

Azura Amid · Sarina Sulaiman  
Dzun Noraini Jimat  
Nor Fadhillah Mohamed Azmin *Editors*

# Multifaceted Protocol in Biotechnology

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 Springer

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ISBN 978-981-13-2256-3      ISBN 978-981-13-2257-0 (eBook)  
<https://doi.org/10.1007/978-981-13-2257-0>

Library of Congress Control Number: 2018963298

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# Preface

*Multifaceted Protocol in Biotechnology* consists of methods and techniques commonly used in biotechnology studies. There are four sections covered in this book – bioprocess, whole cells and isolated biocatalyst, characterization of biochemical products, and cell isolation and culture. A brief introductory supported with each protocol/method designated for better understanding. The first part of this book consists of recent methodologies for discovering the whole cell and isolated biocatalysts as to improve their current properties which significantly contribute to the development of new industrial processes. It encompasses five chapters covering topics on the mutagenesis of cyanobacteria, site-directed mutagenesis on a plasmid, construction of metagenomics DNA libraries, and isolation of bacteria producing enzymes. It is expected that these chapters will bring about the current status of research and developments in biotechnology area with a critical perspective. The second part covers four chapters covering researches in modern biotechnology focusing in bioprocess areas such as biofuel, catalyst, microbial, and enzyme. It is expected that these chapters will bring about the current methods of research and developments in bioprocess areas. The third part of this book encompasses topics such as preparation and characterization of some biochemical products such as nitrocellulose, proteins, poly(lactic acid), and electrochemical transducer for the biosensor. It consists of five chapters covering reviews of the current state of the characterization of biochemical products that may be useful in identifying biochemical products with a different approach. Characterization of biochemical products is an important part of the early stage of process development of biochemical products. It is due to ensuring specification of the product especially its quality and consistency of the product is met before commercialized. It is expected that these chapters will benefit other researchers to apply in determining the characteristics of their biochemical products. The final part covers cell isolation and culture, encompassing a wide range of multifaceted areas including chemistry, biology, engineering, and statistics. This part comprises of five chapters covering the method for isolation of bacterial strain from contaminated soil, monitoring the growth of plant cell suspension culture, culturing and maintaining mammalian cell culture,

integrated data analysis model for screening cell line producer, and skim latex serum as an alternative nutrition for bacterial growth. Advances in cell isolation and cultures technology discussed in these chapters are not a means to an end in this research area but as a paved path for other researchers to explore.

Kuala Lumpur, Malaysia

Azura Amid

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# Chapter 1

## Site-Directed Mutagenesis on Plasmid Using Polymerase Chain Reaction



Abdul Aziz Ahmad, Hamzah Mohd Salleh, and Ibrahim Ali Noorbacha

**Abstract** Site-directed mutagenesis (SDM) is a very useful technique to study changes in protein function that may occur as a result of the DNA manipulation. A detail procedure to employ SDM on whole plasmid using polymerase chain reaction (PCR) with successful application of the method on a pET28a plasmid harboring an endoglucanase I gene from *Fusarium oxysporum* is described here.

**Keywords** Site directed mutagenesis · Endoglucanase · *Fusarium oxysporum*

### 1.1 Introduction

Mutagenesis is the process of making a genetic mutation which may occur spontaneously or be stimulated by mutagens. Researchers use a number of methods to create mutations, including transposon mutagenesis to generate random gene knockouts, and site-directed mutagenesis, which makes use of the polymerase chain reaction (PCR) to introduce specific mutations. Site-directed mutagenesis (SDM), is a very useful technique to study gene and protein functions relationship. For instance, one may mutate the specific location of a protein and observed the activity increases by many folds. SDM enables researchers to study other properties of proteins such as thermostability. For example, Akcapinar et al. (2015) had identified three thermostabilizer mutations (Q126F, K272F, Q274V) in endoglucanase I from *Trichoderma reesei*, through molecular dynamics simulations. These mutations were then introduced into the endoglucanase I gene, using site-directed

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mutagenesis, and the thermostability of the enzymes was found to increase. The experimental method for introducing mutations in endoglucanase from *Fusarium oxysporum* (EGuia) will be described in this chapter. The experimental method to analyze enzymes by enzyme assays have been presented elsewhere (Salleh 2011), and hence it will not be repeated here.

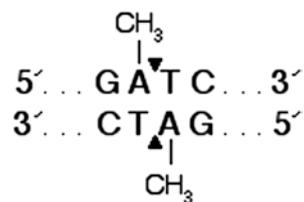
## 1.2 Site-Directed Mutagenesis on Plasmid

The initial methods for SDM required single-stranded DNA (ssDNA), as the template (Kunkel 1985; Sugimoto et al. 1989; Taylor et al. 1985; Vandeyar et al. 1988). These methods need subcloning and are labor intensive, in addition to the necessity of specialized vectors and the presence of unique restriction sites. The method proposed by Papworth et al. (1996), allows site-specific mutation in virtually any double-stranded plasmid, thus eliminating the need for subcloning into M13-based bacteriophage vectors and for single-stranded DNA rescue. Plasmid DNA isolated from almost all of the commonly used *E.coli* strains (*dam*<sup>+</sup>) is methylated and is a suitable template for mutagenesis, whereas the plasmid DNA isolated from the exceptional (*dam*<sup>-</sup>) *E.coli* strains are not suitable.

