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Formulation of an Efficient Combinatorial Cellulase Cocktail by Comparative Analysis of Gibson Assembly and NEBuilder HiFi DNA Assembly Modus Operandi

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ABSTRACT: Keeping in view the importance of combinatorial cellulase genes expression for bioethanol production, the current study was aimed to identify appropriate DNA fragments assembly approach. Home-made Gibson Assembly Master Mix and NEBuilder HiFi DNA Assembly Master Mix were utilized for codon optimized synthetic cellulase genes constituted of endoglucanase (*Trichoderma harzianum*), cellobiohydrolase (*Penicillium oxalicum*) and β-glucosidase (*Aspergillus niger*). These assembly methods were modified by using restriction enzyme (*HindIII/BamHI*) ligation method for cloning of assembled DNA cassettes. The evaluation results of potential and efficacy of these assembly techniques indicated that both the techniques are favorable for scarless assembly of multiple fragments simultaneously. However, the NEBuilder HiFi DNA Assembly Master Mix exhibited appreciable results of transformation, colony screening and quantification of cloning efficiency that may be utilized in bioethanol production for a consolidated bioprocessing (CBP) host confronting the challenge of multiple genes expression with the loss of gene fragments from microbial cell. In addition, this molecular cloning and assembly technique will be a valuable tool for protein engineering and synthetic biology that extends the range of applications of DNA assembly strategies.

Keywords: Gibson Assembly, Cellulase, Endoglucanase, Cellobiohydrolase, β-glucosidase, Bioethanol.

Abbreviations: DNA, Deoxyribonucleic acid; CBP, consolidated bioprocessing; IPTG, Isopropyl β-D-1-thiogalactopyranoside; OE-PCR, overlap extension polymerase chain reaction; X- Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

I. INTRODUCTION

Gene synthesis enables creation and modification of genetic sequences at an unprecedented pace, offering enormous potential for new biological functionality [1]. Recent advances in DNA manipulation techniques and synthetic biology are producing significantly successful outcomes in the field of life sciences, including the metabolic engineering for drug production, minimal bacterial cells construction, and the synthetic assembly of eukaryotic chromosomes [2].

Presently, numerous synthetic methods are available for successful DNA construct assembly of larger size. However, these methods have certain shortcomings, for instance: presence of restriction sites (scars) within the assembled sequences or multiple-step strategy for large number of DNA fragment assembly [3].

Multiple gene construct assembly and expression mechanism is critical to rapid prototyping of the metabolic pathways and desired genetic circuits. The traditional restriction digestion and ligation methods for instance Golden Gate method [4] and BioBrick™ [5] that allow the sequential assembly of standard biological parts but may not support larger DNA constructs or make the selection of unique restriction sites extremely difficult which cripples the modularity of DNA assembly [6].

There are numerous protocols designed for combinatorial DNA fragments including overlap extension polymerase chain reaction (OE-PCR) that enables scarless assembly of DNA parts [7]; sequence and ligation-independent cloning (SLIC) to generate recombination intermediates with endogenous DNA repair machinery [8]; DNA assembler, that enables design and rapid construction of large biochemical pathways in one-step fashion by exploitation of in vivo homologous recombination mechanism [9]; Gibson Assembly [10]; serine integrasere combinational assembly (SIRA) which involves recombination machinery of C31 integrase from phage [11]; ligase cycling reaction (LCR), a sequence homology-based method [12]; promoter-based gene assembly and simultaneous over expression (PGASO) technique [13], and NEBuilder HiFi DNA assembly method [14]. The researchers round the globe are grabbing the fruitful results of improved efficiency, fidelity, and modularity

with continuous modification in these DNA assembly protocols which have simplified both design and bench-side operation.

Gibson Assembly and NEBuilder HiFi DNA assembly master mix as suitable alternatives to iteratively concatenate, amplify multiple oligonucleotides in order to generate large sequencing reads. Both of these assembly techniques are isothermal and single-reaction methods for assembly of multiple DNA sequences. These master mix contain a set of three enzymes: (i) T5 Exonuclease which creates single-strand DNA 3' overhangs by chewing back from the DNA 5' end. Complementary DNA fragments can subsequently anneal to each other; (ii) Phusion DNA Polymerase which incorporates nucleotides to "fill in" the gaps in the annealed DNA fragments; and (iii) Taq DNA Ligase - covalently joins the annealed complementary DNA fragments, removing any nicks and creating a contiguous DNA fragment [15].

