonging to a family with available information on their diverse thermostability such as xylanase from *Bacillus stearothermophilus* No. 236 (Jeong, Kim, Yun, Choi, & Cho, 2007) and acetylcholinesterase from *Drosophila melanogaster* (Siadat, Lougarre, Lamouroux, Ladurantie, & Fournier, 2006). Twenty-five residue pairs having possibility to form the disulfide bonds in xylanase from *Bacillus stearothermophilus* No. 236 by DbD program, for example, were further filtered by multiple sequence alignment with other 11 family xylanases and select only one residue pair which are evolutionarily conserved cysteines for site-directed mutagenesis. The mutant with one additional disulfide bonds from the residue pair increased 5°C in half-survival temperature after 20 minutes and increased 3.5-fold in half-life inactivation at 65°C compared (Jeong, Kim, Yun, Choi, & Cho, 2007). For development of enzymes that are not belong to a family with diverse thermostability or have little information about their family or enzymes showing too many possible residue pairs for disulfide bond formation from MODIP and DbD, a general tool to identify the promising residue pairs to increase enzyme's thermostability was developed. Residual flexibility analysis which was firstly proposed by our group is one of example as a general selection tool for selecting the promising residue pairs from the predicted programs. The flexibility analysis of the predicted residue pairs by using both B-factor values from enzyme's crystal-structure and FRODAN dynamics for given residue pairs before and after *in-silico* mutagenesis was used for selection of the promising residue pairs for experimental site-directed mutangesis in engineering disulfide bonds of lipase B from *Candida antarctica* in our previous study (Le, Joo, Yoo, & Kim, 2012). The B-factor values, the atomic displacement parameters which determined by high-resolution X-ray crystallographic studies represent smearing of atomic electron densities around ther equilibrium positions due to thermal motion and positional disorder (Parthasarathy S. & Murthy M.R.N., 2000). Beside B-factor values which indicates the flexibitility of static states and flexibility motions which predict the flexibility changes of local structures is also essential for evalution and selection of the residue pairs for site-directed mutagenesis (Joo et al., 2011), (Le, Joo, Yoo, & Kim, 2012). In addition, energy stability evaluation based on MD Simulations was also used as a general tool for selection of the promising residue pairs in engineering thermostability of organophosphate hydrolase from *Pseudomonas diminuta* (Farnooshgholamreza, Khajeh, Latifi, & Aghamollaei, 2016). The rational introduction of disulfide bonds into proteins has demonstrated its usefulness to enhance the thermal stability of many industrial enzymes. However, the introduction of disulfide bonds does not always get expected results. Destabilization of protein's stability through introduction of additional disulfide bonds has been reported in one or some mutants of several enzymes such as T4 lysozyme (Wetzel, Perry, Baase, & Becktel, 1988), acetylcholinesterase from *Drosophila melanogaster* (Siadat, Lougarre, Lamouroux, Ladurantie, & Fournier, 2006), and lipase B from *Candida antarctica* (Le, Joo, Yoo, & Kim, 2012).

