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Review article

Novel organisms by genetic engineering to improve recombinant drug therapy in biotechnology by molecular cloning

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Abstract

Study design: Study design of this research Systematic review, Meta-analysis study design. A subset of systematic reviews; a mode for systematically consoldating pertinent qualitative and quantitative study datum from several selected treatise to develop a single conclusion that has major statistical power. This conclusion is statistically sturdy than the analysis of any single study because increased numbers of object, greater diversity through subjects, or accumulated effects and results.

Background: Genetic engineering, also recognized genetic modification or genetic manipulation is the direct manipulation of an organism's genes in biotechnology via transmit genes within species to produce ameliorate or novel organisms.

Objective: The goal of the article is to produce a new organism that carries new characteristics that differ from the original organism through genetic

manipulation with genetic engineering and the use of molecular color technology in order to produce hybrid drugs used for treatment in biomedical sciences.

Results: Improve restriction enzyme via using Blunt ends generated via *Eco*R V and 5' Cohesive end begeted by *Bln* I 3' Cohesive end created by *Kpn* I. Restriction enzymes can cut DNA in two route to generate blunt ends, cut precisely at opposite sites, e.g., HpaI and staggard ends, cut at asymmetrical position, e.g., Eco RI with short single stranded preside at each end. A large numberal of restriction enzymes have been identified and distributing into three categories (type I, II, III) on the ground of their site of cleavage. All genetically engineered organisms ameliorate the activity of T4 DNA ligase, in scenarios that are pertinent for molecular biologists and Cloning assay, a 739-bp, blunt-ended introduce was cloned into pUC18 and the ligation output were utilized to transform *E.coli* DH5α-E. Colony enumeration are the means (±SEM) of independent triplicates.

Conclusions: First: The process of molecular chlorination in genetic manipulation to produce new organisms bearing new characteristics is very important in the production of medicines that are less productive compared to the original organisms. Second: The molecular chlorination process has proven to be successful in producing many enzymes used in the treatment of many difficult diseases.

Key words: Gene cloning, enzyme pharmaceuticals, artificially synthesis and genetic manipulation.

Introduction and review

1- Genetic engineering

Genetic engineering, also known genetic modification or genetic manipulation is the direct manipulation of an organism's genes in biotechnology via transmit genes within species to produce ameliorate or novel organisms [1].

An organism that is created main while genetic engineering is theorized to be genetically modified (GM) and the resulting subsistence is a genetically modified organism (GMO). Genetic engineering has been utilized in numerous fields inclusive research, medicine, manufacturing biotechnology and agriculture. In research GMOs are utilized to study gene function and expression meanwhile loss of function, gain of function, tracking and expression experiments via knocking out genes answerable for certain conditions it is potential to create animal model organisms of human diseases. As well as resulting hormones, vaccines and another drugs, genetic engineering has the possibility to cure genetic diseases meanwhile gene therapy. The same techniques that are utilized to produce drugs can also have industrial applications for example producing enzymes for lavement detergent, cheeses and another products. The increase of commercialized genetically modified crops has supplied economic benefit to farmers in several different countries, but has also been the provenance of utmost of the controversy embracing the technology [2].

2- Gene excommunication and molecular cloning

The steps are to isolate the nominee gene, the cell containing the gene is unfolded and the DNA is purified [3]. The gene is detached via utilizing restriction enzymes to cut the DNA till fragments [4] and amplify gene fragment via polymerase chain reaction (PCR) [5]. These fragment can then be extracted during gel electrophoresis. If the chosen gene or the donor organism's genome, it might already be accessible from a genetic library. If the DNA sequence is recognize, but no copies of the gene are obtainable, it can also complicated [6]. Once insulated the gene is ligated till a plasmid that is then introduced into a bacterium. The plasmid is replicated when the bacteria division, ensuring unlimited copies of the gene are obtainable [7].

A previously the gene is introduced into the target organism it utmost be combined with another genetic elements. These inclusive a promoter and terminator district which initiate and end transcription. A single out gene is added that in most cases accords antibiotic resistance. The gene may also be modified at this stage for better expression or performance. These manipulations are accomplished using recombinant DNA techniques for example restriction digests, ligations and molecular cloning [8].

3- Inserting DNA into the host genome

There are a numeral of techniques utilized to insert genetic material till the host genome. Several bacteria can naturally elevate foreign DNA. This competence can be influenced in other bacteria via stress for example thermal or electric shock that increases the cell membrane's permeability of DNA, up-taken

DNA may either consolidate with the genome or subsist as extra chromosomal DNA. DNA is mostly inserted into animal cells via using microinjection, where it can be pollinated during the cell's nuclear envelope immediately into the nucleus or during the use of viral vectors [9].

As only a single cell is transformed with genetic materiality, the organism utmost be regenerated from that single cell. In plants this is carried out during the use of tissue culture [10, 11]. In animals it is necessity to ensure that the inserted DNA is existing in the embryonic stem cells [12]. Bacteria composed of a single cell and reproduce clonally so regeneration is not necessity. Selectable markers are utilized to readily differentiate transformed from untransformed cells. These markers are ordinarily present in the transgenic organism, though a numeral of strategies have been improved that can remove the selectable marker from the overripe transgenic plant [13].

The technique utilized in genetic engineering inclusive PCR, Southern hybridization, and DNA sequencing is managed to confirm that an organism comprise the new gene [14]. These tests can else confirm the chromosomal position and copy numeral of the inserted gene. The existence of the gene does not mortgage it will be expressed at convenient levels in the target tissue so mods that look for and mensuration the gene products RNA and protein are else used, these inclusive northern hybridization, quantitative RT-PCR, Western blot, immunofluorescence, ELISA and phenotypic analysis [15].

The novel genetic material can be inserted randomly through the host genome or targeted to a specific position. The technique of gene targeting employs homologous recombination to fabricate desired changes to a specific endogenous gene. This resort to happen at a relatively low frequency in plants and animals and mostly demand the utilize of selectable markers. The indecision of gene targeting can be greatly influenced meanwhile genome editing. Genome editing utilizes artificially engineered nucleases that generate specific double-stranded breaks at desired positions in the genome and utilize the cell's endogenous mechanisms to rehabilitation the induced break via the natural processes of homologous recombination and non homologous end-joining [16,17], zinc finger nucleases [18, 19], transcription activator-like effector nucleases (TALENs) [20, 21] and the Cas9-guide RNA framework (adapted from CRISPR) [20]. TALEN and CRISPR are the two utmost commonly used and each has its own characteristic [22]. TALENs have greater target specificity whilst CRISPR is easier to design and more functional. In addition to enhancing gene objective, engineered nucleases can be used to insert mutations at endogenous genes that create a gene knockout [23, 24].