### 1.2.1 Principle

The fundamental process makes use of a supercoiled double-stranded deoxyribonucleic acid (dsDNA) vector with the site of interest to be mutated and two synthetic oligonucleotide primers having the needed mutation. The oligonucleotide primers, each complementary to each other, are extended in the temperature cycling by high-fidelity DNA polymerase. The high-fidelity DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. Integration of the oligonucleotide primers produces a mutated plasmid containing staggered nicks. Subsequent to temperature cycling, the PCR product is digested with *DpnI*. The *DpnI* endonuclease target sequence is specific for methylated and hemi-methylated DNA (Fig. 1.1) and is employed to digest the parental DNA template.

**Fig. 1.1** *DpnI* endonuclease recognition site



### 1.2.2 Objective of Experiment

The objective of this procedure is to introduce the desired mutation at a specific predetermined location into a plasmid (a vector with gene insert of interest) using PCR approach which will afford site-directed mutation(s) (Fig. 1.2).

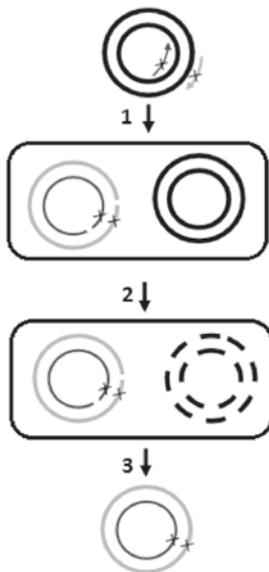
## 1.3 Materials and Skills Required

### Required Basic Skills

1. DNA Primer design
2. Basic lab equipment operation

### Materials

1. PCR reagents
2. Thin-walled 200  $\mu$ l PCR tubes
3. Petri dishes
4. Inoculating loop
5. 15 ml falcon tube
6. *DpnI* restriction enzyme
7. Competent cells
8. Mini-prep plasmid extraction kit (Qiagen)



#### 1. Mutant Strand Synthesis

Perform PCR to:

- Denature DNA template
- Anneal DNA mutagenic primer containing desired mutation
- Extend DNA primers with high-fidelity DNA polymerase
- Some parental DNA may remain as such

#### 2. *DpnI* Digestion

- Digest parental methylated and hemimethylated DNA.
- Mutant strand will not be digested by *DpnI* restriction enzyme.

#### 3. Transformation

Transform the mutated molecule into competent cells for nick repair.

**Fig. 1.2** Flow of the overall mutagenesis procedure. The dark thick circles refer to parental methylated DNA. The broken thick circles refer to parental methylated DNA degraded by *DpnI*. The dark thin and grey circles refer to the forward and reverse strand of the mutant DNA respectively. The “x” mark refers to the mutation site (Image derived from Agilent Technologies QuikChange Mutagenesis workflow)

## Equipment

1. PCR thermocycler
2. Incubator shaker
3. Microcentrifuge
4. Centrifuge

## 1.4 Methodology

### 1.4.1 Template Preparation

The double-stranded plasmid DNA template is obtained by extraction from an *E.coli* (dam<sup>+</sup>) strain with miniprep plasmid extraction kit (Qiagen GmbH, Germany).

### 1.4.2 Mutagenic Primer Design

The mutagenic DNA primer to be used in this method should be designed individually according to the desired mutation. Consider the following when designing the mutagenic DNA primers:

1. The forward and reverse mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid template.
2. The desired mutation should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.
3. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

### 1.4.3 Polymerase Chain Reaction (PCR)

Prepare two sets of 50 µl reaction mixtures containing forward and reverse primers in 200 µl PCR tubes as follows (Table 1.1 and Fig. 1.3):

Part A: Initial denaturing of the **double helix** DNA template at 94 °C–95 °C.

