Determination of Plasmid Stability, pRSOD Extraction and SOD Production by Recombinant *Escherichia coli*

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ABSTRACT

A preliminary study of Escherichia coli harbouring superoxide dismutase (ESOD) was carried out to ensure the capability of the recombinant to achieve protein production in large scale cultivation process. The objectives of this study are first, to determine the stability of the plasmid in the recombinant E. coli, and later, the pRSOD and SOD production After the confirmation of the availability of the plasmid, the determination of medium composition was performed to choose the best medium for highest dry cell weight (dcw). The findings indicate that the ESOD successfully carried the plasmid (pRSOD) compared with other control references, E. coli BL21 (DE3) pLysS and E. coli BL21 (DE3) pLysS pRSET A. The ESOD also achieved 100% stability over 48 generations. The SOD was also found capable to produce a reasonable amount of SOD. Thus, the study has established that the ESOD is clearly comparable to other recombinant E. coli and suitable to pursue large scale process for high cell density cultivation.

Keywords: *plasmid stability, recombinant E. coli, Superoxide dismutase, SOD*

Introduction

The superoxide dismutase or SOD is an enzyme that catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. As such,

it is an important antioxidant defense in nearly all cells exposed to oxygen. Naturally, SOD would be the antioxidants in aqueous compartments, for example, in the cytosol and the extracellular fluids in human cell that defends against reactive oxygen species (Valentine et al., 1998). This enzyme is used in pharmaceutical products and also in cosmetic products as antioxidant that can help to protect against cell destruction.

Hence, manipulation of microorganism nowadays makes the production of recombinant protein possible. Therefore, the amendment of producing SOD in *Escherichia coli* was selected as the microbes capable as hosts for the production of the heterologous proteins and its genetics are far better characterised than those of any other microorganisms. Tan (2003) successfully constructed a recombinant *E. coli* into which carrying gene expression of SOD from *Lactoccocus lactis* M4. The expression of the protein in laboratory scale cultivation was, therefore, come into highlight as the potential of SOD in the therapeutic and pharmaceutical, as well as cosmetic products.

Experimental studies in molecular biology with transformed *E. coli* assume that the recombinant plasmids are stable. However, different growth and storage condition can evoke changes in the transformed population (Smith & Bidochka, 2004). Shiloach and Fass (2005), reviewed growing recombinant *E. coli* and method development of the microbes to achieve high cell density cultivation. They also mentioned that the overall productivity of the recombinant protein depends on numerous factors such as plasmid stability, promoter response to inducer, post transcriptional inhibition events, and post-translation inhibition caused by proteolysis and improper folding.

Plasmid is maintained under a subtle quasi-equilibrium condition in the host cell, and it is easily supposed that the recombinant plasmid carrying cloned genes would behave in a different manner compared to the original vector plasmid (Imanaka, 1993). Reasons for a general inclination towards instability of recombinant plasmid are explained by many previous researchers such as Schumann and Ferreira (2004), Hoffmann and Rinas (2004), Francois (1999), Mcloughlin (1994), Bailey (1993), and Zabriskie and Arcuri (1986).

The maintenance of a plasmid often induces a stress response especially when a target protein is highly expressed (Hoffmann & Rinas, 2004). Such stress responses resemble environmental stress situations such as heat shock, amino acid depletion or starvation. Stress induced by plasmid maintenance is often related to plasmid copy number (Bailey, 1993), while the main perturbation can be attributed to genes encoded

by the plasmid and even constitutively expressed genes such as antibiotic resistance genes (Hoffmann, Weber, & Rinas, 2002).

Materials and Methods

Bacterial Strains and Plasmids

E. coli BL21 (DE3) pLysS, carrying pRSOD (ESOD) gene (Tan, 2003) from locally isolated *Lactoccocus lactis* M4 was used in this study. *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 (DE3) pLysS pRSET A were used for comparison purposes. The frozen stocks of each strain were streaked onto LB selective agar and transferred into 100 ml LB selective broth.

Medium and Cultural Condition

The media used for the preliminary study were Luria Bertani (LB) and Terrific Broth (TB). Each medium was used independently and also treated with 20 g/L of glucose or 0.4% v/v of glycerol as carbon source. The pHs of all medium was initially determined at 7 with pH meter Metler Toledo MP320U.

Batch Cultivation

All batch experiments were conducted using 250 ml shake flasks and the total volumes of the cultivation were 50 ml for each flask. The ESOD was strictly cultivated with filtered sterile antibiotics, which are chramphenicol and ampicillin at a concentration of 35 μg/ml and 50 μg/ml, respectively. Glycerol and glucose were autoclaved separately as the stock. The culture was cultivated for 24 h on a shaker with a controlled temperature and agitation at 37°C and 250 rpm respectively.

