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جامعة الملك سعود
King Saud University
1957
College of Science

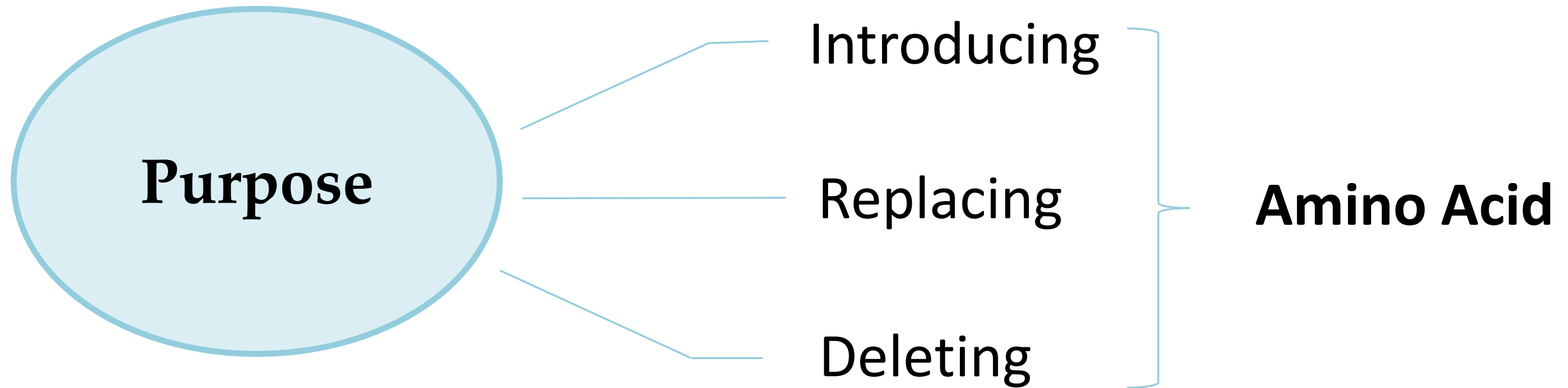
Protein Engineering

Site-Direction Mutagenesis

Site-Direction Mutagenesis

- Site-directed mutagenesis was described by Hutchinson et al. in 1978.
- It is e a powerful tool to study the molecular structure and function of proteins.
- The technique enables a desired modification to be achieved with exquisite precision

Purpose of SDM



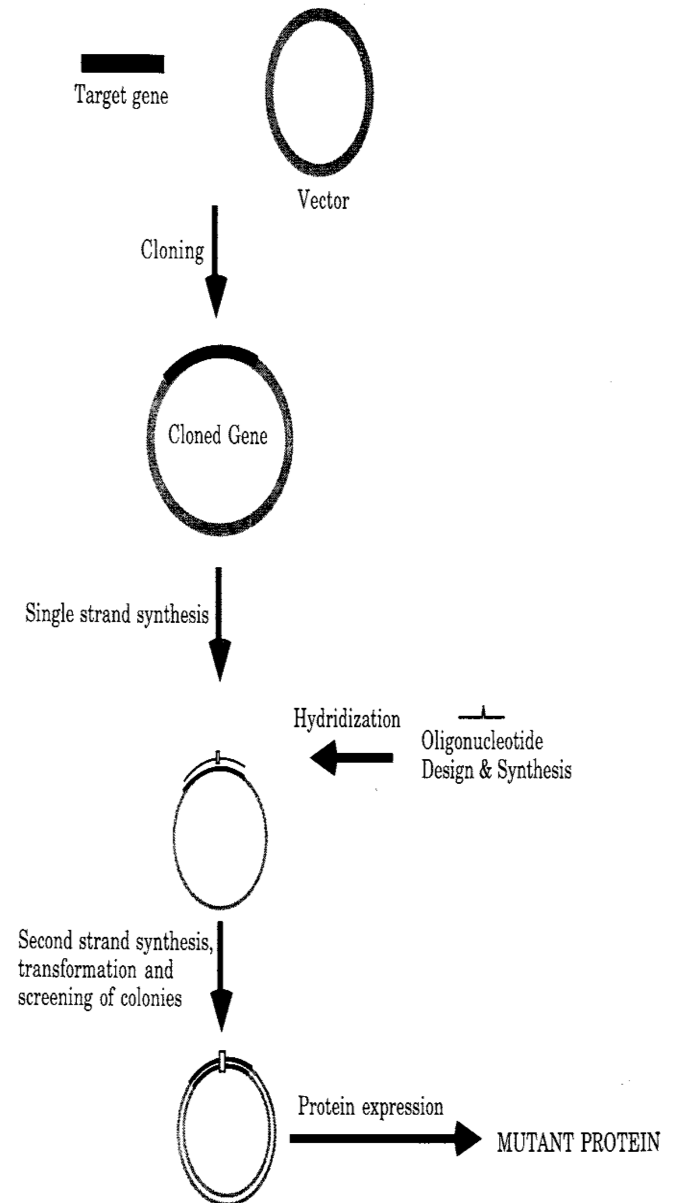
Site-Direction Mutagenesis

- SDM has been used to change the activity and stability of enzymes, as well as substrate specificity and affinity.