This study was performed in perspective of recent challenges faced by bioethanol industry, combating gradual depletion of fossil fuels. High cost of commercial cellulase enzymes has been hindering the efficacy of biofuel production and raised demand for genetically manipulated cellulase enzymes producing micro-organisms for lignocellulolysis [16]. Consolidated bioprocessing (CBP) is taken into account an economical substitute [17], but an efficient host for it is still enigma. For this purpose, a supportive synthetic biology technique that can transform multiple genes into a genome of a CBP host is mandatory [18]. Therefore, in this study two most applied and demanding DNA construct assembly methods (i) Gibson assembly (ii) NEBuilder HiFi DNA assembly were compared by assembling three cellulase genes consisting of CMCase (*Trichoderma harzianum*, 687 bp), cellobiohydrolase (*Penicillium oxalicum*, 1374 bp) and β-glucosidase (*Aspergillus niger*, 1464 bp) in perspective of downstream applications of expression in prokaryotes (*E.coli*) and eukaryotes (*Saccharomyces cerevisiae)* for bioethanol production.

II. MATERIALS AND METHODS

Reagents and Chemicals: *E. coli* DH5 α competent cells were prepared at Fermentation Technology Research Center (FTRC), Kasetsart University, Bangkok, Thailand by following the protocol of Mandel and Higa [19]. Overnight grown, 50 ml cultures of *E. coli* DH5α cells were pelleted at high speed centrifuge and resuspended with gentle pipetting in 25 ml of ice-cold 0.1 M $CaCl_2$ (sterilized at 121℃ for 15 min) and incubated on ice for 6 h. The suspension was re-pelleted by centrifugation at high speed for 10 min with subsequent resuspension in 4 ml of 0.1 M $CaCl_2$+15% (v/v) glycerol (sterilized at 121℃ for 15 min) and stored in 100 µl aliquots at –80℃ [20].

NEBuilder HiFi DNA assembly master mix was purchased from New England BioLabs (NEB, UK) while for Home-Made Gibson assembly master mix, the molecular grade chemicals were obtained from Thermo Fisher Scientific, USA. pUC18 plasmid (Thermo Scientific, SD0051) was used for cloning. For assembly, pUC18 was linearized with *HindIII* (Thermo Scientific, ER0501)/*BamHI* (Thermo Scientific, ER0051) restriction enzymes. The stitched cellulase gene cassette was ligated in pUC18 (named pUC-PMP) at *HindIII*/*BamHI* restriction sites. T5 Exonuclease (NEB, M0363S) was used to create single-stranded 3´ overhangs that facilitated the annealing of fragments and shared complementarity at one end (the overlap region); gaps between each annealed fragment were filled with Phusion® High-Fidelity DNA Polymerase (NEB, M0530S) and the T4 DNA Ligase (NEB, M0202) was used to seal the nicks in assembled DNA.

Apparatus: The quality and quantification of gene fragments and plasmids were determined by NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific, USA). Spectrophotometer Schimadzu was used for OD600 measurement to analyze consistent cell growth results. T-Gradient thermal cycler (Biometra, Germany) was used for amplification of colonies and gene fragments. Quantification of gene copy numbers was performed on Light Cycle 480 (Roche, USA). The nucleic acid bands on gel, after gel electrophoresis were viewed through Gel Documentation and Imaging System (Bio-Rad, Germany).

Primer Design and Overlapping DNA Fragments: According to New England BioLabs (NEB, UK), the rules for designing primers are similar for both Gibson assembly and NEBuilder HiFi DNA assembly. Any primer designed for Gibson assembly can be used with NEBuilder HiFi. Nevertheless, some primers that work with NEBuilder HiFi may not work with Gibson assembly method. Therefore, NEBuilder assembly tool was used for designing primers (Table 1) for both types of assembly methods with specific features.

Table 1: Primers designed for NEBuilder HiFi DNA assembly master mix and Gibson assembly master mix.

