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Optimization of Media and Growth Conditions Improve Overall Quality of Yeast Superoxide Dismutase in *Escherichia coli* Periplasm.

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Abstract
Superoxide dismutase (SOD), an antioxidant enzyme is one of the leading required cosmetic industry protein as it prevents skin damage. Small proteins with few disulphide bonds are generally targeted to the oxidizing periplasmic space of *Escherichia coli* for proper folding. However, it is difficult to achieve good protein concentration without any optimization. In this study, Yeast Cu-Zn Superoxide dismutase is cloned in *Escherichia coli* to be expressed in periplasm. Yeast Cu-Zn SOD is homodimer in holo form and single subunit is 15.7 kDa in size. For cloning Yeast Cu-Zn SOD, mRNA was isolated from *Saccharomyces cerevisiae* and cDNA was generated in two-step RT-PCR. PelB was selected as a signal peptide and pET21b as a cloning vector. To resolve problems related to periplasmic expression, techniques such as codon optimization of signal peptide, and media and growth condition optimization were used. SOD activity assay has been used to analyse expression efficiency. SOD protein expressions were done in shake flask and lab scale fermenter. In both the cases, it was found out that slow growth conditions and longer harvesting time results in increased SOD activity. Among various media additives tested, combination of L-arginine, NaCl, and sorbitol were found helpful and caused 64% increase SOD enzyme activity.

Keywords
Periplasmic protein expression, Cu-Zn Superoxide dismutase, Optimization, Media additives, shake flask cultivation.

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INTRODUCTION:
Expression of small size proteins in periplasm of *E. coli* are common in recent times. Still it is observed that the production of functional protein with proper folding are frequently low and determined by the type and sequence of the protein. Recombinant protein concentration at the end of the expression varies from ng to gm per Litre at shake flask level and

12-25 mg active protein per litre are found decent [1], [2].

Reducing environment of cytoplasm make it difficult to form disulphide bonds [3], [4]. Many alternatives have been used to make cytoplasm preferable to achieve active recombinant protein. One of them being construction of redox mutant strains. However, these strains fail at achieving desirable cell concentration and production at high scale fermentation [5], [6]. In such cases recombinant proteins are joined with signal peptide to transfer them in periplasmic space that is oxidising in nature. There are lots of signal peptide from various sources are explored for this purpose [7], [8], [9]. To secrete protein with single subunit is quite simple as compared to the protein with two or more subunits. Dimeric protein that might be expressed separately, required to assemble with proper conformation with one or more disulphide bonds. This is considered big challenge in the case of heterodimers like Fab molecules [10].

There are two major ways to increase periplasmic protein yields (i) optimize growth conditions and culture medium, and (ii) improve vector construct. The second approach includes improvement of the target protein and expression construct at DNA level. This can be achieved by expression of fusion proteins that can increase the solubility and/or yields; and co-expression of chaperons, dithiol-disulphide oxidoreductases, and/or prolyl cis-trans isomerases that can increase different qualities of protein. In many studies these approaches have given better yields [11]. First approach on which current study is focused on utilise parameters related to cell growth. Optimization of media seems more simple and rational approach in getting active protein with correct conformation. Various media components can be used to tackle specific problems at different stages of expression. Most of the times it is not needed to modify whole media, just addition of few components into already established culture media have seen effective in increasing overall quality of the recombinant protein expression in periplasmic space [12]. Addition of Sorbitol [13] and glutathione [14] has been found to be effective for expression of protein with disulphide bonds. In other study, reducing conditions generated with dithiothreitol (DTT) has been successful in giving active protein in both periplasm and cytoplasm [15]. By optimizing growth conditions involving pH, temperature, and rpm resulted in better expression [6], [16], [17].

Superoxide dismutase (SOD) (EC 1.15.1.1) belongs to class of oxidoreductases. It is an important enzyme that protects all living cells exposed to oxygen. SOD is an anti-oxidative enzyme, which safeguard the cell from oxidative damage by reactive oxygen species [18]. SOD acts against degenerative diseases, aging and increase longevity [19]. Many commercially available skin care and hair products contains Superoxide dismutase [20]. Yeast derived SOD has shown brilliant antioxidant activity than common cosmetic antioxidants, and it has better temperature stability when a copper/zinc-rich nutrient media is used during fermentation [21]. Yeast SOD have been widely used commercially as moisturizes, sunscreens, skin-lightening creams, eye creams, nail polish and anti-hair fall sprays [22].