Stability of ESOD

The ESOD was cultivated in 100 ml LB broth with 20 g/L glucose and its proper concentration of antibiotics. Initial pH was determined at 7 prior to autoclave. The cultivation was extended for 72 h after it reached 24 h. 4 ml of sample was taken out at 16 h and 72 h of cultivation. Plasmid stability was estimated by manual picking of 100 bacteria colonies aseptically from the master plate by using sterilised toothpicks onto the

LB agar with antibiotics as selective agar. Putative non-recombinant and recombinant cells grew and appeared on the surface of agar. The fraction (F_n) of plasmid-containing cells (P) after n generations may be calculated from the experimental measurements of P and plasmid-lacking cells (N) as the formula shown below:

$$Fn = \frac{P}{P + N}$$

Note that the total of P + N is 100. The percentage is simply gained by multiplying with 100 (Ho, 2002; Worden & Kinney, 2002).

Determination of pRSOD

Plasmid extraction was performed to determine the existence of pRSOD in the recombinant. The culture of ESOD was incubated overnight at 37°C and 250 rpm. The culture was then transferred into 1.5 ml eppendorf tubes and centrifuge for 10 000 rpm for 1 min. The pellets were then washed with saline solution and centrifuged once again. The pellets were suspended with 200 µL of solution A and left for 5 minutes at room temperature. After that, 400 µL of solution B and followed by 300 µL of solution C was added into the eppendorf tube. For each addition, the solution was gently inversed and placed in ice for 5 minutes.

Later, the tube was centrifuged at 12 000 rpm, 11 min and 4°C. Clear supernatant was gently transferred to a new eppendorf tube. Double volume of 96% ethanol was added in the eppendorf tubes and placed in -20°C for 10 minutes for precipitation. After that, the tube was centrifuged at high speed and the supernatant removed. Next, the tube was added with 70% cold ethanol, centrifuged and the supernatant was later discarded. The pellet was dried in laminar ^{air} flow for 2 h until it was completely dry. Lastly, 20 µL of distilled water and 1 µL of RNAase were added in the tube. The plasmid solution was then mixed with 6XDNA loading dye and loaded into 7% agarose gel with 100 volt and 500 mA. 1 kb marker from Fermentas was used as the size marker. Gels were then destained in water for 15 min, and photographs were taken under UV light by using the Gel Doc 2000 gel documentation system (Bio-Rad).

Determination of SOD

ESOD, *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 (DE3) pLysS pRSET A were cultivated separately in LB with 20 g/L glucose and

appropriate concentration of antibiotics. Initial pH was determined at 7 prior to autoclaving. The ESOD was then induced with 1 mM IPTG at OD ~ 0.5. The cultures were harvested at 24 h cultivation. The total protein and SOD was visualized by performing 12% SDS-PAGE. SOD was purified using Ni-NTA Qiagen Spin Column.

Analytical Procedures

Colony forming unit assay was then carried out to the samples by applying appropriate dilution with its reading at OD_{600nm}. The cell concentration of ESOD was determined by conversion from standard equation of absorbance at 600nm versus dry cell weight (g/L). One optical density at 600nm is equal to 0.541 g/L dcw.

Results and Discussion

Stability of ESOD

Table 1 illustrates ESOD growth by dcw, log cfu and percentage of plasmid stability cultured in LB with antibiotics, chramphenicol and ampicillin (LBCA) and without antibiotics. Growth was assayed after 72 hours equivalent of about 200 generations of ESOD. From the table, ESOD cultured with the antibiotics gave higher dcw but lower in log cfu than ESOD cultured without the antibiotics. Even though, both media given 100% stability out of 100 colonies transferred to the second agar with the antibiotics (Figure 1). Hence, the ESOD was confirmed to be a stable recombinant although without antibiotics stress.

