Genetic Manipulation to Increase Lipase Production in Microorganisms – A Recent Review

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Genetic Manipulation to Increase Lipase Production in Microorganisms – A Recent Review

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Abstract: Enzymes are important biocatalysts that exist naturally in all living organisms. Nowadays, enzymes have been widely applied to modern industries because biological catalysts are substrate-specific, highly active and operate within mild environments. However, the cost of culturing protein-rich microorganisms as well as extracting and purifying the enzymes are high. One solution is to genetically modify the microorganisms for high protein expression. Therefore, this review discusses the mechanism of various mutagenesis techniques (physical and chemical) as well as the potential genetic modification to increase protein yields in microorganisms.

Keywords: Enzymes, genetic engineering, micro-organisms, protein expression.

1. INTRODUCTION

Enzymes are important biocatalysts that occur in all living organisms, from micro-organisms such as bacteria and yeasts, to macro-organisms such as animals and plants. Enzymes exist in many different forms, with a slight difference in amino acid composition in their primary structure leading to a variety of secondary, tertiary and/or quaternary structure, hence explaining the specificity of enzymes toward substrates [1]. The strong dependence of the final structure, function and efficacy of an enzyme upon its primary structure has been observed, in contrast to normal proteins, in human diseases caused by hereditary genes or cell mutations such as Alzheimer’s disease and sickle cell anemia [2]. On normal operation, enzymes function to lower the activation energy of chemical reactions by binding to the targeted substrates via active sites to weaken the bonds in the substrates, thus allowing a greater reaction rate. Enzymes have been used extensively since the ancient era. Some examples include the fermentation of rice, fruits and honey into alcohol in a Neolithic village in Jiahu, China in 7000-6600 BC as well as making a type of blue cheese named Fourme d’Ambert using moulds by the Arverne tribe in France as early as 52 BC [3, 4].

A lipase (triacylglycerol acylhydrolases, EC 3.1.1.3) is an enzyme that catalyzes two reactions: the hydrolysis of fats into fatty acids and glycerol at the water-lipid interface and the synthesis of esters from fatty acids and glycerol at the water-insoluble substrate interface. This special feature of reaction restricted to the interface between aqueous and non-aqueous phases distinguishes lipases from esterases. Lipases have been used extensively in both domestic and industrial areas such as organic synthesis, paper manufacture, oleochemistry, dairy industry, cosmetics, perfume, biosensors and detergents [5].

In 1973, Professors Stanley Cohen (Stanford University) and Herbert Boyer (University of California, San Francisco) performed the first transfer of recombinant DNA and documented the gene splicing and transfer method used [6]. Since then, rapid advancement in the fields of genetic engineering and microbial screening has been reported by numerous researchers around the world. The main reasons for the cultiva-
tion of microbial enzymes include: 1) the capability of micro-organisms to produce a wide diversity of enzymes, 2) the microbial strains can be manipulated to give highly specialized enzymes with high activity, 3) the enzymes are stable across a larger range of pH and temperature compared to animal-derived enzymes and 4) animal friendly [7]. Lipases are especially helpful in domestic and industrial applications due to numerous technological breakthroughs. The various lipase purification techniques and microbial strains used have been reviewed in detailed by Taipa et al. (1992) [8], Sharma et al. (2001) [9] and Saxena et al. (2002) [10].

Researchers and scientists have been focusing on using genetic engineering techniques to select and manipulate lipase producing micro-organisms toward the ideal strain, which: 1) has high growth rate, 2) possess the ability to hyper-produce lipase, 3) have zero excretion of proteases which degrade the product lipase, and 4) the produced lipase is tolerant against large changes in pH and temperature [11].