Cu-Zn SOD is more studies class of SOD and in *Saccharomyces cerevisiae* it is coded by Sod1 gene. The enzyme is homodimer and ~31.4 kDa in size. Each subunit corresponds to 15.7 kDa and 153 amino acids long without N-terminal methionine. Mature protein subunit do not contain first methionine at N-terminal and it is removed during protein processing [23], [24].

This research tests the effect of media additives and growth conditions on expression of Yeast Cu-Zn SOD in *E. coli* periplasm. To determine the effect, SOD activity assay was used. Media additives were selected specifically to improve Cu-Zn SOD protein yield. To achieve highest possible protein activity using combination of these parameters was the main aim of this study.

MATERIALS AND METHODS:

Acquiring the Strains and Vector

Two isolates of *Saccharomyces cerevisiae* were obtained from Department of Biotechnology, Guru Nanak Khalsa College, Mumbai. *Escherichia coli* DH5α, *Escherichia coli* Rosetta (DE3) and pET21b vector were obtained from Micro test Innovations Pvt. Ltd, Bangalore.

Growth media and chemicals

YPD (YEPD) growth media (HIMEDIA) was used to grow *Saccharomyces cerevisiae*. Luria Bertani media (HIMEDIA) was used to grow all *Escherichia coli* strains except when other growth media is mentioned. All chemicals were purchased from Sigma Aldrich, unless mentioned otherwise.

PelB Signal peptide codon optimization

PelB signal peptide DNA acid and protein sequence obtained from literature search and confirmed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Six less frequent codons at the position 3, 4, 6, 7, 11 and 15 detected using Bioline Rare Codon Search Tool (www.bioline.com/media/calculator/01_11.html) and changed manually to more frequent codons to express in *E. coli.* Figure 1 shows codon sequence of

PelB signal peptide with original and optimized codons. Codon optimization was done without altering amino acid sequence.

Original codons	ATG	AAA	**TAC**	**CTA**	TTG	**CCT**	**ACG**	GCA	GCC	GCT	**GGA**	TTG	TTA	TTA	**CTC**	GCG	GCC	CAG	CCG	GCC	ATG	GCC
Optimized codons	ATG	AAA	**TAT**	**TTA**	TTG	**CCG**	**ACC**	GCA	GCC	GCT	**GGC**	TTG	TTA	TTA	**TTA**	GCG	GCC	CAG	CCG	GCC	ATG	GCC
Amino acid	M	K	Y	L	L	P	T	A	A	A	G	L	L	L	L	A	A	Q	P	A	M	A
Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22

Figure 1: Codon optimisation of PelB signal peptide. Codons in red have been changed. Codons in green are optimized codons.

Primer designing and ordering

The designed Gene construct for *Saccharomyces cerevisiae* Cu-Zn SOD with optimized PelB is shown in figure 2. Based on this gene construct, primers for *Saccharomyces cerevisiae* Cu-Zn SOD were designed to attach the PelB signal peptide to SOD gene and getting its amplification with NdeI and BamHI restriction sites. Primer designing was done manually and checked using pDRAW32 software. Table 1 shows the sequences for designed primers. Primers were ordered from Eurofins scientific and with HPLC grade purity.

Figure 2: Schematic representation of the Cu-Zn SOD expression construct to be clone in pET21b vector. 462 bp Cu-Zn SOD gene was arranged in single reading frame. SOD was equipped with 66bp long PelB signal peptide in the N-terminus. T7 P: T7 promoter, SD: Shine-Dalgarno ribosome binding site.

Table 1: Designed primers for cloning Yeast Cu-Zn SOD with optimized PelB signal peptide in pET21b with NdeI and BamHI restriction sites.