4- Applications

Genetic engineering has implementation in medicine, research, industry and agriculture of plants, animals and microorganisms. Bacteria, the headmost organisms to be genetically modified, ability have plasmid DNA inserted comprise new genes that code for medicines or enzymes that practicability food and other substrates [25, 26,27]. Utmost commercialized GMOs are insect resistant neither herbicide tolerant crop plants [28]. Genetically modified

animals have been utilized for research, model animals and the production of agricultural or pharmaceutical products. The genetically modified animals inclusive animals with genes knocked out, raised susceptibility to disease, hormones for extra growth and the capability to express proteins in their milk [29].

4-1 In Medicine

Genetic engineering has several applications to medicine that inclusive the manufacturing of drugs, habit of model animals that mimic human status and gene therapy. One of the earliest utilizes of genetic engineering was to mass manufacture human insulin in bacteria. This application has now been utilized to human growth hormones, follicle catalyzing hormones for treating infertility, human albumin, monoclonal antibodies, antihemophilic employee, vaccines and several other drugs [30, 31]. Mouse hybridomas, cells fused simultaneously to create monoclonal antibodies have been adapted meanwhile genetic engineering to create human monoclonal antibodies [32]. Genetically engineered viruses are being advanced that can still confer immunity, but absence the infectious sequences [33].

Genetic engineering is else used to create animal models of human diseases. Genetically modified mice are the utmost common genetically engineered animal model [34]. They have been utilized to treatise and model cancer (the oncomouse), obesity, heart disease, diabetes, arthritis, materiality abuse, anxiety, aging and Parkinson disease [35]. Potential cures can be experienced against these mouse models. Gene therapy is the genetic engineering of humans, generally via replacing defective genes with effective

ones. Clinical research utilizing somatic gene therapy has been conducted with many diseases including X-linked SCID [36]. Chronic lymphocytic leukemia (CLL) [37,38] and Parkinson's disease [39]. In 2012, Alipogene tiparvovec became the headmost gene therapy treatment to be approved for clinical use [40,41]. In 2015 a virus was utilized to insert a healthy gene till the skin cells of a boy suffering from a infrequent skin disease, epidermolysis bullnose in order to grow, and then graft healthy skin onto 80 percent of the boy's body that was affected by the illness [42].

Germline gene therapy would consequence in any change being inheritable that has raised concerns within the scientific community [43,44]. In 2015, CRISPR was used to edit the DNA of non-viable human embryos [45,46]. leading scientists of essential world academies to call for a temporary on inheritable human genome edits [47]. There are also intervest that the technology could be utilized not just for treatment, but for enhancement, modification or modification of a human beings' appearance, adaptability, intelligence, representative or behavior [48]. The distinction between cure and enhancement can also be complicated to establish [49]. In November 2018, He Jiankui announced that he had omitted the genomes of two human embryos, to attempt to macerate the *CCR5* gene which codes for a receptor that HIV utilizes to enter cells. The work was exceedingly condemned as unethical, severe and premature [50]. Currently, germ line modulation is banned in 40 countries [51,52].

Scientists are generating "gene drives" revision the genomes of mosquitoes to manufacture them immune to malaria and then looking to prevalence the

genetically altered mosquitoes throughout the mosquito inhabitance in the hopes of eliminating the disease [53].

4-2 Applications of enzymes in pharmaceuticals

Enzymes are the functional proteins neither nucleic acids (Ribozymes), also known as biocatalysts that facilitate the fulfillment of biochemical reactions at the rates which are appropriate for the normal functioning, growth and proliferation of any living system, including unicellular or multicellular plants as well as animals [54]. The capability of enzymes to exist viable and perform catalytic activities even outside their resources organism under in situ conditions [55] permits them to be exploited for carrying out a numeral of industrial processes which rely on chemical transformations of substrates to their corresponding products. The reactions catalyzed via enzymes are highly efective that occur beaneath ambient environmental conditions inclusive temperature, pH and pressure (the conditions upon on the physiological status of the source organisms and its environmental conditions). For example, an enzyme gain from a mesophilic organism (optimal growth temperature is 37 °C and growth temperature range from 20 to 40 °C), inhabiting neuter environments [56,57]. As enzymes are functional outgoing the cells or organisms, the are utilized in a number of industrial applications such as synthesis of pharmaceuticals for example drugs, process grain juices into lager and wine, leaven dough for baking production, production of agrochemicals, artificial flavors, biopolymers, garbage remediation and many others [58].

Majority of the industrial enzymes derive from microorganisms as they are the most convenient sources that gives the tendency of faster production, easy scale up, recovery and purification, strain modification for over-expression, enzyme activity, specificity modulations [59,60]. Almost 75% of the total enzymes are produced via three top enzyme companies inclusive Denmark-based Novozymes, US-based DuPont and Switzerland-based Roche [61].

Insights related to the market prediction of the most widely used enzymes like proteases, carbohydrases and lipases marketed of pharmaceutical and healthcare sectors is presented here for the indication of the readers [62]. The main growth in proteases' market shall be in the health industry because many benefits offered by them such as, boosting the immune system, prohibition inflammatory bowel diseases, curing skin burns and stomach ulcers. Morever, other sectors like animal feed segment where protease are used for ameliorate the nutritional or digestive properties of fodder and upkeep of animal gut health shall carry a great share of protease market. Carbohydrases crossed USD 2.5 billion in 2016 and are contemplated to show a good increase of more than 1/3 over the present figure via 2024. The prominent application sectors shall be in food, drinkable, and pharmaceutical industry. Lipases are expected to achieve the increase in sale via about 6.8% by 2024. The major area of lipase market shall be in the field of healthcare for the therapy of obesity which is becoming an emerging issue in evolved countries. As lipase break down fats into glycerol and fatty acids under natural status, its demand is expected to rise in healthcare industry as an aid for weight surveillance and management in obese people [69].

Several drugs or pharmaceutical formulazation are comprised of active pharmaceutical ingredients (APIs) that are synthesized utilizing enzymes as important components of the manufacturing practicability [63]. The usage of biocatalysts for pharmaceuticals' production as well as increasing interest in the production of chiral intermediates and green synthetic practicability has substantiated the interest in the applications of biocatalysts in these domain [64].

