A Simple Two-Stage PCR Based Method to Construct Gene Disruption Cassette

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ABSTRACT

Use of microorganisms in fabricating products of commercial importance has been practiced for several years. Genes in microorganism codes for selected metabolic pathway through which the end product is obtained. It cannot be assuned that all genes in an organism will be useful in serving the stated cause. In order to achieve the desired product, some genes coding the vital pathways have to be silenced. Several methods have been used for constructing gene disruption cassettes, but some prove ineffective because of the complexity in methodology and instability of the construct. Here we have described a two-stage PCR based method for the construction of gene disruption cassette with following advantages: (1) stability of the construct; (2) simple protocol; (3) cost effective and feasibility. Thus this protocol paves way for stable, easy and quick construction of disruption cassettes.

Keywords — Candida Tropicalis, Disruption Cassette, Gene Silencing, PCR, Restriction Enzymes

1. INTRODUCTION

Since the birth of industrial microbiology, fermentation process has been carried out to obtain metabolites of interest [1]. In generic fermentation process, selected microorganisms were used for specific purpose [2]. But this scenario has been changed by modern day science with many advanced techniques and vast exposure in research. With the help of genetic engineering, genes in microorganisms can be manipulated to give a specific end product. This gene control can be at performed at any stage starting with gene expression till the gene knock out. Several methods have been carried out for gene silencing i.e. selective gene inhibitors [3], multi stage PCR techniques etc which incur higher cost, complex protocols and extensive material requirement.

In the current research work we have developed a two-stage PCR based method for the construction of gene disruption cassette. To experiment the protocol, cassette for disrupting POX4 gene (a gene coding for acyl Co-A oxidase, primary enzyme for beta oxidation pathway) in *Candida tropicalis* (yeast) has been constructed. Nullifying the primary pathway will route the metabolism via the secondary pathway i.e. omega oxidation. It has been reported that 95% of the metabolism in this yeast occurs though beta oxidation and only 5% is through omega oxidation [4]. Beta oxidation blocked *Candida tropicalis* is widely used for the production of Di-Carboxylic Acids (DCA) [5] which has its application in paint, perfume, adhesives and a monomer for polymer production [6-7]. Thus the objective of current research is to create a simple protocol for gene silencing which can yield a stable construct ensuring the manipulation of genetics in microorganisms.

2. MATERIALS AND METHODS

2.1 Culture maintenance and growth conditions

Candida tropicalis (MTCC 230) was grown on YPD agar plates (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, pH 7.2 and 15 g/L agar) for 48 h at 32ºC. For isolation of genomic DNA from liquid culture *C. tropicalis*

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cells were grown for 48 h at 120 rpm in YPD medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, pH 7.2). 5 mL culture was used for genomic DNA isolation.

2.2 Genomic DNA isolation and quantification

Yeast genomic DNA was isolated using standard large scale DNA isolation protocol [8]. Purity and yield of gDNA were assessed spectrophotometrically (Nanophotometer, Implan) by calculating A_{260}/A_{280}, A_{260}/A_{230} ratios and A_{260} values to determine the protein impurities and DNA concentration.

2.3. PCR and cloning

Restriction enzyme sites included POX4 gene specific primers were designed using Primer3 software (Table 1). Two sets of primers were used which corresponds to the amplification of front franking and rear flanking region respectively. Polymerase chain reaction (Veriti, Life Technologies) was carried out in two stages.

- Stage 1: Set 1 primers were used to amplify the front flanking region of the gene
- Stage 2: Set 2 primers were used to amplify the rear flanking region of the gene

To test whether the amplified products are specific, restriction enzyme analysis was performed using AciI and Tsp509I enzymes.

Table-1: PCR primers for the amplification of POX4 gene products

Region	Primer sequence	Size
Front Flanking Primers (Set – 1)	**F -** 5'**ATCGAT**GCCCAACAAATGGAAAGAGA3' Restriction enzyme site – ClaI	209bp
	R 5'**ATGCAT**CCGAGGTTGACACCAATTCT3' Restriction enzyme site – NsiI	
Rear Flanking Primers (Set – 2)	**F -** 5'**GCTAGC**ACCGACTCCTTCCAACAAT3' Restriction enzyme site – NheI	211bp
	R - 5'**GGATCC**AGCAGCGGTTTCATCAGACT3' Restriction enzyme site – BamHI	

YIp30 (ATCC 37109) isolated from *E. coli* using alkaline lysis method [9] was used as vector for cloning the amplified gene fragments. Set 1 & Set 2 restriction enzymes were used to restrict the plasmid in upstream and downstream of URA3 gene in the plasmid vector. The restriction and ligation was done in two steps.