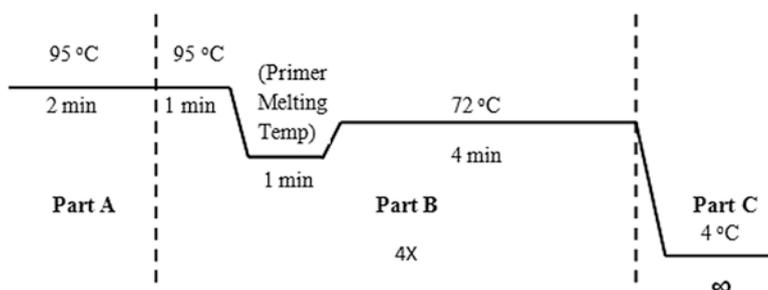
Part B: Starts by denaturation at 94 °C–95 °C followed by cooling the sample to moderate temperature, approximately 54 °C, which facilitates the annealing of the mutagenic DNA primers to the single-stranded DNA templates. In the third step of the cycle, the sample is reheated to 72 °C, the ideal temperature for thermostable DNA polymerase, for elongation process. Part B is repeated four times in the first cycle.

Part C: This is simply for storage at 4 °C, no reaction occurs in this part.

**Table 1.1** Reaction mixtures containing forward and reverse primers

Reagent	Forward ( $\mu$ l)	Reverse ( $\mu$ l)
1. 10X reaction buffer	5.0	5.0
2. Plasmid	1.0	1.0
3. Forward mutagenic primer	2.5	–
4. Reverse mutagenic primer	–	2.5
5. DMSO	1.5	1.5
6. dNTP mix	1.0	1.0
7. Distilled water	38.5	38.5
8. High-fidelity polymerase	0.5	0.5

Note: Always keep the reagents on ice while preparing the reaction mixture

**Fig. 1.3** Cycling parameters for 1st cycle

In the second cycle, mix 25  $\mu$ l of forward and 25  $\mu$ l of reverse reaction mixtures from the first cycle in a new 200  $\mu$ l PCR tube and repeat the whole process (Part A, B and C) with the same parameters, except the Part B is repeated 12–18 times according to the type of mutation desired as follows (Tables 1.2 and 1.3):

#### 1.4.4 *DpnI* Digestion

Add 1  $\mu$ l of the *DpnI* restriction enzyme (10 U/ $\mu$ l) to each PCR product. Gently mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures using a microcentrifuge for 1 min and incubate each reaction at 37 °C for 1 h to digest the parental supercoiled double-stranded DNA.

#### 1.4.5 Transformation: Nick Repair

To repair the nick caused by the non-strand replacing high-fidelity DNA polymerase during PCR, the plasmid needs to be transformed into competent cells.

**Table 1.2** Type of mutation and number of times Part B repeated

Type of mutation desired	No. of repeats
Point mutations	12
Single amino acid changes	16
Multiple amino acid deletions or insertions	18

**Table 1.3** Designed mutagenic primer

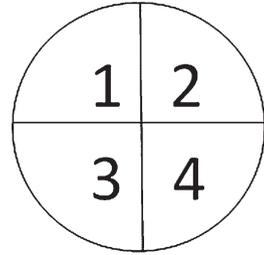
Primer name (length)	Sequence (5' ----- 3')
T224Ef (27nt)	CTC TAC GGC TGC <b>G</b> AA GGC GAT GAG TGC
T224Er (27nt)	GCA CTC ATC GCC <b>T</b> TC GCA GCC GTA GAG

1. Gently thaw chemically competent cells (e.g., DH5 $\alpha$ ) on ice for 20–30 min.
2. Mix 10  $\mu$ l of the *DpnI* digested mixture with 10  $\mu$ l TE buffer and add the mixture to competent cells. Swirl the transformation reaction mixture very gently and incubate on ice for another 20–30 min.
3. Heat shock the transformation reactions for 45 s at 42 °C and then place the reactions back to ice immediately and allow the cells to recover on ice for 10 min and add 1 ml of Luria Bertani (LB) liquid media.
4. Place the tube in an incubator shaker at 37 °C for 90 min.
5. Plate on 1-2 LB agar plates containing appropriate selection antibiotics.
6. Grow plates overnight at 37 °C.
7. Randomly choose 4–10 single colonies from the plate(s) and streak on a plate divided into four sectors as shown in Fig. 1.4.
8. Incubate overnight and randomly select few sectors and isolate the plasmid using mini-prep plasmid extraction kit. To isolate the plasmid, it is possible to scratch the fully grown sectors or prepare a culture broth with appropriate antibiotics inoculated with the randomly selected sectors. Optional: Check the presence of the plasmid on 1% agarose gel.

### 1.4.6 Confirmation by DNA Sequencing

To confirm the success of the mutagenesis, the plasmid harboring the mutated gene needs to be sequenced. This step can be outsourced by sending the plasmid harboring the mutated gene along with the appropriate sequencing primers to DNA sequencing service providers.

**Fig. 1.4** One Petri dish divided into four sectors



## 1.5 Case Study: Mutating EGuia

EGuia is an endoglucanase I from *Fusarium oxysporum* and it is termed as wild-type in this current procedure. We have successfully mutated EGuia amino acid at position 224 from Threonine to Glutamic acid (T224E) following the method described above.