Gene	Primer
NEBuilder HiFi DNA Assembly Master Mix	
EG_F	tatcacgaggccctttcgtcATGAAAGCTACATTGGTTTTAG
EG_R	caacaatcatTGGTAAACATTGAGAGTAC
CBH_F	atgtttaccaATGATTGTTGGTATTTTGACTAC
CBH_R	ctgaacccatCAAAAATGATGGATTAGCG
BGL_F	atcatttttgATGGGTTCAGCTACTGCATC
BGL_R	gtcatcaccgaaacgcgcga TTATTTCTTTTCAATGTATTGAGAGAAAATTTG
Gibson Assembly Master Mix	
EG_F	cgacggccagtgccaATGAAAGCTACATTGGTTTTAG
EG_R	acaatcatTGGTAAACATTGAGAGTAC
CBH_F	caatgtttaccaATGATTGTTGGTATTTTGACTAC
CBH_R	gaacccatCAAAAATGATGGATTAGCG
BGL_F	catcatttttgATGGGTTCAGCTACTGCATC
BGL_R	agctcggtacccgggTTATTTCTTTTCAATGTATTGAGAGAAAATTTG

The PCR mixture (Thermo Scientific, USA) contained 100 ng DNA template, 5µl PCR buffer (10X), 0.5 µl dNTPs (50 mM), 1 µM of each primer, 1 µl Taq Polymerase (1 unit/µl) and deionized water to make a final volume up to 50µl. PCR reactions were carried out under the following conditions: the initial denaturation of 30s at 98℃, followed by 35 cycles of 10s at 98℃, 30s at 62℃, 40s per kb at 72℃, followed by a final elongation of 5min at 72℃. All amplified fragments purified using the GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo scientific, K0832).

Gibson Assembly Based In Vitro Recombination: The three clean-up PCR fragments (EG, CBH and BGL) were assembled according to the one-step isothermal DNA assembly method described by Gibson [10]. In a tube on ice, a 20-ml reaction consisting of 5 ml DNA (0.025µL of three gene fragments) and 15 ml Gibson assembly master mixture was prepared. This homemade master mixture was prepared by combining 320 ml of 5X reaction buffer [3 ml of Tris–HCl (1 M, pH 7.5), 150 ml of $MgCl_2$ (2 M), 60 ml of dGTP (100 mM), 60 ml of dATP (100 mM), 60 ml of dTTP (100 mM), 60 ml of dCTP (100 mM), 300 ml of DTT (1 M), 1.5 g PEG-8000, and 300 ml of NAD (100 mM)], 0.64 ml of 10 U/ml T5 exonuclease, 20 ml of 2 U/ml Phusion High-Fidelity DNA Polymerase, 160 ml of 40 U/ml T4 DNA Ligase, and water up to a final volume of 1.2 ml. The mixture was incubated at 50℃ for 1 hour in a thermal cycler (Biometra, Germany). In order to control DNA molecules assembly, a 0.8% agarose gel electrophoresis was performed in 1X TE buffer, and gel was stained with ethidium bromide (5 µg/mL) for visualization of DNA.

NEBuilder HiFi DNA Assembly Based In Vitro Recombination: Like for Gibson assembly, the three clean-up PCR fragments (EG, CBH and BGL) were assembled according to the protocol by NEBuilder HiFi DNA assembly master mix (NEB, England): 0.05 pmol of each DNA fragments were pooled in 10µl NEBuilder HiFi DNA Assembly Master Mix. The total reaction volume was set to 20µl with deionized H_2O. The reaction mixture was incubated in a thermal cycler at 50℃ for 60 minutes and stored at –20℃ for downstream applications.

Transformation, Blue-White Screening and Selection of Positive Clones

E. coli DH5 α competent cell heat-shock transformation was carried out according to standard protocol of Sambrook and Russell [21]. The competent cells were thawed on ice and 50 µL aliquots were transferred to prechilled Eppendorf tubes. 100 ng DNA was mixed with 50 µL of competent cells in a microcentrifuge tube. The bottom of the tubes were flicked with finger and incubated on ice for 30 min. They were then heat pulsed in a 42℃ water bath for 30s followed by incubation on ice for 2 min. Subsequent to this 500 µL of preheated (42℃) LB growth medium was added to the above and the samples were incubated at 37℃ for 1 h with shaking at 250RPM. The transformation was plated onto LB + ampicillin agar plates and incubated at 37℃ overnight.

For Blue-White screening, in 100mL LB agar media, 100 µL IPTG (100mM), 100 µL X-Gal (20mg/mL) and 100 µL ampicillin (100mg/mL) were added and poured in disposable petri dishes for screening of transformants at 37℃ overnight [22]. Recombinant colonies were selected for downstream applications.