Table 2

The Computational protein engineering to enhance thermostability of enzymes

Engineering strategies	Target Enzymes	Computational methods	Improved property of mutant	References
Introduction of proline	Neutral protease from *Bacillus stearothermophilus*	Engineering the alpha-helix (residues 140-153) which combines N- and C-terminal domains in the active sites of the enzymes.	Increased 7.5°C in half-survival temperature after 30 minutes. increased 4.4° in Tm	(Nakamur, Tanaka, Yada, & Nakai, 1997).
Introduction of proline	D-xylose isomerase from *Streptomyces diastaticus* No.7 (M1033)	Multiple Sequence alignment of the enzyme with other glucose isomerases of thermophilic bacteria.	Increased 2.5-fold in half-life inactivation at 35°C	(Zhu et al., 1999).
Introduction of proline	Liquefing α-amylase from *Bacillus* sp. Strain KSM-1378 (LAMY)	Multiple sequence aligment of LAMY with alpha-amylases from other *Bacillus* sp.	Increased 4°C in half-survival temperature at 50°C after 10 minutes and 2.6-fold increased in half-life at 50°C.	(Igarashi et al., 1999).
Introduction of proline	Methyl parathion hydrolase from *Ochrobactrum* sp. M231 (Ochr-MPH)	MD Simulations to find the greatest conformation fluactuation.	Increased 5°C in T_{50}^{10} (half-survival temperature at 50°C after 10 minutes) and 3.3°C in T_m	(Tian et al., 2010).
Proline or glutamic acid replacement	Xylanase from *Streptomyces* sp. strain S9	Multiple-sequence analysis and molecular dynamics simulations predicted 4 redisudes affecting thermostability of the xylanase.	Increased >9-fold increase in half-life at 70°C and 7.0°C higher in T_m	(Wang et al., 2014).
Introduction of disulfide bonds	Acetylcholinesterase from *Drosophila melanogaster*	**Prediction**: Choosing residue pair which has their C_β distance of 3.6 –4.0 Å. **Selection**: n Multiple sequence alignment.	Increased 170-fold in half-life at 50°C.	(Siadat, Lougarre, Lamourou, Ladurantie, & Fournier, 2006).
Introduction of disulfide bond	Xylanase from *Bacillus stearothermophilus*	**Prediction:** Disulfide by Design. **Selection**: Multiple	Increased 5°C in half-survival temperature after 20 minutes and	(Jeong, Kim, Yun, Choi & Cho, 2007).

	No. 236	sequence alignment	increased 3.5-fold in half-life inactivation at 65°C.	
Introduction of disulfide bond	Lipase from *Rhizomucor miehei*	**Prediction:** Disulfide by Design. **Selection**: Selecting the residue pairs in lid region which are reported to be related to activation of the enzyme.	Increased 5-fold in half-life inactivation at 60°C	(Han, Han, Zheng, & Lin, 2009).
Introduction of disulfide bonds	Cellulase C (a member of GH5 endoglucanses)	**Prediction**: Disulfide by Design **Selection**: MD Simulations analysis at various temperatures	Increased 4°C in half-survival temperature after 10 minutes and increased >5-fold in half-life at 65°C.	(Badieyan, Bevan, & Zhang, 2012).
Introduction of disulfide bonds	*Candida antarctica* lipase B (CalB)	**Prediction:** MODIP and Disulfide by Design. **Selection**: Residual flexibility analysis which combines B-factor values of residues and FRODAN dynamics	Increased 8.5°C in half-survival temperature at 50°C for 60 minutes and a 4.5-fold increase in half-life at 50°C.	(Le, Joo, Yoo, & Kim, 2012).
Introduction of disulfide bonds	Organophosphate hydrolase from *Pseudomonas diminuta*	**Prediction**: Disulfide by Design. **Selection**: Stability energy evalution using ERIS database and molecular dynamics Simulation (i.e., Gromacs).	Increased 1.5-fold in half-life at 65°C, but it only remained 30% catalytic efficient (kcat/Km).	(Farnooshgholamreza, Khajeh, Latifi, & Aghamollaei, 2016).

Tm: Melting temperature is a temperature at which protein remains 50% of its native structure

Half-life (t_{50}): The time at which protein remain 50% its activity at given temperature

4. Conclusion and perspectives

The rational protein engineering has demonstrated its usefulness to successfully increase the thermostability of various industrial enzymes. Some mutants from the rational protein engineering also exhibited the decrease in the thermostability. Identification of the promising residues for experimental site-directed mutagenesis is critical for success of the engineering and dependent both on the enzyme and applied computational strategies. Several protein-stabilizing factors such as disulfide bonds and proline residues at critical positions were applied to increase the thermal stability of the enzymes by the support of several computational strategies such as multiple

sequence alignment and residual flexibility. Multiple sequence alignment is very helpful and limited in the engineering of a given enzyme belonging to a family with available information on their diverse thermostability. More general and direct computational tools such as FRODAN dynamics and MD Simulations to evaluate residual fluctuation and energy's stability have been increasingly used to work individually or collaboratively with simpler computational tools such as multiple sequence alignment for engineering the thermostability of enzymes. In addition, searching for better and simpler computational strategies would be beneficial to get better thermal stability of the enzymes with lesser effort■

References

Akoh, C. C., Chang, S. W., Lee, G. C., & Shaw, J. F. (2007). Enzymatic Approach to biodiesel production. *J. Agric. Food. Chem., 55*, 8995-9005.