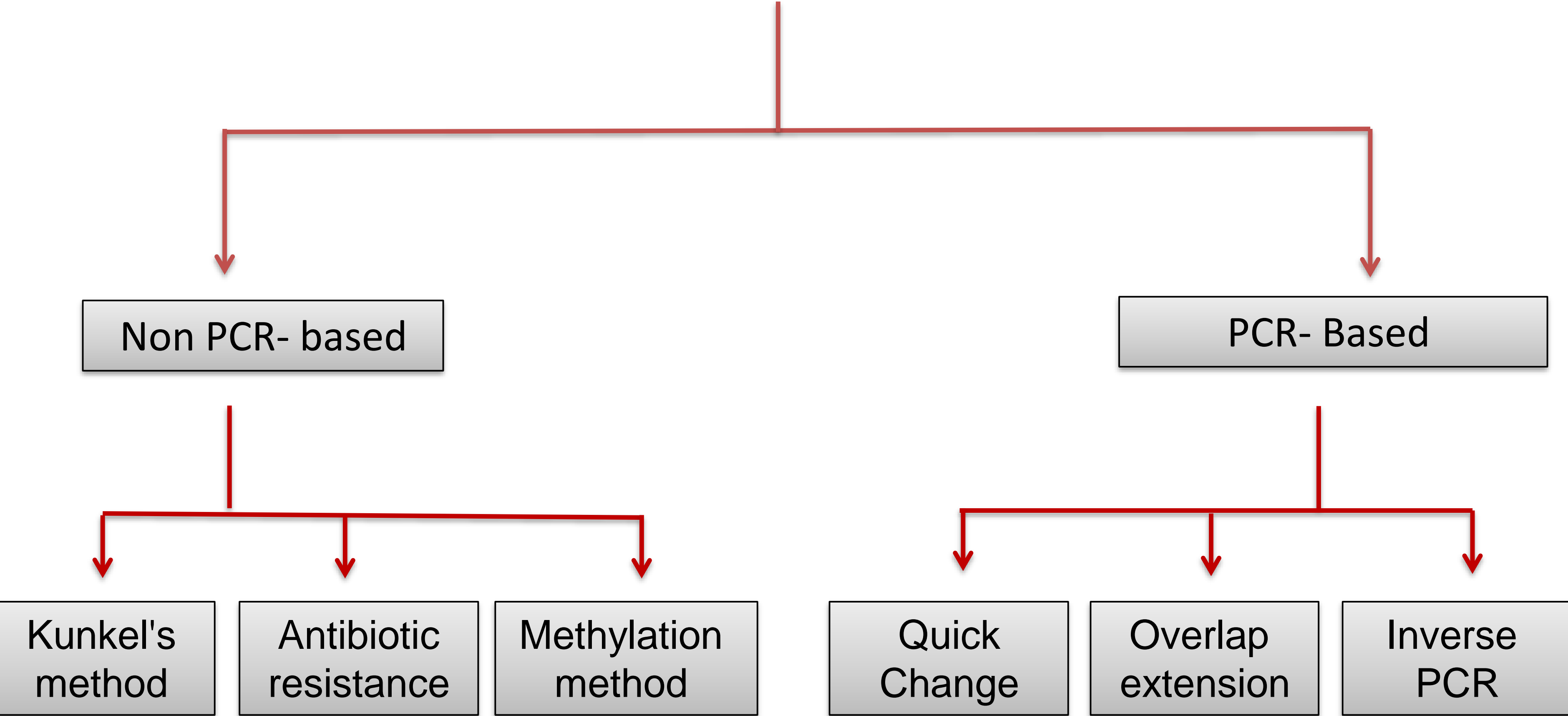
- Indeed, much of the biologic detergent enzyme sold in the United States (~$200 million per year) is a protein-engineered variant of the native enzyme.

Overview of site-directed mutagenesis

1. The first step of SDM involves cloning the gene for the protein into a vector.
2. Next, an oligonucleotide is designed and synthesized with desired mutation (usually a mismatch).
3. Thus the oligonucleotide is designed to bind to a single region of the target gene.
4. The mutation can then be introduced into the gene by hydridizing the oligonucleotide to the single-stranded template.



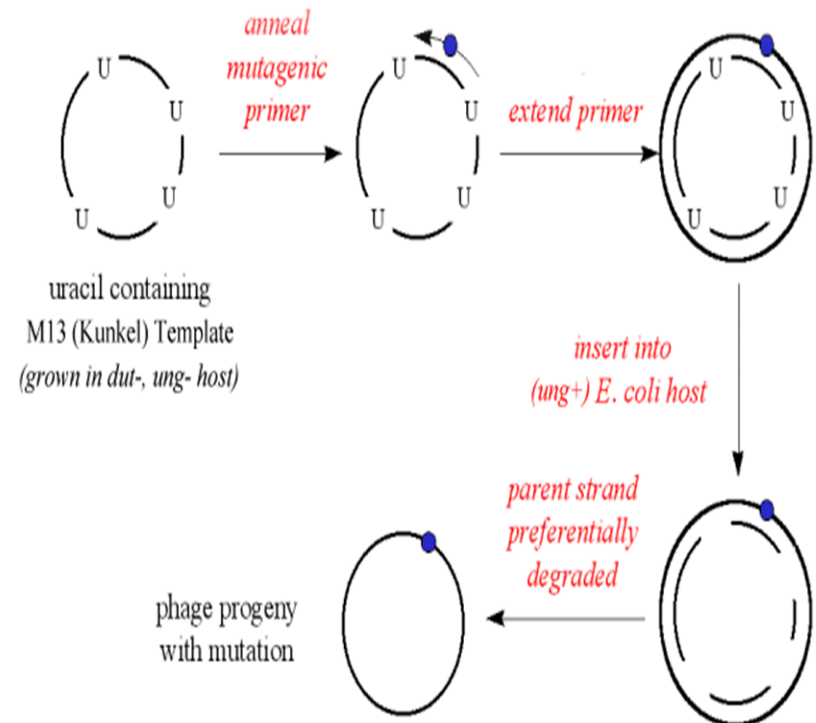
Methods for Site-specific Mutagenesis



Kunkel's method

- This method developed by Kunkel in 1985, that takes advantage of a strain deficient in dUTPase and uracil deglycosylase so that the recipient E.
- Kunkel method is carried out in three steps which are follows:

1. Step1: In Vivo Process Plasmid containing the target sequence is placed to be mutated into an E.coli strain that lacks **uracil deglycosidase** and **dUTPase** (ung- dut- strain). As a result the plasmid is transformed into DNA that contains U's instead of T's.
2. Step2: In Vitro Process Target DNA containing uracil is placed with a synthetic oligo that does not pair at the location of the desired mutation.