Primer	Primer name	Sequence (5' – 3')	Length
Forward primer	PelB SOD_F	ATAAATTATACATATGAAATATTTATTGCCGACCGCAGCCGCTGGCTTG TTATTATTAGCGGCCCAGCCGGCCATGGCCGTTCAAGCAGTCGCA	94bp
Reverse primer	SOD_R	TTCGCACCATGGATCCTTAGTTGGTTAGACC	31bp

mRNA isolation

RNA isolation from two isolates of *Saccharomyces cerevisiae* was carried out using Wei Zheng method of RNA isolation based on phenol-chloroform [25]. Cells were grown and at mid log phase (OD_{600nm} between 0.4 - 0.6), 10 ml culture was centrifuged, and supernatant was discarded. Pellet was reconstituted in 1 mL nuclease free water, centrifuged at 10,000 rpm for 5 minutes. Supernatant was discarded. To the pellet, 0.5 ml of buffer A was added. 600µL of pre-warmed phenol A was added and kept in water bath at 65°C and vortexed for 5 seconds. Tubes were centrifuged at 10,000 rpm for 2 minutes and kept in water bath at 65°C. Phenol layer was carefully removed and transferred in a new tube. To it, 600µL of phenol was added. Tubes were vortexed for 6 minutes and centrifuged for 2 minutes at 10,000 rpm. In fresh tube 200µL water and 500µL phenol (TE)/chloroform was added. Aqueous layer was separated and transferred into tubes containing water and phenol (TE)/chloroform. It was stored in water bath at 65°C.

Tubes were then vortexed for 5 seconds and centrifuged for 2 minutes at 10,000 rpm. The aqueous layer was again transferred in new tube and tubes were kept in water bath at 65°C until all the transfer was complete. To it 50µL of 3M NaAC and 1ml ethanol was added, mixed, and kept on ice for 15 minutes. Tubes were then centrifuged for 15 minutes at 4°C. Tubes were then washed with 1 mL of 70% ethanol and centrifuged for 10 minutes at 4°C. Ethanol was discarded and 400 µL of water was added. Tubes were kept in water bath for 5 minutes. 40µL NaAC and 800µL ethanol was added and mixed and kept on ice for 15 minutes. Tubes were then washed with 70% ethanol and dried. Lastly, RNA was reconstituted in 50 µL of Nuclease free water.

Amplification of Cu-Zn SOD with PelB signal peptide

Double stranded DNA was generated in two steps RT-PCR. In the first step, reverse transcription was carried out using isolated mRNA. For this, Revert Aid Reverse Transcriptase (Thermo Scientific) kit was utilized. First Strand of cDNA was synthesized according to manufacturer recommendations. Gene specific primer (SOD_R) was used during synthesis. In second step, mRNA-DNA hybrid was converted in dsDNA using DFS-Taq DNA Polymerase (Bioron). PCR conditions were initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 46°C for 45 seconds, extension at 72°C for 60 seconds, final extension at 72°C for 7 minutes, 35 cycles. The primers used for the amplification were forward and reverse primers mentioned in table 1.

Cloning

All remaining PCR amplifications were done using DFS-Taq DNA Polymerase (Bioron) with cycle conditions mentioned earlier. PCR product and pET21b vector were digested separately with Fast Digest NdeI and BamHI (Thermo Scientific). These digested products were ligated using T4 DNA Ligase (1 U/µL) (Invitrogen) and transformed in *Escherichia coli* DH5α using calcium chloride method developed by A. Y. Chang et. al. [26]. The positive clones were confirmed using PCR utilising both vector specifics and gene specific primers, and restriction enzyme digestion. Cloned pET21b vector with Cu-Zn SOD was isolated from positive clone and transformed in *Escherichia coli* Rosetta (DE3) expression host.

DNA sequencing was done on both strands to determine errors in the frame. DNA sequencing was done through Eurofins Genomics India Pvt. Ltd. Sequencing data was analysed and edited in Chromas software (version 2.6.6).

SureSpin Plasmid Mini Kit (Genetix Biotech Asia Pvt. Ltd, cat. no. NP-37107) was used for all plasmid purification. All purification from agarose gels were performed using the gel extraction kit (Bio Basic, cat. no. BS353) according to the manufacturer's instructions. All agarose gel electrophoresis were carried out at 100V using horizontal gel electrophoresis apparatus (Techno Source).