4-3 Production pharmaceutical drug of industrial enzymes

Most of the industrial enzymes produced through fermentation of suitable microbial strains mainly belonging to bacteria and fungi due to their easy handling, fast growth rates, and convenient scale up in large vessels (fermenters) [65,66]. Bacteria like *Escherichia coli*, *Bacillus subtilis*, lactic acid bacteria, and the filamentous fungi such as *Aspergillus oryzae*, *Aspergillus niger*, *Trichoderma atroviride* and Yeasts, for example, *Saccharomyces cerevisiae, Pichia pastoris* and so on are the most exploited microorganisms for enzyme production by the biotechnology industries over the world [67, 78]. Selection of appropriate microbial strains for the production of various industrial enzymes is a very paramount aspect for their successful industrial applications, the enzymes produced should be excrete out in the fermentation medium by the producing microbial strain as it manufactures the downstream processing more convenient and economically feasible, morever, this may not be the case with most of the industrial strains. Pharmaceutical enzymes are produced via utilizing the fermentation technology, mainly utilizing the

microorganisms bacteria and fungi which comes under the Generally Recognized as Safe denomation [68]. This production is mainly carried out utilizing two processes, submerged fermentation (SmF) and solid-state fermentation (SSF) [69]. Utmost industries have adopted SmF process for enzyme production, but there has been a regenerate interest in SSF for certain specific industries [70].

5- Enzyme Therapy

Enzyme therapy indicate to the applications of enzymes for treating enzyme deficiencies and other medical status in human beings. In humans, enzymes assist in food digestion, body detoxification, strengthening of the immune system, muscle contraction, decreasing of stress on the vital organs like pancreas and others. In this regard, enzyme therapy has considerable possible medical applications, for instance treatment of pancreatic insufficiency and cystic fibrosis (CF), metabolic disturbance, lactose intolerance, eliminate of dead tissues, cancers or tumors, and so on. The therapy may be systemic or no systemic and may be administered by multiple mode of administration, utmost often orally, topically or intravenously [71]. A summary of enzymes utilizes as therapeutic agents is presented in Table 1.

Table 1. Enzymes for therapeutic utilizes

Trade Name	Generic Name	Indication
ADAGEN	Pegademase bovine	For enzyme replacement therapy for ADA in patients with SCID
CEREDASE	Alglucerase injection	For replacement therapy in patients with Gaucher's disease type I
PULMOZYME	Dornase alpha	To reduce mucous viscosity and enable the clearance of airway secretions in patients with CF
CEREZYME	Imiglucerase	Replacement therapy in patients with types I, II, and III Gaucher's disease
SUCRAID	Sacrosidase	Treatment of congenital sucrase-isomaltase deficiency
ELITEK	Rasburicase	Treatment of malignancy-associated or chemotherapy-induced hyperuricemia
FEBRAZYME	Agalsidase beta	Treatment of Fabry's disease
NATTOKINASE NSK-SD	Nattokinase	Support healthy blood clotting, circulation, and platelet function

6- Enzymes for the treatment of contagious diseases

Lysozyme a bactericidal enzyme created naturally in human body is added in many food products. It has been discovered that this enzyme has activity against HIV, similar to RNase A and urinary RNase U, it selectively contaminate viral RNA [72] and therefore is a promising candidate for the therapy of HIV infection. Other examples of antimicrobial enzymes are chitinases. Chitin is a pretty target for antimicrobials because it is a main composition of the cell wall of different pathogens like fungi, helminths, and protozoa [73]. On the other

hand, lytic bacteriophage-derived enzymes have been called for killing bacteria for example Streptococcus pneumonia, Clostridium perfringens, and Bacillus anthracis , consequently, the application of lytic bacteriophages as a treatment for bacterial infections is else under development and is supposed to be effective contra antibiotic resistant bacterial strains [74,75].

7-Enzymes for the treatment of cancer

Application of therapeutic enzymes in cancer therapy is an emerging field of research. Lately, it has been detected that PEG immobilized arginine deaminase (an arginine-degrading enzyme) can prohibit human hepatocellular carcinomas and skin cancers which are deficient in arginine because lack of arginosuccinate synthetase activity [76]. A better enzyme treatment has been developed utilizing PEGylated L-asparaginase with the denotation Oncaspar (pegaspargase). It has shown better produces for the treatment of acute lymphoblastic leukemia, acute myeloid leukemia, and non-Hodgkin's lymphoma [77]. In other words, the naturalistic cells, that is, the noncancerous cells can synthesize asparagine while the cancer cells cannot, and die in the presence of enzymes that degrade asparagine. Though the cost of the PEG–asparaginase formulations is higher than the nature enzyme the overall cost of the treatment is very similar in both cases. Actually, asparaginase and PEG-asparaginase are better alternatives to gauge chemotherapy [78].

Results and discussions

Genetic engineering turn into possible with the discovery of mainly two kinds of enzymes including the cutting enzymes known restriction endonucleases and

the joining enzymes called ligases inclusive Restriction Endonuclease and DNA Ligase.

1. Restriction Endonuclease

Restriction endonucleases or restriction enzymes as they are known generally, recognize unrivaled base sequence motifs in a DNA strand and cleave the backbone of the molecule at a place within, at some milage from the recognition site Whereas ligase is the enzyme that connects a 5′ end of a DNA with a 3′ end of the similar or of another strand [79].

Average nucleases are endonucleases or exonucleases. The former cleaves the DNA backbone between two nucleotides for example it cleaves the double stranded DNA at any point except the ends, but it include only one strand of the duplex. The latter eliminate or digest one nucleotide at a time starting from 5′ or 3′ end of a DNA strand. The restriction endonucleases cleave only at specific districts in a particular DNA, so that discrete and defined fragments are gain at the end of total digestion. The name 'restriction' endonuclease created from an observation of a system of restriction of the growth of the phage lambda in specific strains of the *E. coli* host cell [80].

Utmost restriction enzymes distinguish only one short base sequence in a DNA molecule and manufacture two single strand breaks, one in each strand, generating 3'OH and 5'P groups at each situation. The sequences recognized by restriction enzymes are often palindromes for example inverted repetition sequences that are symmetrical [81].

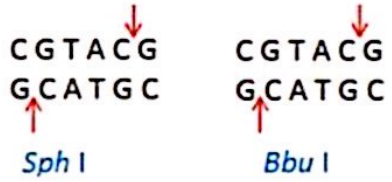
1-1 Employee affecting on the activity of restriction enzymes

Depending on the substrate DNA and the reaction status, restriction enzymes have a wide variation of cleavage and potential star activity. In order to obtain the desired cleavage, it becomes paramount to control the following factors:

1. **Star activity:** Under sub-optimal reaction status, some restriction enzymes cleave base sequences at sites diverse from the defined recognition sequence, the cleave at non-specific sites. This phenomenon is known star activity. Some of the factors that enhance star activity are high salt and glycerol concentration, presence of imperfection, excessive enzyme compared to substrate DNA, raised incubation time or incompatible buffer and cofactor.
2. **Methylated DNA:** Many DNA molecules are methylated at the recognition site, manufacturing them resistant to cleavage via specific restriction enzymes. For instance, most *E. coli* strains express Dam or Dcm methyltransferases which methylate certain recognition sites to form G6mATC and C5mCA/TGG, respectively. G6mATC is resistant to cleavage by *Mbo* I.
3. **Temperature:** Utmost endonucleases optimally digest the target DNA at 37 °C. However, there are several exceptions with lower or higher optimal temperatures. For example, *Taq* I optimally assimilate at 65 °C and Apa I (Catalog No. 10899208001) assimilate at 25 °C [82].