### 1.5.1 Results and Discussion

T224Ef and T224Er are the forward (sense) and reverse (antisense) DNA mutagenic primers respectively, designed for introducing Threonine to Glutamic acid mutation at position 224 (single amino acid change) in wild-type EGuia endoglucanase I. Codon changed is bold and underlined.

#### 1.5.1.1 DNA Sequencing Result

The following is the nucleotide sequence comparison between the wild-type (WT) EGuia and the T224E mutant. (DNA sequencing service provided by AIT Biotech (Singapore) via NextGene Sdn. Bhd.)

#### 1.5.1.2 Discussion

Figures 1.5 and 1.6 are the aligned nucleotide and amino acid sequence for wild-type EGuia and the expected T224E mutant. An asterisk (\*) denotes that conservation of nucleotide and amino acid. At nucleotide position 673 and 674 and the amino acid at position 224, the absence of an asterisk indicates the nucleotides in both the sequence are not identical. The DNA sequencing result above indicates the intended point mutation of changing threonine (T) to glutamic acid (E) was successfully introduced at position 224 of EGuia. A set of five positive samples were sent for DNA sequencing; three of the samples contained the mutation at the intended position. There was no mutation in the 4th sample and the final sample, additional

```

WT      1  ATGCAGACCCCGACAAGGCCAAGGAGCAACCCCAAGCTCGAGACCTACCCTGCACC
T224E   1  ATGCAGACCCCGACAAGGCCAAGGAGCAACCCCAAGCTCGAGACCTACCCTGCACC
*****

WT      61  AAGGCCTCCGGCTGCAAGAAGCAGACCAACTACATCGTCGCCGACGCAGGTATTACCGGC
T224E   61  AAGGCCTCCGGCTGCAAGAAGCAGACCAACTACATCGTCGCCGACGCAGGTATTACCGGC
*****

WT      121  ATCCACCAAAAGAACGGCGCCGGCTGCGGTGACTGGGGACAAAAGCCCAACGCCACAGCC
T224E   121  ATCCACCAAAAGAACGGCGCCGGCTGCGGTGACTGGGGACAAAAGCCCAACGCCACAGCC
*****

WT      181  TGTCCCGATGAGGCTTCTCGCCGAAGAACTGTATCCTCAGTGGTATGGACTCAAACGCT
T224E   181  TGTCCCGATGAGGCTTCTCGCCGAAGAACTGTATCCTCAGTGGTATGGACTCAAACGCT
*****

WT      241  TACAAGAACGCTGGTATCACTACTTCTGGCAACAAGCTTCGTCTCAGCAGCTTATCAAC
T224E   241  TACAAGAACGCTGGTATCACTACTTCTGGCAACAAGCTTCGTCTCAGCAGCTTATCAAC
*****

WT      301  AACCAGCTTGTTCCTCCTCGAGTTTATCTGCTTGAGGAGAACAAAGAAGTATGAGATG
T224E   301  AACCAGCTTGTTCCTCCTCGAGTTTATCTGCTTGAGGAGAACAAAGAAGTATGAGATG
*****

WT      361  CTTCACTCACTGGCACTGAGTTCTCTTTTGATGTTGAGATGGAGAAGCTTCCTTGTGGT
T224E   361  CTTCACTCACTGGCACTGAGTTCTCTTTTGATGTTGAGATGGAGAAGCTTCCTTGTGGT
*****

WT      421  ATGAATGGTCTCTGTACCTTTCTGAGATGCCCAAGGATGGCGGTAAAGCAGCAGCCGA
T224E   421  ATGAATGGTCTCTGTACCTTTCTGAGATGCCCAAGGATGGCGGTAAAGCAGCAGCCGA
*****

WT      481  AACAGCAAGGCTGGCGCTACTATGGTGTGGATACTGTGATGCCCAAGTGTACGTCACT
T224E   481  AACAGCAAGGCTGGCGCTACTATGGTGTGGATACTGTGATGCCCAAGTGTACGTCACT
*****

WT      541  CCTTTCATTAACGGAGTTGGAACATCAAGGGACAGGGTGTCTGCTGTAACGAGCTCGAC
T224E   541  CCTTTCATTAACGGAGTTGGAACATCAAGGGACAGGGTGTCTGCTGTAACGAGCTCGAC
*****

WT      601  ATCTGGGAGGCCAACTCCCGGCAACTCACATTGCTCCTCACCCCTGCAACAAGCCCGGC
T224E   601  ATCTGGGAGGCCAACTCCCGGCAACTCACATTGCTCCTCACCCCTGCAACAAGCCCGGC
*****

WT      661  CTCACGGCTGCAAGGCGATGAGTGGCGAGCTCCGGTATCTGCGCAAGGCTGGTGC
T224E   661  CTCACGGCTGCAAGGCGATGAGTGGCGAGCTCCGGTATCTGCGCAAGGCTGGTGC
*****

WT      721  GGCTGGAACCAACAACCGCATCAACGTGACCGACTTCTACGGCCCGGCAAGCAGTACAAG
T224E   721  GGCTGGAACCAACAACCGCATCAACGTGACCGACTTCTACGGCCCGGCAAGCAGTACAAG
*****

WT      781  GTCGACAGTACCCGCAAGTTCACCGTGACATCCAGTTCGTGCGCAACAGCAGGGCGAC
T224E   781  GTCGACAGTACCCGCAAGTTCACCGTGACATCCAGTTCGTGCGCAACAGCAGGGCGAC
*****

WT      841  CTCATCGAGCTGCACCGCCACTACATCCAGSACAACAGTTCATCGAGTCAAGTTCGCTC
T224E   841  CTCATCGAGCTGCACCGCCACTACATCCAGSACAACAGTTCATCGAGTCAAGTTCGCTC
*****

WT      901  AACATCTCCGGCCCTCCCAAGATCAATTTTCATCAACGACAAGTACTGCGCTGCCACTGGA
T224E   901  AACATCTCCGGCCCTCCCAAGATCAATTTTCATCAACGACAAGTACTGCGCTGCCACTGGA
*****

WT      961  GCTAACGAGTACATGCGCCTCGGCGTACTAAGCAAATGGGCGATGCCATGTCCCGCGGA
T224E   961  GCTAACGAGTACATGCGCCTCGGCGTACTAAGCAAATGGGCGATGCCATGTCCCGCGGA
*****

WT      1021  ATGTTTCTCGCCATGAGCGTCTGTTGGAGCGAGGGTATTTCATGGCTGGTTGGATCAG
T224E   1021  ATGTTTCTCGCCATGAGCGTCTGTTGGAGCGAGGGTATTTCATGGCTGGTTGGATCAG
*****

WT      1081  GGCCTTGCTGGACCTGTGACGCCACTGAGGCGATCCCAAGAACATCGTCAAGTGCAG
T224E   1081  GGCCTTGCTGGACCTGTGACGCCACTGAGGCGATCCCAAGAACATCGTCAAGTGCAG
*****

WT      1141  CCCAACCCCTGAAGTGACATTCAGCAACATCCGAATTGGAGAGATTGGATCTACTTCGTG
T224E   1141  CCCAACCCCTGAAGTGACATTCAGCAACATCCGAATTGGAGAGATTGGATCTACTTCGTG
*****

WT      1201  GTC AAGGCTCCTGCGTATCCTGGTCTCACCGCTTGT AAGGGCTGAGC
T224E   1201  GTC AAGGCTCCTGCGTATCCTGGTCTCACCGCTTGT AAGGGCTGAGC
*****