Many methods have been developed to fully utilize the technology of genetic engineering to induce higher production of lipases through gene transfer and manipulation, as well as mutate existing micro-organism strains to induce new characteristics which yields more products. The mutagenesis methods developed are divided into two broad categories, physical and chemical mutagenesis. Physical mutagenesis involves ultraviolet (UV) irradiation, fast neutron irradiation, gamma (γ) rays, X-rays and neodymium-doped yttrium aluminum garnet (Nd:YAG) laser. Chemical mutagenesis includes application of nitrosoguanidine, dialkyl sulphates and nitrous acid. All the mutagenesis methods damage the DNA structure by altering or breaking the double helix strands. But this does not directly cause mutations in the cells. The damaged DNA in turn undergoes enzymatic repair by using one or more of three mechanisms: photoreactivation, excision repair and postreplication repair. These repair mechanisms are error-prone when dealing with specific DNA lesions, causing misrepair of the DNA strands. It is this DNA misrepair that constitutes mutations in cells [12]. This paper will review the mechanism of various mutagenesis techniques as well as the potential genetic modification to increase protein yields in microorganisms.

2. PHYSICAL MUTAGENESIS

2.1. Ultraviolet (UV) Irradiation

Ultraviolet (UV) irradiation is a method of bringing about mutations in organisms by bombarding the targets with the right wavelength and intensity of UV light. The UV radiation damages the DNA in living cells by inducing two frequently-occurring genotoxic and cytotoxic DNA lesions: the formation of cyclobutane–pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), as well as their Dewar valence isomers [13]. Three factors that influence the precise yields and type of DNA damage are the sequence and configuration of DNA [14, 15], curvature of DNA [16] and folding of DNA into nucleosomes [17]. The distribution of CPDs and 6-4 PPs are commonly observed as 70 to 80% and 20 to 30% respectively [18]. CPDs and 6-4 PPs are formed when a pyrimidine in a geometrically favorable position is excited by absorbing UV radiation and photoreacts with adjacent pyrimidine bases. These favorable positions are sites for DNA bending and unwinding, hence UV damage is more distinct in single-stranded DNA and flexible poly (dA).poly(dT) ends [15, 19]. The absorption of UV photons cannot alter the geometrical positions of the pyrimidines. In a DNA double helix, the bases are locked in place, so non-adjacent pyrimidines cannot photoreact. In contrast, non-adjacent pyrimidines photoreact easily in a single-stranded DNA [20]. A small fraction of CPDs also induces mutations [21].

The natural way to induce this mutation is by constant reception of UV radiation from the Sun, and these UV rays are categorized into 3 types by their wavelength: UV-A (315–400 nm), UV-B (280–315 nm) and UV-C (<280nm) [13]. UV-C is actively absorbed by both the ozone layer and atmospheric oxygen, hence it is relatively harmless. Although UV-B and UV-A are also absorbed by the ozone layer, the small amount that passes through the ozone layer can damage DNA in living cells. UV-B is the main culprit behind the dangers of UV radiation because this damaging agent is absorbed by DNA. Despite not being absorbed by DNA, UV-A can also cause secondary photoreactions to damage DNA [22]. Alternatively, UV rays can be artificially applied with the intended wavelength and intensity so as to induce the targeted mutagenesis. Germicidal lamps are generally used to produce short-wave UV light [15].

Kanimozhi and Arulpandi (2014) had isolated an extracellular lipase producing wild strain Pseudomonas sp. Lp1 from edible oil contaminated soil. The Pseudomonas sp. was subjected to UV irradiation in order to enhance the lipase production capabilities. Six different mutants were created via UV irradiation, namely Pseudomonas sp. Lp2 – Lp7. The wild strain Pseudomonas sp. produced 68.0 U/ml of lipase after fermentation for 48 h, while the mutant Pseudomonas sp. Lp6 achieved 115.6 U/ml of lipase. This showed that UV irradiation caused the mutant strain to generate 1.7 fold greater lipase activity than the wild strain [23].

Suribabu et al. (2014) had applied UV irradiation on wild strain Brevibacillus borstelensis R1 to enhance its production of amylase. After 80 minutes of exposure to UV, the percentage survival of B. borstelensis was 42%. The UV mutants that showed more than 20 mm zone of starch hydrolysis were screened and selected. The wild strain produced 3000 U/ml of amylase when cultured under fixed parameters. Out of ten mutants isolated, two mutants (UV-3 and UV-10) showed an amylase activity of 3000–4000 U/ml, which was an improvement compared with the wild strain. The highest amylase activity of 3967 U/ml was obtained by the mutant UV-3 [24].