1-2 Isoschizomers and Neoschizomers

Isoschizomers are restriction enzymes together the same recognition sequence and cleavage sites. For instance: *Sph* I (CGTAC/G) and *Bbu* I (CGTAC/G)



Neoschizomers are restriction enzymes together the same recognition sequence but cleave the DNA at a diverse site within that sequence. For instance: *Tai* I (ACGT/) and *Mae* II (A/CGT) [83].

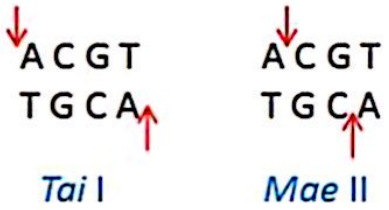


Figure (1): Recognition sequence and cleavage site of spI and BbuI of isoschizomers and neoschizomers.

1-3 Out put of restriction enzymes

Restriction digestion of double-stranded DNA out put two kinds of ends: Sticky ends and Blunt ends.

1-3-1 Blunt ends own a 5'-phosphate group that allow ligation. They are universally compatible with other blunt-ended DNA.

Blunt ends created by *Eco*R V

*Eco*R V

5'...GAT|ATC...3'
3'...CTA|TAG...5'

Figure (2): Blunt ends via cleavage of restriction enzyme of EcoRV.

1-3-2 Sticky ends are small stretches of single-stranded DNA enable of self-ligation or ligation with a complementary part from another DNA molecule. The sticky ends possess 3'- or 5'-overhangs of 1–4 nucleotides.

5' Cohesive end generated by *Bln* I (Catalog No. 11558170001)

Bln I

5'...C|CTAG G...3'
3'...G GATC|C...5'

3' Cohesive end created via *Kpn* I

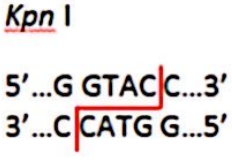


Figure (3): Sticky ends via cleavage of restriction enzyme of KpnI and BlnI.

Restriction enzymes able cut DNA in two mods to generate blunt ends (cut precisely at opposite sites, e.g., HpaI) and staggard ends (cut at asymmetrical position, for example Eco RI) with short single stranded overhangs at each end. A large number of restriction enzymes have been specified and classified till three categories (type I, II, III) on the basis of their site of cleavage [84].

1-4 Lineaments of Restriction enzymes

1. Restriction enzymes fabricate breaks in palindromic sequences.

2. The breaks are generally not directly opposite to one another.

3. The enzymes create DNA fragments with complementary ends.

The capability of restriction endonucleases to cleave DNA at particular recognition sites has enabled extensive utilize of these enzymes as fundamental tools in several molecular biology techniques. Some of the main applications are explained below:

1-4-1 Molecular cloning

A widespread application of restriction enzymes has been in the creation of recombinant DNA molecules. The process contain the cutting of the donor DNA (generally a plasmid) and the vector DNA (generally a gene from other organism) via a restriction enzyme to yield appropriate ends. These ends could be either 'blunt' or "sticky". The two cleaved DNAs are joined simultaneously utilizing an enzyme called DNA ligase to create a recombinant DNA molecule. This recombinant DNA can, then, be introduced into a host organism for replication.

1-4-2 DNA mapping

Else known as restriction mapping contain the use of restriction endonucleases to obtain structural acquaintance of the DNA fragment or genome. Mapping involves definition of the order of the restriction enzyme sites in the genome. The DNA of interest whose structure is to be limited is cleaved with a series of restriction endonucleases to produce DNA fragments different in size. These fragments are separated on an agarose gel to define the structure of the DNA of interest based on the known restriction enzyme sites of a certain DNA fragment, restriction endonucleases can be used to verify the symmetry of that DNA fragment.

1-4-3 Restriction landmark genomic scanning

is a genome dissection method that utilizes a incorporation of restriction enzymes to visualize differences in methylation levels towards the genome of a given organism. It is a useful technique to distinguish deviations from naturalistic in any DNA. It is very effective in detecting hyper/hypomethylation

in tumors, deletions or amplifications of genes or alterations in gene expression during the development of an organism [85, 86].

2- DNA Ligase: Ends of DNA strands may be joined via the enzyme polynucleotide ligase known 'glue' of the recombinant DNA molecule. The enzyme stimulate the formation of a phosphodiester bond between the 3'OH and 5'P terminals of two nucleotides. The enzyme is consequently capable to join unrelated DNA, repair nicks in single strand of DNA and join the sugar phosphate backbones of the recently repaired and resident region of a DNA strand. The enzyme that is extensively utilized for covalently joining restriction fragments is the ligase from *E. coli* which encoded by T4 phage. As the major resources of DNA ligase is T4 phage, as a consequence, the enzyme is known as T4 DNA ligase. The ligation reaction is controlled via many factors for example pH, temperature, concentration and kinds of sticky ends. As ligase utilizes the ends of DNA molecules as substrates rather than the entire DNA, the kinetics of joining upon on the number of ends (concentration) obtainable for joining. DNA ligases catalyze the formation of new phosphodiester linkages in DNA during the condensation of adjacent 3′-hydroxyl and 5′-phosphate termini. The first step in the ligation reaction is the formation of a ligase-AMP covalent intermediate. DNA ligases are categorized into two families—ATP-dependent and NAD^+-dependent according to the identity of their AMP donor. DNA ligases are foundamental for DNA replication, repair and recombination in commonalty organisms [87].

DNA ligases are climacteric for several applications in molecular biology and biotechnology. For decades, they have been utilized in the construction of recombinant DNA molecules for instance, cloning and for mutation detection utilizing the ligation chain reaction. More recently, a mode for the isothermal assembly of very large DNA fragments that utilizes the thermostable *Taq* DNA ligase has been described and widely adopted. DNA ligases can also be utilized in gene synthesis. They are essential in many next-generation sequencing (NGS) methods, either for adapter ligation through sample preparation for instance, Illumina, 454 and Ion Torrent sequencing or for the sequencing reaction onself for example, SOLiD sequencing [88].

2-1 Construction of ligase fusion proteins

The T4 DNA ligase gene (*lig*) was of plasmid pRBL that was a gift from Assoc. Prof. Ichiro Matsumura (Emory University, Atlanta, GA, USA). The *E.coli* DNA ligase gene (*ligA*) was from the ASKA summation of open reading frames. T4 DNA ligase was consolidated to DNA-binding proteins from a variety of sources (Table 4); *E.coli* DNA ligase was consolidated to NF-κB p50 only. Standard cloning techniques were used to erect a total of 17 plasmids, for the expression of T4 DNA ligase, *E.coli* DNA ligase and 15 fusion proteins. In all cases, the IPTG-influencable expression vector used was pCA24N. Full details of the cloning strategies used to construct each plasmid are supplied in the Supplementary data [89].