- Step 1: Set 2 restriction enzymes cut a region downstream of URA3 gene and amplified PCR product from stage 2 was ligated using standard ligation protocol. This was applied to 1% agarose gel electrophoresis.
- Step 2: Above run sample was eluted from the gel (Fermentas gel extraction kit) and restricted using Set 1 restriction enzymes to cut a region upstream of URA3 gene. Amplified PCR product from stage 1 was ligated and applied to 1% agarose gel electrophoresis.

2.4. Transformation and confirmation

POX4-URA3-POX4 construct was identified as POX4 gene disruption cassette. YIp30 vector containing POX4 gene disruption cassette was transformed to URA^- *Candida tropicalis* using $CaCl_2$ transformation protocol [9-10]. These clones were sub cultured in optimal conditions and stored in 4ºC to check the stability of the cassette.

2.5. Stability analysis

PCR based analysis was done to check the stability and integrity of the construct. The genomic DNA isolated from the clone (30 days after incubation) was subjected to PCR. POX4 primers were used to amplify the gene followed by nucleotide sequencing.

3. RESULTS AND DISCUSSION

The standard methods of gene silencing include the usage of specific inhibitor and multi stage PCR methods. But the problems in these protocols were on the stability of the construct and procedural complexity. Here we have developed a simple two-stage PCR based method to construct gene disruption cassette for gene silencing (Fig 1).

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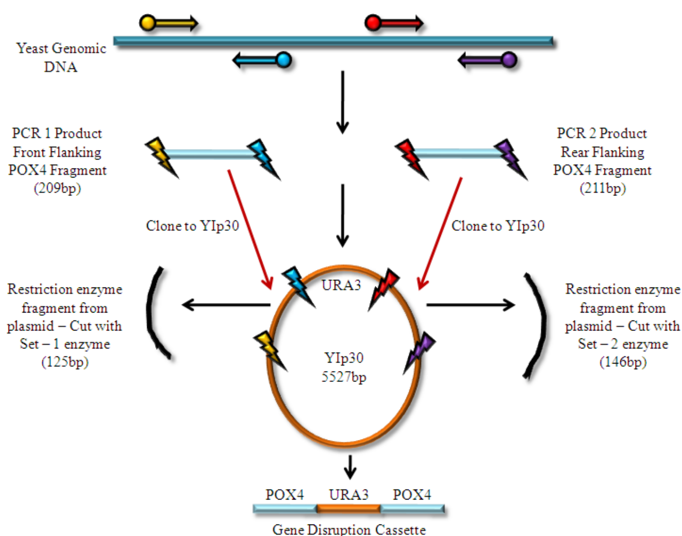

Figure-1: Two-Stage PCR based protocol to construct gene disruption cassette

The protocol started with genomic DNA isolation with the yield of 140ng/mL culture and high purity (A_{260}/A_{280} - 1.74 and A_{260}/A_{230} - 2.193). Two-stage PCR was performed to amplify the front flanking region (209bp), rear flanking region (211bp) of POX4 gene. By computational gene analysis, two enzymes (AciI and Tsp509I) were identified to have potential restriction sites in amplified products. Experimental confirmation of these products was done using restriction enzyme analysis which cuts the amplified products at specific places to give restricted fragments (Fig 2).