Shake flask cultivation

For shake flask, 250 ml sterile LB broths were inoculated with 1% culture of *Escherichia coli* Rosetta (DE3) with Cu-Zn SOD having OD_{600nm} of 1. For growth condition optimization, temperature was the first point chosen for optimization, then rpm and finally harvesting time. Earlier parameters that were found optimum were used while optimizing next parameter. The basal level of protein expression was done without any optimization. Varied IPTG (HiMedia) concentrations ranging from 0.01mM to 1mM were tested for protein expression. Mid log phase (0.6 OD_{600nm}) was kept as an induction point. Media additives such as L-arginine (HiMedia), Betaine, NaCl (HiMedia), Sorbitol (HiMedia), Sucrose (HiMedia), Ethanol (HiMedia), Glutathione and DTT were added individually and in different combinations. Media additives with desired concentration were added into the media just before the induction.

Fermentation cultivation

Fed-batch fermentation was carried out on Sartorius BLite 5L fermenter with parameters optimized at shake flask level. The initial media volume was kept 3 L and supplemented with 500 mL of total feed. Mixture of 50% dextrose (HiMedia) and 30% yeast extract (HiMedia) was used as a feeding agent. Temperature (30°C) and pH (7.2) were controlled by the automated fermenter system while DO_2 (above 30%) and glucose concentrations were controlled manually. Samples were collected after every hour and pH, glucose concentration and OD_{600nm} were checked. Glucose concentration was maintained above 250 mg/L and was monitored with Accu-chek Active Glucometer Kit. Volumes of all samples were adjusted to 1 OD_{600nm} for periplasmic extraction.

Protein expression in periplasm

Superoxide dismutase was extracted from periplasm of *Escherichia coli* Rosetta (DE3) with Cu-Zn SOD using modified TSE extraction method [27]. At selected time interval, 10 mL culture was taken and centrifuged at 6000 rpm for 10 minutes. Supernatant was stored at -20°C as a medium fraction and pellet was resuspended in 0.4 ml of 100 mM Tris-Cl, 20% sucrose and 1 mM EDTA. Incubated on ice for 15 minutes. Centrifuged at 6000 rpm for 10 minutes at 4°C and supernatant was stored. To the pellet, 0.4 mL of 0.5 mM $MgSO_4$ was added. Tube was incubated on

ice for 15 minutes. Centrifuged at 6000 rpm for 10 minutes at 4°C. Supernatant was collected in new tube and if not used immediately then stored at -20°C until further used.

SOD activity determination

Superoxide dismutase activity was determined from samples extracted from periplasm by commercially available assay kit (SOD Assay kit Sigma, cat. no. 19160) according to manufacturer's guidelines. Unknown SOD concentrations (U/mL) were determined using inhibition curve.

Western blot assay

After growing at optimised media and growth conditions, periplasm proteins were extracted from *Escherichia coli* Rosetta (DE3) with Cu-Zn SOD after 10 hours of induction. Cell free supernatant was collected to determine any protein leakage. Samples were separated on 12% poly-acrylamide gel and proteins from the gel were transferred to nitrocellulose membrane. The membrane was blocked by incubating overnight at 4°C with 1.5% BSA prepared in TBST (Tris-buffered saline having 0.05% Tween-20) and washed thrice with TBST. Membrane was incubated with an anti-SOD1 rabbit antibodies (StressMarq Biosciences Inc, cat. no. SPC-115) at 1:1000 for 2 hours at room temperature. After washing thrice with TBST, the membrane was then incubated for 1 hour at room temperature with HRP conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, cat. no. G-21234) secondary antibodies at 1:10000. Membrane was developed with the Pierce ECL Western blotting substrate (Thermo Fisher Scientific, cat. no. 32134).

RESULTS AND DISCUSSION:

Cloning

Extracted mRNA of *Saccharomyces cerevisiae* from isolate 2 (lane 3 in Figure 3a) was subjected to reverse transcription using gene specific primer to get RNA-DNA hybrid, which is then amplified into dsDNA in second step, showed a band at ~557bp on agarose gel (Figure 3b). To get periplasmic expression of the recombinant Cu-Zn SOD protein, the protein-coding region of the Cu-Zn SOD gene was inserted between the NdeI and BamHI restriction sites of the pET21b plasmid. This resulted in a recombinant plasmid pET21b- Cu-Zn SOD that contained the desired SOD gene sequence.