```

Fig. 1.5 Nucleotide comparison between wild type (WT) EGuia and T224E mutant

```

WT      1  MQTPDKAKEQHPKLETYRCTKASGCKKQTNYIVADAGIHGIHQKNGAGCGDWGQKPNATA
T224E  1  MQTPDKAKEQHPKLETYRCTKASGCKKQTNYIVADAGIHGIHQKNGAGCGDWGQKPNATA
*****

WT      61  CPDEASCAKNCILSGMDSNAYKNAGITTSGNKLRLLQQLINNLVSPRVYLLEENKKKYEM
T224E  61  CPDEASCAKNCILSGMDSNAYKNAGITTSGNKLRLLQQLINNLVSPRVYLLEENKKKYEM
*****

WT      121  LHLTGTEFSFDVEMEKLPCGMNGALYLSEMPQDGGKSTSRNSKAGAYYGAGYCDACQCYVT
T224E  121  LHLTGTEFSFDVEMEKLPCGMNGALYLSEMPQDGGKSTSRNSKAGAYYGAGYCDACQCYVT
*****

WT      181  PFINGVGNIKGQGVCCNELDIWEANSRATHIAPHPCNKPGLYGCTGDECGSSGICDKAGC
T224E  181  PFINGVGNIKGQGVCCNELDIWEANSRATHIAPHPCNKPGLYGCEGDECGSSGICDKAGC
*****

WT      241  GWNHNRINVTFYGRGKQYKVDSTRKFTVTSQFVANKQGDLLIELHRHYIQDNKVIESAVV
T224E  241  GWNHNRINVTFYGRGKQYKVDSTRKFTVTSQFVANKQGDLLIELHRHYIQDNKVIESAVV
*****

WT      301  NISGPPKINFINDKYCAATGANEYMLGGTKQMGDAMSRGMVLAMSVVWSEGDFMAWLDQ
T224E  301  NISGPPKINFINDKYCAATGANEYMLGGTKQMGDAMSRGMVLAMSVVWSEGDFMAWLDQ
*****

WT      361  GVAGPCDATEGDPKNIVKVQPNPEVTFNSNIRIGEIGSTSSVKAPAYPGPHRL
T224E  361  GVAGPCDATEGDPKNIVKVQPNPEVTFNSNIRIGEIGSTSSVKAPAYPGPHRL
*****
    
```