Irfan et al. (2011) performed UV irradiation on Aspergillus niger to study the effects of UV on the production of two enzymes, namely CMCase (Carboxymethyl-cellulase) and FPase (Filter-paparse). The parent strain showed CMCase and FPase activities of 1.8 IU and 0.92 IU. After 15 min of UV exposure, the mutant strain achieved 2-fold increase in CMCase (2.6 IU) and 3-fold increase in FPase (2.5 IU). On the other hand, avicelase and xylanase showed no enhancement in activities even after 45 min of UV exposure. It was
also found that the mutant with 15 min UV exposure achieved the highest biomass production of 2.8 g/L. The highest biomass was observed after 6 days of fermentation in Vogel’s medium [25].

2.2. Neutron Irradiation

Neutron radiation is the release of a stream of free neutrons from atoms when the atoms undergo nuclear fission, nuclear fusion, radioactive decay or nuclear reactions with other radiation and ionizing particles. When this stream of free neutrons bombards the nuclei of other atoms, they react to form isotopes and/or some form of radiation such as free neutrons or gamma waves. Neutron irradiation refers to the exposure of a targeted organism to neutron radiation in order to study the extent of DNA damage or to allow mutagenesis. Neutron radiation is also commonly known as ionizing radiation due to the ability of free neutrons to cause direct or indirect ionization to atoms. Free neutrons are divided based on their speed. Slow neutrons have lower energy, but they are still efficient ionizing agents. Upon bombardment of the slow neutrons with the nuclei of atoms, the neutrons are absorbed and the nuclei changed into unstable ionized isotopes. Thus, slow neutrons are said to be indirectly ionizing. In contrast, fast neutrons have higher energy and are very penetrating. It is their high penetration that enables direct ionizing of atoms. One such mechanism involved the bombardment of a fast neutron on the nucleus of an atom, effectively altering the original structure of the atom. The chemical bonds of the atom break so suddenly that it leaves behind one or more electrons. This results in the production of free radicals. Another mechanism is that the fast neutrons cause high energy proton emissions upon collision with the nuclei of atoms, ionizing the atoms.

Karanam and Medicherla (2008) investigated the influence of three mutagenesis methods (UV, nitrous acid and nitrosoguanidine (NTG)) on the lipase production of fungal strain Aspergillus japonicas MTCC 1975. The parent strain was subjected to each mutagenesis treatment, where the best lipase producing mutant (Ant II R. oligosporus) sp. TL-12 from a pool of 150 fungal isolates from oily soil samples. The selected fungal strain was subjected to UV, EMS and NTG treatment. The best lipase producing mutant Aspergillus sp. TL-12(3) was obtained from NTG treatment. The highest lipase production was found to be 34.5 U/ml, which was an increase of 5.75 fold compared to the wild strain [30].

Iftikhar et al. (2010) had studied the effect of physical mutagens (UV and γ rays) and chemical mutagens (NTG, nitrous acid and ethidium bromide) on the capability of fungal strain Rhizopus oligosporus to produce extracellular lipases. A total of 167 fungi cultures were isolated from various environments such as soil, air, milk, pickle, oily bread, decayed fruits and vegetables using serial dilution method. The most potent strain was identified as R. oligosporus IIB-63 which generated 3.20 U/ml of lipase. After the mutagenetic treatments, the mutant IIB-63 NTG-7 displayed the highest lipase production of 10.37 U/ml and showed a zone size of 12.3 mm on Luria-Bertani-tributyrin agar plates. This mutant achieved 325% increase in lipase productivity compared to the wild strain [31].

3. CHEMICAL MUTAGENESIS

3.1. Nitrosoguanidine

Nitrosoguanidine, also known as N-Methyl-N-nitroso-N′-nitroguanidine (MNNG or MNG) or N-Methyl-N′-nitro-N-nitrosoguanidine (NTG), is a powerful carcinogen and mutagen. MNNG damages the DNA by the addition of an alkyl group to the O2 and O6 of guanine, which are the replication sites of the double helix DNA. MNNG also causes comutations around the main sites of attack, but since it only distorts the double helix DNA strands slightly, it is difficult for the DNA mismatch repair system to detect the damage, resulting in induced mutation [29].