2-2 Cloning assay

DNA ligase activities were estimated via utilizing blue-white screening in a 'vector + insert' ligation assay. The vector foundamental in the assay was pUC18, linearized with restriction enzyme *SmaI* to create blunt ends. A appropriate blunt-ended insert fragment was generated in two stages. Mosthead, the ASKA plasmid pCA24N-*cpdB* was amplified in a PCR with the primers pCA24N and pCA24N.rev (Supplementary Table SI). Next, the PCR product was assimiliate with MscI to liberate a blunt-ended fragment of the *cpdB* gene (739 bp) that was purified from an agarose gel utilizing the Qiaquick Gel Extraction Kit. Each 20 µl ligation reaction include 100 ng of the pUC18 vector and 83 ng of the *cpdB* insert (a three-fold molar overflowing of insert over vector). Each reaction also contained a ligase (1 µM) and was accomplished in 1× New England Biolabs T4 DNA Ligase Reaction Buffer. Reactions were brood at 22°C for 1 h, then heat-inactivated at 65°C for 15 min. Proteinase K was added to 1 mg ml^{-1} and the samples were incubated for a moreover 20 min at 50°C. Next, the products of each reaction were purified utilizing the EZNA Cycle Pure Kit from Omega Bio-Tek (Norcross, GA, USA). The reaction outputs were treated with SmaI (20 U, 25°C, 1.5 h) to linearize any pUC18 that had been recircularized absence the *cpdB* insert. The SmaI was inactivated via heating at 65°C for 20 min and then aliquots of every reaction were utilized to transform *E.coli* DH5α-E by electroporation. The modified cells were allowed to retrieve in SOC medium for 1 h at 37°C, before aliquots were prevalence on LB-agar plates include ampicillin (100 µg ml^{-1}), IPTG (100 µM) and X-gal (40 µg ml^{-1}). Successful ligation of the *cpdB* introduce to the pUC18 vector gave

increase to white colonies that were counted next plates had been incubated at 37°C for 16–18 h. Each ligase was examined in triplicate [162].

2-3 Design rationale for T4 DNA ligase fusion proteins

The designing of Sso7d-ligase fusion protein, analogous to the chimeric DNA polymerases that had been substantive previously. Sso7d is a small (7 kDa), monomeric and highly thermostable protein that connects dsDNA in a sequence-independent manner with a dissociation constant (K_D) of <10 μM. The anticipated that the Sso7d domain would effectively recruit its ligase fusion associate to dsDNA. Morever, we also predicted that a high measure of conformational flexibility for the ligase moiety, relative to Sso7d, would be characteristic for effecting ligation. Therefore, the utilized a glycine-rich sequence (Gly-Thr-Ser-Gly-Gly-Gly-Ser-Gly-Gly-Gly) as the linker between the two fusion partners. This linker was else used in all subsequent fusion proteins expecting noted otherwise [91].

Sso7d has an unfolding temperature overhead 90°C and it is highly resistant to chemical denaturation. The anticipated which may be problematic because most ligation protocols contain thermal inactivation and chemical denaturation of the ligase to dissociate it from its ligated product and to prohibit it from inhibiting downstream steps such as bacterial transformation. Therefore, the examine alternative fusion partners for T4 DNA ligase. The p50 segment of the human transcription factor NF-κB associates strongly with dsDNA *in vitro* and it able tolerate the presence of fusion partners at each terminus. A detailed biophysical analysis showed that p50 binds a 10-bp palindromic consensus

series as a dimer with extremely high affinity (K_D = 8 pM), but that it also binds non-specific dsDNA strongly (K_D = 5.7 nM). The same study showed that a monomeric murine homolog of p50, nuclear agent of activated T-cells (NFAT), bound dsDNA lower tightly and with lower sequence specificity (K_D = 40 nM and K_D = 11 nM, for non-specific DNA and the 5 bp NF-κB half-site, respectively). The authors else constructed a chimera including the DNA-binding domain of NFAT and the dimerization domain of p50, termed this chimera NFAT-Ala-p50, but for brevity that abridgement it 'cTF' (for 'chimeric transcription factor'). cTF bound DNA obsessivly as a monomer and showed utmost no preference for the p50 palindome (K_D = 28 nM) over non-specific DNA (K_D = 40 nM). The various DNA binding process, affinities and specificities of p50, NFAT and cTF made it likely that would alter the performance of T4 DNA ligase, although in ways that were difficult to predict *a priori*. Hence, organized the six corresponding fusion proteins (p50-ligase, ligase-p50, NFAT-ligase, ligase-NFAT, cTF-ligase and ligase-cTF) [89].

The identified additional fusion partner nomine from pathways of double-strand break repair in bacteria. One such nomine was the PprA protein from the radiation-resistant bacterium, *Deinococcus radiodurans*. Absence of PprA sensitizes *D.radiodurans* to DNA deterioration and the protein appears to localize to broken DNA ends *in vitro*. It has else been reported that free PprA can induce the activities of the T4 and *E.coli* DNA ligases in *trans*. Similarly, the *Mycobacterium tuberculosis* Ku protein connects preferentially to dsDNA ends. Ku recruits the ATP-dependent *M.tuberculosis* DNA ligase and induces

double-strand break repair. The constructed the PprA-ligase, ligase-PprA, Ku-ligase and ligase-Ku fusion proteins.

Finally, a large numeral of sequence non-specific DNA-binding proteins include helix-hairpin-helix (HhH) motifs, utmost often in the context of larger $(HhH)_2$ domains. Latterly, it was shown which the activity of the φ29 DNA polymerase could be improved by fusing it to two $(HhH)_2$ domains of the *Methanopyrus kandleri* topoisomerase V enzyme. Thus, the constructed the $[(HhH)_2]_2$-ligase and ligase-$[(HhH)_2]_2$ fusion proteins.

In total, the expressed and purified 14 His_6-tagged proteins involving T4 DNA ligase itself, for our premier activity assays. IMAC was used to recover each protein from the soluble cell lysate after IPTG-stimulated over-expression. Proteins were purified to >95% homogeneity (as judged by SDS-PAGE) and yields were in most case 10–20 mg per litre of bacterial culture. The exceptions were ligase-NFAT, Ku-ligase, ligase-Ku and cTF-ligase, the yields of that were 1–5 mg l^{-1} of culture. All four of these proteins were prone to aggregate through storage at 4°C with precipitation being most rapid (<1 h) in the case of cTF-ligase. Thus, these proteins were stored at −80°C and thawed instantly prior to their use [92].