**Fig. 1.6** Translated amino acid sequence comparison between wild type (WT) EGuia and T224E mutant

nucleotide was present at the intended mutation region. The 4th sample might be the parental strand which escaped the *DpnI* digestion, and since it has the antibiotic resistance gene (kanamycin), it survives on the plate. In the final sample, the reason this happened is that the primers are complementary each other. In the PCR amplification, the primers dimerized into double-strand small DNA fragments while the PCR products are blunt end fragment (produced by proofreading thermal DNA polymerase). When transformed the PCR product into a cell, the cell DNA repair system did a blunt-end ligation of the PCR fragment with primer dimer rather than do a nick repair.

## 1.6 Conclusion

An *in-vitro* mutagenesis technique presented. The technique was successful in introducing site-specific mutation as described in the case study. A point mutation from threonine to glutamic acid at position 224 (T224E) was introduced to the endoglucanase I gene from *Fusarium oxysporum*.

**Acknowledgement** The authors would like to acknowledge the Ministry of Higher Education, (MoHE) Malaysia for awarding fund via Fundamental Research Grant Scheme: Grant No: FRGS-13-070-0311 and Professor S.G. Withers (UBC, Vancouver) for providing the original wild-type plasmid construct.

## List of Abbreviations

SDM	Site-directed mutagenesis
PCR	Polymerase chain reaction
M13	Is a filamentous bacteriophage composed of circular single-stranded DNA
DNA	Deoxyribonucleic acid
ssDNA	single-stranded DNA
<i>dam</i>	gene encoding Dam. Dam stands for DNA adenine methyltransferase, an enzyme of ~32 kDa that does not belong to a restriction/modification system. The target recognition sequence for <i>E. coli</i> Dam is GATC and the methylation occurs at the N6 position of the adenine in this sequence (GmATC). <i>dam</i> <sup>+</sup> has the ability for methylation and <i>dam</i> <sup>-</sup> does not have this ability.
<i>DpnI</i>	<i>DpnI</i> is a restriction enzyme from <i>Diplococcus pneumoniae</i> . This enzyme recognizes the double-stranded DNA <u>methylated</u> sequence GATC. It works well in the presence of Zn <sup>2+</sup> ions. <i>DpnII</i> recognizes the double-stranded DNA <u>unmethylated</u> sequence GATC and cleave before G-1.

## List of Nucleobases

Nucleobase	Abbreviated names
Adenine	A
Thymine	T
Guanine	G
Cytosine	C

## List of Naturally Occurring Amino Acids

Amino acid	Abbreviated names	
Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Proline	Pro	P
Cysteine	Cys	C
Methionine	Met	M
Serine	Ser	S
Threonine	Thr	T
Asparagine	Asn	N
Glutamine	Gln	Q
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Histidine	His	H
Lysine	Lys	K
Arginine	Arg	R
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Tryptophan	Trp	W

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# Chapter 2

## Isolation of Halophilic Bacteria



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**Abstract** Protease the hydrolytic enzyme has very broad applications in industries. Identifying new protease source from food by isolating the protease producing microbe is consider a safe approach compare to isolating it from other sources which may be dangerous for human consumption. Therefore, this chapter discusses the method of isolating the microbial strain that can produce safe protease enzyme from fermented food.

**Keywords** Halophilic · Protease · Salt-tolerant · Milk-salt agar · Halotolerant

### 2.1 Introduction

Proteases are hydrolytic enzymes that break peptide bonds between amino acid residues and are widely used in various industries, such as those involving food, detergents, pharmaceuticals, leather, waste management and diagnostic reagents (Kumar and Takagi 1999). Halophiles are microorganisms that can grow in highly saline conditions. Previous researches have shown that halophilic microorganisms can be isolated from saline water, saline oil, salty food, salt lakes and seawater (Setati 2010; Karan et al. 2012). Halophilic microorganisms are expected to produce salt-tolerant enzymes owing to their ability to perform catalysis under high salinity. These microbes can be classified into three groups based on their salt requirement (Ventosa et al. 1998):

1. Slight halophiles which grow optimally at 0.20–0.85 M (1–5% sodium chloride; NaCl).
2. Moderate halophiles which show rapid growth at 0.85–3.40 M (5–20% NaCl).
3. Extreme halophiles which grow optimally at 3.4–5.1 M (20–30% NaCl).

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## 2.2 Principles

The purpose of isolating bacteria from the sample is to obtain pure cultures of bacteria. Culture methods can be divided into two: culture on solid media or liquid media. Some solid culture media are nonselective (e.g., nutrient agar) and allow a wide variety of bacteria to grow on it. Meanwhile, some types of agar are more selective (e.g., MacConkey agar). MacConkey agar with added bile salts is specifically used to isolate bacteria like *Escherichia coli* and *Enterococcus faecalis*, both of which are found in large intestines of humans and are known as ‘bile-tolerant’ bacteria.