Tagore and Narasu (2014) had also isolated an extracellular lipase producing fungal strain Aspergillus sp. TL-12 from a pool of 150 fungal isolates from oily soil samples. The selected fungal strain was subjected to UV, EMS and NTG treatment. The best lipase producing mutant Aspergillus sp. TL-12(3) was obtained from NTG treatment. The highest lipase production was found to be 34.5 U/ml, which was an increase of 5.75 fold compared to the wild strain [30].

3.2. Dialkyl Sulphates

Dialkyl sulphates are alkylating agents, capable of inducing mutagenic effects similar to ionizing irradiation; hence they are also categorized as radiomimetic agents. Both dimethyl sulphate (DMS) and diethyl sulphate (DES) are popular monofunctional alkylating agents used extensively for carcinogenic and mutagenic purposes in experiments. Other common alkylating agents used include methyl methanesulphonate (MMS), ethyl methanesulphonate (EMS), methylnitrosourea (MNU) and ethylnitrosourea (ENU) [32].

(Table 1) shows the reaction mechanism of various common alkylating agents. It has been shown that alkylating agents confined to S2 mechanism are very inefficient at producing O′-alkylguanine [33-35] and alkylating other oxygen sites in the nucleotides [36], unlike those undergoing S1 mechanism. This is due to the specificity of S2 agents toward the highly nucleophilic ring nitrogen groups [160]. S1 agents alkylate oxygen sites effectively, as seen in the case of ENU, with more than 80% of the total activity observed to be on oxygen sites of phosphate groups [36]. S1 agents are regarded as more alkylating but S2 agents are observed to be more carcinogenic and mutagenic [37].
DMS (S<sub>2</sub> agent) and DES (S<sub>N1</sub>/S<sub>N2</sub> agent) induced mutations by attacking the nitrogen and oxygen positions in the DNA base pairs, producing 7-methylguanine residues [49] and 7-ethylguanine residues [249] respectively as the main products. This indicated the favored site of attack by DMS and DES is the N-7 position of guanine [33-35]. However, it has been observed that there exist other sites also damaged by alkylation, which include N-3 and O<sup>6</sup> of guanine, the N-3 of cytosine, the N-1, N-3 and N-7 of adenine, as well as the N-3 and O<sup>6</sup> of thymine [33, 34, 42].

One of the contributing mutagenic effects of dialkyl sulphates is the formation of adducts with DNA bases, which can be reversed by the excision repair systems of both species. The repair process involves the removal of the damaged DNA bases and the insertion of intact nucleotides. This process is important for maintaining genomic stability and preventing the formation of mutations.

Table 1. Reaction type of various alkylating agents.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Type</th>
<th>Ref.</th>
<th>Chemical</th>
<th>Type</th>
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<tr>
<td>Methyl bromide</td>
<td>S&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[38]</td>
<td>DES</td>
<td>S&lt;sub&gt;N1&lt;/sub&gt;/S&lt;sub&gt;N2&lt;/sub&gt;</td>
<td>[39]</td>
</tr>
<tr>
<td>MMS</td>
<td>S&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[40]</td>
<td>MNU</td>
<td>S&lt;sub&gt;3&lt;/sub&gt;</td>
<td>[41]</td>
</tr>
<tr>
<td>DMS</td>
<td>S&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[40]</td>
<td>iPMS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S&lt;sub&gt;3&lt;/sub&gt;</td>
<td>[38]</td>
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<sup>a</sup> Isopropyl Methanesulphonate.

DMS, a potent mutagen, has been extensively used in genetic studies to induce mutations in various organisms. It is also used as a tool to study the repair mechanisms in DNA. In the study of Yarrowia lipolytica DSM3286, a yeast species, the effect of DMS exposure on protein production was investigated. The yeast was exposed to DMS for 120 minutes, and the production of amylase was measured. The results showed a significant increase in amylase production compared to the control, indicating the potential of DMS as a mutagenic agent in genetic studies.