Colony PCR, a Quick Way of DNA Manipulation Analysis: The transformants were identified by colony PCR. For 2µL (100 ng) DNA template, PCR Mix (50 µL reaction) comprised 5 µL Taq Polymerase Buffer ($MgCl_2$ included), 0.5 µL dNTP, 1 µL forward primer (CCC AAG CTT ATG CACACCTGTCGTTGTTGTCC), 1 µL reverse primer (CGC GGA TCC TTA CCTTGACGTTGAGGAAGCTC), 39.5 µL molecular water, and 1 µL Taq Polymerase. PCR conditions for temperature, time and cycles were following: 95℃ for 15 min, followed by 35 cycles of 30s at 94℃, 1 min at 62℃, and 1 min at 72℃ with final elongation for 10 min at 72℃. The PCR product was analyzed through gel electrophoresis using 1% agarose gel with 1 Kb Plus DNA Ladder (Thermo Fisher Scientific, 10787026) for DNA fragment size determination.

Plasmid Extraction and Restriction Enzyme Digestion: Plasmid extraction was carried out by GeneJet Plasmid Minipreparation kit (Thermo Fisher Scientific, USA). About 300ng plasmid was digested with *HindIII/BamHI* in a water-bath at 37℃ for 40 minutes (Manufacturer's protocol followed). The digested product was separated by 1% agarose gel electrophoresis and purified with GeneJet Gel Purification Kit (Thermo Fisher Scientific, K0691).

DNA Fragments Assembly Efficiency Measurements: The assembly efficiency was determined by two ways: (i) percentage of white colonies and (ii) percentage of Colony PCR containing the desired construct. In Blue-White Screening, white colonies indicate successful assembly with the insert; blue colonies indicate the absence of insert and vector assembly. 06 white colonies were randomly picked for PCR and run DNA gel to check the insertion fragment. The efficiency was calculated as the number of colonies containing the desired construct divided by the total number of colonies. The following formulae were used for calculating the cloning efficiency:

Cloning efficiency (CE, %)

$$= \frac{\text{No. of while colonies}}{\text{Total colonies}} \times 100 \qquad (1)$$

$$\frac{\text{No. of colonies with desired construct}}{\text{Total colonies}} \times 100 \qquad (2)$$

III. RESULTS AND DISCUSSION

In Vitro Recombination: The three cellulase genes i.e. CMCase (*Trichoderma harzianum*), cellobiohydrolase (*Penicillium oxalicum*) and β-glucosidase (*Aspergillus niger*) were assembled by using Homemade Gibson assembly and NEBuilder HiFi DNA assembly master mix and ligated in pUC18 plasmid for cloning at *HindIII/BamHI* restriction sites.

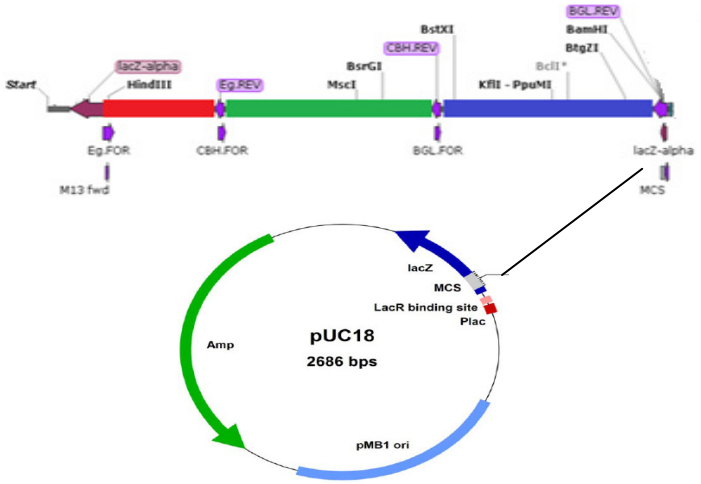

Fig. 1. Gene fragments assembly and ligation in pUC18 at *HindIII/BamHI* restriction sites.

Transformation, Blue-White Screening and Selection of Positive Clones: A total of 10 LB-agar/ampicillin (10g/L bacto peptone, 5g/L yeast extract, 10g NaCl, replicate plates of each kind of Master Mix were used to count the number of transformed colonies. Table 2 indicate that NEBuilder HiFi DNA Assembly Master Mix was comparatively more efficient producing 274 white colonies.