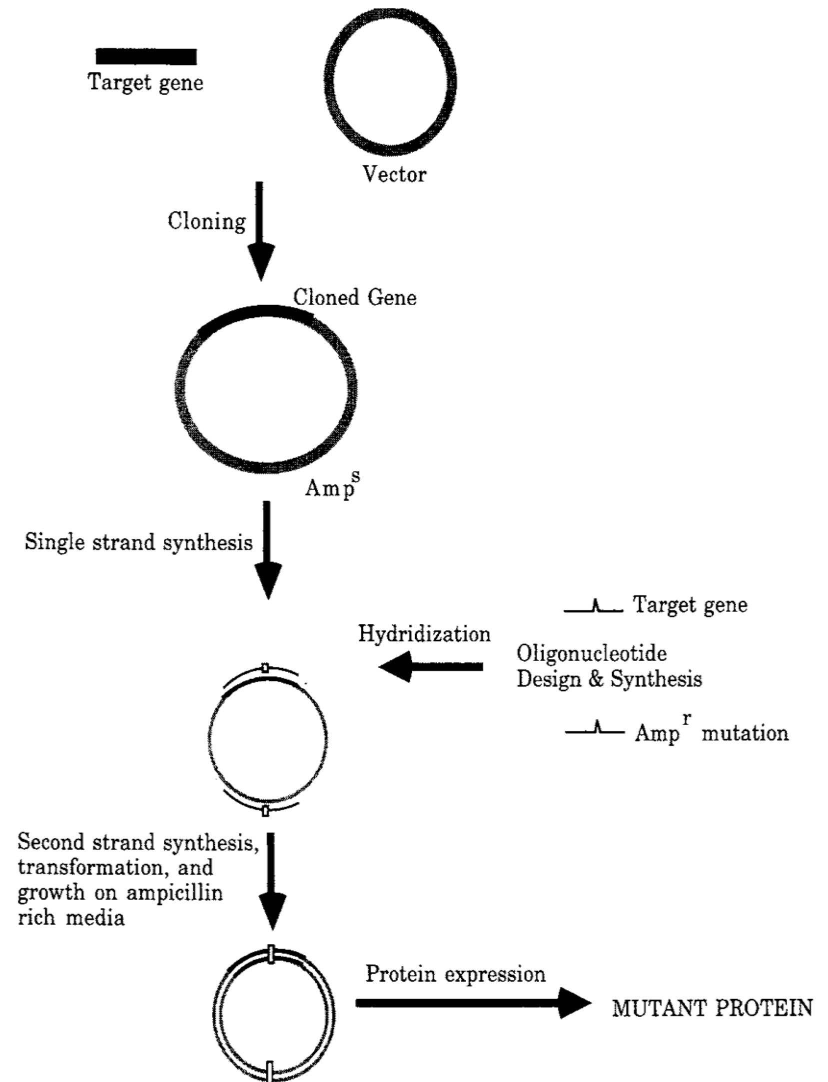


Cont.

Step3: In Vivo Process The hybrid DNA is transformed in bacteria by degrading the older DNA strand containing uracil to produce a strand containing T using the new and mutagenized DNA strand as a template. Thus, all the plasmids will contain the newly mutated sequence

Antibiotic resistance

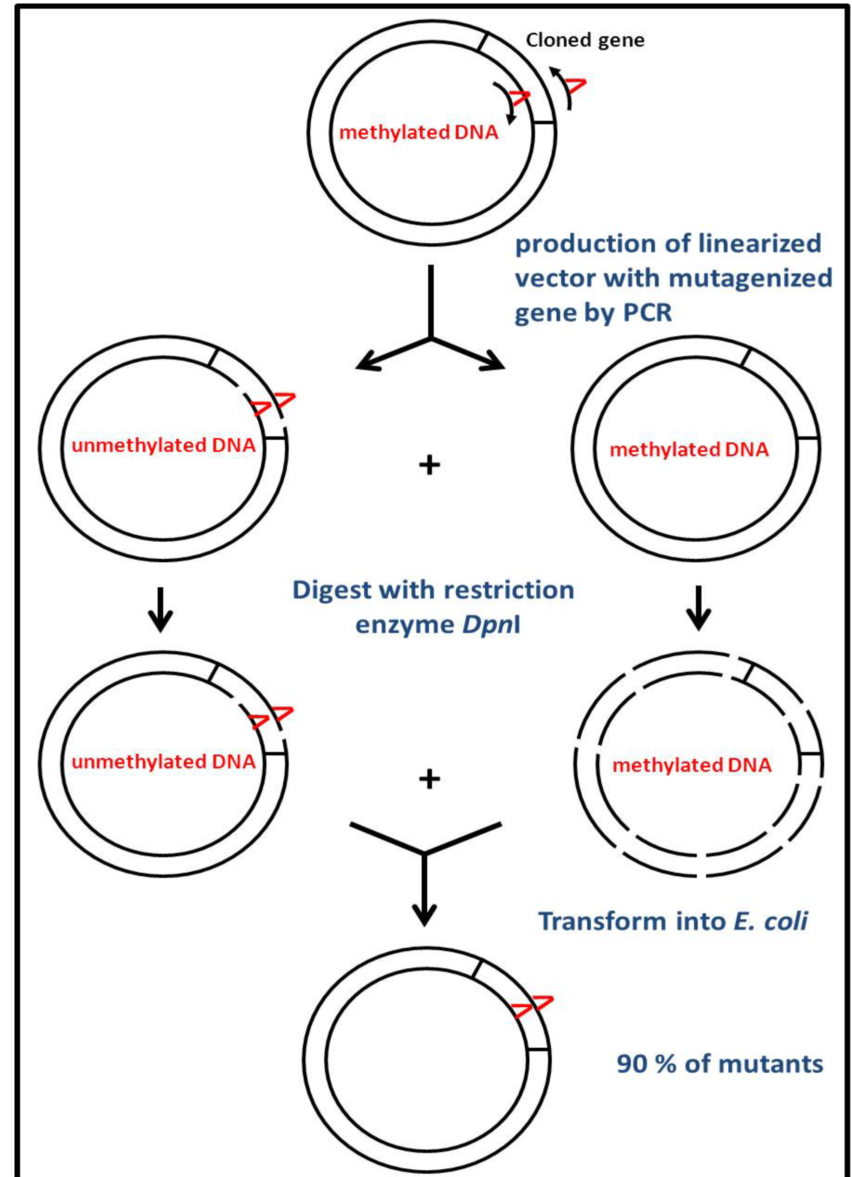
- Simultaneous site-directed mutations alter the antibiotic resistance gene of the plasmid along with the target gene by specific oligonucleotides. Enrichment of the desired mutants is by antibiotic resistance