4. BACKGROUND OF POPULAR MICROBES FOR GENE MANIPULATION

Pichia pastoris, a methylotrophic yeast strain, is now a standard eukaryote utilized in the fields of recombinant protein expression and cell biology research. P. pastoris attracted numerous researchers due to its: 1) capability of post-translational modifications similar to higher eukaryotic organisms such as disulphide bond formation, glycosylation and proteolytic folding, 2) ability to express recombinant proteins at high yields and 3) more rapid, easy and inexpensive gene expression compared to higher eukaryotes. Apart from this, P. pastoris is specifically used in cell biology to study the mechanisms of secretory pathways in eukaryotes as well as the uptake, manufacture and selective autophagic decomposition of peroxisomes [50]. In the 1970s, the Phillips Petroleum Company first started cultivating P. pastoris using methanol as the sole carbon source in the hope of industrializing production of P. pastoris for use as animal feedstock [51]. In the 1980s, Phillips Petroleum worked with the Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, CA, USA) to identify the potential of P. pastoris for heterologous protein expression, contributing to the current reputation of P. pastoris [52].

Schizosaccharomyces pombe, a fission yeast, is another commonly used eukaryote in cell biology study, recombinant protein production and eukaryote protein analysis. The species is unicellular and rod-shaped, with cells measuring 3-4 (width) by 7-14 (length) micrometers. Its genome is about 14.1 million base pairs containing 4,970 genes for protein expression and more than 450 non-coding RNA [53]. In addition to capabilities of protein expression identical to P. pastoris, Sz. pombe alone contains sophisticated Golgi bodies and galactosyltransferase, making the species ideal for eukaryote protein expression [54]. In 1893, Paul Lindner first isolated Sz. pombe from East African millet beer when working in Brewery Association Laboratory in Germany. The strain’s name ‘Schizo’ means different and ‘pombe’
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5. MODIFICATION OF LIPASE GENE FOR BETTER YIELD

5.1. Stronger Lipase Gene Promoter

A promoter is a part of DNA which marks the start of transcription of a specific gene. A promoter can span 100-1000 base pairs. Promoters can be found upstream and on the same DNA strand of the gene they represent, located near the 3’ region of the template strand. Normally a promoter is turned off due to a repressor binding to the active site of the promoter. But when specific substrates are present, the substrates bind with the repressor and change its active site, releasing it from the promoter. Hence the promoter is turned on. RNA polymerase can then bind to the promoter and transcription of the gene starts. Once the produced enzyme used up all the substrates, the repressor is freed and binds to the promoter again, stopping further transcription of the gene [61].

In *P. pastoris*, alcohol oxidase (AOX) is generated and kept within peroxisomes where it breaks down methanol into formaldehyde and hydrogen peroxide. Hydrogen peroxide, being toxic to the cell, is broken down by catalase into oxygen and water in the peroxisomes. Formaldehyde released from peroxisomes is broken down by two dehydrogenases into formate and carbon dioxide in the cell cytoplasm, with those two reactions providing energy for the cell. There are two alcohol oxidase promoters, namely AOX1 and AOX2, with AOX1 being more active. To induce high levels of transcription of AOX1 and AOX2, the presence of methanol as the sole carbon source would be ideal [62]. Since methanol is a fire hazard at large industrial quantities and a petroleum-derived product, there have been researches into alternative promoters to AOX. One of the alternatives is a promoter gyceraldehyde 3-phosphate dehydrogenase (GAP) which is significantly produced in *P. pastoris* in glucose broth. The advantages of GAP are the elimination of methanol requirement and exchanging of different carbon sources for the cultures [62].

5.2. Codon Change for Recombinant Lipase Gene

Universal codon codes were thought to govern DNA of all living organisms following the ‘Frozen Accident Theory’ which proposes that ‘the code is universal because any change would be lethal or at least strongly selected against’ [63]. However, there have been cases where different microorganisms express different amino acids when coding for the same codons. To solve this issue in gene transferring, the codons from the recombinant gene will have to change so that the host organism also codes for the same amino acids, preventing undesired structural changes to the expressed protein. An organism that shows non-universal coding is the *Candida* species which recognizes the universal codon CUG/CTG that codes for leucine as serine [64]. After the conversion of 19 non-universal CTG-serine codons into universal TCT-serine codons in the lipase gene (*lip1*) of *Candida rugosa* by using overlap extension PCR-based multiple site-directed mutagenesis, the *lip1* gene was successfully expressed in *P. pastoris*. The lipase activity recorded was 253.3±18.8 U/ml [65].