Anbar, M., Gul, O., Lamed, R., Sezerman, U. O., & Bayer, E. A. (2012). Improved Thermostability of Clostridium thermocellum Endoglucanase Cel8A by Using Consensus-Guided Mutagenesis. *Appl. Environ. Microbiol.*, 3458–3464.

Anders Hamberg, Steffen Maurer and Karl Hult (2012). Rational engineering of *Candida antarctica* lipase B for selective monoacylation of diols. *Chem. Commun., 48*, 10013–10015.

Anfinsen, C., & Scheraga, H. (1975). Experimental and theoretical aspects of protein folding. *Adv. Protein Chem., 29*, 205–300.

Arnold, F. H. (2001). Combinatorial and computational challenges for biocatalyst design. *Nature, 409*, 253-257.

Badieyan, S., Bevan, R. D., & Zhang, C. (2012). Study and design of stability in GH5 Cellulases. *Biotechnol. Bioeng., 109*, 31-44.

Bornscheuer, U. T., & Pohl, M. (2001). Improved biocatalysts by directed evolution and rational protein design. *Curr. Opin. Chem. Bio., 5*, 137-143.

Bornscheuer, U. T., Huisman, G. W., Kazlauskas, R. J., Lutz, S., Moore, J. C., & Robins, K. (2012). Engineering the thirdwave of biocatalysis. *Nature, 485*, 185-194.

Clarke J, F. A. (1993). Engineered disulfide bonds as probes of the folding pathway of barnase: Increasing the stability of proteins against the rate of denaturation. *Biochemistry, 32*, 4322–4329.

Chan, C. H., Yu, T. H., & Wong, K. B. (2011). Stabilizing salt-bridges enhances protein thermostability by reducing the heat capacity change of unfolding. *PlosOne, 6*(6), 1-8.

Dani, V., Ramakrishnan, C., & Varadarajan, R. (2003). MODIP revisited: Reevaluation and refinement of an automated procedure for modeling of disulfide bonds in proteins. *Protein Eng., 16*(3), 187-93.

Didier Rotticci, Johanna C. Rotticci-Mulder, Stuart Denman, Torbjörn Norin, Karl Hult (2001). Improved Enantioselectivity of a Lipase by Rational Protein Engineering. *ChemBioChem, 2*, 766 - 770.

Dombkowski, A. (2003). Disulfide by Design TM: A computational method for the rational design of disulfide bonds in proteins. *Bioinformatics, 19*(14), 1852-1853.

Eijsink, G. V., Bjork, A., Gaseidnes, S., Sirevag, R., Synstad, B., Bertus, B. V., & Vriend, G. (2004). Rational engineering of enzyme stability. *J. biotechnol., 113*, 105-120.

Farnooshgholamreza, Khajeh, K., Latifi, M., & Aghamollaei, H. (2016). Engineering and introduction of de novo disulphide bridges in organophosphorus hydrolase enzyme for thermostability improvement. *J. Biosci., 41*, 577–588.

Frances H., Arnold (2001). Combinatorial and computational challenges for biocatalyst design. *Nature, 409*, 253-257.

Global Markets for Enzymes in Industrial Applications (2017). *[https://www.bccresearch.com]Business Communications Company, Inc;*.

Han, Z., Han, S., Zheng, S., & Lin, Y. (2009). Enhancing thermostability of a *Rhizomucor miehei* lipase by engineering a disulfide bond and displaying on the yeast cell surface. *Appl. Microbiol. Biotechnol., 85*, 117–126.

Hans, M., Keul, H., & Moeller, M. (2009). Ring-Opening Polymerization of DD-lactide catalyzed by Novozyme 435. *Macromol. Biosci., 9*, 239-247.

Hans, M., Keul, H., & Moeller, M. (2009). Ring-opening polymerization of DDlactide catalyzed by novozyme 435. *Macromol Biosci, 9*, 239–247.