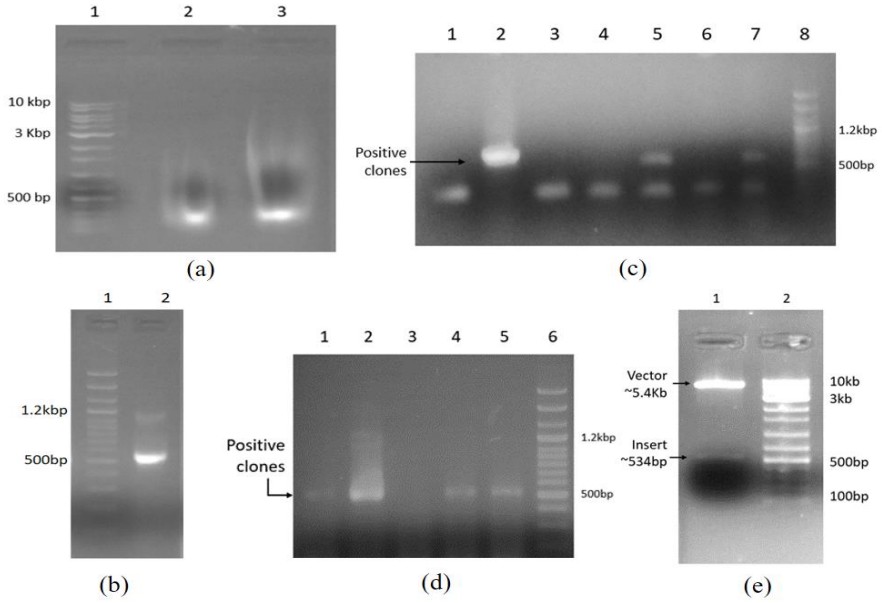
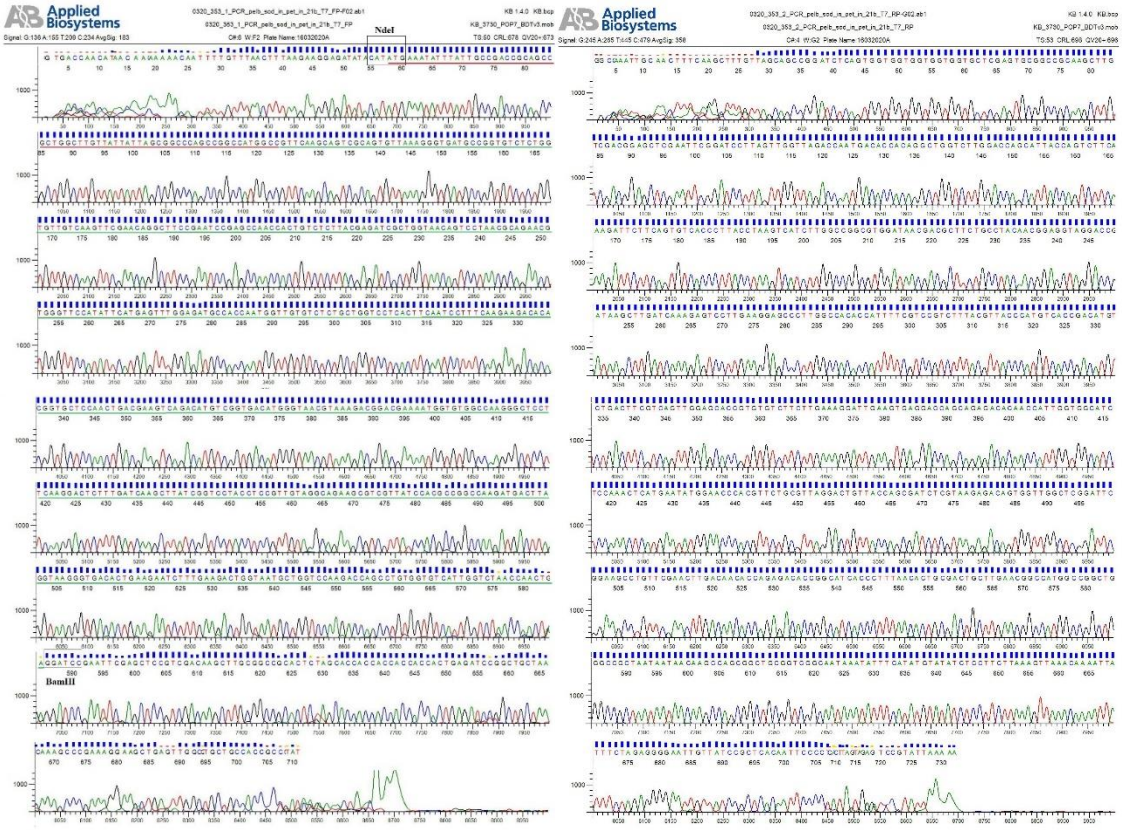