5.3. Replacement of Leader Sequence of Lipase Gene

A leader region is a sequence of DNA located at the 5’ end of an mRNA molecule which is not involved in protein translation. The leader sequence is also called signal sequence or more properly known as five prime untranslated region (5’ UTR). The 5’ UTR begins at the transcription start site of an mRNA molecule and ends right before the start codon of the protein coding region. A Shine-Delgarno sequence (AGGAGGU), also called a ribosome binding site (RBS) is found within the 5’ UTR. The main function of the 5’ UTR is to act as a regulatory sequence, which can increase or decrease gene expression. One or more of several different types of regulatory elements can be found in the 5’ UTR, which includes RBS, protein binding sites (e.g. transferrin which is an iron-transferring molecule in human blood), riboswitches (a regulatory segment that regulates its own gene expression rate following the concentration of its effector molecules) and microRNA (abbr. miRNA, which is found in eukaryotic mRNA, acting as a gene silencer in both transcription and post-transcription stages).

The 5’ UTR is susceptible to proteolytic splicing which can produce variations of the 5’ UTR. The different variations include varying combinations of the regulatory sequences, changes to the length of the 5’ UTR and mutations of the sequences within the 5’ UTR. These alterations will change the activity of the mRNA. Fabrizia Fusetti and three co-workers reported the expression of the lipase I gene from *C. rugosa* in the host *Saccharomyces cerevisiae*. The recombinant gene expression yielded high activity only after the original endogenous leader sequence of the enzyme was replaced by the signal peptide of a killer toxin from *Kluyveromyces lactis*. The final lipase yield was over 1 g/l [66].

5.4. Addition of Fusion Tag to Lipase Gene

A fusion tag is a short peptide, protein domain or whole protein that fuses with a targeted protein to construct a fusion protein. The targeted protein gene is joined with one or more protein genes and translation of this fusion gene creates a single protein with functional properties from the original
proteins. This is called fusion protein or chimeric protein. Addition of fusion tags can bring the following advantages to the targeted proteins:

- Enhanced expression – Fusion of the C-terminus of a highly expressed protein to the N-terminus of the targeted protein increases the expression of the targeted protein.
- Enhanced purification – Affinity tags that bind specifically to resins, ligands or antibodies enables easy purification of the targeted protein.
- Enhanced detection – Fusion of a marker gene enables easy detection of the targeted protein upon expression and purification.
- Enhanced solubility – Fusion of the C-terminus of a soluble protein to the N-terminus of the targeted protein often increases the solubility of the targeted protein.
- Enhanced localization – Tags, normally located at the N-terminus of the targeted protein, can act as a medium for protein transfer to specific organelles in cells.

Thioredoxin was used as a fusion tag with lipase B from *Candida antarctica* (CalB). The thioredoxin-CalB fusion protein was expressed in *E. coli*, yielding 17 U mg⁻¹, compared to the most efficient *cspA* promoter yielding 2 U mg⁻¹ for CalB in *C. antarctica* [67].

### 5.5. Molecular Chaperones

Molecular chaperones are proteins that aid in the folding, assembly and degradation of macromolecules such as the folding of other proteins and assembly of nucleosomes from histones and DNA [68]. Chaperones mainly function to stop the formation of nonfunctional proteins from the aggregation of newly synthesized polypeptide chains and protein subunits. As proteins lean toward denaturation at elevated stress, most of the chaperones are heat shock proteins, meaning their expression increases in response to high temperature or other stresses [69]. Another function of chaperones is the assistance of formation of unique protein structures, but this function only exist in some highly specific steric chaperones.