2-4 Activity characterize identify improved DNA ligases

Assay design, the designed and implemented agarose gel-based assays for cohesive and blunt ended segment joining, in which two substrate molecules could be ligated, but further concatemerization was not potential. This simplified downstream data analysis because there were single bands corresponding to the substrate and the ligated output in each assay. For cohesive-ended segment joining, the utilized PCR to amplify ompC from the ASKA library of open reading frames. This open reading frame was chosen solely due to it contained an SpeI restriction site, positioned such that the PCR out put could be cut into two utmost identically-sized fragments 638 and 639 bp that co-migrated on agarose gels due to the PCR primers were not phosphorylated, ligase-catalyzed re-joining of the PCR out put could only happen at the cohesive ends that had been created via SpeI digestion. The substrate for blunt-ended segment joining assays was a SmaI/SfiI restriction fragment. SfiI cleaves a non-palindromic sequence to leave a 3-base overhang that is not a substrate for cohesive end joining. Hence, ligation of this substrate could only occur at the blunt ends generated via SmaI digestion. The conditions in each assay were designed to convergent those used in most molecular biology protocols, the concentration of the dsDNA substrate was comparatively low (15 ng μl^{-1}) and the concentration of the DNA ligase was comparatively high (2 μM) [93].

2-5 Cohesive-ended fragment ligation

The results of the screen for cohesive-ended ligation activity are shown in Fig (4). Under the status of the assay, T4 DNA ligase was impact at ligating most of the substrate (lanes 14 and 19 of Fig. 4). The ligase fusion proteins showed a range of activities, from almost undetectably low (Ku-ligase and ligase-Ku) to catalyzing the near-complete conversion of substrate to out put (Sso7d-ligase, ligase-cTF and p50-ligase). The poor performance of the Ku fusion proteins is proportionate with the observation that Ku prohibits T4 DNA ligase in trans, presumably by hindering the access of the ligase to the DNA ends that are to be connected. Unsurprisingly, the other aggregation-prone ligases (cTF-ligase and ligase-NFAT) also disapled weak activity. On the other hand, the alternate fusion proteins (ligase-cTF and NFAT-ligase) both possessed major activity than T4 DNA ligase [90].

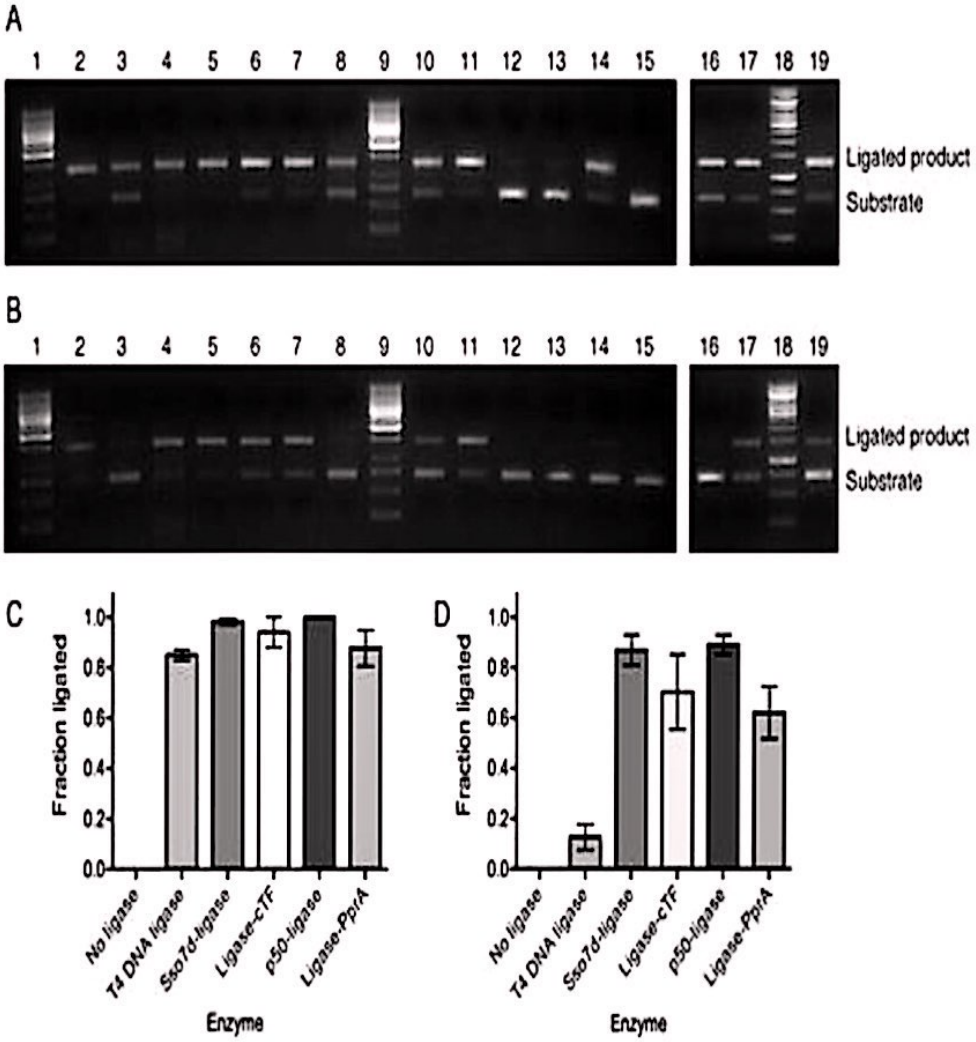


Figure (4): Gel-based screens for ligase activity: (**A**) Ligation of cohesive-ended PCR outputs (638/639 bp; lower band) to yield output of 1277 bp (upper band). Lanes are numeral and loaded as follows: 1, molecular weightiness marker; 2, ligation catalyzed by Sso7d-ligase; 3, cTF-ligase; 4, ligase-cTF; 5, p50-ligase; 6, ligase-p50; 7, NFAT-ligase; 8, ligase-NFAT; 9, molecular weightiness marker; 10, PprA-ligase; 11, ligase-PprA; 12, Ku-ligase; 13, ligase-Ku; 14, T4 DNA ligase; 15, no ligase control; 16, $[(HhH)_2]_2$-ligase; 17, ligase-$[(HhH)_2]_2$; 18, molecular weightiness marker; 19, T4 DNA ligase. (**B**) Ligase-catalyzed binding of a blunt-ended 717-bp restriction fragment (lower band) to give a 1434-bp output (upper band). Lanes are numeral and loaded as described in (A). (**C**) Fraction of cohesive-ended substrate which was ligated to product, for selected ligases from (A) and (B). Datum

are the means (±SEM) of two independent experiments conducted beneath identical status to the assay in (A). **(D)** Fraction of blunt-ended substrate that was ligated to output for selected ligases from (A) and (B). Data are the means (±SEM) of two independent experiments managed under identical status to the assay in (B) [91].