Another way to culture bacteria is by using liquid media (broth). Bacterial growth can be easily observed as the clear liquid medium turns cloudy or turbid within 24–48 h. Broth culture is significantly more sensitive compared to the direct culture on agar. However, mixed growth can occur, and this makes it difficult to determine the type of bacteria present. Thus, broth media must be subcultured in solid agar to obtain pure cultures.

In this case, the spread plate technique can be used to isolate individual colonies. The advantage of this technique is that it distributes bacteria evenly on a petri dish. Moreover, it is also a quantitative technique which allows the determination of the number of bacteria in a sample.

Milk salt agar is composed of standard nutrient agar with the addition of NaCl and skim milk powder. This agar is used to screen and identify colonies that show proteolytic activity and can tolerate high concentrations of NaCl. Proteases (extracellular enzymes) produced by these microorganisms are secreted into the surrounding media, where they break down the casein in the said agar to give small peptides and individual amino acids which will then be taken up by the microorganisms to produce energy or undergo anabolism. This hydrolysis reaction gives rise to clear zones around the colonies. The presence of NaCl in skim milk agar inhibits the growth of non-halophiles and selects for bacteria that can grow in high salt content. Halophiles may produce halotolerant proteases which can withstand extreme condition, i.e., high NaCl concentrations (Karan et al. 2012).

### 2.2.1 Objective of Experiment

This experiment aims to isolate from fermented food halophilic bacteria which produce halotolerant proteases.

## 2.3 Materials and Methods

### 2.3.1 Materials (Tables 2.1, 2.2 and 2.3)

**Table 2.1** Consumable items used

No	Equipment	Aims of usage
1	Pipettes (200 $\mu$ L, 1 mL, 5 mL)	To add solutions into cuvettes
2	Felcon Tube (50 mL)	To culture microorganisms
3	Microcentrifuge tube (2 mL)	To perform enzyme assay and determine total protein
4	Sterilized hockey-stick and loop wires	To spread and streak culture onto plate
5	Plate/Petri Dish	To contain culture medium (agar)

**Table 2.2** Equipment used

No	Equipment	Usage
1	Weighing balance	To weigh chemicals and reagents
2	Shaking incubator Brand: Stuart SI500 (Bibby Scientific)	To grow and culture microorganisms
3	Microplate Spectrophotometer Brand: Multiskan	To measure absorbance during determination of enzyme activity and total protein
4	GO (Fisher Scientific) pH meter Brand: Accumet Basic AB15 Plus Fisher Scientific	To measure pH

**Table 2.3** Chemicals and reagents used

No	Chemicals	Manufacturer
1	Bacteria lysate	NA
2	Nutrient Broth	Sigma-Aldrich, USA
3	Nutrient Agar	Sigma-Aldrich, USA
4	Skim milk agar	Sigma-Aldrich, USA
5	Casein	Sigma-Aldrich, USA
6	Trichloroacetic Acid	Sigma-Aldrich, USA
7	Sodium Carbonate	Sigma-Aldrich, USA
8	Folin & Ciocalteu's Phenol	Sigma-Aldrich, USA
9	Reagent Quick Start Bradford 1x Dye Reagent	Bio-rad, USA

### ***2.3.2 Isolation of Strains for Proteolytic Activities***

1. From 1 L of nutrient broth, 25 g of it is taken out, the weight of which is measured using an electronic balance. The pH of the culture medium is adjusted to 7 using HCl or NaOH with the help of a pH meter. Then, distilled water is added until the volume reaches 1 L.
2. 0.1 ml of the sample is diluted with 1 ml of sterilized distilled water and incubated in 4.9 ml of nutrient broth (pH 7) at 37 °C for 24 h in a shaker incubator at 250 rpm.
3. To prepare 1 L of 5% NaCl nutrient agar, 28 g of nutrient agar powder and 50 g of NaCl are weighed using an electronic balance. The pH of the agar is adjusted to 7 using HCl or NaOH with the help of a pH meter. Then, distilled water is added until the volume reaches 1 L.
4. Samples in the nutrient broth are serially diluted, and 0.1 ml of suspension is spread uniformly on the 5–10% NaCl nutrient agar plates using a sterile hockey stick wire. The plates are then incubated at 37 °C for 24 h to isolate the colonies.
5. Colonies are selected and grouped based on their differences in morphology, size, and color.