### 5.6. Directed Evolution, Like Error Prone PCR and DNA Shuffling

Directed evolution is a powerful biochemical tool used to improve and tailor proteins and DNA molecules. The two most popular techniques utilized are error-prone PCR and DNA shuffling [70]. Polymerase chain reaction (PCR) is a biotechnological advancement in molecular biology to multiply a single or a small number of DNA fragments or sequence thousands to millions of times, effectively obtaining a yield of several orders of magnitude. This technique was first introduced by Kate Mullis in 1983, and is now indispensable across a wide range of fields including forensics, parental testing, infectious microbes identification, analysis of hereditary diseases as well as DNA cloning and study [71]. PCR is a three-step process, and is similar to the natural process of DNA replication. PCR takes place in a thermal cycler, which is a programmable machine that allows automatic temperature control and changes over the entire duration of the required number of PCR cycles. The three steps of PCR are denaturation, annealing and extension. The targeted DNA sequence is stored under oil in the presence of primers and four nucleotide bases (adenine, guanine, thymine and cytosine). The DNA double-stranded helix is broken down into two separate strands by heating to above 90°C. This process breaks the hydrogen bonds linking both strands together and is termed denaturation. Upon cooling to 40-65°C, primers bind to both strands, a process called annealing. The DNA is then heated up to 72°C where *Taq* polymerase binds to the primers and synthesizes two new DNA double helices by utilizing the free nucleotides. The new DNA molecules are identical to the original double helix. This step is called extension, and these three steps constitute a PCR cycle. The PCR cycles are repeated as much as needed to reach the required order of amplification [72, 73].

Error-prone PCR is the modified protocols of the normal PCR procedures, specifically altered to increase the error rate of DNA polymerase [74]. *Taq* polymerase has been found to have a naturally high error rate, favoring AT to GC alterations [75]. To sustain non-complementary pairs introduced by error-prone PCR, higher concentrations of MgCl₂ (7 mM) are required, unlike normal PCR (1.5 mM) [76]. Apart from this, the addition of MnCl₂ [77], alternating the ratio of nucleotides [78, 79] and using a nucleotide analog such as dIPT or 8-oxo-dGTP [80] also improved the error rate.

On the other hand, DNA shuffling refers to an in vitro technique which propagates mutations rapidly via the recombination of homologous DNA fragments. W.P.C. Stemmer invented and first commercialized DNA shuffling when he founded a biopharmaceutical company called Maxygen in 1997. Firstly the targeted DNA strands and genes are randomly sectioned by DNaseI, where the fragments with the correct sizes were specifically purified from an agarose gel. These fragments undergo random reassembly following many PCR cycles. When fragments from different parent DNA anneal at regions of identical nucleotide sequence, this is termed recombination. Another PCR step with primers generates new full-length DNA called chimeras, which are suitable for cloning into a vector [81, 82].

Yamada *et al.* (2015) used a directed evolution approach using error-prone polymerase chain reaction to enhance the stability of homodimeric BPO-A1 haloperoxidase in organic solvents. The haloperoxidase was produced by *Streptomyces aureofaciens*. From 1000 mutant BPO-A1 haloperoxidases, an organic solvent-stable mutant OST48 with P123L and P241A mutations and a high activity mutant OST959 with H53Y and G162R mutations were selected. After immersing in 40% (v/v) 1-propanol for 1 h, the residual activity of mutant OST48 was 1.8-fold greater than the original enzyme BPO-A1. Apart from this, the mutant OST48 was also more stable in methanol, ethanol, dimethyl sulfoxide and N,N-dimethylformamide compared to the original. On the other hand, the residual activity of the mutant OST959 was 4.6-fold greater than the original after being incubated at 80°C for 1 h. Based on the analysis of single amino acid-substituted mutant models, the higher organic solvent-stability was attributed to P123L mutation which stabilized the hydrophobic core of the enzyme, whereas the higher thermostability was due to G162R mutation which enhanced the amount of hydrogen bonds within the enzyme structure [83].
5.7. Heterologous Expression of Lipase Gene

Heterologous expression is the expression of a recombinant gene in a host micro-organism. Many researchers have successfully cloned and expressed recombinant protein genes in suitable and easily cultivated host micro-organisms in laboratories. The need for heterologous expression is due to the low protein yields and difficulty of breeding the native micro-organisms in laboratories. The need for heterologous expression is due to the low protein yields and difficulty of breeding the native micro-organisms in laboratories [84]. A comprehensive table listing the various recombinant protein genes expressed by P. pastoris as well as their respective type of secretion (intracellular or extracellular), yields and signal sequences used has been produced by James M. Cregg et al. [50].