Quick Change

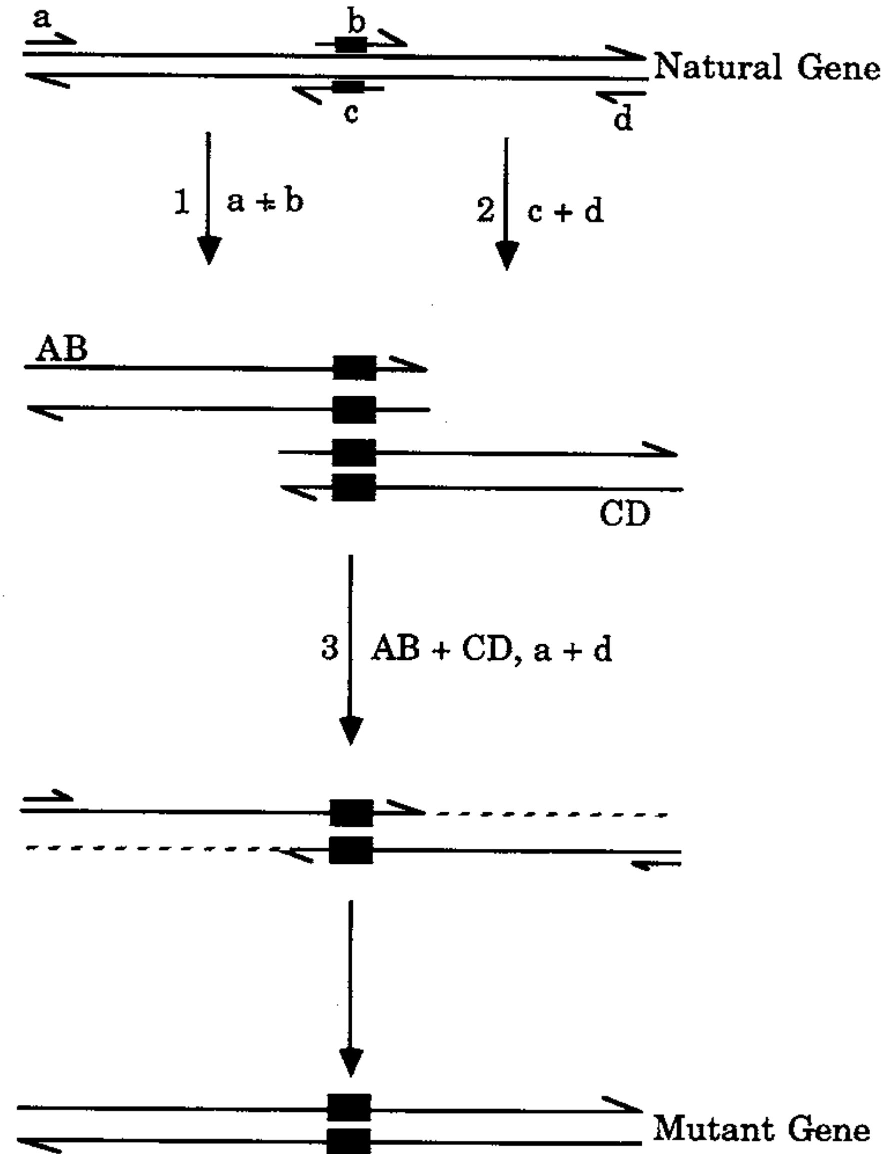
Quick change method is carried out in main three steps which are following:

1. The first step of mutagenesis is using mutagenic **complementary primers**, both containing the desired mutation, a mutated plasmid containing staggered nicks are generated.
2. The second step by digestion with the **restriction** enzyme **DpnI** the methylated parental DNA template is removed.
3. The third step the nicked mutated vector is transformed into an E. coli strain