2-6 Blunt-ended segment ligation

As with the cohesive-ended segments, the ligases showed marked difference in their abilities to join blunt-ended DNA (Fig. 4B). On average, T4 DNA ligase only ligated a minority of the substrate molecules over the path of the 20 min assay. Eight of the 13 engineered ligases inclusive at least one of the fusions to each DNA-binding protein apart from Ku, out-performed T4 DNA ligase in this screen. The best were Sso7d-ligase and p50-ligase (lanes 2 and 5 in Fig. 4B), each of which converted almost all of the substrate to output. Specially, the optimal direction for each fusion varied. For example, p50-ligase was most active than ligase-p50 (compare lanes 5 and 6 in Fig. 4B), while ligase-PprA out-performed PprA-ligase (lanes 10 and 11). The implications are that T4 DNA ligase is tolerant of fusions at every of its termini and that the optimal direction of the fusion partner, relative to the ligase, requirments to be determined empirically [92].

2-7 Densitometry

The agarose gel-based screens (Fig. 4 A and 4 B) proposition that the most active ligase variants were Sso7d-ligase, ligase-cTF, p50-ligase and ligase-PprA. Fresh batches of these enzymes were reexamined under identical status and densitometry was used to quantify their improvements over T4 DNA ligase. Improvements in cohesive end joining were small due to T4 DNA ligase was active enough to ligate ~85% of the substrate in the screening (Fig. 4 C). In contrast, T4 DNA ligase only converted ~13% of the blunt-ended substrate to product (Fig. 4 D). Sso7d-ligase and p50-ligase reproducibly ligated ~90% of this substrate, congruous to improvements of almost 7-fold in blunt-ended ligation. These refinements over T4 DNA ligase were highly important in two-tailed unpaired *t*-tests ($P = 0.01$ for Sso7d-ligase; $P < 0.01$ for p50-ligase). On average, ligase-cTF and ligase-PprA every ligated ~5-fold most of the blunt-ended substrate than T4 DNA ligase, morever, the results for these fusion proteins were most

variable. This variability was reverberated in the decreased statistical support for their elaboration over T4 DNA ligase ($P = 0.07$ for ligase-cTF; $P = 0.05$ for ligase-PprA) [93].

2-8 Blunt end cloning screenings

In utmost laboratories, the primary use for T4 DNA ligase reside in the construction of recombinant plasmids. The utilized T4 DNA ligase and the four better fusion proteins to ligate an qualitative, blunt-ended dsDNA fragment into a standard cloning vector (pUC18) that had been linearized by assimilation with SmaI. Blue-white screening enabled the enumeration of succeeded cloning events (Fig. 5). On average in independent triplicates, ligase-cTF gave the greatest numeral of white colonies (mean = 1234 colonies). This was an elaboration of ~160% over T4 DNA ligase which gave an average of 748 colonies per replicate screening. All three of the ligase-cTF colony counts were higher than any of the three T4 DNA ligase counts, though the assay-to-assay variation in the T4 DNA ligase datum intended that the adverse between the means was only moderately important in a two-tailed unpaired *t* test ($P = 0.10$). Sso7d-ligase also yielded highly in constant colony counts, resulting in no statistically significant elaboration over T4 DNA ligase ($P = 0.31$). This variability is similarly to have arisen because the maximum stability of Sso7d that makes it difficult to remove from the ligated reaction products. During screening development, Sso7d-ligase appeared to perform 3- to 10-fold worst than T4 DNA ligase. Incorporating a Proteinase K step into the reaction clean-up, in an potential to disrupt the protein-DNA interaction, recovered the colony counts to the levels described here. Even with the Proteinase K treatment,

however, cloning with ligase-PprA gave an average of 55% as several colonies as T4 DNA ligase, although this adverse also lacked statistical support (P = 0.21) [90].

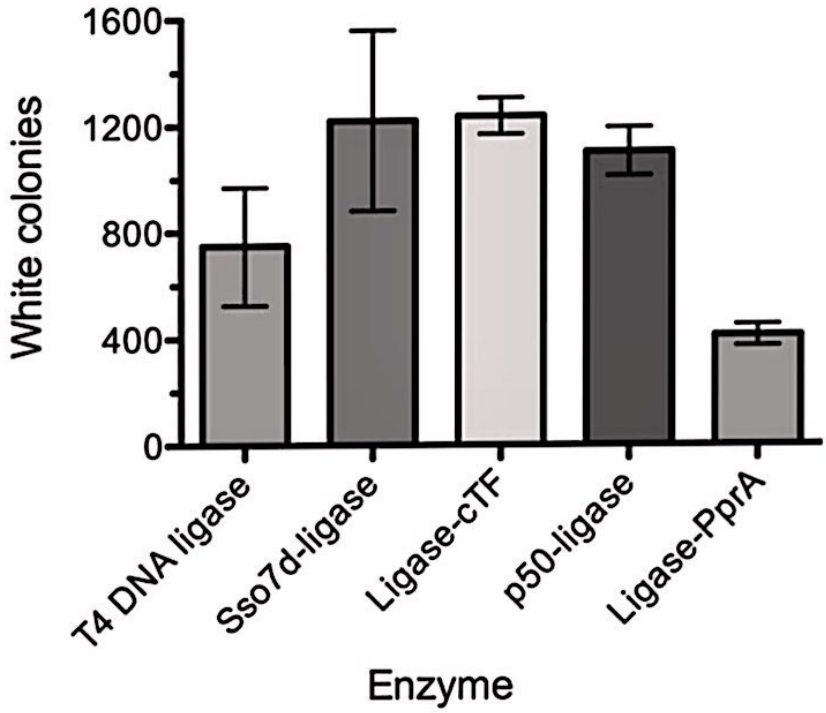


Figure (5): Cloning screening, a 739-bp, blunt-ended introduce was cloned into pUC18 and the ligation outputs were utilized to transform *E.coli* DH5α-E. Colony counts are the means (±SEM) of independent triplicates [90].

2-9 Engineered ligases in NGS library elaboration

There is a growing send for for DNA ligases in NGS library construction. For Illumina sequencing, dsDNA samples are ready via random fragmentation, end repair and then the increment of a single nucleotide dA overhang at the 3′ end of every strand. A DNA ligase is required that able effectively join these substrates

with adaptors that carry single nucleotide 3′ dT overhangs. The resembled the activities of T4 DNA ligase, ligase-cTF and p50-ligase in this scenario. Every of the ligases was utilized to ligate three diverse sets of bar-coded adapters to randomly fragmented *E.coli* genomic DNA. The samples were combine after the ligation step, and then sequenced, so that the numeral of reads with each bar code represented the relative efficiency of the DNA ligase that was utilized with that adaptor. The Illumina sequencing run gave a total of 3.10×10^7 bar-coded reads. The utmost efficient ligase was p50-ligase that participated 49.1% of the total reads to this pool (mean ± SEM for the three p50-ligase bar codes was $(5.1 \pm 0.4) \times 10^6$ reads). This was an elaboration of ~160% over T4 DNA ligase (Fig. 6) and this divergence was highly significant in a two-tailed unpaired *t*-test (P= 0.01). In contrast, ligase-cTF was comparatively inoperative in this test (Fig. 6) [91].