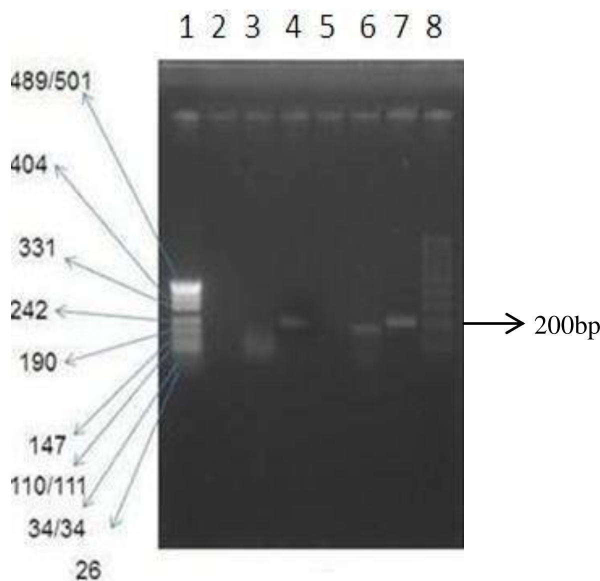

Figure-2: Experimental verification of amplified gene products; Lane 1 – pUC19/Msp1 Digest; Lane 3 – Restricted Front Flanking POX4 Gene (84bp & 125bp); Lane 4 – Uncut Front Flanking POX4 Gene (209bp); Lane 6 – Restricted Rear Flanking POX4 Gene (34bp & 177bp); Lane 7 – Uncut Rear Flanking POX4 Gene (211bp); Lane 8 – 100bp Ladder

YIp30 containing URA3 marker gene was used as the vector to construct the disruption cassette. Cloning was facilitated by two sets of restriction enzymes and DNA ligase. Set 2 restriction enzyme was used to remove 146bp downstream of URA3 gene and 211bp rear flanking (amplified by stage 2) PCR product was ligated to the vector. This product was applied to 1% agarose gel electrophoresis. The band was eluted and subjected to set 1 restriction enzyme which removed 125bp upstream of URA3 gene. A 209bp front flanking (amplified by stage 1) PCR product was ligated to the vector and applied to 1% agarose gel electrophoresis (Fig 3). This gave the construct POX4-URA3-POX4 which was the gene disruption cassette.

The construct was transformed to URA^- *Candida tropicalis* host for the positive selection of clones. By the phenomenon of homologous recombination, the construct (POX4-URA3-POX4) will replace the native POX4 gene in the yeast thus nullifying the expression of POX4 gene. When the gene gets disrupted beta oxidation pathway will not be active and metabolism gets shifted to omega oxidation. To check the efficiency of transformation and to verify the integrity of the construct, confirmation/stability analysis was performed using PCR. Genomic DNA was isolated from the clones and gene specific primers were used to amplify the POX4 gene. Nucleotide sequencing analysis showed the integrity of cassette after transformation and stability after storage. In-silico work was done to compare the sequence information from experimental result and theoretical result. It has been verified that the disruption cassette was stable and has recombined effectively with the yeast genomic DNA.

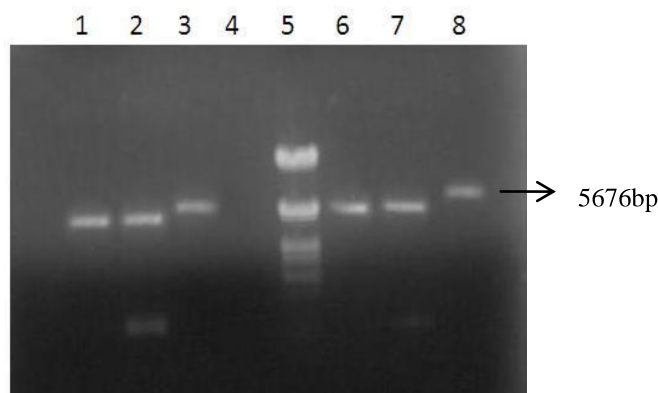

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Figure-3: Cloning and construction of gene disruption cassette; Lane 1 – Plasmid restricted with Set-2 enzymes (5381bp); Lane 2 – Control (without DNA ligase); Lane 3 – Ligated rear flanking region (5592bp); Lane 5 – Lambda DNA marker; Lane 6 – Plasmid restricted with Set-1 enzymes (5477bp); Lane 7 – Control (without DNA Ligase); Lane 8 – Ligated front flanking region (Gene Disruption Cassette – 5676bp)

4. CONCLUSION

We have developed a simple two-stage PCR based method to construct gene disruption cassette. This procedure has the following advantages: (1) Very stable construct (2) Simple procedure (3) Cost effective & feasible (4) Less instrumentation. This protocol can be used effectively to construct many recombinant strains so as to obtain the products of commercial importance.

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