Igarashi, K., Ozawa, T., Ikawa-Kitayama, K., Hayashi, Y., Araki, H., Endo, K.,… Ito, S. (1999). Thermostabilization by proline substitution in an alkaline, liquefying alpha-Amylase from *Bacillus* sp. Strain KSM-1378. *Biosci. Biotechnol. Biochem, 63(9)*, 1535-1540.

Ivens, A., Mayans, O., Szadkowski, H., Jurgens, C., Wilmanns, M., & Kirschner, K. (2002). Stabilization of a (ba)8-barrel protein by an engineered disulfide bridge. *Eur. J. Biochem., 269*, 1145–1153.

Jeong, M., Kim, S., Yun, C., Choi, Y., & Cho, S. (2007). Engineering a de novo internal disulfide bridge to improve the thermal stability of xylanase from *Bacillus stearothermophilus* No. 236. *J. Biotechnol., 127*, 300–309.

Joo, J. C., Pack, S. P., Kim, Y. H., & Yoo, Y. J. (2011). Thermostabilization of *Bacillus circulans* xylanase: Computational optimization of unstable residues based on thermal fluctuation analysis. *J. Biotechnol., 151*, 56–65.

Kanaya, S., Katsuda, C., Kimura, S., Nakai, T., Kitakuni, E., Nakamura, H., . . . Ikehara, M. (1991). Stabilization of Escherichia coli ribonuclease H by introduction of an artificial disulfide bond. *J. Biol. Chem., 266*, 6038–6044.

Kim, H., Le, QAT., & Kim, Y. (2010). Development of thermostable lipase B from *Candida antarctica* (CalB) through in silico design employing Bfactor and Rosetta Design. *Enzyme Microbiol. Technol., 47*, 1–5.

Kim, S. C., Nam, D. H., Kim, Y. H., & Song, B. K. (2010). Enzymatic Acrylation of 2-hydroxy-gamma-butyrolactone to synthesize the Gamma Butyrolactone Methacrylate (GBLMA) for photoresist. *Biotechnol. Bioprocess Eng., 15*, 208-212.

Kim, S., Kim, Y., Lee, H., Yoon, D., & Song, B. (2007). Lipase-catalyzed synthesis of glycerol carbonate from renewable glycerol and dimethyl carbonate through transesterification. *J. Mol. Catal B: Enzym., 49*, 75–78.

Ko¨se, O., Tu¨ter, M., & Aksoy, H. (2002). Immobilized Candida antarctica lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. *Bioresour. Technol., 83*, 125–129.

Korkegian, A., Black, M. E., Baker, D., & Stoddard, B. L. (2005). Computational thermostabilization of an enzyme. *Science, 308(5723*, 857–860.

Kuhad, C. R., Gupta, R., & Singh, A. (2011). Microbial cellulases and their industrial applications. *Enzyme Research*.

Le, QAT., Joo, J., Yoo, Y., & Kim, Y. (2012). Development of Thermostable *Candida antarctica* Lipase B Through Novel In Silico Design of Disulfide Bridge. *Biotechnol. Bioeng., 109*, 867-876.

Lutz, S. (2010). Beyond directed evolution - semi-rational protein engineering and design. *Curr. Opin. Biotechnol., 21*(6), 734-743.

Maarel, v. d., Veen, v. d., Uitdehaag, J. C., Leemhuis, H., & Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the alpha-amylase family. *J. Biotechnol., 94*, 137-155.

Magnusson, A. O.-M. (2005). Creating space for large secondary alcohols by rational redesign of *Candida antarctica* Lipase B. *ChemBioChem, 6*, 1051 – 1056.

Nakamura, S., Tanaka, T., Yada, R., & Nakai, S. (1997). Improving the thermostability of *Bacillus stearothermophilus* neutral protease by introducing proline into the active site helix. *Protein Eng., 10*(11), 1263–1269.

Ole Kirk, T. V. (2002). Industrial enzyme applications. *Curr. Opin. Biotechnol., 13*, 345–351.

Parthasarathy, S., & Murthy, M. (2000). Protein thermal stability: insights from atomic displacement parameters (B values). *Protein Eng., 13*(1), 9-13.