Figure 3: Agarose gel electrophoresis results (a) isolated mRNA, Lane 2 and 3 represent mRNA from *Saccharomyces cerevisiae* isolate 1 and isolate 2 respectively (b) Amplified Cu-Zn SOD DNA, Lane 2 represents dsDNA obtained after two step RT-PCR (c) Positive clone confirmation with PCR using vector specific T7 primers (d) Positive clone confirmation with PCR using gene specific primers. (e) RE confirmation of positive clone with NdeI and BamHI.

After transformation, the positive clones which were detected with the help of vector specific PCR showed ~ 751 bp size bands (Figure 3c), and ~ 557 bp bands for gene specific PCR (Figure 3d). Further confirmation of positive clone with restriction digestion showed two bands, one for insert at ~ 534 bp and one for vector backbone at ~5.4 kbp (Figure 3e) that confirms the Cu-Zn SOD gene has been inserted in the pET21b plasmid.

DNA sequencing done on both strands verified the gene has set in correct reading frame and there are no amplification errors in sequence of the cloned SOD gene. Figure 4 shows DNA sequencing results of PelB Cu-Zn SOD done with the T7 forward and reverse primers. DNA sequencing results also confirmed the codon optimization of PelB signal peptide. After analysing both sequences, the final Cu-Zn SOD DNA sequence was submitted to GenBank (GenBank accession number MT779809).



(a) **(b)**

Figure 4: DNA sequencing result of PelB Cu-Zn SOD done with the (a) T7 forward and (b) reverse primers. In (a) Sequence underlined in red colour represents PelB signal peptide DNA sequence. Sequence underlined in green colour represents SOD gene sequence. Restriction sites (NdeI and BamHI) are represented in the boxes.

Growth conditions optimization

In order to observe the expression of the Cu-Zn SOD protein, a single colony of *E. coli* Rosetta (DE3) carrying the pET21b-Cu-Zn SOD plasmid was grown and induced with IPTG for several post induction periods and its periplasmic proteins were subjected to SOD protein activity assay. During optimization of growth conditions, it was observed that SOD activity was found less at 37°C and at 25°C than 30°C (figure 5a). Indicating that high and low than temperature than 30°C are not effective for SOD production in periplasm. When subjected to different rpm after induction, it was noticed that SOD activity is inversely proportional to rpm and SOD activity increased significantly as rpm decreases until 150 rpm (figure 5b). Bellow 150, activity reduced with reduction in OD. Broth was harvested at different time intervals after induction and better protein activity was observed by harvesting the broth at 10 hours (figure 5c). After 10 hours, SOD activity gradually decreased as culture reaches stationary phase. SOD activity increased by 83% when expressed in lab scale fermenter compared to shake flask expression (figure 5g).