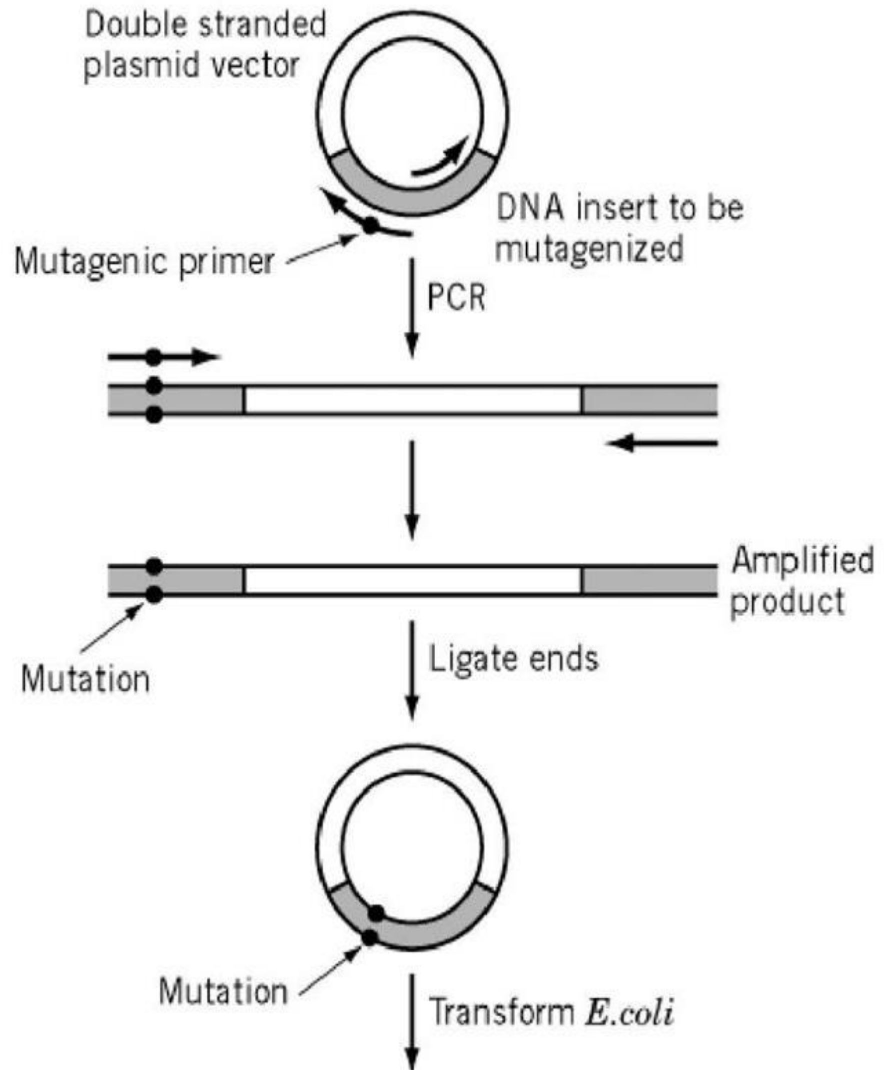
Overlap extension

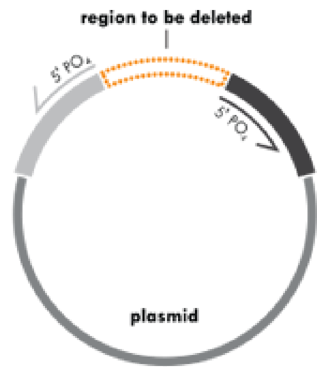
- Overlap extension was developed by **Ho** and colleagues in 1989.
- Four oligonucleotides are used as primers.
- Two of the primers containing the mutant are complementary to each other.
- The two other primers are complementary to the opposite strand of the ends of the cloned genes.
- Polymerase chain reactions are performed three times.
- The two DNA products are overlapped in the mutated region.
- The advantage of this method is that it can be done quickly with nearly 100% efficiency.



Inverse PCR

- In the inverse PCR, the primers amplify the fragment other than the target sequence
- The primers are oriented in the reverse direction, facing "outwards," away from each other.
- The method is used for inserting mutation into the plasmid having the gene of interest.



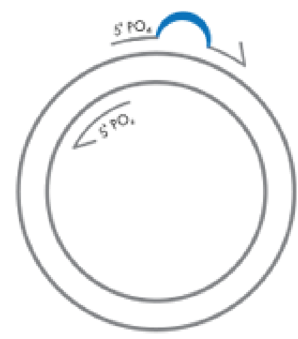


PCR

intramolecular ligation

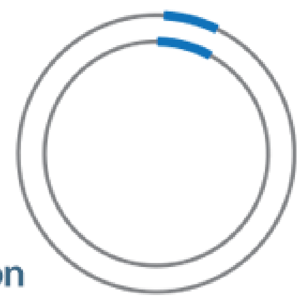
A. Deletion

final product



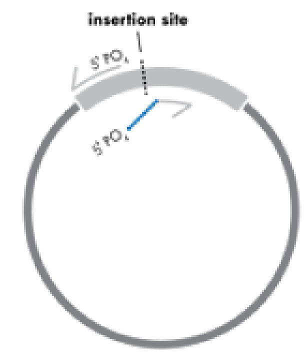
amplify target DNA

intramolecular ligation



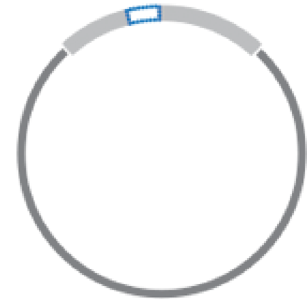
B. Substitution

final product



PCR

intramolecular ligation



C. Insertion

final product

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Improving the Thermostability and pH Stability of *Aspergillus niger* Xylanase by Site-directed Mutagenesis[1]

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Abstract—To increase the thermostability and pH stability of the recombinant xylanase MxynB from the *Aspergillus niger* nl-1, site-directed mutagenesis method was selected by rational approaches based on the three-dimensional structures using a homology modeling of *mxynB* constructed by SWISS-MODEL and BLAST. The effect of disulphide in the structure of xylanase was studied to increase the optimum tempera ture and thermostability of enzyme, the variant MxynB-116-135 which was created with amino acid residues substitutions G116C+Y135C by site directed mutagenesis had its reaction temperature for the maximum activity at 50°C, which corresponded to a basic 10°C unit increase compared with the wild type enzyme. Moreover, the thermostability of *A. niger* nl 1 xylanase after mutation MxynB G116C Y135C D76R N28H N29D-Y45M-N47L was clearly increased by 570% as compared with the wild-type enzyme under 70°C. In addition, the optimal activity pH of the enzyme after the mutations MxynB G116C Y135C D76R N28H N29D-Y45M-N47L and MxynB-G116C-Y135C-S58H-D76R-N28H-N29D-Y45M-N47L raised from 4.5 to 6.0, meanwhile, the pH stabilities were increased from 120 to 194% compared with the wild-type enzyme under pH 5.0 to 7.0. All the results suggest that these amino acid residues are important in determining the thermostability and pH stability of MxynB from *Aspergillus niger* nl-1, which will raise its potential interest for the industrial applications.