Peterson ME, D. R. (2007). The dependence of enzyme activity on temperature: Determination and validation of parameters. *Biochem. J., 402*, 331–337.

Petsko, G., & Ringe, D. (2004). *Protein structure and function.* London: New Science Press.

Plaza, d. P., Ibarra-Molero, B., & Sanchez-Ruiz, J. (2000). Lower kinetic limit to protein thermal stability: A proposal regarding protein stability in vivo and its relation with misfolding diseases. *Proteins, 40*, 58–70.

Saha, B. C. (2003). Hemicellulose bioconversion. *J. Ind. Microbiol. Biotechnol., 30*, 279-291.

Shimada, Y., W., Y., S., T., S., A., N., H., F., . . . Y. (1999). Conversion of vegetable oil to biodiesel using immobilized Candida antarctica lipase. *J. AmOil. Chem. Soc., 76*, 789–793.

Siadat, R. O., Lougarre, A., Lamouroux, L., Ladurantie, C., & Fournier, D. (2006). The effect of engineered disulfide bonds on the stability of Drosophila melanogaster acetylcholinesterase. *BMC Biochem., 7*, 12-19.

Sowdhamini, R., Srinivasan, N., Schochet, B., Santi, D., Ramakrishman, C., & Balaram, P. (1989). Stereochemical modeling of disulfide bridges. Criteria for introduction into proteins by site-directed mutagenesis. *Protein Eng., 3*, 95-103.

Suzuki, Y. (1989). A general principle of increasing protein thermostability. *Proc. Japan Acad.Ser. B, 65*, 146-148.

Suzuki, Y. (1999). The proline rule- A strategy for protein thermal stabilization. *Proc. Japan Acad., 75*(6), 133-137.

Tian, J., Wang, P., Gao, S., Chu, X., Wu, N., & Fan, Y. (2010). Enhanced thermostability of methyl parathion hydrolase from Ochrobactrum sp. M231 by rational engineering of a glycine to proline mutation. *FEBS J., 277*, 4901–4908.

Turner, N. J. (2009). Directed evolution drives the next generation of biocatalysts. *Nature Chem. Biol., 5*, 567-573.

Turner, P., Mamo, G., & Karlsson, N. E. (2007). Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microbiol. Cell Fact., 6*, 9-31.

Turunen, M. L. (2007). Protein engineering: opportunities and challenges. *Appl. Microbiol. Biotechnol., 75*, 1225–1232.

Wang, K., Luo, H., Tian, J., Turunen, O., Huang, H., Shi, P., . . . Yao, B. (2014). Thermostability Improvement of a Streptomyces Xylanase by Introducing Proline and Glutamic Acid Residues. *Appl. Environ. Microbiol., 80*, 2158–2165.

Watanabe, K., & Suzuki, Y. (1998). Protein thermostabilization by proline substitutions. *J. Mol. Cat. B: Enzym., 4*, 167-180.

Wells, J., & Powers, D. (1986). In Vivo formation and stability of engineered disulfide bonds in Subtilisin. *J. Biol. Chem., 261*(14), 6564–6570.

Wetzel, R., Perry, L., Baase, W., & Becktel, W. (1988). Disulfide bonds and thermal stability in T4 lysozyme. *Proc. Natl. Acad. Sci. USA, 85*, 401-405.

Woodley, D. J. (2006). Biocatalysis for pharmaceutical intermediates: the future is now. *TRENDS Biotechnol., 25*, 66-73.

Xiao, Z., Bergeron, H., Grosse, S., Beauchemin, M., Garron, M.-L., Shaya, D., . . . Lau, P. C. (2008). Improvement of the Thermostability and Activity of a Pectate Lyase by Single Amino Acid Substitutions, Using a Strategy Based on Melting-Temperature-Guided Sequence Alignment. *Appl. Environ. Microbiol., 74*(4), 1183–1189.

Zhu, G., Xu, C., Teng, M., Tao, L., Zhu, X., Wu, C., . . . Wang, Y. (1999). Increasing the thermostability of D-xylose isomerase by introduction of a proline into the turn of a random coil. *Protein Eng., 12*(8), 635-638.