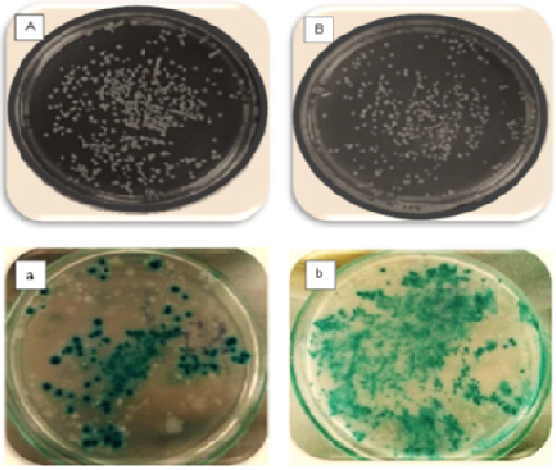

Fig. 2. (A) Antibiotic selection and (a) Blue-White screening of NEBuilder HiFi DNA Assembly; (b) Antibiotic selection and (b) Blue-White screening of Gibson Assembly.

Colony PCR for DNA Manipulation Analysis: The colony PCR for endoglucanase gene was performed for six randomly picked colonies of pUC-PMP plasmids assembled with each type of test assembly protocol. For every screened colony, replica plates were also produced on LB-Agar/Ampicillin petri plates. The gel documentation of each purified PCR product assembled with Gibson Assembly and NEBuiled DNA Assembly method exhibited bands that indicated the presence of each DNA fragment presence in all the colonies.

The bands were prominent and equal to the amplified gene size. Conversely, the gel documentation of colony PCR products that were stitched with Homemade Gibson Assembly protocol, despite expressing positive results for all the amplified colonies, exhibited the bands that were very weakly visible which may be attributed to very low expression of genes that depending on the DNA used; the incubation time required could differ and that optimization is required for each individual hairpin design. For enhanced assembly, it is suggested to test a longer incubation time of up to 60 min, depending on the size of the fragments being assembled. A longer incubation time could increase the amount of positive clones obtained but too long incubation might lead to unspecific or unwanted assembly reactions [23]. The reason for weak bands could also be related with lower DNA concentration [24].

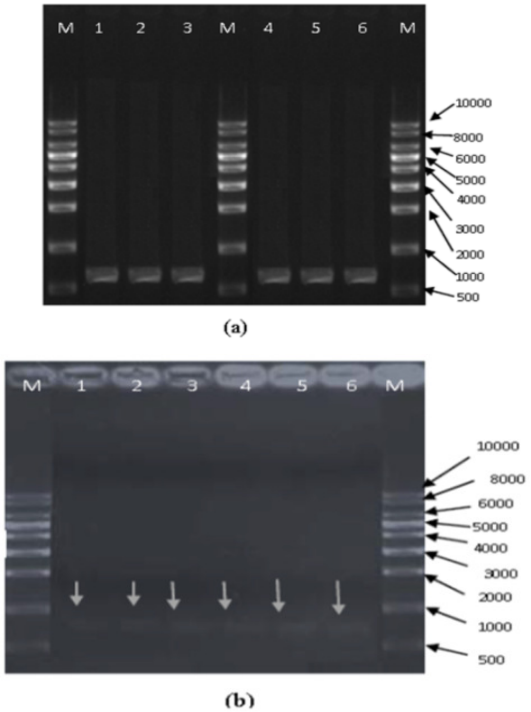

Fig. 3. Gel electrophoresis of the colony PCR for endoglucanase gene (EG). M: 1KB DNA marker, 1 to 6: endoglucanase gene (EG). (a) NEBuilder HiFi DNA assembly master mix; (b) Gibson Assembly master mix.

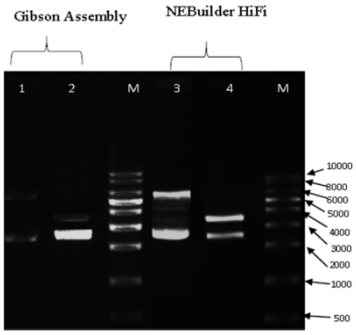

Fig. 4. Electrophoretic analysis of plasmid constructs.

Double Digestion: The results obtained with double digestion of both types of stitched constructs revealed that in double digestion, there were faint bands of constructs assembled with Gibson assembly method, whereas, the NEBuilder assembled fragments were visible as sharp and accurate bands which rejects the possibility of gel electrophoresis related issues like voltage and buffer because the NEBuilder assembled products could produce very sharp bands, hence it may be perceived that the issue of weak bands could be because of degraded gene fragments or low quality DNA (Thermo Fisher Scientific).