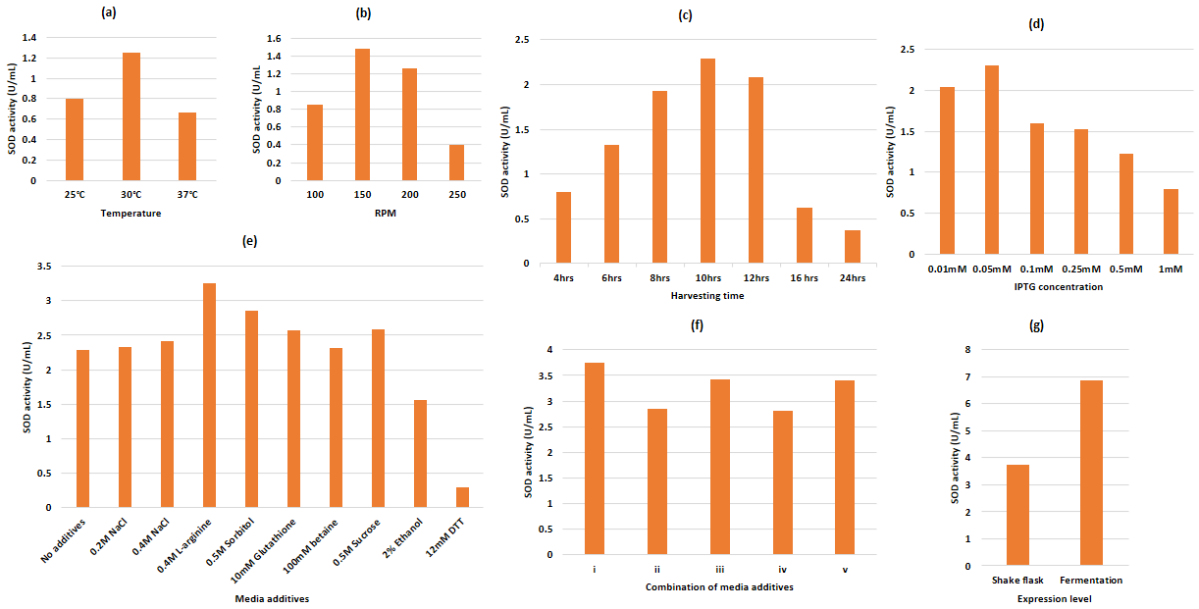


Figure 5: SOD activity at different growth parameters and with different media additives tested at shake flask level expression - (a) Temperature (b) RPM (c) Harvesting point (d) IPTG concentrations (e) Media additives (f) Combination of media additives - (i) 0.4M NaCl, 0.4M L-arginine, 0.5M Sorbitol (ii) 0.4M NaCl, 0.4M L-arginine, 0.5M Sorbitol, 10mM Glutathione (iii) 0.4M NaCl, 0.4M L-arginine, 0.5M Sucrose (iv) 0.4M NaCl, 0.4M L-arginine, 0.5M Sucrose, 10mM Glutathione (v) 0. 4M NaCl, 0.4M L-arginine, 0.5M Sucrose, 100mM Betaine and (g) Comparison between SOD activity at shake flask and lab scale fermenter expression.

Media optimization

In optimization of inducer amount, it was found out that concentration of IPTG has direct effect on OD of the culture and with higher IPTG concentration SOD activity decreases exponentially (figure 5d). This suggest that induction with minimal IPTG found to be beneficial while SOD periplasmic expression. Optimum SOD activity was observed with 0.05 mM IPTG.

Comparison of effect of different media additives on SOD activity was shown in figure 5e and 5f. In all media additives tried, L-arginine found most effective when used alone and in combination to give better activity. Glutathione seems helpful in increasing SOD activity by small proportion when used alone, but not when used in the combination with other media additives. Reducing environment created by DTT addition effect the SOD activity in a negative manner. Hence reducing condition does not benefit in periplasmic expression of Cu-Zn SOD. Betaine in combination of L-arginine, NaCl and Sucrose helps in increasing SOD activity, but absence of betaine does not have much effect. Most media additives caused depletion in cell growth (figure 6). Addition of mixture of media additives such as NaCl and sorbitol or sucrose caused cells to grow slow. This could be due to osmotic stress created by these compounds. However, these conditions resulted in a better SOD activity. In various combination of media additives used, combination of L-arginine, NaCl, and sorbitol was found most effective and caused 64% increase SOD activity than media with no additives.