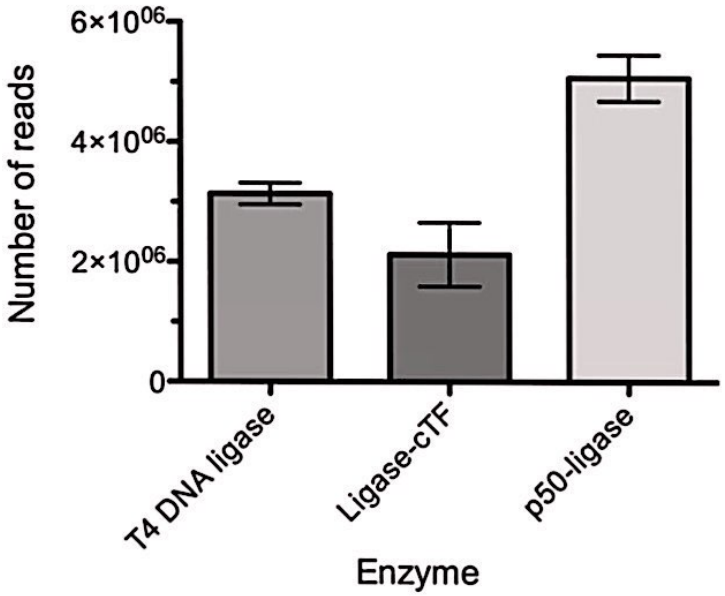


Figure (6): Ligase competence at adaptor ligation for subsequent Illumina sequencing. Each enzyme was utilized to ligate three bar-coded adaptors onto dA-tailed dsDNA fragments. For each ligase, the mean numeral of reads per barcode (±SEM) is plotted [91].

It has been shown which fusing dsDNA-binding proteins to DNA polymerases able ameliorate their performances *in vitro*. In this work, the demonstrated that the activities of DNA ligases can be influenced via taking a similar method. Our primary goal was to improve the activity of T4 DNA ligase, in scenarios that are pertinent for molecular biologists. The assumed that the unfavourable kinetic parameters of T4 DNA ligase could be overcome by artificially raising its overall affinity for dsDNA. At the outset, morever, it was unclear how DNA binding affinity would commerce off with other factors that might impact ligation efficiency such as the sequence specificity of the protein-DNA interaction, the prospect for the fusion partner to block access of the ligase to its substrates e.g. to the DNA ends, if overly stable protein-DNA interactions might inhibit downstream steps in generic protocols. The protein design problem was also rendered more difficult due to the three-dimensional structure of T4 DNA ligase has not yet been specified [92].

With these foresight in mind, the undertook a broad and empirical search for suitable DNA-binding fusion partners. The particular seven candidates, from all three domains of life. The initial activity screen showed that the preponderance of these fusion partners could increase the activity of T4 DNA ligase, mostly in joining blunt-ended dsDNA fragments. The most committed fusion proteins from the screen, in rank order according to the data include p50-ligase, Sso7d-

ligase, ligase-cTF and ligase-PprA. The composition of this set emphasized the value of our somewhat heuristic engineering strategy, it involved chimeras with each possible direction of ligase and DNA-binding protein and the DNA-binding fusion partners were a eukaryotic transcription agent (p50), an archaeal protein (Sso7d), a bacterial DNA repair protein (PprA) and an artificial protein which was oneself a chimera (cTF) [94].

Further testing appeared that different ligases were best for different applications. The two best fusion proteins in the gel-based screen (p50-ligase and Sso7d-ligase) were out-performed by ligase-cTF in the blunt end cloning screening (Fig. 2). Moreover, the 5- to 7-fold improvements over T4 DNA ligase in binding blunt-ended fragments were reduced to <2-fold ameliorate in blunt end cloning. Fragment joining comprises a single intermolecular ligation event while 'vector + insert' cloning demands an intermolecular ligation step, followed via an intramolecular ligation step, to circularize the insert-containing plasmid. The datum suggest that the ligase fusion proteins can be better, proportionately at the intermolecular phase [95].

The ligase-cTF was the preferable ligase for cloning blunt-ended segments, it was less efficient at adaptor ligation for NGS library elaboration. In contrast, p50-ligase significantly out-performed T4 DNA ligase at converter ligation via approximately 160%. Overall, these two ligases with eukaryotic DNA-binding partners appeared as the utmost promising for further development with additional evidence of their utility coming from the raised activity of a linker variant (ligase-NLS-cTF) and the ability of p50 to induce the activity of the *E.coli* DNA ligase. On the another hand, Sso7d-ligase was difficult to

dissociate from its ligated output and this reduced its usefulness. Similarly, none of the chimeras that involved the bacterial DNA repair proteins (PprA and Ku) systematically out-performed T4 DNA ligase, though ligase-PprA showed some promise. The placement of PprA and Ku at the ends of DNA duplexes appears to provide a steric block that interacts with the capability of the fused ligase to catalyze end joining [96].

The demonstrated of the interest of our ligases in mutul molecular biology protocols. However, more detailed kinetic characterization of the end-joining reactions that they catalyze will be required to understand the underlying mechanisms of their ameliorate performance. To datum, an analysis of this type has only been reported for the nick-sealing activity of T4 DNA ligase and not the end-joining activities. The authors obtained burst phase kinetics for the nick-sealing reaction, consistent with output release or a post-ligation conformational alter being the rate limiting step. It is different that the same post-ligation phase will be rate limiting for the much slower end-joining reactions. Therewith, assessing the mechanistic affects of the fusion partners will be valuable for informing future designs [97].

There is raising interest in the utilize and optimization of application-specific DNA polymerases, particularly for active and unbiased amplification of NGS libraries. Novel DNA polymerases have been engineered for a numeral of specific applications and many companies now offer inclusive portfolios of highly specialized polymerase products, have display that our protein design planning is a facile one for engineering bespoke, application certain DNA ligases [98].

Conclusions

1-The process of molecular chlorination in genetic manipulation to produce new organisms bearing new characteristics is very important in the production of medicines that are less productive compared to the original organisms.

2-The molecular chlorination process has proven to be successful in producing many enzymes used in the treatment of many difficult diseases such as SCID, Gaucher's disease, Parkinson's disease and PKU.

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American Award For the invention of 2020 by the American GOIDI the World Investment Commission in America.

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