Improving the Thermostability and pH Stability of Aspergillus niger Xylanase by Site-directed Mutagenesis

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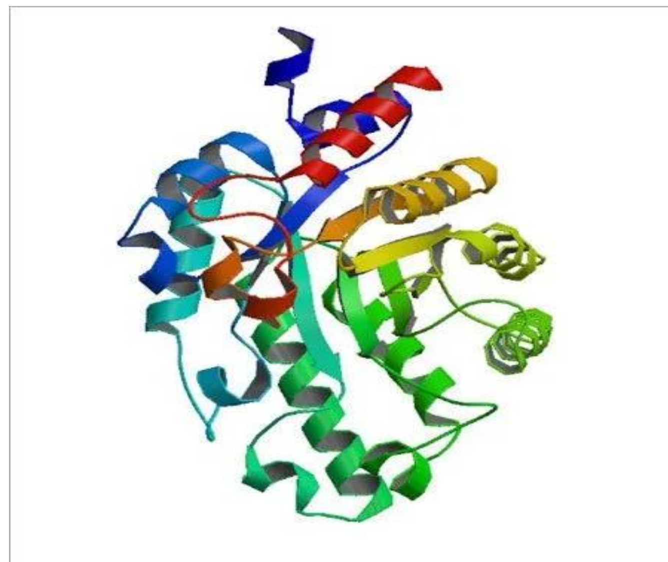
Biochemistry and Microbiology. 2019



Xylanase

- Xylanase is the class of enzymes produced by microorganisms which hydrolyze the internal xylans, major hemicellulosic components in plant cell walls.

- They have been widely applied in feed, paper pulp, food, brewing and textile industries

Aim

The aim of this study was using a sited-directed mutagenesis technique to enhance the thermal tolerance and improve pH stability of xylanase.

Methodology

Primers were designed for PCR

Construction of site-directed mutants

The mutations were generated by inverse-PCR and Quick Change SDM

Expression, purification and enzyme assay of mutant enzymes

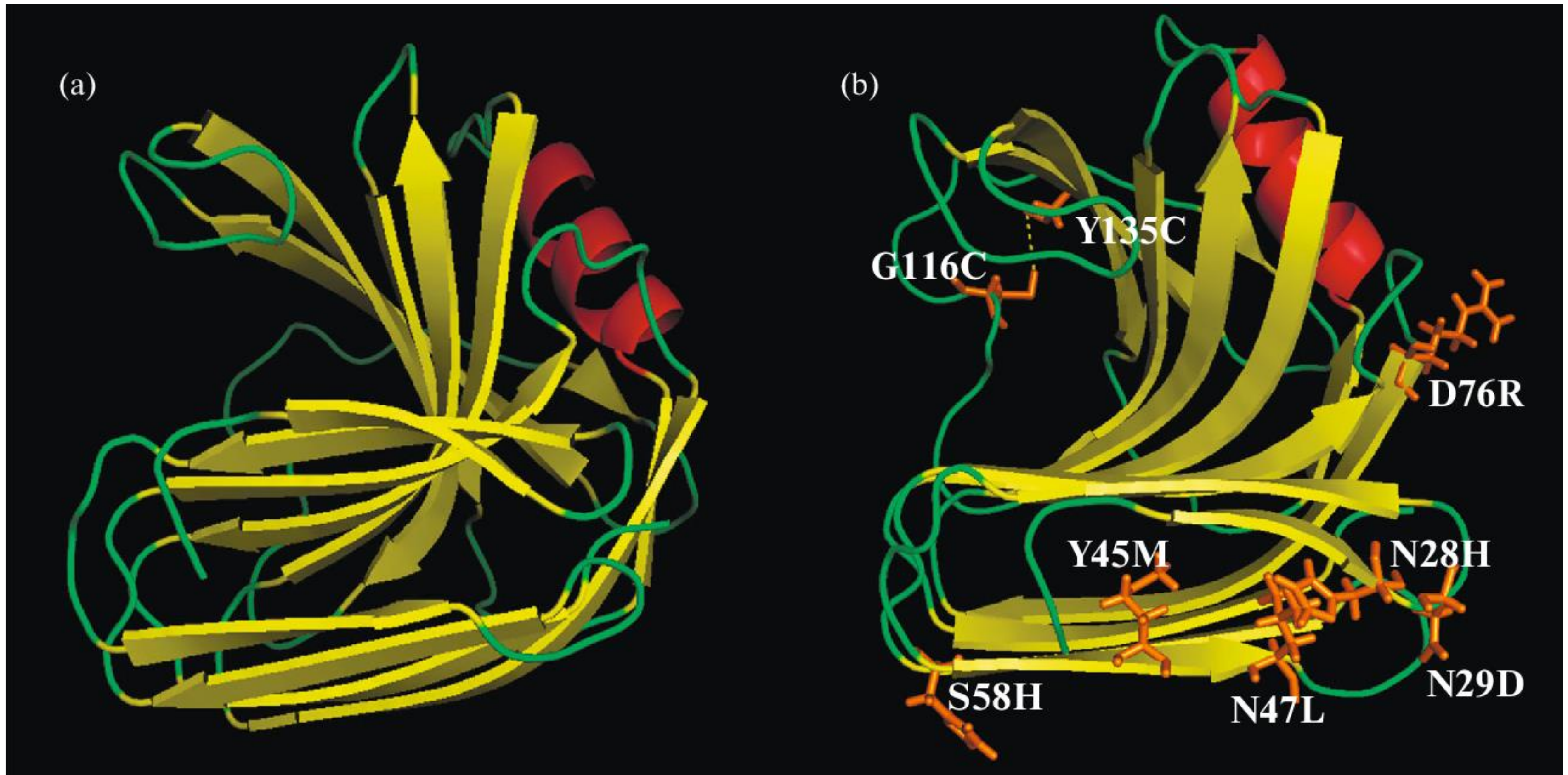
Properties of the mutant xylanases

Table 1. Sequences of oligonucleotides used for site-directed mutagenesis of MxynB

Target sites	Oligonucleotide sequences (5'–3')*
G116C	5'-**<u>TGT</u>**TCTGGAGGTACTTACAAGGGA-3' 5'-AGGATTATAGTCTCCGTAACTCTCAACG-3'
Y135C	5'-**<u>TGT</u>**ACAGCCACCAGAACTAACGCT-3' 5'-GATGTCGTAGACGGAACCATCAGA-3'
S58H	5'-**<u>CAC</u>**AACGTTGGTAATTTTGTCGGA-3' 5'-CCATTCGACTGTATATGAACCAGC-3'
D76R	5'-**<u>CGC</u>**ATTACTTACTCTGGAACTTTCAC-3' 5'-TTGGGCGGAACCTGGGTTCCAT-3'
N28H	5'-**<u>CAC</u>**AATGGTTTTTACTATAGTTTCTGG-3' 5'-CTCTCCGGTGGAAGATGGTGTAC-3'
N29D	5'-**<u>GAC</u>**GGTTTTTACTATAGTTTCTGGACT-3' 5'-GTGCTCTCCGGTGGAAGATGGT-3'
Y45M/N47L	5'-**<u>ATG</u>**ACC**<u>CTC</u>**GGAGATGCTGGTTC-3' 5'-TGTAACGTCACCTCCACCATCAG-3'

* The nucleotide changes are underlined in bold.