Hence, it may be perceived that the issue of weak bands could be because of degraded gene fragments or low quality DNA (Thermo Fisher Scientific).

The amplicons and restriction enzyme-digests of plasmid constructs were separated on a 0.8% agarose gel in 1X TAE. Lane M 1KB DNA ladder, lane 1 and Lane 3 exhibit uncut plasmid carrying the stitched constructs with Gibson assembly and NEBuilder HiFi assembly techniques, whereas, Lane 2 and 4 are *HindIII /BamHI* digests of the aforementioned techniques.

DNA Fragments Assembly Efficiency Measurements: The analytical methods such as transformation, colony PCR and DNA concentration are the vital points for the assessment of assembly efficacy of various gene fragments assembled through NEBuilder HiFi DNA assembly and Gibson assembly approaches. The assembly quality assessment results are depicted in Table 2.

Table 2: Assembly quality assessment with Blue-White Screening.

Assembled genes	Colony counts	Validation method	Confirmation
Assembled by using the Gibson Assembly Kit			
EG-CBH-BGL	310	Amp selection	Colonies with smaller size observed
		Blue White Screening	White Colonies: 116 Blue Colonies: 168
		Colony PCR	Faint bands with appropriate size
		Plasmid concentration 260/280	~2.0
Assembled by using NEBuilder HiFi Assembly Kit			
EG-CBH-BGL	375	Amp selection	Colonies with larger size observed
		Blue White Screening	White Colonies: 274 Blue Colonies: 89
		Colony PCR	Sharp bands with appropriate size
		Plasmid concentration 260/280	~2.0

IV. CONCLUSION

Bioethanol industry as ecofriendly, cost-effective and productive technology, is combating the challenges of demand and supply, food versus fuel crises (1stgeneration bioethanol), protocol efficacy, genetic makeup manipulation and synthetic assembly. On industrial scale, the recombinant cellulolytic enzyme producing ethanolgenic microbe (particularly, *Saccharomyces cerevisiae*) may carry multiple genes such as endoglucanase, cellobiohydrolase, β-glucosidase and xylanase. Though there are numerous available techniques for gene fragments assembly, nevertheless, the efficacy of these techniques vary from gene isolation techniques to specific species, expression vector, and the type of Open Reading Frame (ORF). Thereby, the two mostly used recently invented gene fragment assembly methods i.e. Gibson Assembly and NEBuider HiFi DNA Assembly, were comparatively analyzed with little modification in protocol i.e. assembling genes together and later ligating in plasmid. The three cellulase genes were assembled with Gibson assembly and NEBuilder HiFi DNA assembly reagents but the ligation in plasmid was done with restriction enzymes *HindIII/BamHI* which makes this study prominent from previous studies where one-pot assembly approach was followed for combing genes and plasmid together in one step but it may reduce the stability and expression of genes in a cassette. From previous studies, the NEBuilder HiFi Assembly is found to have perfectly assembled sequences up to 45 fragments [25] and high copy number [26], however between 30 to 45 fragments is observed to be the practical limit of Gibson Assembly and beyond that point the success rate dropped significantly and it no longer remains a feasible approach to attempt higher-number assemblies. In Gibson assembly, endonuclease activity could cleave the single-stranded 3' overhangs revealed by the exonuclease activity, resulting in undesired 3' single-stranded DNA (ssDNA) ends which could assemble improperly or not at all [25]. The NEBuilder HiFi DNA used for CRISPR-δ-integration and multiple promoter shuffling displayed efficient results than Gibson assembly [27]. Similarly, in the current study, all the results supported NEBuilder HiFi DNA Assembly technique for three cellulase genes stitching and cloning. It could generate more correct clones with greater accuracy.

V. FUTURE SCOPE

This study gives an insight for future genetic engineering synthetic tools for assembling seamless, scarless and stable stitching of multiple genes,

irrespective of size and numbers; particularly, in industrial biotechnology related to the integration of new sets of genes in host microbes for biofuel production. This research can also be used as a model study for comparing the other existing gene assembly techniques such as OE-PCR [7], SLIC [8], LCR [12] and PGASO [13] that may face DNA fragments loss or mutation.

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Conflict of Interest. The Authors declare that there is no conflict of interest. This study is based on a comparative analysis of two different methods of genes assembly.

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