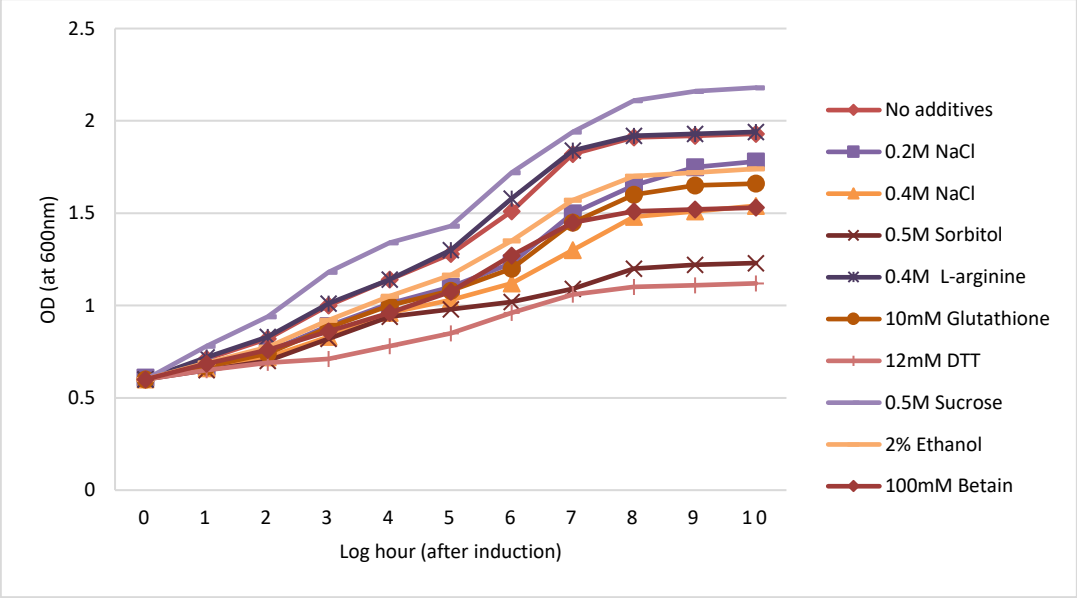


Figure 6: Determination of effect of different media additives on growth, tested at shake flask. X-axis shows the OD_{600nm} and Y-axis shows time in log hour after induction at 0.6 OD_{600nm}. ODs were monitored until 10 hours post induction. All growth parameters were kept constant for all flasks (Inoculum - 1%, Temperature - 30°C, IPTG - 0.05mM, RPM - 150).

Western blot assay

Identification of Cu-Zn SOD protein was done by performing western blot assay. In western blot analysis done on the periplasmic and medium fractions, SOD protein was detected (figure 7). Periplasmic fraction showed two bands, one at ~31.4 kDa for homodimer and other at ~15.7 kDa for free SOD subunits. Presence of small band at ~31.4 kDa was also observed in the medium fraction, which indicates SOD leakage from periplasm in the culture medium during cultivation.

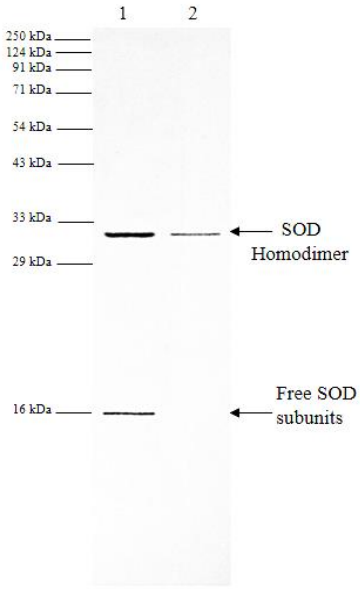


Figure 7: Western blot analysis of periplasmic and medium fraction. Lane 1 represents Periplasmic fraction, Lane 2 represents Medium fraction.

CONCLUSION:

Recombinant protein localization in all three compartments i.e. cytoplasm, periplasm and medium, have their own advantages and disadvantages, and choice of any compartment requires additional optimization and standardization

that varies from protein to protein. Mostly, periplasm or medium expression is selected for protein that required in an active form. High periplasm protein expression depends upon lot of parameters and achieving optimum expression is accomplished through trial and errors. For production of recombinant protein, fermentation is perhaps the utmost important process. To attain optimized efficiency without lacking on product quality, it is crucial to optimize media components and process parameters. Design of cultivation medium along with the growth conditions decide cell's microenvironment, which predominantly decides the quality of recombinant protein. Though it is essential to understand role of reach media component with their possible interaction with each other, it is not possible to find all probable concentration and combination.

In this study, we found out that inducer concentration, growth conditions, media additives, and the mixture of these factors have effect on overall quality of yeast Cu-Zn Superoxide dismutase protein while expressing it in periplasm. Increase in Cu-Zn SOD activity was achieved in both shake flask and lab scale fed-batch fermentation in an IPTG based induction. We also observed that slow growth rate is one of the ways to achieve active protein while expressing periplasmic SOD.

Presence of free subunits of Cu-Zn SOD in periplasmic space suggest optimizing growth conditions and culture medium improve active protein concentration in periplasm but are not enough to tackle unprocessed free SOD subunits. Such problems can be solved through further optimization with respect to co-expression [11].

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