Homology modeling and structure comparison of the wild type MxynB and xylanase from Mutant 8



Mutation sites from Mutant 1 to Mutant 8

Table 2. Amino acid substitutions of mutated xylanases

Mutant	Amino acid mutation sites
Mutant 1	G116C-Y135C
Mutant 2	G116C-Y135C-S58H
Mutant 3	G116C-Y135C-S58H-D76R
Mutant 4	G116C-Y135C-D76R
Mutant 5	G116C-Y135C-S58H-D76R-N28H-Y45M-N47L
Mutant 6	G116C-Y135C-D76R-N28H-Y45M-N47L
Mutant 7	G116C-Y135C-D76R-N28H-N29D-Y45M-N47L
Mutant 8	G116C-Y135C-S58H-D76R-N28H-N29D-Y45M-N47L

Results

Temperature characteristics of MxynB and xylanases of mutants

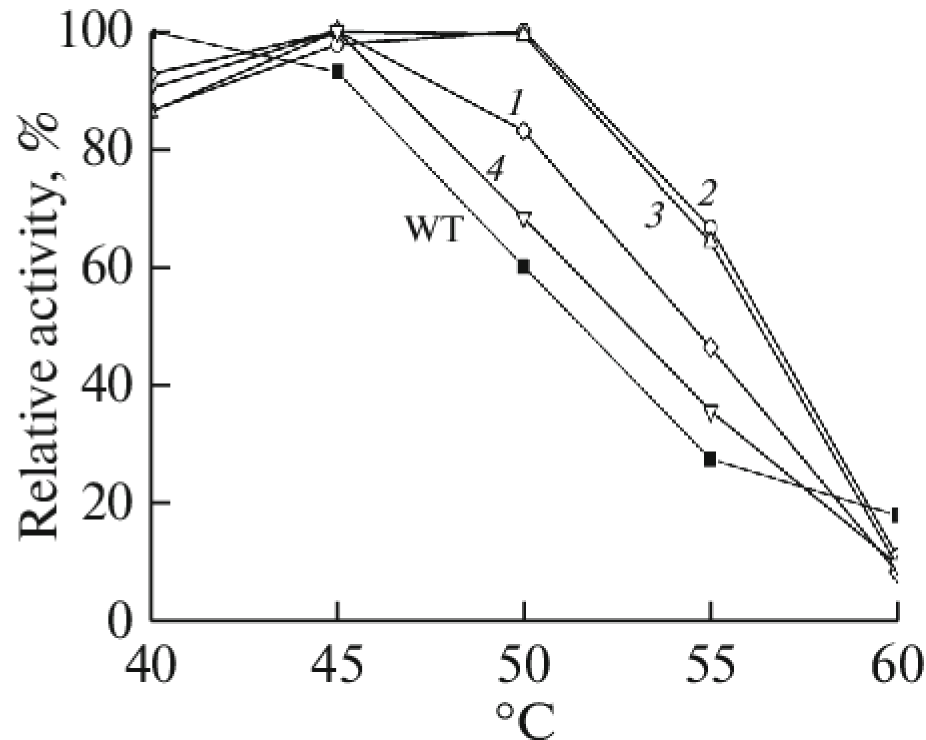


Fig. 2. Temperature optima for the A. niger nl-1 xylanase activity of the wild-type (WT) and mutants (for Mutants 1–4;