### ***2.3.3 Screening of Strains for Extracellular Protease Activity***

1. The colonies are further screened on skim milk agar, which contained concentrations of NaCl ranging from 5 to 10% (w/v), to identify the presence of proteolytic activity in the isolates.
2. To prepare 1 L of 1.5% (w/v) skim milk agar containing 5% (w/v) NaCl, skim milk solution is prepared separately from the nutrient agar and NaCl solution. 28 g of nutrient agar is mixed with 50 g of NaCl. The pH is adjusted to 7, and distilled water is added until the volume of the agar reaches 800 ml. After that, 15 g of skim milk is weighed and distilled water added until the volume reaches 200 ml. The skim milk solution is autoclaved at 121 °C for 5 min. Both solutions (skim milk agar and NaCl nutrient agar) are then mixed to give a combined volume of 1 L.
3. Individual colonies on the 5% NaCl nutrient agar are streaked onto the skim milk agar using a sterile loop. Colonies which hydrolyze the skimmed milk will give rise to clear zones around them and will be considered as positive for protease production.

### ***2.3.4 Gram Staining***

1. A few drops of distilled water are placed on the surface of a glass slide, and bacterial colonies are smeared on it.
2. The water is dried and the specimen heat-fixed by moving the slide in and out of a flame for 2–3 times. Heat-fixing is needed to make sure the culture does not move about during visualization later.

3. A few drops of crystal violet are added to the fixed culture and left for 1 min. After that, it is washed off by using distilled water.
4. A drop of iodine is applied to the culture for 1 min and drained with distilled water after that.
5. A few drops of 95% ethanol are added for 10 s and washed off with distilled water later.
6. A few drops of safranin (a counterstain) are added for 45 s and then washed off with distilled water.
7. A few drops of oil are added to the slide to increase its resolution when viewed under a light microscope.

### ***2.3.5 Preparation of Crude Enzyme***

1. Selected colonies which show proteolytic activity are cultured in 5 ml of nutrient broth containing NaCl at 37 °C for 24 h in a shaking incubator at 250 rpm.
2. The cultures are centrifuged at 10,000 × g for 45 min at 4 °C. Extracellular enzymes which are produced from the microorganisms are in the solution mixture, so the supernatant fluid is collected when the pellet is removed.
3. The supernatant fluid is filtered using a 0.45 µm sterile syringe filter to remove the contaminants.

### ***2.3.6 Protease Assay***

1. A quantitative assessment of the volume (U/ml) of protease produced is determined using casein at 37 °C as a substrate. This procedure follows Sigma's non-specific protease activity assay (Sigma-Aldrich, Germany). 0.1 ml of the enzyme is added to 1 ml of casein (0.65% w/v in 50 mM potassium phosphate buffer, pH 7.5) and the mixture is incubated for 10 min.
2. 1 ml of trichloroacetic acid (110 mM) is added to terminate the reaction, and the mixture is incubated for 30 min.
3. The supernatant fluid is collected by centrifuging the mixture at 5000 rpm for 15 mins.
4. 0.5 ml of the supernatant fluid is mixed with 1.25 ml of sodium carbonate solution and 0.25 ml Folin & Ciocalteu's phenol reagent.
5. The reagents are mixed by gently inverting the tube several times. It is then centrifuged for 5 min at 14,000 rpm.
6. The supernatant fluid is collected by using a Millipore membrane filter and syringe. Absorbance is measured at 660 nm.
7. 1 unit of protease is defined as the amount of the enzyme required to liberate 1 µmol of tyrosine per minute under the predefined assay conditions.
8. The standard curve of tyrosine is constructed to calculate the amount (in µmol) of tyrosine equivalents released by using a standard curve linear equation.

9. Enzyme activity is calculated using the equation:

$$\text{Enzyme activity } U / ml = \frac{(\mu\text{mole tyrosine equivalent released}) \times (\text{total volume})}{(\text{Enzyme solution}) \times (\text{Colometric volume}) \times (\text{time})}$$

10. The specific activity of the enzyme is calculated using the equation:

$$\text{Specific activity } \frac{U}{\mu\text{g}} = \frac{\text{Enzyme activity } (U / ml)}{\text{Total protein } (\mu\text{g} / ml)}$$

### 2.3.7 Protein Determination

1. The protein concentration in the supernatant is determined by Bradford method using bovine serum albumin (BSA) as the standard (Bradford 1976). This procedure is in accordance with the manual instruction of Quick Start Bradford protein assay.
2. About 1 ml of 1x dye reagent (Quick Start Bradford) is added to microcentrifuge tube (or cuvette) and mixed with 0.2 ml of sample. Then, the tube is vortexed (or inverted).
3. The tube is incubated for 5 min – 1 h at room temperature. The absorbance of protein content is read at 595 nm.

## 2.4 Results and Discussion

### 2.4.1 Results (Figs. 2.1 and 2.2)

Sample	Picture	Colony morphology	Bacterial shape	Gram staining
B1		Irregular Off-white	Rods and groups in chains.	+ve (purple)
T1		Circular Cream	Rods	+ve (purple)

**Fig. 2.1** Colony and bacterial morphology