Table 1: Result of Dry Cell Weight, Log cfu and Plasmid Stability of ESOD in LB with Antibiotics and LB without Antibiotic

Media	Dcw (g/L)	Log cfu/ml	Plasmid stability %
LBCA	1.92	7.38	100
LB	1.82	7.7	100

The ESOD would be comparable to *E. coli* pELCHis24 containing gene for levansucrase (Kim et al., 1998). The population of plasmid harboring cells was maintained above 95% of the total cell for more than 100 generations. Lee et al. (1994) also showed 100% stability of plasmid

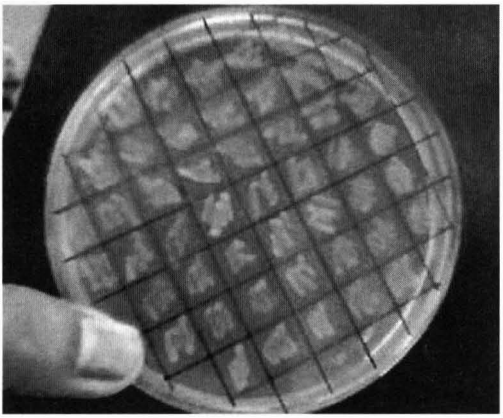

Figure 1: Plate Used for Determination of pRSOD Stability

in the recombinant *E. coli* harboring poly (3-hydoxybutyric acid) (PHB) after 110 generations.

Determination of pRSOD

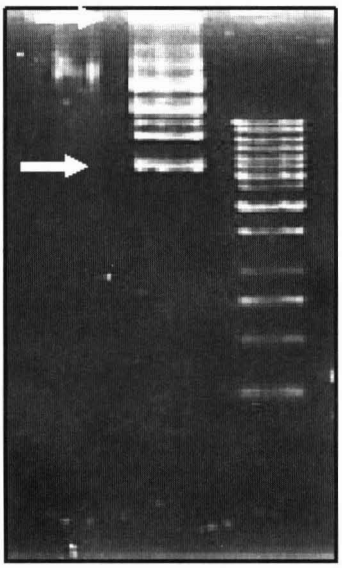

Figure 2: Result of Plasmid Extraction. Lane 1, ESOD Cultured in 72 Hours with Antibiotics; Lane 2, ESOD Cultured in 16 Hours with Antibiotics; Lane 3, 1 Kb DNA Marker

Figure 2 shows the result of plasmid extraction obtained from the agarose gel viewed under UV light. Lane 2 clearly shows that the target plasmid, pRSET A, was obtained at 3.5 kb as compared to the 1 kb marker. Lane 2 suggests that the ESOD was actively carrying the plasmid throughout the cultivation. Lane 1, however, fails to show any plasmid at the desired target, due to the extension period of cultivation. The extension up to 72 h without appropriate antibiotics feeding explained that the culture was segregating and was less likely to carry the recombinant plasmid. Figure 3 shows the ESOD under fluorescence light microscope at X1000 total magnification power by using Leica DMLB Spot Advance Software.

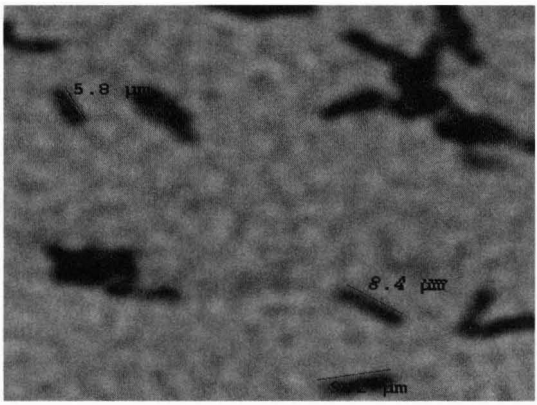

Figure 3: ESOD Photographed under Fluorescence Light Microscope (X1000)

SOD Determination

Figure 4 below shows the result of protein extraction of ESOD, *E. coli* BL21 (DE3) pLysS and also *E. coli* BL21 (DE3) pLysS pRSET A. The target protein size for the extraction was about 27 kDa. Lanes 3 and 4 show that the protein (SOD) were successfully secreted at the desired target. The result suggests that the ESOD was capable of producing SOD after it was induced with 1 mM of IPTG.

Medium Composition

The media used in the preliminary study were LB and TB respectively resulted to more than 2 g/L of ESOD (Figure 5). Significantly, the TB media gave higher dcw compared to LB media in all shake flasks treatment. The highest dry cell weight of ESOD was obtained at 3 g/L in