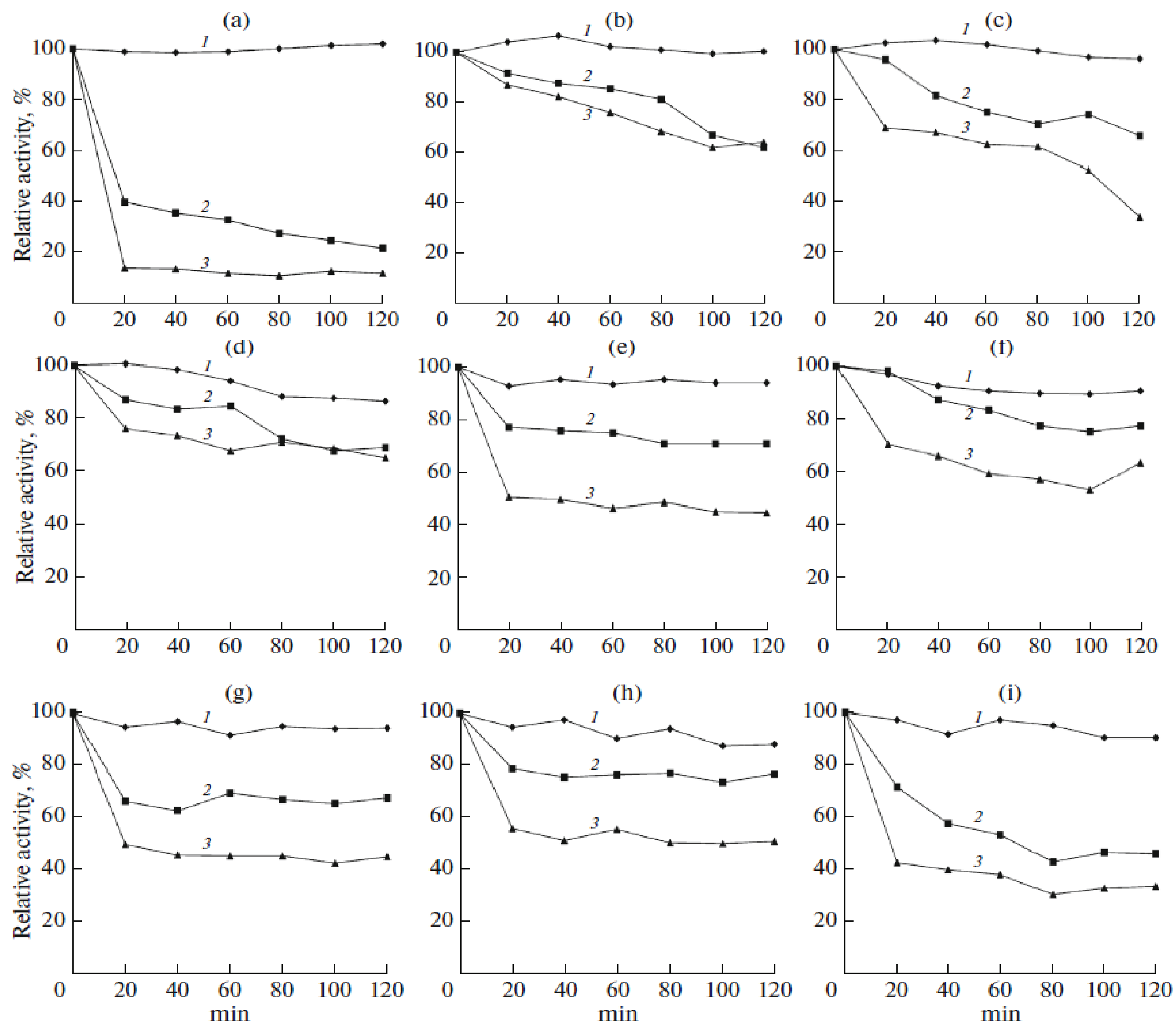


Fig. 5. Temperature stability for the *A. niger* nl-1 xylanase activity of the wild-type (WT) (a) and mutants (b: Mutant 1; c: Mutant 2; d: Mutant 3; e: Mutant 4; f: Mutant 5; g: Mutant 6; h: Mutant 7; i: Mutant 8). The activity of the enzyme without pre-incubation was defined as 100%. *1*—50°C; *2*—60°C; *3*—70°C.

Optimum pH and pH stability of MxynB and xylanases of mutants

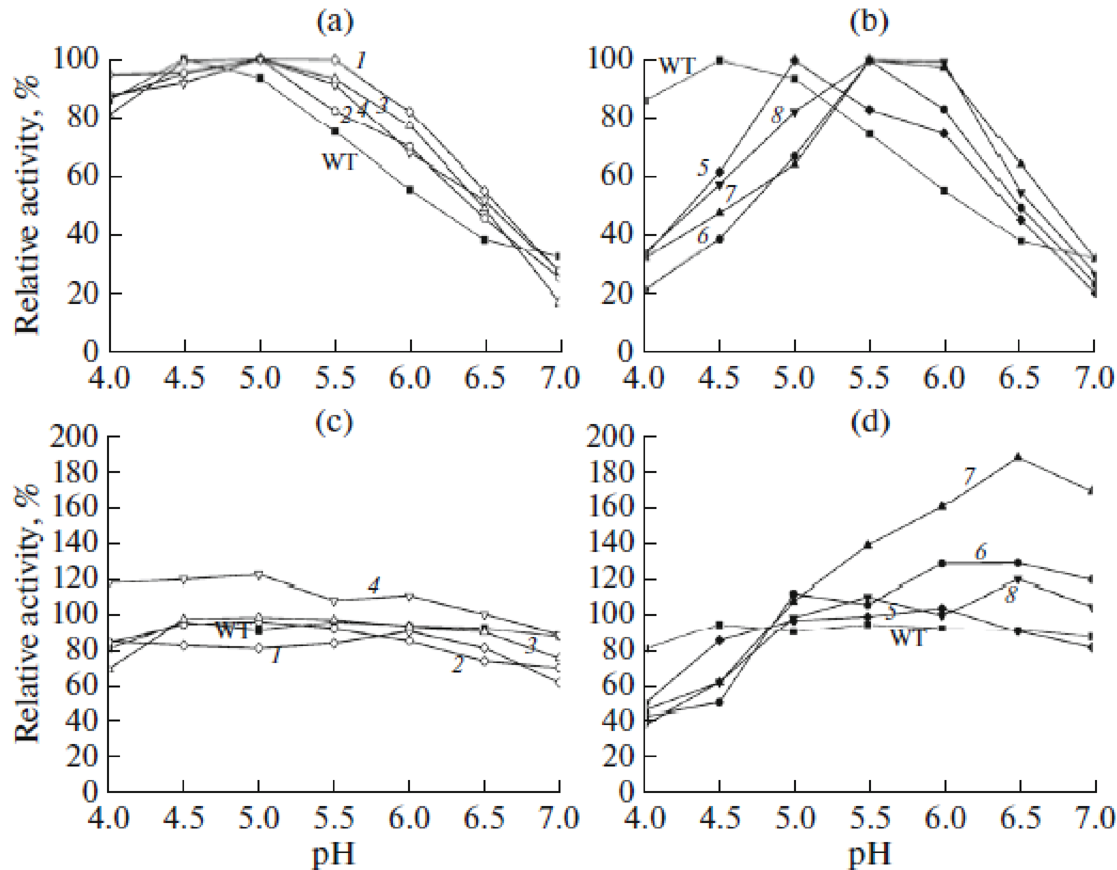


Fig. 6. pH optima (a—for Mutants *1–4*; b—for Mutants *5–8*) and pH stability (c—for Mutants *1–4*; d—for Mutants *5–8*) for the *A. niger* nl-1 xylanase activity of the wild-type (WT) and mutants. The highest xylanase activity was taken as 100% in an optimum pH assay. In (c) and (d), the enzyme activity without pre-incubation was defined as 100%.

Conclusion

- ✓ The results suggested that the technology of homology modeling and site-directed mutagenesis enhanced thermal and pH tolerance of the enzyme.
- ✓ Mutants with improved xylanase properties could be obtained by further manipulations at other effective positions via site-directed mutagenesis.
- ✓ These present results will promote the industrial application of this enzyme.

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