ABSTRACT

Present study was conducted for the production, characterization and utilization of oxyrase from *E. coli* as part of its cytoplasmic membrane fragments. This enzyme system is known for its antioxidant potential to reduce dissolved oxygen from medium and generating microanaerboic conditions thereof. For this purpose, 30 intact poultry intestinal samples were collected from thirty sites/butcher shops located in Ichhra, Anarkali and Rehmanpura, Lahore. Total 88 strains of *E. coli* were isolated from these samples which were preliminary identified microscopically and by biochemical profiling. Downstreaming of membrane fragments was initially performed with buffer-mediated sonicated lysis of *E. coli* cells and purification of membrane fragments from cell debris by centrifugation and sterilization by membrane filtration (pore size 0.45µm). Qualitative methylene blue reduction assay and quantitative dissolved oxygen probe meter were employed for screening of best *E. coli* isolate for active oxyrase. Seventy six isolates of *E. coli* were found to contain active oxyrase in membrane fragments. *E. coli* oxyrase strain-4 (EC4) was found the best for its potential to reduce dissolved oxygen with maximum oxyrase activity of 0.41±0.008U/mL/min with 41% reduction in dissolved oxygen at pH 7.5, Temperature 37°C, 25mM lactate as H^+ donor after 20 minutes. Molecular confirmation of EC4 was done by ribotyping of 16S rRNA conserved region using Universal Primers 27F and 1492R. Evolutionary relation drawn by using Molecular Evolutionary Genetics Analysis (MEGA) 6.0 tool confirmed Oxyrase producing isolate EC4 as *E. coli*. Growth optimization studies revealed maximum EC4 growth with cell density 2.78±0.04 in modified RF medium (pH 7.5) with 1.5% glucose as carbon and 0.2% $NH_4(SO_4)_2$ as nitrogen source after 8 h of incubation at 37°C with agitation rate of 225rpm when 8 h old 4% (v/v) inoculum was used. Effect of H^+ donor/substrate and its various concentrations at different time intervals was evaluated on the oxygen reduction activity of membrane fractions. Lactate (25mM) was best substrate which showed maximum Oxyrase activity of 0.40±0.07 U/mL/min with 40.4±1.22% reduction in dissolved oxygen after 20 minutes of incubation. Oxyrase worked best at pH 8.5 with maximum 45% reduction in dissolved oxygen and activity 0.45±0.08 U/mL/min was

observed. Moderate thermophilic nature of oxyrase was revealed because temperature profiling of enzyme showed maximum activity of 0.65±0.09 U/mL/min at 57°C. Modification in downstreaming strategy for extraction of oxyrase from *E. coli* was adopted by using Avestin Emulsiflex-C3® resulted in drastic improvement in oxygen reduction activity. Chromatographic analysis using Q-Sepharose® Fast Flow anion exchange column of Emulsiflex mediated oxyrase membrane preparation showed significant ~45 kDa bands using Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis profile. This preparation of eluted fractions with 65% buffer comprised 50 mM Tris pH 8.0 + 1.0 M NaCl. These membrane fractions caused complete reduction in blue colour of methylene blue within 1 minute. Avestin Emulsiflex-C3® mediated membrane fractions showed 100% reduction in dissolved oxygen at 57°C in presence of 25 mM lactate as H^+ donor using pH 8.5 after 1.5 minutes with activity 13.3±0.19 U/mL/min and specific activity of 8.0 U/mg. Kinetic parameters derived from double reciprocal Lineweaver-Burk plot showed K_m values 8.49×10^{-3} M^{-1} and 4.75×10^{-3} M^{-1} of sonication and Emulsiflex prepared oxyrase membrane fragments respectively for lactate as substrate. Enhanced activity of Emulsiflex membrane fragments to reduce dissolve oxygen is because of 55 folds improvement in affinity of oxyrase enzyme for substrate as compared to sonication prepared membrane fragments. *E. coli* NADH Dehydrogenase-II (EC NDH-II), one of the ubiquinone reductase in cell's membrane was cloned and expressed with Histidine tags using pMA507 vector due to its relevance in oxyrase activity. Nickel chelating ligand nitrilotriacetic acid (NiNTA) column chromatography was employed for purification and extraction of His-tagged fusion protein with Lauryldimethylamine oxide, Triton X-100 (TrinX-100) and Tween-20 detergents. TrinX-100 detergent suited best for elution of protein. Further purification of His-tagged EC NDH-II by using BIORAD® BioLogic DuoFlow™ Liquid Chromatography System with HiPrep Sephacryl S-200 HR size exclusion column with buffer comprised 50 mM Tris pH 8.0 + 300 mM NaCl + 0.1% Triton X-100 demonstrated a significant band of 43 kDa. Characterization of purified EC NDH-II was performed; maximum activity of 46±1.1 U/mL was obtained at standard assay conditions when 225 µM NADH was used as substrate. Determination of kinetic parameters showed inhibitory effect of EDTA and Mg^{+2} with different concentrations; highest V_{max}/K_m (8.35×10^5) was observed when

dehydrogenase activity was characterized in the absence of inhibitory agents, with K_{cat} value of 1.2×10^{19} s^{-1}. Antioxidant potential of Oxyrase in Emulsiflex prepared membrane fractions can be utilized in variety of industrial applications. In present study, efficacy of oxyrase was evaluated for improved cultivation of anaerobic bacteria. Antioxidant potential of oxyrase was compared with common reducing agent Cysteine-HCl (Cys-HCl) using Hungate Technique. Four anaerobic bacterial strains (*Anaerobaculum hydrogeniformans* OS1, *Akkermansia muciniphila*, *Bilophila wadsworthia*, and *Roseburia intestinalis*) were selected as experimental models for this purpose. Significant improvement in cell density of *Anaerobaculum hydrogeniformans* culture with maximum OD_{600nm} of 0.80±0.0017 reached after 4 days of incubation when oxyrase used as reducing agent, and maximum OD_{600nm} 0.65±0.0016 in the presence of Cys-HCl. *Akkermansia muciniphila* culture reached maximum OD_{600nm} value of 2.1±0.07 in the presence of oxyrase after 18 h of incubation. While with Cys-HCl, *Akkermansia muciniphila* culture reached maximum cell density OD_{600nm} of 2.0±0.05 after 27 h of incubation. Oxygen reducing potential of oxyrase also suited the growth of *Bilophila wadsworthia* because it reached maximum cell density OD_{600nm} of 2.0±0.05 after 73 h of incubation. Whereas, in the presence of Cys-HCl *Bilophila wadsworthia* cells only achieved maximum OD_{600nm} of 1.54±0.07 after 28 h of incubation. Cultivation studies of *Roseburia intestinalis* in the presence of oxyrase also exhibited noteworthy improvement in the yield of cell density with OD_{600nm} of 2.1±0.06 after 25 h of incubation. With Cys-HCl, maximum cell density of *Roseburia intestinalis* with 2.0±0.06 OD_{600nm} was observed after 73 h.