Vaquero et al. (2015) had expressed a recombinant protein in P. pastoris that coded for the production of a versatile sterol-esterase (OPE). The enzyme OPE is highly efficient in both hydrolysis and synthesis of triglycerides and sterol esters. OPE is originally secreted by Ophiosoma piceae, but the enzyme aggregates in aqueous solutions. However, the recombinant OPE secreted by P. pastoris was highly soluble in aqueous solutions, thereby enhancing its catalytic activity. The higher solubility was due to an integration of 4-8 additional amino acids in the N-terminal sequence of the enzyme expressed in P. pastoris. These additional amino acids affect the aggregation behavior of the enzyme, making it more soluble. The same recombinant protein was alternately expressed in the yeast Saccharomyces cerevisiae and the bacterium Escherichia coli, which corroborated the fact that the additional amino acids increased the solubility of the enzyme. This study illustrated the importance of choosing a suitable heterologous host for successful expression and enhancement of recombinant proteins [85].

5.8. Creation of Protease-Deficient Strain for Better Yield

The microbial lipase product is prone to proteolysis by various proteases secreted by the host microbes. Despite the high production rate of lipase, a small amount of proteases can reduce the final yield of lipase significantly. This fact pushes for the search or creation of protease-deficient microorganism strains.

The complete mapping of Sz. pombe genome in 2002 [86] has allowed the identification of most proteases produced by Sz. pombe, at the same time paving the way for genetic manipulation in order to achieve a protease-deficient strain. Idiris and his colleagues successfully developed and reported the first protease-deficient Sz. pombe strain ARCO10 using a well-established fusion extension PCR coupled with gene disruption method, using ura4 as the marker gene [87]. Each open reading frame (ORF) of all protease genes of the parent cell is extracted. A first PCR step cuts the 5' and 3' flanking sequences of each ORF which spanned 200-300 base pairs (bp). A second PCR joined the flanking sequences to the 5' and 3' terminals of ura4 (1762 bp), yielding a disruption gene. The ura4 genes were then inserted into the strain using lithium acetate-based transformation method [54, 88].

Idiris et al. (2006) had set out to develop a protease-deficient Sz. pombe host strain that can be used for effective production of distinct protease-sensitive heterologous proteins and peptides. Since there were few documented reports regarding the functionalization of proteases in Sz. pombe, the team chose the method gene disruption to screen and isolate active protease genes. The recombinant gene for human growth hormone (hGH), a secretory protein expressed in the human pituitary gland with 22 kDa weight and 191 amino acid molecules when matured [89], was used in Sz. pombe to analyze the extent of reduced proteolytic degradation for the protease-deficient mutants. Out of the 52 gene disruptants obtained, 49 hGH-producing mutants were isolated. It was found that 8 gene disruptants were reproducibly effective in minimizing hGH degradation, namely isp6, pp1, ps3, sxa2, pps51, pps53, pps60 and pps80. Of these, pps80 is a cysteine protease, isp6, sxa2 and ps3 are serine proteases, while pp1, pps51, pps53 and pps60 are categorized under metallo-protease family. Most of the proteases, whose deletion boosted the production of hGH, were intracellular enzymes [90].

6. CONCLUSIONS

Enzymes are important biocatalysts to both living organisms and the modern industries. In order to lower the cost of producing and purifying enzymes on a large scale, numerous research have been done on genetic modification of protein-rich microorganisms. The efficiency of the physical and chemical mutagenesis techniques reviewed in this paper could be further enhanced by combining different techniques. More research is needed to develop and analyze the commercial feasibility of genetic manipulation on protein-rich microorganisms.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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