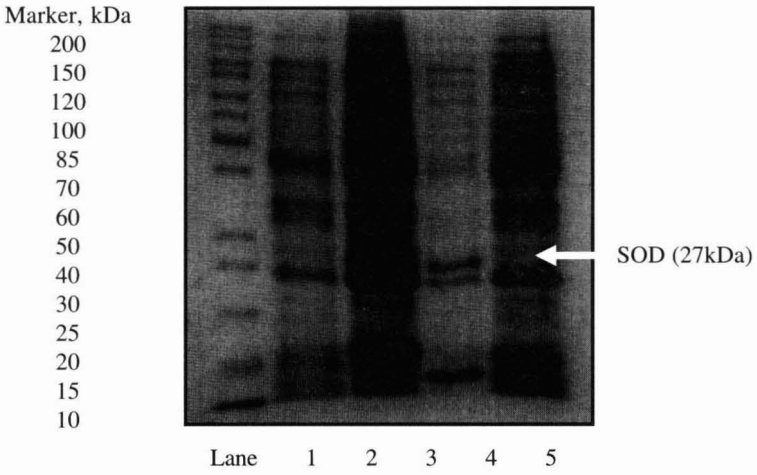

Figure 4: Superoxide dismutase (SOD) Protein Result Visualized by SDS-PAGE 12%. Reference: Lane 1, Protein Marker (Fermentas); Lane 2, *E. coli* BL21 (DE3) pLysS; Lane 3, ESOD Total Protein; Lane 4, ESOD Partially Purified; Lane 5, *E. coli* BL21 (DE3) *pLysS* pRSET A

medium that contained TB with 20 g/L of glucose. Generally, TB media is much more concentrated with yeast extract and tryptone as compared to LB. The nitrogen source from both tryptone and yeast extract helps to raise up the cell growth faster. In spite of that, adding glucose and glycerol as the carbon source into culture medium, also helps to improved the ESOD growth.

Yeast extract has often been utilised for cultures of *Bacillus sphaericus*, as it is an excellent source of amino acid and vitamin for growth and sporulation (Sasaki, Jiaviriyaboonya & Rogers, 1998). The TB medium was said to be the suitable medium to maintain the plasmid of a recombinant *E. coli* (Tartoff & Hobbs, 1987). Lee (1996) reported that some nutrient, including carbon and nitrogen sources can inhibit *E. coli* cell growth when present above a certain concentration. In *E. coli* cultivation, the growth is inhibited when the glucose is used above 50 g/L (Riesenberg et al., 1991).

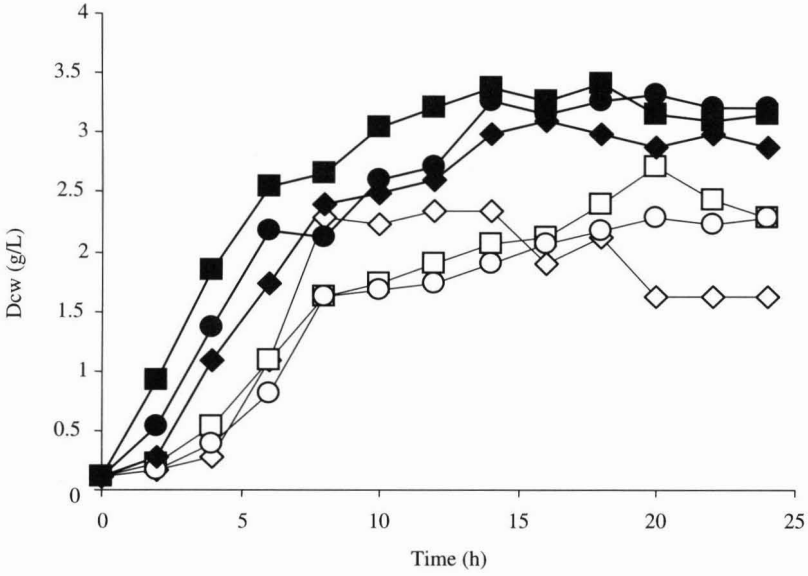

Figure 5: Profile of ESOD Growth in Different Composition of Media.
TB (Closed Symbols) ♦ - TB, ■ - TB with 20 g/L Glucose, • - TB with 0.4% v/v Glycerol; LB (Open Symbols) ◊ - LB, □ - LB with 20 g/L Glucose, ○ - LB with 0.4% v/v Glycerol

Conclusion

The preliminary studies of ESOD were included to determine a few factors that are important especially in culturing a recombinant *E. coli.* The studies were to determine the suitable and optimised medium for the ESOD in large scale cultivation such as bioreactor. In the present study, the findings show that the ESOD is capable in carrying the recombinant plasmid up to 48 generations (16 h) and the stability of the plasmid is stable 100% for about 200 generations (72 h) although without antibiotic pressure. The ESOD is also capable of producing SOD after it is induced by the synthetic inducer, IPTG at the early exponential phase. The ESOD is capable in maintaining the pRSOD and producing the SOD in shake flasks cultivation.

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